STRATEGIES FOR EFFECTIVE BIOREMEDIATION OF WATER CO-CONTAMINATED WITH 1,2-DICHLOROETHANE AND HEAVY METALS

BY

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As the candidate's supervisors, we have approved this dissertation for submission.

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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 March 2009 to March 2011, 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

The production of 1,2-Dichloroethane (1,2-DCA) exceeds 5.44 billion kg per year, and is higher than that of any other industrial halogenated chemical. Improper disposal practices or accidental spills of this compound have made it a common contaminant of soil and groundwater. 1,2-DCA has been classified as a priority pollutant by the Environmental Protection Agency owing to its toxicity, persistence and bioaccumulation in the environment. It has also been shown to have mutagenic and potential carcinogenic effects on animals and humans. Bioremediation is emerging as a promising technology for the clean-up of sites contaminated with chlorinated hydrocarbons. However, sites co-contaminated with heavy metals and 1,2-DCA may pose a greater challenge for bioremediation, as the former pollutant could inhibit the activities of microbes involved in bioremediation. Therefore, this study was undertaken to quantitatively assess the effects of heavy metals on 1,2-DCA biodegradation and to investigate the use of biostimulation, bioaugmentation, dual bioaugmentation, and biosorption for remediation of water co-contaminated with 1,2-DCA and heavy metals in microcosms. The combined effect of 1,2-DCA and the respective heavy metals on the microbial population and diversity was also investigated. The minimum inhibitory concentrations (MICs) and concentrations of the heavy metals (arsenic, cadmium, mercury and lead) that caused half-life doubling (HLDs) of 1,2-DCA as well as the degradation rate coefficient (k_1) and half-life ($t_{1/2}$) of 1,2-DCA were measured in two different wastewater types. An increase in heavy metal concentration from 0.01–0.3 mM, resulted in a progressive increase in the $t_{1/2}$ and relative $t_{1/2}$ and a decrease in k_1. The MICs and HLDs of the heavy metals were found to vary, depending on the heavy metals and wastewater type. In addition, the presence of heavy metals was shown to inhibit 1,2-DCA biodegradation in a dose-dependent manner, with the following order of decreasing inhibitory effect: $Hg^{2+} > As^{3+} > Cd^{2+} > Pb^{2+}$. For the bioremediation experimental set-up, 150 ml wastewater was spiked with 1,2-DCA (2.5 mM) and the respective heavy metal in air-tight serum bottles (Wheaton). The bottles were biostimulated, bioaugmented, dual-bioaugmented or undergoing biosorption. The microcosms were incubated at 25 °C and the 1,2-DCA concentration was measured weekly. Co-contaminated water undergoing biostimulation, bioaugmentation and, in particular, dual bioaugmentation were observed to exhibit higher degradation of 1,2-DCA in the presence of the heavy metal, compared to co-contaminated water receiving none of the treatments. Dual bioaugmentation, proved to be most effective, resulting in up to 60% increase in 1,2-DCA

degradation after 4 weeks, followed by bioaugmentation (55%) and biostimulation (51%). In addition, an increase in the total number of 1,2-DCA degrading bacterial population was observed in the bioaugmentated microcosms compared to those biostimulated, which corresponds to an increased 1,2-DCA degradation observed in the bioaugmentated co-contaminated microcosms. Dominant bacterial strains obtained from the co-contaminated microcosms were identified as members of the genera, *Burkholderia, Pseudomonas, Bacillus, Enterobacter* and *Bradyrhizobiaceae*, that have been previously reported to degrade 1,2-DCA and other chlorinated compounds. Some of these isolates also belong to genera that have been previously shown to be resistant to heavy metals. PCR-DGGE analysis revealed variations in microbial diversity over time in the different co-contaminated microcosms, whereby the number of bands was reduced, the intensity of certain bands increased, and new bands appeared. Agricultural biosorbents (AB) were found to adsorb heavy metals effectively when utilized at a concentration of 2.5%, with the level of biosorption found to be dependent on the type of AB as well on the type of heavy metal present. OP proved to be the most efficient biosorbent for the heavy metals tested, followed by CNF, and corn cobs (CC) least efficient; therefore CC was not used in further bioremediation experiments. Both orange peel (OP) and coconut fibre (CNF) were found to be excellent at removing heavy metals from co-contaminated microcosms, with OP removing 14.59, 74.79, 60.79 and 87.53% of As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+}, respectively, while 10.03, 40.29, 68.47 and 70.00% of As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+}, respectively, was adsorbed by CNF. Consequently, a higher degradation of 1,2-DCA was observed in the presence of OP and CNF, compared to the untreated control. It can be concluded that the remediation approaches utilized in this study proved effective in the bioremediation of water co-contaminated with 1,2-DCA and heavy metals and may provide the foundation for new and innovative treatment strategies for co-contaminated sites.

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CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Rapid industrialisation, economic expansion and human activities are the key factors that led to widespread pollution of the natural global environment (Ang *et al*., 2005). Chlorinated hydrocarbons are of foremost environmental concern given that these contaminants are repeatedly found in groundwater, soil and the atmosphere due to improper disposal of wastes, accidental spillage, or deliberate release. Among the short-chained chlorinated aliphatic compounds, 1,2-dichloroethane (1,2-DCA) represents one of the world's most significant toxic chlorinated aquifer pollutant (Marzorati *et al*., 2007), and it is produced industrially in larger volumes than any other halogenated compound (Laturnus, 2003). Improper disposal practices or accidental spills of these compounds have made them common contaminants of soil and groundwater (McCarty, 1997) causing serious environmental and human health problems as a result of their persistence and toxicity (Squillace *et al.*, 1999). The use of microbes to clean up polluted environments, i.e. bioremediation, is a rapidly changing and expanding area of environmental biotechnology, and microorganisms have demonstrated the ability to detoxify chlorinated organic compounds, including 1,2-DCA. 1,2-Dichloroethane has been reported to be biodegraded *via* 2-chloroethanol, 2-chloroacetaldehyde, and chloroacetic acid to glycolate (Janssen *et al*., 1985; Janssen *et al*., 1987).

Although bioremediation is a promising technology, remediation of sites co-contaminated with organic and metal pollutants is a complex problem, as the two components must often be treated differently (Sandrin and Maier, 2003). The rate of heavy metal influx into the environment far exceeds their removal by natural processes, thus leading to their accumulation in the environment, with aquatic ecosystems normally at the receiving end (Shirdam *et al*., 2006). This may adversely affect potentially important biodegradation processes (Said and Lewis, 1991). The inhibitory effect of heavy metals on microbial processes, such as methane metabolism, growth, nitrogen and sulphur conversions and reductive process, are well documented (Baath, 1989). However, the effects of metal toxicity on organic compound bioremediation when both types of contamination are present in water and soil environments are challenging to define quantitatively or qualitatively (Sandrin and Maier, 2003). This is because

metals may be present in a variety of different physical and chemical forms, namely, as separate-phase solids, soil-adsorbed species, colloidal solutions, soluble complexed species, or ionic solutes. Related complications stem from the fact that the physical and chemical state of metals are affected by environmental conditions such as pH and ionic strength of the water phase as well as soil properties that include ion exchange capacity, clay type and content, and organic matter content (Sandrin and Maier, 2003). Therefore, bioremediation of chlorinated xenobiotic compounds in the environment must take into consideration the effect of metals on bacterial processes and of bacterial species on the metals themselves (Kuo and Genthner, 1996).

Previous studies have focused extensively on the biodegradation of 1,2-DCA in several contaminated soil and water environments (Marzorati *et al*., 2010; Van Der Zaan *et al*., 2009) while toxic effects of heavy metals on microorganisms have also been studied (Gikas *et al*., 2009; Rajapaksha *et al*., 2004). Conversely, 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 co-contaminated sites, it is necessary to evaluate 1,2-DCA biodegradation in co-contaminated water and ascertain the effects of heavy metals on 1,2-DCA biodegradation. Furthermore, due to the widespread use and release of organic pollutants and heavy metals, determining their combined effects on microbial communities is essential. This study therefore focused on the quantitative assessment of the effects of heavy metals on the 1,2-DCA biodegradation profile in co-contaminated water. Remediation strategies such as biostimulation, bioaugmentation and biosorption were investigated for their potential to alleviate the co-contamination problem. Denaturing gradient gel electrophoresis and direct plate counts of the 1,2-DCA degrading population were used to assess the impact of heavy metals on the microbial population and diversity during 1,2-DCA degradation in co-contaminated water.

1.2 Chlorinated hydrocarbons: A cause for concern

Chlorine compounds are a huge assembly of environmental pollutants that negatively affect the well-being of humans and wildlife, thus bringing chlorine chemistry to the forefront of public concern (Hileman, 1993). Large quantities of chlorinated compounds are manufactured annually for a wide range of commercial, agricultural and industrial uses. There are about 15000 chlorinated compounds in commerce, including; pesticides, pharmaceuticals, disinfectants for drinking water, and many modern consumer products such as items made of polyvinyl chloride that have improved the quality of life in both developed and developing countries (Gribble,

1996). In addition to the 15000 chlorine products manufactured by humans, about 1500 to 2000 chlorine compounds are released to the environment from natural sources (Gribble, 1996). Chlorinated organic compounds are known to be persistent and thus able to reach high concentrations both in the environment and in human tissues due to their inertness to biotic or abiotic transformation reactions (Hocheolsong and Carraway, 2005). Their interactions with hormonal systems have created escalating interest, and they are known to be predominantly harmful to the developing embryo, foetus and toddler (Hanberg, 1996). In addition, they cause cancer in experimental animals (Hocheolsong and Carraway, 2005).

1.3 1,2-Dichloroethane

1.3.1 Properties

1,2-Dichloroethane also called 1,2-ethylene dichloride, dichloroethylene or ethylene dichloride is a manufactured chemical that is not found naturally in the environment. 1,2-DCA has a molecular formula of $C_2H_4Cl_2$ and a molar mass of 98.97 g/mole. It appears as a clear, colourless, oily liquid, and has a sweet taste and pleasant chloroform-like odour (IPCS, 1995). It has a melting point of -35.5 °C and a boiling point of 83.5 – 84.1 °C and moderate solubility in water (8000 mg/l) (Baden, 2008).

1.3.2 Production and uses

1,2-DCA was reported as the first chlorinated hydrocarbon to be synthesized and commercial production was first reported in 1922 (IARC, 1979). The production of 1,2-DCA exceeds 5.44 billion kg per year, and is larger than that of any other industrial halogenated chemical (Janssen *et al.,* 1989). 1,2-DCA pollution in rivers is due largely to anthropogenic sources (De Rooij *et al*., 1998).

1,2-dichloroethane was previously used as a general anaesthetic as an alternative to chloroform, particularly in ophthalmic surgery (HSDB, 2000). It was also used as an insect fumigant for stored grain and as a lead scavenger in leaded petrol (Falta, 2004). At present, the major use (90%) of 1,2-DCA is as an intermediate in the production of vinyl chloride. Less significantly, 1,2-DCA is also used in the production of vinylidene chloride, 1,1,1-trichloroethane, trichloroethene, tetrachloroethene, ziridines, and ethylene diamines and in

chlorinated solvents (USATSD, 2001). It is also used as an extractant and cleaning solvent, in the synthesis of other chlorinated solvents.

1.3.3 Human health effects

1,2-DCA has acute toxic, mutagenic, and carcinogenic effects on humans and have been reported to pose various health problems (Baker *et al*., 1996; Nouchi *et al*., 1984). From case studies of poisoning incidents, the lethal dose of 1,2-DCA is estimated to be 20-50 ml (WHO, 2003). Workers exposed to 1,2-DCA levels of 4 mg/m^3 were found to exhibit an increased lymphocyte sister chromatid exchange frequency (Cheng *et al*., 2000). 1,2-DCA can negatively affect the reproductive system in humans. Female workers and wives of male 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; Zhao *et al*., 1989). Respiratory and circulatory failures have resulted in death due to ingestion or inhalation of 1,2-DCA in humans. Anorexia, nausea, abdominal pain, mucous membranes irritation, liver and kidney dysfunction and neurological disorders are all due to repeated exposure to 1,2-DCA in the occupational environment (IARC, 1999). 1,2-DCA is acutely nephrotoxic in humans following inhalation exposure. A 51-year-old male died after inhaling concentrated vapour for only 30 minutes (Nouchi *et al*., 1984). An increased incidence of colon and rectal cancer has been reported when men, aged 55 and over, were exposed to 1,2-DCA in drinking water (Isacson *et al*., 1985).

1.3.4 Biodegradation of 1,2-dichloroethane

A broad range of chlorinated aliphatic compounds are susceptible to biodegradation under a variety of physiological and redox conditions. Microbial degradation of a wide variety of chlorinated aliphatic compounds have been shown to occur under five different physiological conditions; however, any given physiological condition could only act upon a subset of the chlorinated compounds (Field and Sierra-Alvarez, 2004). Firstly, chlorinated compounds are used as electron donors and carbon sources under aerobic conditions. Secondly, they are cometabolized under aerobic conditions while the microorganisms are growing (or otherwise already have grown) on another primary substrate. Thirdly, they are also degraded under anaerobic conditions in which they are utilized as electron donors and carbon sources. Fourthly, they can serve as electron acceptors to support respiration of anaerobic microorganisms utilizing

simple electron donating substrates. Lastly, chlorinated compounds are subject to anaerobic cometabolism becoming biotransformed while the microorganisms grow on other primary substrates or electron acceptors (Field and Sierra-Alvarez, 2004).

Studies focusing on the fate of 1,2-DCA in the environment have suggested that it can be transformed through both abiotic and biotic mechanisms (aerobic and anaerobic) mechanisms. However, microbial enzyme systems capable of its degradation have not evolved sufficiently to make the compound widely biodegradable (Van Der Zaan *et al*., 2009). Abiotic degradation of 1,2-DCA has been reported; however it proceeds at a very slow rate. Under abiotic conditions, the half-life of 1,2-DCA at pH 7 and 25 °C was found to be 72 years. At neutral pH, a hydrolytic substitution takes place and ethylene glycol is the product, however, when alkaline hydrolysis of 1,2-DCA occurs vinyl chloride is detected (Jeffers *et al*., 1989). The half-life of 1,2-DCA was reported by Barbash and Reinhard (1989) to be 23 years when the conditions matched that of groundwater (15 °C, pH 7, and in the presence of 1 mM total sulfide); however certain anions found in aqueous environments were found to increase the abiotic reaction rate, at 15 °C and pH 7, the half-life of 1,2-DCA in 50 mM phosphate buffer is reduced from 170 to 23 years when 1 mM total sulfide is present. Dissolved 1,2-DCA is transformed slowly under abiotic conditions and the resulting products may even be more toxic than 1,2-DCA itself (Gallegos *et al*., 2007). Therefore, recent methods of degradation are focusing on microbial biodegradation.

1.3.4.1 Aerobic biodegradation of 1,2-dichloroethane

The aerobic biodegradation of 1,2-DCA has been extensively studied and was first demonstrated in 1983 by Stucki *et al*. (1983). Pure cultures of *Xanthobacter autotrophicus* GJ10 and *Ancylobacter aquaticus* AD20 and AD25 can degrade 1,2-DCA to 2-chloroethanol by a constitutively produced haloalkane dehalogenase (Janssen *et al*., 1989; Van Den Wijngaard *et al*., 1992). 2-Chloroethanol is subsequently converted *via* chloroacetaldehyde to chloroacetic acid by a quinoprotein alcohol dehydrogenase and an NAD-dependent aldehyde dehydrogenase (Janssen *et al*., 1985; Van den Wijngaard *et al*., 1992). The second dehalogenase, specific for halocarboxylic acids, dechlorinates 2-chloroacetic acid to glycolic acid (Janssen *et al*., 1985) which can enter the central metabolic pathways. The haloalkane dehalogenase nucleotide sequences in these strains were found to be identical. The enzymes alcohol dehydrogenase and the chloroacetate dehalogenase appear to be widespread in nature, however, the haloalkane dehalogenase and chloroacetaldehyde dehydrogenase appear to be particularly adapted for the

degradation of xenobiotic substrates (Klecka *et al*., 1998).

Pseudomonas sp. strain DCA1 has a strong affinity for 1,2-DCA. This bacteria makes use of a novel pathway for 1,2-DCA degradation. Only the initial attack of the 1,2-DCA molecule seems to be different from the 1,2-DCA degradation pathway in *X. autotrophicus* GJ10 and *Ancylobacter aquaticus* AD25. In contrast to the hydrolytic dehalogenation of 1,2-DCA in *X. autotrophicus* GJ10 and *A. aquaticus* AD25, an oxidation reaction seems to be the first step in 1,2-DCA degradation in *Pseudomonas* sp. strains DE2 and DCA1 (Hage and Hartmans 1999) The differentiation in the first step is shown in Figure 1.1.

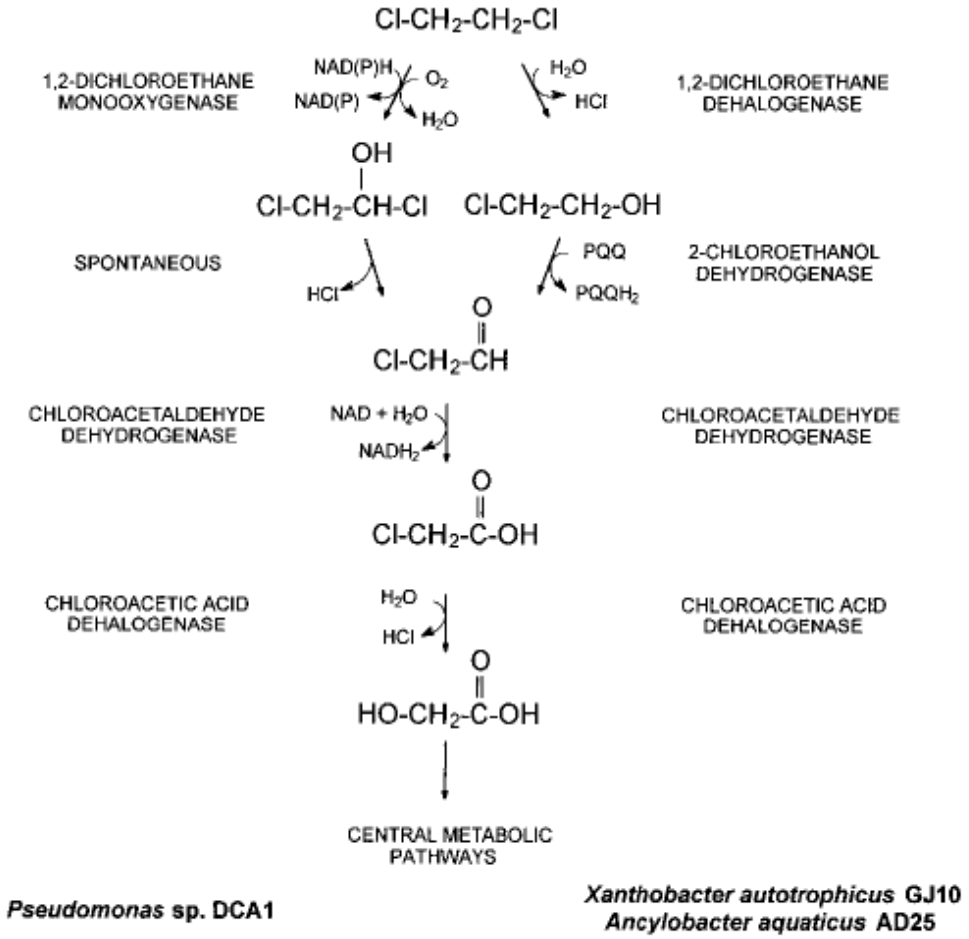


Figure 1.1: 1,2-DCA degradation pathway in *X. autotrophicus* GJ10 and *A. aquaticus* AD25 (right) and the proposed pathway in *Pseudomonas* sp. strain DCA1 (left) (Hage and Hartmans, 1999).

1.3.4.2 Anaerobic biodegradation and halorespiration of 1,2-DCA

1,2-DCA can be biotransformed under anaerobic conditions as described by Belay and Daniels (1987) and Egli *et al*. (1987). These authors suggested that methanogenic or sulfate reducing bacteria transform 1,2-DCA to ethane. This was different to the findings of Holliger *et al*. (1990) who suggested that methanogenic bacteria reductively dechlorinated 1,2-DCA *via* two dissimilar reaction mechanisms; a dihaloelimination reaction yielding ethene as well as two successive hydrogenolysis reactions yielding chloroethane and ethane. Nitrate can also serve as an alternate electron acceptor in the oxidation of 1,2-DCA when there is a deficiency of oxygen (Dinglasan-Panlilio *et al*., 2006; Gerritse *et al*., 1999). Dehalorespiration, using 1,2-DCA as the terminal electron acceptor by a bacteria to support its growth has been demonstrated. This was first reported by Maymo-Gatell *et al*. (1999) who found that *Dehalococcoides ethenogenes* strain 195 could grow by means of oxidizing H^{2+} to H^{+} and by reducing 1,2-DCA principally to ethene and only <1% to vinyl chloride.

1.4 Metal toxicity to microbes

In nature, living organisms come into contact with heavy metals, frequently existing in their ionized species. All metals, in spite of whether they are essential or non-essential, can exhibit toxic effects at elevated concentrations (Silver, 1996). Toxicity of metals to an organism can be defined as the intrinsic potential or ability of a metal to cause negative effects on living organisms and depends on the bioavailability of the metals (Rasmussen *et al*., 2000). Metals in the environment can be separated into two groups: bioavailable (soluble, nonsorbed and mobile) and non-bioavailable (precipitated, complexed, sorbed and non-mobile). It is the bioavailable metal concentration that is taken up and is toxic to biological systems by exerting diverse toxic effects on microorganisms (Cervantes *et al*., 2006). In co-contaminated sites, metal toxicity inhibits the processes of organic-degrading microorganisms (Roane *et al*., 2001). Numerous metal ions can simultaneously cause toxicity on biological systems by more than one biochemical pathway. These reactions can be separated into five mechanistic categories of which the first entails substitutive metal-ligand binding, in which one metal ion replaces another at the binding site of a specific biomolecule, thereby altering or destroying the biological function of the target molecule (Nieboer *et al*., 1996). Second is the covalent and ionic reduction–oxidation (redox) reaction of metal ions with cellular thiols (R-SH) (Stohs and Bagchi 1995; Zannoni *et al*.,

2007), in particular, glutathione (Turner *et al*., 2001). Studies have shown that metals, including iron, copper, chromium, and vanadium undergo redox cycling, while cadmium, mercury, and nickel, as well as lead, deplete glutathione and protein-bound sulfhydryl groups (-SH), resulting in the production of reactive oxygen species (ROS) such as superoxide ions, hydrogen peroxide, and hydroxyl radicals and as a consequence, enhanced lipid peroxidation, DNA damage, and altered calcium and sulfhydryl homeostasis occur. Fenton-like reactions may be commonly associated with most membranous fractions including mitochondria, microsomes, and peroxisomes. The third category involves the participation of transition metals, such as Cu, Ni and Fe, in Fenton-type reactions that produce ROS, such as H_2O_2, hydroxyl radicals and O_2^- (Geslin *et al*., 2001; Inoaoka *et al*., 1999; Stohs and Bagchi, 1995). In general, ROS are transient and highly reactive compounds that can damage all biological macromolecules (Pomposiello and Demple, 2002). The fourth category entails interference with membrane transport processes, in which a toxic metal species can competitively inhibit a specific membrane transporter by occupying binding sites or by using and/or interfering with the membrane potential that is normally reserved for an essential substrate (Foulkes, 1998). The fifth category involves the indirect siphoning of electrons from the respiratory chain by thiol-disulphide oxidoreductases (Borsetti *et al*., 2007) thereby destroying the proton motive force of the cell membrane (Lohmeier-Vogel *et al*., 2004).

1.5 Microbial resistance to heavy metals

Even though metals are known to incapacitate microorganisms that biodegrade organic pollutants, a number of microbial metal-resistance mechanisms are recognized (Roane *et al*., 2001). In an endeavor to safeguard susceptible cellular components, a cell can build up resistance to metals. Metal containing environments have built up selective pressures which eventually lead to the expression of resistance mechanisms in microbes to practically all heavy metals (Rouch *et al*., 1995). Numerous mechanisms by which microorganisms resist metal toxicity exist, and five mechanisms have been hypothesized (Bruins *et al.*, 2000). These include: I) active or dynamic transport; II) development of a permeability barrier; III) enzymatic detoxification; VI) reduction in sensitivity; and V) sequestration (Bruins *et al.*, 2000). Microbes may either use one or more of these methods to eliminate non-essential metals and normalize concentrations of vital metals.

1.5.1 Active or dynamic transport

In active or dynamic transport, metals are exported away from the microorganism or more specifically from the microorganism's cytoplasm. These mechanisms can be chromosomal or plasmid-encoded while efflux systems can be non-ATPase or ATPase-linked and extremely precise for the cation or anion they export (Bruins *et al*., 2000). An active transport or efflux mechanism embodies the principal category of metal resistance systems and is the most well studied metal resistance system. Three main detoxification mechanisms are known to function in bacteria: (i) P_{1B}-type ATPases, which use ATP energy to pump metals out of the cytoplasm to the periplasm; (ii) CBA transporters (three-component transenvelope pumps, consisting of subunits C, B, and A), which extrude metals from the cytoplasm to the periplasm to outside the outer membrane (Franke *et al*., 2003); and iii) and cation diffusion facilitator (CDF) family transporters which function as chemiosmotic ion-proton exchangers (Grass *et al*., 2001). P-type ATPases and CDF transporters can functionally replace each other, however they cannot replace CBA transporter (Scherer and Nies, 2009). Examples of active transport include the *ars* operon used in the export of arsenic from *E. coli, cop* operon for the removal of excess copper from *Enterococcus hirae* and the *cad* system for removing cadmium from *Staphylococcus aureus* (Silver and Phung, 1996; Tsutomu and Kobayashi, 1998). *Ralstonia* sp. CH34 encompasses resistance determinants that can detoxify heavy metal ions by inducible ion efflux systems that reduce the intracellular concentration of a given ion by active export (Nies, 2000).

1.5.2 Development of a permeability barrier

For microbes that lack a specific active transport system, metal exclusion by a permeability barrier can be an alternative mechanism whereby the essential cellular components which are metal sensitive are protected by altering the cell wall, membrane or envelope of the microorganism (Bruins *et al*., 2000). Examples of such alterations include: prevention of entry of heavy metals by diminishing production of membrane channel proteins (Rouch *et al.,* 1995); possible saturation of metal-binding sites in the membrane and periplasm with non-toxic metals (Mergeay, 1991); prevention of metals from reaching the surface of the cell by the formation; and binding of an extracellular polysaccharide coat (Scott and Palmer, 1990). Gram-negative bacteria possess a cell wall that is a more effective barrier to heavy metals than Gram-positive bacteria, this was demonstrated in a study by Balestrazzi *et al*. (2009) where tetra-resistant

bacterial isolates were more frequent among the Gram-negative bacteria belonging to the genus *Pseudomonas*.

1.5.3 Enzymatic detoxification

In microbes without a permeability barrier, enzyme detoxification of the metal to a less toxic form can also be a method to reduce heavy metal toxicity. In contrast to thermophilic or psychrophilic organisms, heavy metal-resistant bacteria do not supply enzymes that are active under harsh conditions, but are themselves tools for the evaluation and remediation of heavy metal-contaminated environments (Nies, 2000). Several bacteria utilize a metal resistance operon, i.e. a set of genes that can not only detoxify the heavy metal but can transport and self-regulate resistance (Ji and Silver, 1995; Misra, 1992). The mercury resistance system is the most renowned example of enzyme detoxification. Bacteria that possess the *mer* operon include *E. coli, Thiobacillus ferrooxidans, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus* sp. and *Serratia marcescens*. Two different metal resistance mechanisms are encoded by the *mer* operon, mercury reductase encoded by merA, which can reduce Hg^{2+} to the volatile, more stable and less toxic elemental Hg^0 (Barkay *et al*., 2003). Other genes allow for active transport of elemental mercury out of the cells by membrane proteins which are encoded in the operon (Misra, 1992; Wireman *et al.,* 1997; Rasmussen *et al.,* 2000). Other examples include the $As^{3+} \rightarrow As^{5+}$ oxidase and the $Cr^{6+} \rightarrow Cr^{3+}$ reductase. In other cases, such as As^{5+}, Ag^{+} and Cd^{2+}, no change in redox state occurs but, rather, uptake and transport differences accompany resistance determinants (Jeffrey and Silver, 1984). Sulfate-reducing bacteria such as *Desulfovibrio vulgaris* strain Hildenborough are able to reduce heavy metals by a chemical reduction *via* the production of H_2S and by a direct enzymatic process involving hydrogenases and c3 cytochromes (Goulhen *et al*., 2006).

1.5.4 Reduction in sensitivity

A fourth mechanism of resistance is the reduction in metal sensitivity whereby some degree of natural protection from heavy metal toxicity can be provided to the microorganism by reducing the metal sensitivity of cellular targets. Microorganisms can do this by altering the sensitivity of essential cellular components, thus allowing for adaptation to the presence of heavy metals (Bruins *et al*., 2000).

1.5.5 Sequestration

Microbes that lack enzyme protection may be capable of sequestering heavy metals either inside or outside the cell. Intracellular sequestration of metals by protein binding, leads to the accumulation of metals within the cytoplasm thus averting contact to vital cellular components. Metals generally sequestered are Cd^{2+}, Cu^{2+}, and Zn^{2+}. The two most widespread molecules used for intracellular sequestration are metallothioneins and cysteine-rich proteins (Rouch *et al.,* 1995; Silver and Phung, 1996). *Alcaligenes eutrophus* CH34 strains internally sequester metals by forming carbonate precipitates within the cell or extracellular sequestration outside the cell where heavy metals bind to outer cell membrane proteins (Mahvi and Diels, 2004). Resistance by extracellular sequestration occurs once the toxic metal is bound in a complex and cannot go through the cell membrane. Examples of molecules used for extracellular sequestration are glutathione and ionic phosphate, these molecules are the foundation for metals to form insoluble complexes (McEntee *et al*., 1986). Sequestration is also a detoxification mechanism for Pb^{2+}. For example *Vibrio harveyi* can bind Pb^{2+} intra- and extracellularly, thus avoiding toxicity by lowering the concentration of free lead ions by precipitating lead as a phosphate salt (Hynninen et *al*., 2009).

A comparison of heavy metal resistance systems by Nies (2003) shows that it is the result of multiple layers of resistance systems with overlapping substrate specificities, but unique functions. Some microbial systems of metal tolerance have the potential to be used in biotechnological processes, such as the bioremediation of environmental metal pollution (Cervantes *et al*., 2006). Even with all of these mechanisms in place for metal resistance, microbes are still susceptible to heavy metals at high concentrations.

1.6 Effect of heavy metals on the bioremediation process

In the environment, heavy metal toxicity affects many important microbial processes, including litter decomposition, methanogenesis, acidogenesis, nitrogen transformation, and biodegradation of organics (Baath, 1989). Human health and wildlife are becoming increasingly compromised due to the various organic and heavy metal contaminants situated at hazardous waste sites around the globe (Fierens *et al*., 2003; Järup, 2003). Chlorinated organic solvents and heavy metals are two frequently encountered classes of pollutants often observed in contaminated subsurfaces (Lien *et al*., 2007). Two techniques that are commonly used for eradicating organic wastes from these sites; mining and ignition, have been established to be

ineffective and expensive (Lehr *et al.,* 2001). The presence of heavy metals may inhibit biodegradation by either inhibiting enzymes involved in both biodegradation and microbial metabolism, thus compounding the problem of chlorinated organic contamination (Kuo and Genthner, 1996). The effects include extended acclimation periods, reduced biodegradation rates, and failure of target compound biodegradation (Kuo and Genthner, 1996).

Metals are known to exist in several diverse chemical and physical states such as soil-adsorbed species, ionic solutes, soluble complexed species, separate- phase solids or colloidal solutions, thus making it difficult to be able to adequately define the effects of heavy metal toxicity on organic pollutant biodegradation in co-contaminated sites. The fact that the chemical and physical forms of heavy metals is influenced by environmental surroundings such as pH and ionic strength of the water phase as well as soil characteristics such as organic matter content, ion exchange capacity as well as clay type and content, further complicates remediation in these co-contaminated sites (Sandrin and Maier, 2003).

An extensive list of reported metal concentrations that cause inhibition of biodegradation of organic contaminants has been presented by Sandrin and Maier (2003). There is also a wide range of reported inhibitory metal concentrations in the literature. For instance, five orders of magnitude separate lowest reported concentrations of zinc that inhibit biodegradation. Said and Lewis (1991) demonstrated that copper, mercury, chromium (II), cadmium and zinc are able to inhibit biodegradation of 2,4- dimethyl ether in lake water samples inoculated with either a sediment or an aufwuch (floating algal mat) sample. In the sediment samples, zinc was most toxic, with an MIC of 0.006 mg total zinc.l^{-1}, however, in samples that contained aufwuchs, mercury was most toxic, with an MIC of 0.002 mg total mercury.l^{-1}. In a study, using a pure culture of naphthalene (NAPH)-degrading *Burkholderia* sp., a MIC of 1 mg solution-phase cadmium.l^{-1} was reported (Sandrin *et al*., 2000). A comparable MIC was reported by Said and Lewis (1991) for cadmium (0.1 mg total cadmium.l^{-1} for sediment samples and 0.629 mg total cadmium.l^{-1} for aufwuch samples). The variation between these MICs is thought to be organism/community specific. Springael *et al.* (1993) demonstrated that metals will possibly inhibit biodegradation of pollutants by several bacteria genera under pure culture settings. However, the MICs reported in this study were 2-4 orders of magnitude greater than those formerly described by Said and Lewis (1991). This huge inconsistency is possibly owing to discrepancies in test conditions. Also, the broad range of reported inhibitory concentrations can be attributed to divergences in experimental protocols that affect solution-phase metal

concentrations. For example, many laboratory media contain metal-binding (e.g. yeast extract) and metal precipitating (e.g. phosphate or sulfate salts) components that can reduce solution-phase metal concentrations (Hughes and Poole 1991). Medium pH can also considerably impact on solution-phase metal concentrations which become diminished as the pH is raised. This is due to metals forming insoluble metal oxides and phosphates (Crannell *et al*., 2000). Hughes and Poole (1991) stress the importance of understanding metal speciation in the test system. Unfortunately, few studies provide speciation information and as a result, an enormous range of metal concentrations has been reported to inhibit organic biodegradation.

The effects of Hg^{2+}, Cu^{2+}, Cd^{2+} or Cr^{6+} at concentrations ranging from 0.01 to 100 ppm on degradation of 2-chlorophenol (2CP), 3-chlorobenzoate (3CB), phenol, and benzoate by anaerobic bacterial consortia was investigated by Kuo and Genthner (1996). Three effects were observed, including extensive acclimation times (0.1 to 2.0 ppm), reduced dechlorination or biodegradation rates (0.1 to 2.0 ppm) and failure to dechlorinate or biodegrade the target compound (0.5 to 5.0 ppm). The biodegradation of 3CB was established to be the most susceptible to Cd^{2+} and Cr^{6+}. Biodegradation of benzoate and phenol was most susceptible to Cu^{2+} and Hg^{2+}, in that order. Either specific or general variations in the bacterial populations could account for differences in metal sensitivity between the phenol and benzoate consortia. The biotransformation of 2-CP and 3-CP was examined by Kong (1998) in anaerobic fresh and accustomed sediments in the occurrence and deficiency of $CuCl_2$, $CdCl_2$ and $K_2Cr_2O_7$ ranging from 10 to 200 $mg.l^{-1}$. Various inhibitory effects of certain metals were noticed on dechlorination of CPs in sediment slurries. In the case of fresh unacclimated slurry, addition of 20 mg $Cu.l^{-1}$ resulted in no substantial reduction of dechlorination for tested CPs. In contrast, 20 $mg.l^{-1}$ of Cd and Cr reasonably affected lag periods preceding dechlorination. In this case, Cd was more inhibitory than either Cu or Cr (Cd > Cr ≥Cu), which demonstrates almost comparable outcomes. In the case of the accustomed slurry, the dechlorination activity of 2- and 3-CP was inhibited in the presence of 20 $mg.l^{-1}$ of three tested metals. Metals amended to 2- and 3-CP acclimated sediments exhibited inhibition degree in the order of Cd > Cu > Cr, which are different patterns compared to fresh slurry The effect of a transition metal, cadmium (Cd), on reductive dechlorination of a model chlorinated organic, 2,3,4-trichloroaniline (2,3,4-TCA), was determined in the laboratory by Pardue *et al*. (1996) in three anaerobic, flooded soil types with varying properties. Dechlorination of 2,3,4-TCA was inhibited over a range of pore-water-soluble Cd concentrations (10-200 $\mu g.l^{-1}$) in mineral-dominated rice paddy and bottomland

hardwood soils. In organic-dominated marsh soil, however, a critical inhibitory level of pore-water-soluble Cd (200 $\mu g.l^{-1}$) was observed. Lower-chlorinated aniline metabolites differed with soluble Cd concentration. Different metabolites (2,3-dichloroaniline and 2-chloroaniline) were observed in some soil types as Cd neared the completely inhibitory concentration. Speciation of soluble Cd was necessary to predict whether inhibition would occur, particularly in the presence of high concentrations of organic ligands such as humic acids.

Not all studies can correlate heavy metal effects and biodegradation inhibition. It has been demonstrated that heavy metals did not negatively impact on the biodegradation of organic compounds. For example, Baldrian *et al.* (2000) showed the degradation of polycyclic aromatic hydrocarbons by the fungus *Pleurotus ostreatus* in the presence of cadmium and mercury. It is possible that the metals were being sequestered by a component of the solid medium used in these studies, therefore not being available to the organism. Said and Lewis (1991) and Kuo and Genthner (1996) have also demonstrated that biodegradation could be stimulated in response to heavy metals. In this situation, a dose-dependent relationship occurs between metal concentration and biodegradation, however, when microbial consortia are faced with selective pressure at higher metal concentrations, the dose dependent relationship can no longer exist. Said and Lewis (1991), observed that an aerobic consortium of microbes degraded 2,4-dichlorophenoxyacetic acid methyl ester at a faster rate when exposed to 100 μM cadmium than when exposed to 10 μM cadmium. This increase may be due to only the most resistant microbes being able to survive when exposed to higher metal concentrations and hence these resistant microbes no longer needed to out-compete less resistant microbes for a carbon source. Another possible explanation is that when faced with higher metal concentrations, the metal resistance mechanisms now become more rapidly induced therefore increasing biodegradation (Worden, 2008).

1.7 Remediation strategies to improve biodegradation in co-contaminated sites

Recent approaches to increasing organic biodegradation in the presence of metals involve reduction of metal bioavailability and the use of metal-resistant bacteria, treatment additives, and clay minerals. The addition of divalent cations and adjustment of pH are additional strategies under investigation. Other recent strategies include the use of biostimulation, bioaugmentation, biosorption, biosurfactants, zerovalent irons and cyclodextrin to enhance the bioremediation of

sites co-contaminated with chlorinated hydrocarbons and heavy metals. Some of these methods are described below:

1.7.1 Biostimulation

Biostimulation refers to the manipulation of the environment in such a way that biodegradation is stimulated and the reaction rates are increased. A study by Olaniran *et al*. (2009) revealed that biostimulation of soil (with glucose), co-contaminted with heavy metals (mercury and lead) and 1,2-DCA was most effective when compared to Kompel fertilizer Chemicult Products, and agricultural fertilizer. Biostimulation using glucose, yeast extract and fructose, added separately, was found to enhance the degradation of a combination of chlorinated aliphatic hydrocarbons (CAH), resulting in 3.28–26.43% increase for CCl_4; 6.1–24.68% increase for dichloromethane and 12.72–26.36% increase for dichloroethane degradation in the microcosms assembled with waste water. Correspondingly, up to 11.25% increase for CCl_4; 41.68% boost for DCM and 32.67% boost for DCA degradation were realized after three weeks in the microcosms constructed with waste water from a different source. Of the nutrients added, glucose seemed to have a major effect in stimulating the degradation of CAHs, followed by fructose in both microcosms (Olaniran *et al*., 2008). This is very noteworthy as these substrates are water soluble, easily metabolizable and non-hazardous (Olaniran *et al*., 2008). A study by Massimo *et al*. (2006) investigated the degradation of 1,2-DCA in microcosms biostimulated with different organic electron donors. It was established that microcosms supplemented with formate, lactate plus acetate exhibited complete removal of 1,2-DCA with release of ethane. However, when cheese whey was added, 1,2-DCA dehalogenation was only stimulated, subsequent to a lag period. Macbeth *et al*. (2004) investigated the indigenous microbial community of a trichloroethene (TCE) contaminated residual source area in deep, fractured basalt at a U.S. Department of Energy site supplemented with sodium lactate to enrich degradation of TCE. This resulted in complete dechlorination of virtually all aqueous-phase TCE to ethane. Since molasses is a well-known energy/carbon source, it could stimulate indigenous microorganisms. This was the basis for the injection of molasses by Grindstaff (1998) into an aquifer as a key substrate for microorganisms to stimulate biodegradation of TCE. The microbes enthusiastically degrade the primary substrate by making use of available dissolved oxygen and therefore generating an anaerobic or reduced state in the system. In this reduced state hexavalent chromium can precipitate out of the aquifer and TCE can be degraded.

1.7.2 Bioaugmentation

There have been numerous reports on bioaugmentation of sites contaminated with only chlorinated organic compounds, however only a small number cover inoculation with metal-resistant bacteria to stimulate organic contaminant biodegradation in a co-contaminated system (Sandrin and Maier, 2003). Roane *et al*. (2001) used an approach which involved the inoculation of a cadmium resistant *Pseudomonas* H1, and organic degrading *Alkaligenes eutrophus* JMP134 into soil microcosms, with the aim to detoxify cadmium so that organic 2,4-dichlorophenoxyacetic acid (2,4-D) degradation was no longer inhibited. It was established that sequestration by heavy metal degrading microorganisms reduced the bioavailable metal concentration and promoted higher 2,4-D biodegradation rates. Another strategy that has the potential to tackle the co-contamination predicament, involves the utilization of a microorganism that can withstand and thrive in sites contaminated with heavy metals and still be able to exhibit unimpaired biodegradation (Springael *et al*., 1993). If microorganisms can display both of these properties they can be considered important for the remediation of those environments contaminated by organic xenobiotic compounds and heavy metals. Plasmid-borne heavy metal resistance and haloaromatic degrading functions were transferred into *A. eutrophus* strain by intra-specific conjugation (Springael *et al*., 1993), resulting in the strain being able to express both metal resistance and degrading functions. This was proven by observing degradation of different polychlorinated biphenyl isomers and 2,4-D in the presence of 2 mM zinc or 1 mM nickel, provided that the metal resistance determinant was present in the catabolizing strain (Springael *et al*., 1993). Another approach was the use of trichloroethane (TCA) degrading rhizobacteria that can survive and thrive in soil polluted with heavy metals. Lee *et al*. (2006), engineered a TCA degrading strain by introducing a gene encoding EC20 (metal-binding peptide) into the bacteria. This resulted in a strain with both heavy metal sequestration and TCE degradation potential. An ice-nucleation protein (INP) anchor allowed EC20 to be displayed onto the cell surface of *Pseudomonas* strain Pb2-1 and *Rhizobium* strain 10320D. Cells that did not exhibit EC20 expression had a reduced rate of TCA degradation. However, cells expressing EC20 showed greater cadmium uptake and fully restored TCA degradation. It was established that EC20 expression can reduce the toxic effect of cadmium on TCA degradation by cadmium accumulation. Growing strains have been shown to be superior for degradation of a xenobiotic compound compared to resting cells. Accordingly, supplying nutrients or carbon sources together with the introduced bacteria can promote biodegradation. Introduction of bacteria that

are sensitive to heavy metals but still retain activity against the organic contaminant in heavy metal-contaminated environments could lead to decreased degradation in time and may require subsequent inoculation of the organisms (Springael *et al*., 1993).

1.7.3 Biosorption

Since the 1990's, the adsorption of heavy metal ions by low cost renewable organic materials has gained momentum. Various types of adsorbents such as activated carbon, zerolites and resins are employed. In view of the high cost of these adsorbents, more research is geared towards biosorption (Azouaou *et al*., 2008). Biosorption, the passive uptake of pollutants such as heavy metals by biological materials, is emerging as a potential alternative to the existing conventional technologies for the removal and/or recovery of metal ions from aqueous solutions (Schiewer and Patil, 2008). The utilization of seaweeds, moulds, yeasts, and other dead microbial biomass and agricultural waste materials for removal of heavy metals has been explored by many researchers and it was found that the major advantages of biosorption over conventional treatment methods include: low cost, high efficiency, minimization of chemical or biological sludge, regeneration of biosorbents and possibility of metal recovery (Sud *et al*., 2008). However the efficiency of biosorption is affected by several factors as described below:

1.7.3.1 Factors affecting biosorption

1.7.3.1.1 Temperature

Temperature plays a key role in the adsorption of heavy metals and is one of the most significant criteria for the efficient removal of heavy metals from wastewater (Addagalla *et al*., 2009). Temperature changes influences a number of forceful factors which are significant in heavy metal ion biosorption, including: (i) the stability of the metal ion species initially placed in solution; (ii) the stability of microorganism-metal complex depending on the biosorption sites; (iii) microbial cell wall configuration; and (iv) the ionization of chemical moieties on the cell wall (Sag and Kutsal, 2000). Temperature has two major effects on the adsorption process. One is that increasing the temperature will increase the rate of adsorbate diffusion across the external boundary layer and in the internal pores of the adsorbate particles because liquid viscosity decreases as temperature increases. The other one is that it affects the equilibrium capacity of the adsorbate depending on whether the process is exothermic or endothermic (Addagalla *et al*.,

2009). The effect of temperature on the sorption of cadmium and chromium by olive stone was found to be insignificant, however when the temperature was raised from 25 to 40ºC an increase in the sorption capacity for lead from 0.596 to 0.728 mg/g was obtained (Calero *et al*., 2009). The optimum biosorption temperature for Pb^{2+}, Cu^{2+} and Ni^{2+} by *R. arrhizus* was determined to be 25 ºC; and that for Cu^{2+}, Cd^{2+} and Pb^{2+} by *S. cerevisiae* was determined to be 25 ºC (Volesky and May-Phillips, 1995). However, the optimum biosorption temperature of the same metal ions by by *Phanerochaete chrysosporium* and by *Streptomyces noursei* was found to be 30 ºC (Say *et al*., 2001). On the other hand, Fe(III) and Pb(II) biosorption by *Zoogloea ramigera* was increased with increasing temperature up to 45 °C (Sag and Kutsal, 2000). It was also reported that at low temperature the binding of heavy metal ions to the microorganisms occurred by a physical adsorption process and because of the low energy utilization, a balance between the cell wall surface and the metal ions was usually quickly and easily reversible (Sag and Kutsal, 2000). Igwe and Abia (2007) studied the design parameters in the bioremediation of arsenic from aqueous solution using modified and unmodified coconut fibre. The result showed that a temperature of 30 °C and particle size of 0.6 mm was most suitable. The thermodynamic parameters evaluated including the sticking probability model indicate that the sorption process was exothermic, physiosorption and diffusion controlled.

1.7.3.1.2 pH

The effect of pH can be explained by the availability of negatively charged groups at the biosorbent surface which is necessary for sorption of metals to proceed. This is unlikely at the highly acidic pH of 2 when a net positive charge is produced in the system due to H^+ and H_3O^+. In such a system, H^+ competes with metal ions, resulting in protonation of active sites to the virtual exclusion of metal binding on the biosorbent surface. This means that at higher H^+ concentrations, the adsorbent surface is further positively charged thus dropping the attraction between adsorbent and metal cations. In contrast, as the pH increases, more negatively charged surface becomes accessible, consequently allowing for greater metal uptake (Abdel-Ghani *et al*., 2007). Abdel-Ghani *et al.* (2007) found the optimum pH for metal removal to range from 4.5-6.5. At these mentioned pH optima, lead removal, using the biosorbents; rice husks, maize cobs and sawdust, reached 99%. The role of pH in As^{5+}, Pb^{2+} and Hg^{2+} ions detoxification by biosorption from aqueous solutions using coconut fibre and sawdust waste biomass, containing chelating agents was studied by Igwe *et al*. (2005). It was shown that adsorption could be

influenced by pH with maximum adsorption found to occur at pH 12, whereas minimum adsorption occurred at pH 6-8. In a study by Vasudevan *et al*. (2003), the Cd^{2+} ion adsorption capacity of sorbent was found to increase with increasing pH, and was maximum at a pH of 6.5. Also, pH was found to influence the sorption capacity of activated sludge for heavy metals, with a pH of 4 reported to be the optimum for the adsorption capacity of the sludge (Addagalla *et al*., 2009). The effect of pH on the sorption capacity of prepared carbon was studied by Khan and Wahab (2007) and the most efficient sorption was found to occur at a pH of 4.5. At this value, the dominant species of copper mainly involved in the sorption process was free Cu^{2+} ions. Further experiments at higher values were hindered owing to the immediate precipitation of blue copper hydroxide. The effect of pH on the removal efficiency of total chromium onto *C. virgatum* was studied at a pH range of 1–8. The highest removal of total chromium was found at pH 1.5, with a decreased removal observed with an increase in pH (Sar and Tuzen, 2008).

1.7.3.2 Agricultural wastes as biosorbents

Recently, attention has been diverted towards byproduct material or wastes from large scale industrial operations and agricultural materials. Cellulosic agricultural waste materials are an abundant source for significant metal biosorption. The functional groups present in agricultural waste biomass viz., acetamido, alcoholic, carbonyl, phenolic, amido, amino and sulphydryl groups have affinity for heavy metal ions to form metal complexes or chelates (Sud *et al*., 2008). The functional groups can be determined by Fourier transform infrared spectrometry (Li *et al*., 2008). Some biosorbents are non-selective and bind to a wide range of heavy metals with no specific priority, whereas others are specific for certain types of metals depending on their chemical composition (Wang and Brusseau, 1995). The mechanisms of the biosorption process include hemisorption, complexation, adsorption on surface, diffusion through pores and ion exchange (Sud *et al*., 2008). The use of fruit wastes for the removal of heavy metal ions from wastewater has been reported. Orange peel was found to be an efficient biosorbent capable of removing heavy metal ions from wastewater (Schiewer and Patil, 2008). Also, another cellulosic material, maize cob, was found to be effective, and the mode of adsorption of the metal ions was established by calculating the intraparticulate diffusivity and the fractional attainment of equilibrium (Abia and Igwe, 2005). It was found that the mode of adsorption could be particle diffusion or film diffusion controlled depending on the metal ion type and the adsorbent type. It was also shown that coconut fibre was capable of removing heavy metals from waste water

(Igwe and Abia, 2007). A study on the ability of some vegetable wastes, released in nature, to remove cadmium from aqueous solution by adsorption was undertaken, using some selected adsorbents; orange bark, olive cores and olive wastes. The results obtained showed that the orange bark are more competitive with a maximum adsorption capacity of 31.01 $mg.g^{-1}$ compared to the olive cores (12.56 $mg.g^{-1}$) and the olive wastes (6.55 $mg.g^{-1}$) (Azouaou *et al*., 2008).

Abdel-Ghani *et al*. (2007) investigated the adsorption efficiency of rice husk, maize cobs and sawdust for the removal of Pb^{2+} from aqueous systems. Removal of the poisonous lead ions from solutions was possible using rice husk, maize cobs and sawdust as adsorbents. Rice husk was the most effective, with 98.15% of Pb removed at room temperature. The removal of Zn, Cd and Pb using maize cob was studied by Akporhonor and Egwaikhide (2007) with the highest sorption rates of 71% for Zn^{2+}, 32% for Cd^{2+}, and 30% for Pb^{2+} observed. The capacity and efficiency of maize cob carbon in removing Zn^{2+}, Ni^{2+} and Cd^{2+} ions from aqueous solutions and the effectiveness of the modified maize cobs in removing the metal ions from solution was found to be Zn >Ni> Cd. A study of the biosorption of cadmium by pectin-rich fruit materials by Schiewer and Patil (2008) revealed that citrus peels were the most suitable biosorbent. This was due to higher stability coupled with good uptake than apple residues and grape skins. Kinetic studies showed that equilibrium was reached within 30–90 min, depending on particle size. The metal uptake rate increased as particle size decreased, indicating mass transfer limitations. There was little impact of particle size on capacity, indicating that biosorption by fruit wastes is not only a surface phenomenon. Schiewer and Balaria (2009) investigated the uptake of Pb^{2+} by processed orange peels, a pectin-rich byproduct of the fruit juice industry. Low pH increased ionic strength, or competing co-ions reduced Pb^{2+} binding at low sorbent dosages, but at high sorbent dosages removal remained above 90%. The Pb^{2+} uptake at 300 ppm was 2 $mmol.g^{-1}$ (40% of the dry weight or 400 $mg.g^{-1}$). Citrus peel biosorbents show high uptake, good kinetics and high stability, properties which hold high promise for industrial applications.

Apart from the use of agricultural biomass for heavy metal removal, studies have shown its potential application to organic pollutants. Imagawa *et al*. (2000) investigated the feasibility of using carbonized rice husk agricultural by-product, for the removal of chlorinated hydrocarbons from air and water by adsorption. It was shown that tetrachloroethylene, trichloroethylene and 1,1,1,-trichloroethane could be adsorbed by the carbonized rice husk. It has also been previously shown that rice husk can be used as an adsorbent for heavy metals (Kumar

and Bandyopadhyay, 2006), thus showing the potential use of agricultural biosorbents for the remediation of co-contaminated sites.

1.7.3.3 Desorption of heavy metal from biosorbents

The ability to recover heavy metals from biosorbents without destroying their sorption capability is also crucial (Lesmana *et al*., 2009). As noted in most studies, heavy metals can be recovered quite easily using various chemicals, although in several cases, the metal binding capability deteriorates after regeneration. Attempts were made by Igwe *et al*. (2005) to regenerate the adsorbent as well as re-solubulize the metal ions from the spent adsorption. Some amount of metal ions were adsorbed at a neutral pH, with an increase in desorption from 20 to 50% when the concentration of NaOH was increased from 10^{-3} to 10^{-2} M.

1.7.4 Use of Zero-Valent iron for remediation

Zero-valent iron metal (ZVI) is one of the most plentiful metals on the globe (Deng *et al*., 2000). The use of ZVI as a reactive medium for water management is one of the most promising methods because iron metal is not expensive, is effortless to get hold of, and has excellent capability and capacity of degrading contaminants (Hardy and Gillham, 1996). ZVI has become a substitute to pump and treat and air-sparging technologies (Gibb *et al.,* 2004). Reactive barriers enclosing iron metals have been previously developed for *in situ* treatment technology (Puls *et al*., 1999). In addition, iron waste particles from industrial filings can be used as a ZVI (Lee *et al*., 2003). As ZVI is a strong reducer; it has been used to remove several contaminants as listed in Table 1.1. ZVI has been used to speedily dechlorinate an extensive array of chlorinated organic compounds following the mechanism described below. The favoured electron acceptor is normally dissolved oxygen under aerobic conditions. Chlorinated hydrocarbons that have equivalent oxidizing potentials to oxygen can contend with dissolved oxygen as an electron acceptor. Aerobic groundwater enters the iron fillings wall, and causes the oxidation of metallic iron (Fe^0) to ferrous iron (Fe^{2+}) with the discharge of two electrons. In addition, trichloroethene was also dechlorinated by iron, although more gradually than carbon tetrachloride. Increasing the clean surface area of iron greatly increased the rate at which carbon solvents respond as electron acceptors, resulting in dechlorination and release of a chloride ion (Junyapoon, 2005). The reaction takes place in a number of steps resulting in reducing halogenated organic compounds *via* intermediates, to non-hazardous compounds. Intermediate compounds like vinyl chloride,

Table 1.1: List of contaminants amenable to zero-valent iron treatment (Junyapoon, 2005).

Organic compounds		**Inorganic compounds**	
Methanes	Tetrachloromethane	**Trace metals**	Arsenic VC
	Trichloromethane		Cadmium
	Dichloromethane		Chromium
			Cobalt
Ethanes	Hexachloroethane		Copper
	1,1,1-trichloroethane		Lead
	1,1,2-trichloroethane		Manganese
	1,1-dichloroethane		Nickel
	1,2-dibromoethane		Selenium
			Uranium
Ethenes	Tetrachloroethene		Zinc
	Trichloroethene		
	cis-1,2-dichloroethene	**Anionic contaminants**	Nitrate
	trans-1,2-dichloroethene		Phosphate
	1,1-dichloroethene		Sulphate
	Vinyl chloride		
Propanes	1,2,3-trichloropropane		
	1,2-dichloropropane		
	Benzene		
	Toluene		
	Ethylbenzene		
Others	Hexachlorobutadiene		
	Freon 113		
	n-nitrosodimethylamine		

which has a higher toxicity than the parent, are not produced in elevated concentrations. The ultimate products of the reaction are simple hydrocarbons like ethylene, ethane and acetylene. High temperature is not a requirement for the reaction to continue but makes it thermodynamically feasible. The lower the degree of chlorination, the slower the rate of dechlorination reaction (Janda *et al*., 2004).

Various heavy metal interactions such as reduction, co-precipitation and surface complexation has led to ZVI technology becoming an effective means for the removal of heavy metals with different chemical properties (Shokes and Moller, 1999). Recent studies suggest that ZVI has a metal removal much higher than conventional sorbents such as activated carbon, zeolites and polymeric ion exchange resins (Li and Zhang, 2007). Mechanisms for metal removal by ZVI are seemingly complex and not well understood (Li and Zhang, 2007). However, the degradation mechanisms are founded on transformation from hazardous to non- hazardous forms or adsorption on the iron surface depending on the type of heavy metals (Junyapoon, 2005). Chromate undergoes a reductive reaction in ZVI systems where chromium serves as the oxidizing agent. The removal of chromium by zero-valent iron is based on transformation from toxic to non-toxic forms. Trivalent chromium [Cr(III)], which is less toxic and less water soluble and connected with solids can be the product of transformation with ZVI from hexavalent chromium (Cr(VI)), which is a strong oxidant, a potential carcinogen and more mobile in soils and aquifers (Junyapoon**,** 2005). The elimination of the reduced chromium species [Cr(III)] occurs through precipitation of the sparingly soluble $Cr(OH)_3$ or precipitation of mixed iron(III)-chromium(III) (oxy)hydroxide solids (Dries *et al*., 2005). Removal of metals can occur without reduction to metallic form, for example, arsenic can be used using ZVI, a method involving surface complexation.

1.7.5 Cyclodextrin as a remediation option

Cyclodextrin (CD), a cyclic oligosaccharide formed from the enzymatic degradation of starch by bacteria, has the potential to remediate contaminated water (Brusseau *et al*., 1997). Cyclodextrins solubilize low-polarity organic compounds by inclusion complexation, wherein the solutes “partition” into the cyclodextrin cavity (Del Valle, 2004) (Figure 1.2). The formation of CD-contaminant complexes significantly increases the apparent solubility of many low-soluble organic contaminants as well as enhance their desorption and transport, which is the

basis for the use of CD in groundwater remediation (U.S. Department of Defense, 2004). Being a sugar, CD is known to be relatively non-toxic to humans, plants, and soil microbes. Therefore, there are minimal health-related concerns linked to the addition of cyclodextrin into the subsurface, which is an intrinsic benefit for its use as a remediation option (Tick *et al*., 2003). Chemical properties of interest such as aqueous solubility and metal complexation potential can be altered by substituting functional groups to the outside of the cyclodextrin. Laboratory and field studies have demonstrated that hydroxypropyl-β-cyclodextrin has high aqueous solubility and strongly enhances solubility of organic compounds (Boving and McCray, 2000).

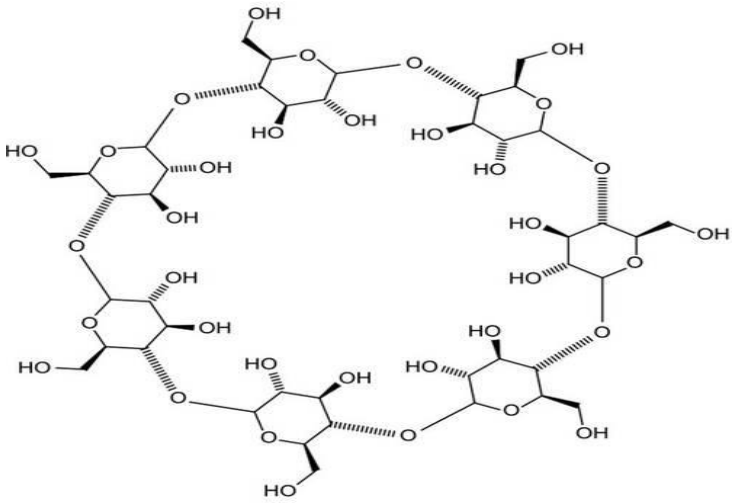


Figure 1.2: Chemical structure of β-Cyclodextrin (http://www.aboutchemistry.com).

Specific CDs, such as modified CD, carboxymethyl-*α*-cyclodextrin (CMCD), can bind cationic metals by complexation interactions between metal ions and the acidic functional groups (e.g., carboxyl) associated with the external surfaces of the CD. Carboxymethyl-β-cyclodextrin enhances the solubility of common metal contaminants making it a potential agent for *in-situ* remediation of metals. The solubility enhancement is greater for heavy metals than for group 2 elements; however, the presence of soluble minerals composed of non-target elements would affect the extent of the solubility of the target metal (Skold *et al*., 2009). Chatain *et al*. (2004) found that CD could be used as an innovative means to facilitate the removal of both organic and inorganic contaminants by dissolving them, thus facilitating easy removal. While cationic metals can be chelated by the oxygen atoms on the rim of the CMCD, hydrophobic organic pollutants can also be included within the nonpolar cavity of CD. If the complexing agent, or mixture of complexing agents is chosen appropriately, both organic and inorganic contaminants can be

removed simultaneously (Mulligan *et al*., 2001). Cathum *et al*. (2008) investigated the effectiveness of a CD-based solid material for the removal of mixed dissolved contaminants in water. The removal efficiency was found to be 70% for total heavy metals (cadmium, lead, chromium, iron, nickel, cobalt, and mercury) to 98% for polychlorinated biphenyls. This identifies CD as a potential complexing agent for cleaning up mixed waste.

1.8 Scope of the present study

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 this toxic organic chemical (Hage and Hartmans, 1999). In addition, heavy metal contamination from both natural (erosion, fires, leaching, volcanic activity, and microbial transformation) and anthropogenic (industrial waste, dumping of sewage, burning of fossil fuels, etc.) sources results in the accumulation of metals in environmental niches (Kuo and Genthner, 1996). Numerous studies extensively address the problem of 1,2-DCA and heavy metal pollutants separately (Gikas *et al*., 2009; Marzorati *et al*., 2010; Rajapaksha *et al*., 2004; Van Der Zaan *et al*., 2009) but not enough work focuses on co-contamination. Even though 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 focused on the subject of co-contamination, and the impact of heavy metals on organic pollutant biodegradation. To the best of our knowledge; no study has focused on the effects of heavy metals on the biodegradation of 1,2-DCA in co-contaminated water. This study therefore investigated the effects of four heavy metals (arsenic, cadmium, mercury and lead) on the biodegradation of 1,2-DCA in co-contaminated water. Various bioremediation options, such as biostimulation, bioaugmentation and biosorption, were employed in this study in order to determine the best way of improving bioremediation of such co-contaminated sites. Microbial diversity and populations were also profiled at the different stages of the degradation process in order to determine the combined effect of chlorinated organics and heavy metal contamination on the indigenous microorganisms.

1.8.1 Hypotheses tested

It was hypothesized that heavy metals will have an inhibitory effect on the biodegradation of 1,2-DCA. It was further hypothesized that biostimulation and/or

bioaugmentation, as well as the addition of biosorbents, will reduce heavy metal toxicity and consequently improve 1,2-DCA biodegradation.

1.8.2 Objectives

The following objectives were established to test the above hypotheses:

1.8.2.1 To determine the kinetics of inhibition of 1,2 DCA degradation in contaminated water in the presence of heavy metals.

1.8.2.2 To evaluate the effects of biostimulation, bioaugmentation and biosorbents on heavy metal toxicity and the 1,2-DCA degradation profile in co-contaminated water.

1.8.2.3 To assess the combined effects of 1,2-DCA and heavy metals on microbial populations and diversity in co-contaminated water.

1.8.3 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 heavy metal concentrations on the biodegradation of 1,2-DCA. Evaluation of the toxic effects of heavy metals were based on the following: (i) degradation rate constants; (ii) estimated MICs; and (iii) concentrations that caused biodegradation half-life doublings (HLDs).

- Chapter Three

This Chapter investigated the effects of bioremediation methods such as biostimulation and bioaugmentation on the biodegradation of 1,2-DCA in water co-contaminated with arsenic, cadmium, mercury and lead. 1,2-DCA-degrading bacterial populations and diversity in the co-contaminated water were profiled using both culture dependent (standard plate count) and culture independent (PCR-DGGE) methods.

- Chapter Four

This Chapter specifically evaluated the efficiency of different agricultural biosorbents in reducing the bioavailability of heavy metals and the consequent impact on 1,2-DCA degradation in water co-contaminated with 1,2-DCA and each of the respective heavy metals (arsenic, cadmium, mercury and lead).

- Chapter Five

This Chapter provides a general discussion and conclusion and places the entire research in perspective. The significant findings of each Chapter, limitations, as well as potential scope for future development of this study in the area of bioremediation of water co-contaminated with 1,2-DCA and heavy metals are highlighted.

CHAPTER TWO

KINETICS OF HEAVY METAL INHIBITION OF 1,2-DCA DEGRADATION IN CO-CONTAMINATED WATER

2.1 Introduction

The industrial sector is one of the most vibrant sectors of the financial system and plays a vital role in economic development, as well as in the mitigation of poverty. However, environmental considerations are not effectively incorporated into the design of industrial processes. This is reflected by the number of environmental problems resulting from the release of large quantities of toxic organic pollutants. Chlorinated organic compounds, in the form of solvents, pesticides and medicine intermediates are produced in huge amounts that instigate important environmental predicaments (Shao-ping *et al*., 2005). Chlorine compounds represent a huge percentage of environmental pollutants that negatively affect the health of humans and wildlife; it is for this reason that chlorine chemistry is at the forefront of public health concern (Hileman, 1993). The production of 1,2-DCA, in particular, is larger than that of any other industrial halogenated chemical (Laturnus, 2003). Inappropriate disposal practices or accidental spills of these compounds have made them common contaminants of soil and groundwater (McCarty, 1997), causing serious environmental and human health problems as a result of their persistence and toxicity (Squillace *et al.,* 1999). Under abiotic conditions, dissolved 1,2-DCA is transformed slowly and toxic products such as vinyl chloride may be formed (Jeffers *et al*., 1989). However, microbial degradation of 1,2-DCA under various conditions have been reported, for example, *X. autotrophicus* and *A. aquaticus* have been shown to degrade 1,2-DCA to nontoxic end products (Janssen *et al*., 1989; Van Den Wijngaard *et al*., 1992).

In addition to chlorinated organic pollutants, heavy metal release into the environment has been increasing continuously as a result of industrial activities and technological development, posing a significant threat to the environment and public health because of their toxicity, accumulation in the food chain and persistence in nature (Parameswari *et al*., 2009). Addition of trace amounts of heavy metals to the environment of microbial cells often stimulate microbial growth (Gikas, 2007; 2008), because they are required for life's physiological processes (Soboley and Begonia, 2008). However, higher concentrations result in severe

reduction of microbial activity (Gikas *et al*., 2009), because excessive accumulation of toxic metals cause enzyme inactivation, damage cells by acting as antimetabolites or form precipitates or chelates with essential metabolites (Soboley and Begonia, 2008). The microbial processes that heavy metals inhibit, such as methane metabolism, growth, nitrogen and sulphur conversions and reductive processes, are well documented (Baath, 1989). However, little is known about the effects of metal toxicity on the bioremediation of organic compound when both types of contaminants are present (Sandrin and Maier, 2003). This makes bioremediation, which is generally a promising technology, much more challenging in sites co-contaminated with organic and metal pollutants, as the two components are treated differently.

A wide range of metal concentrations has been reported to inhibit biodegradation of organic compounds. For instance, five orders of concentration separate lowest reported concentrations of zinc that inhibit biodegradation (Sandrin and Maier, 2003). The large range of reported inhibitory concentrations is due to differences in experimental protocols that affected solution-phase metal concentrations. For example, many laboratory media contain metal-binding (e.g., yeast extract) and metal precipitating (e.g., phosphate or sulfate salts) components that can reduce solution-phase metal concentrations (Hughes and Poole, 1991; Poulson *et al*., 1997). Medium pH also dramatically impacts solution-phase metal concentrations. The variability of each of these factors hampers meaningful comparisons between studies and underscores the need for future studies to report solution-phase metal concentrations.

The ability to estimate biodegradation rates is important for predicting the environmental fate of pollutants and for designing remediation strategies (Wammer and Peters, 2005). However, environmental based rates are difficult to estimate by laboratory studies (Beurskens *et al*., 1993). Biodegradation rate in the real environment depends on characteristics of the system, inorganic concentrations and organic nutrients, presence of particulate matter, temperature, oxygen concentration, redox potential, adaptation of the microbial population and on the concentration of the chemical studied (Howard and Banerjee, 1984). Since microorganisms play a major role in controlling the fate and concentration of environmental pollutants, reliable mathematical descriptions of microbial degradation kinetics are required (Suflita *et al*., 1983). Said and Lewis (1991) developed a test to quantitatively measure the inhibitory effects of metals on the aerobic microbial degradation of organic chemicals. This method could possibly be customized to take into consideration any of the abundant environmental factors that could influence the toxic effects of metals on biologically mediated reactions.

Approximately 40% of the hazardous waste sites currently on the National Priority List of the U.S. Environmental Protection Agency are co-contaminated with organic and metal pollutants (Sandrin and Maier, 2003). Despite the high number of co-contaminated sites, limited studies have been carried out to address this problem that continues to pose a serious environmental health risk. The objective of this study, therefore was to determine the kinetics of inhibition of 1,2-DCA degradation in co-contaminated water in the presence of heavy metals. Four heavy metals: arsenic, cadmium, mercury and lead were used and the effects of various concentrations of these metals on 1,2-DCA degradation were evaluated based on the following: (a) the degradation rate constant, (b) estimating the minimal inhibitory concentrations (MICs), (c) determining the metal concentration that caused an effect on biodegradation parameters (i.e concentrations increasing half life of 1,2-DCA); and (d) concentrations causing biodegradation half-life doublings (HLDs).

2.2 Materials and methods

2.2.1 Sample collection and microcosm set-up

Wastewater samples used in this study were collected from the secondary clarifier of the Northern wastewater (NWW) and New Germany wastewater (NGWW) treatment plants and stored at 4 °C, prior to use. Microcosms were constructed with 150 ml of the wastewater samples in 250 ml serum bottles as previously described (Olaniran *et al*., 2006a). The microcosms were fortified with 1.2-DCA (Merck) to a final concentration of 2.5 mM. Each of the heavy metals (arsenic, cadmium, lead and mercury) was separately added into the microcosms at concentrations ranging from 0.01 to 0.3 mM, using the following salts: arsenic trioxide (Fluka), cadmium nitrate (Sigma), mercuric nitrate (Sigma) and lead nitrate (Saarchem). Microcosms fortified with 1,2-DCA and no heavy metal were used as a positive control while autoclaved wastewater fortified with 1,2 DCA and no heavy metal served as a negative (biologically inactive) control. The bottles were incubated at 25 °C during the course of the experiment.

2.2.2 Measurement of chloride release

Chloride release was determined by the modified spectrophotometric method of Bergmann and Sanik (1957). To 1 ml of the supernatant from the co-contaminated wastewater microcosm, obtained after the removal of cells by centrifugation for 5 min at 12000 rpm, 0.2 ml of 0.25 M $Fe(NH_4)(SO_4)_2.12H_2O$ in 9 M HNO_3, and 0.4 ml of saturated $Hg(SCN)_2$ in 95% ethanol was added. This was then incubated for 10 min at room temperature to allow for colour development, and the absorbance of the $Fe(SCN)^{2+}$ product at 460 nm was measured with a Biochrom, Libra S12 UV-Visible Spectrophotometer. The amount of chloride released was determined by comparing values to a standard curve, derived by plotting absorbance readings against varying concentrations of NaCl (0-100 $\mu g.ml^{-1}$). Chloride released was used as an index of 1,2-DCA degradation. This was done by correlating the amount of chloride relased from 1,2-DCA as an indication of the concentration of 1,2-DCA that was degraded.

2.2.3 Quantitative analysis: determination of k_1, MIC, $t_{½}$ and HLDs

Biodegradation rate coefficient, k_1, was calculated from plots of the natural logarithm of substrate concentration (1,2-DCA) versus time, according to the integrated first-order rate equation: $\ln (C_t/C_0) = -kt$, where C_0 and C_t are the concentrations of 1,2-DCA at time 0 and *t*,

respectively. Minimum inhibitory concentrations (MIC) for the heavy metals were determined from abscissa intercepts of plots of percentage reduction in biodegradation rate coefficients (k_1) versus logarithmic molar concentrations of metals (Said and Lewis, 1991). Percentage reduction in k_1 = [(control k_1- treatment k_1)/control k_1] ×100. Half-life ($t_{½}$) was calculated as 0.693/k_1 (Said and Lewis, 1991). The metal concentration at which a doubling in 1,2-DCA degradation half-life ($t_{½}$) occurred was 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 $t_{½}$. These values were referred to as half-life doubling concentrations (HLDs).

2.2.4 pH measurements and chemical analysis of the wastewater samples

The pH of the wastewater samples was determined using a Beckman 50 pH meter. In addition, wastewater 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.

2.2.5 Measurement of heavy metal concentration

The initial concentration of heavy metals in the two wastewater samples was determined using an inductively coupled plasma-optical emission spectrometer (ICP-OES) 5300 DV. The samples were filtered through 0.45 μm membrane filters (Whatman) and the heavy metal (arsenic, cadmium, mercury and lead) concentration in each filtrate (15 ml) was measured in centrifuge tubes. Concentration of heavy metals was estimated by extrapolating from the standard curve of the absorbance versus known concentrations of heavy metals (Abdel-Ghani *et al*., 2007).

2.3 Results

2.3.1 Wastewater characterization and pH measurement

The pH of the two wastewater samples was slightly acidic, with a pH of 6.7 and 6.0 obtained for NGWW and NWW, respectively. The physico-chemical properties of both wastewater types are listed in Table 2.1. Total Kjeldahl nitrogen concentration in both wastewater types were found to be <3.00 mg $N.l^{-1}$. Wastewater obtained from NGWW showed higher levels of calcium, sodium, potassium and iron, which are 21.53, 13.31, 12.87 and 100% respectively higher than those found in NWW. Conversely Magnesium was 19.02% higher in NWW than NGWW. Also the nitrite, nitrate, sulphate, and total organic carbon levels in NGWW were 11.52, 20.79, 33.88, and 37.85%, respectively higher than in NWW. The phosphate level was 10-fold higher in NGWW than in NWW.

Table 2.1: Physico-chemical properties of the wastewater samples used in this study.

Determinant	NGWW	NWW
Calcium (mg $Ca.l^{-1}$)	17.5	14.4
Magnesium (mg $Mg.l^{-1}$)	4.47	5.32
Sodium (mg $Na.l^{-1}$)	57.9	51.1
Potassium (mg $K.l^{-1}$)	11.4	10.1
Iron (mg $Fe.l^{-1}$)	0.06	0.03
Nitrite (soluble) (mg $N.l^{-1}$)	79.4	71.2
Nitrate (soluble) (mg $N.l^{-1}$)	12.2	10.1
Sulphate (soluble) (mg $SO_4.l^{-1}$)	41.1	30.7
Soluble organic carbon (mg $C.l^{-1}$)	12.2	8.85
Phosphate (µg $P.l^{-1}$)	5010	<500
Total kjeldahl nitrogen (mg $N.l^{-1}$)	<3.00	<3.00

2.3.2 Initial heavy metal concentration in wastewater samples

The initial concentrations of heavy metals in both wastewater types used in this study is shown in Table 2.2. Cadmium was not detected in both wastewater types. However, it should be taken into consideration that the cadmium dection limit on the ICP-OES 5300 DV is 0.23 mg.l^{-1}. Mercury has the highest initial concentration, followed by lead, and then arsenic.

Overall, low amounts of intial heavy metals were present in the wastewater samples at the time of collection.

Table 2.2: Initial concentrations of heavy metals detected in the wastewater used in this study.

Heavy metal ($mg.l^{-1}$)	NGWW ($mg.l^{-1}$)	NWW ($mg.l^{-1}$)
As^{3+}	0.005	0.006
Cd^{2+}	ND*	ND*
Hg^{2+}	0.018	0.026
Pb^{2+}	0.006	0.007

***ND=Not detected, detection limit of 0.23 $mg.l^{-1}$ for cadmium should be taken into consideration

2.3.3 Impact of different concentrations of heavy metals on 1,2-DCA biodegradation in wastewater

The biodegradation profiles of 1,2-DCA (in the presence and absence of heavy metals) for wastewater obtained from NGWW and NWW were determined and represented in Figures 2.1 and 2.2 respectively. In the absence of heavy metals, 1,2-DCA was effectively degraded with up to 65.37% reduction in 1,2-DCA (after 25 days) and 64.97% (after 30 days) observed in microcosms constructed with NGWW and NWW, respectively, above the values observed for the autoclaved wastewater control. However, the inhibitory effects of the heavy metals on 1,2-DCA biodegradation was revealed by a decrease in 1,2-DCA biodegradation observed with an increasing concentration of heavy metals. In NGWW (Figure 2.1 A-D), heavy metal concentration of 0.3 mM was found to be the most inhibitory to 1,2-DCA biodegradation. After 25 days, a 60.30% decrease in 1,2-DCA degradation was observed in the presence of Hg^{2+}, 56. 14% in the presence of As^{3+}, 55.99% in the presence of Cd^{2+} and 52.67% in the presence of Pb^{2+}. However, in the presence of 0.01 mM concentration of heavy metals, 1,2-DCA degradation was inhibited to a lesser extent. After 25 days, only a 6.64%, 3.99% and 2.53% decrease in 1,2-DCA degradation occurred in the presence of 0.01 mM Hg^{2+}, As^{3+} and Cd^{2+}, respectively, compared to the positive control, and no decrease recorded when 0.01 mM of Pb^{2+} was added. The same trend was observed for NWW with the exception that 1,2-DCA degradation occurred at a slower rate. There was a 61.93% reduction in 1,2-DCA degradation in the presence of 0.3 mM Hg^{2+} compared to a 7.61% decrease in degradation with 0.01 mM Hg^{2+} after 30 days (Figure 2.2 C).

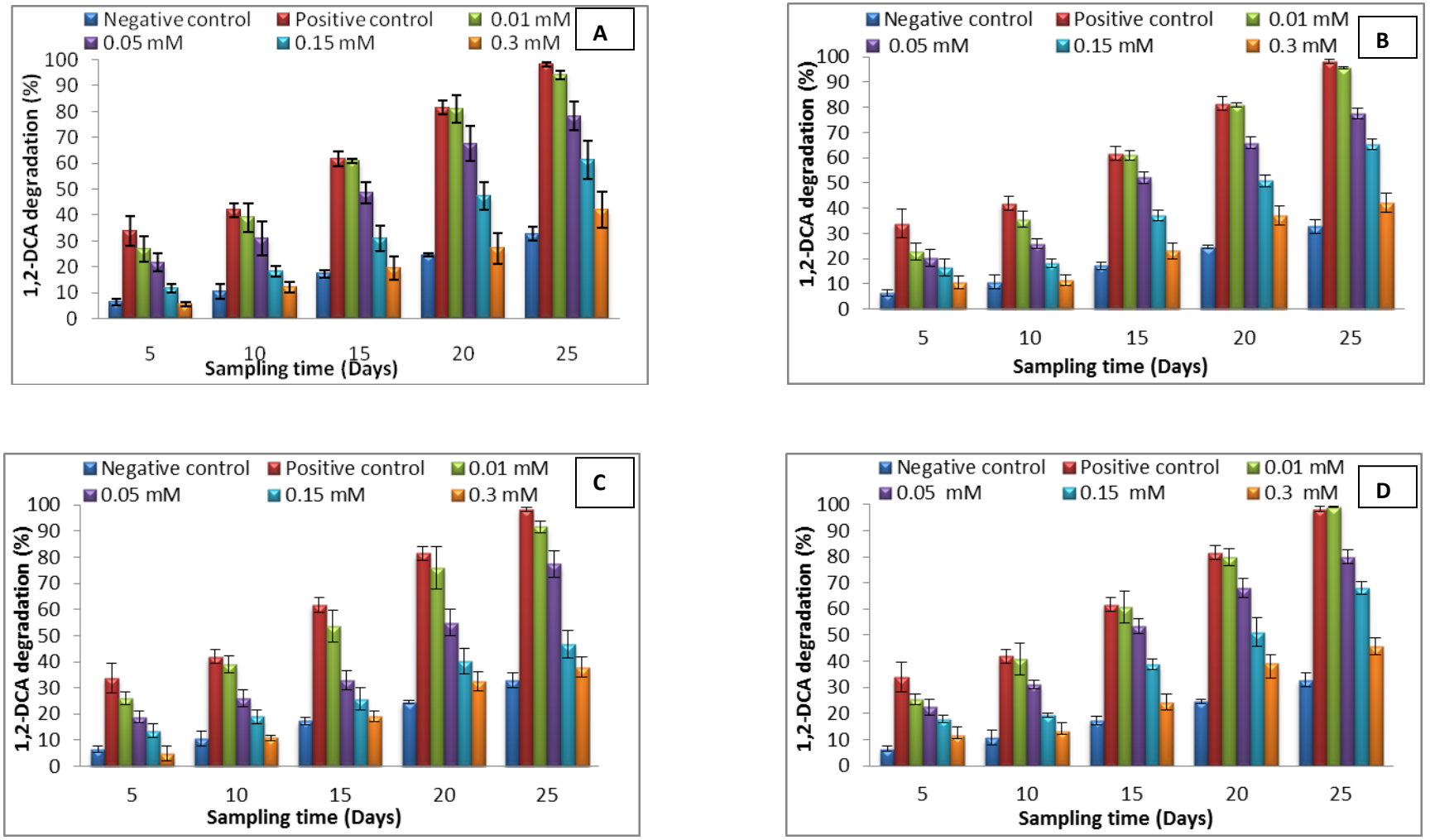


Figure 2.1: Degradation profiles of 1,2-DCA in the presence of arsenic (A), cadmium (B), mercury (C) and lead (D) in wastewater microcosms constructed with the NGWW. Values indicate the average of eight replicates, while error bars indicate the standard deviation.

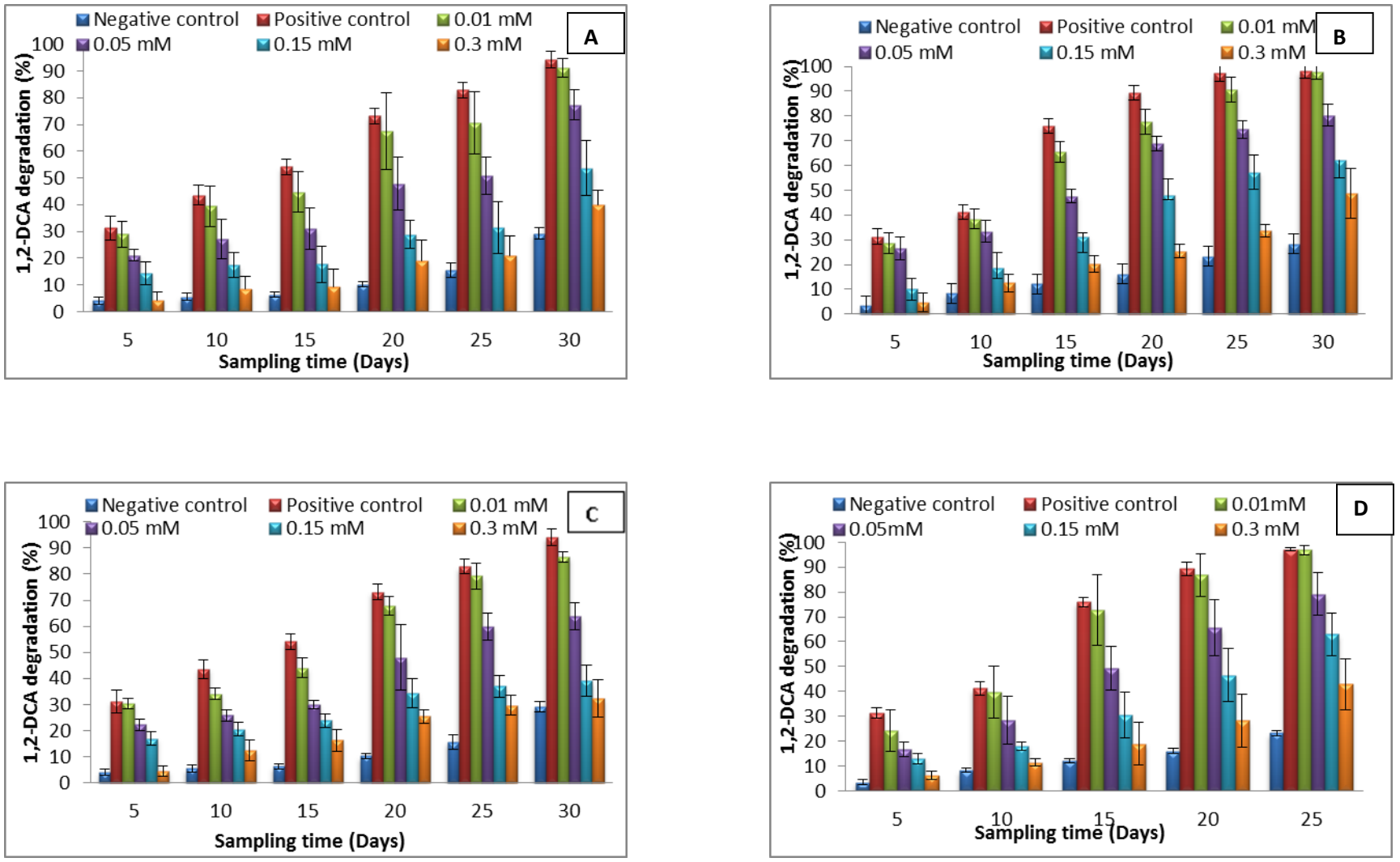


Figure 2.2: Degradation profiles of 1,2-DCA in the presence of arsenic (A), cadmium (B), mercury (C) and lead (D) in wastewater microcosms constructed with the NWW. Values indicate the average of eight replicates, while, error bars indicate the standard deviation.

Pb^{2+} was inhibitory to 1,2-DCA degradation at all concentrations tested (Figure 2.2 D). In summary, the inhibitory effects of metal salts on 1,2-DCA degradation increased as the heavy metal concentration increased and the order of decreasing metal toxicity was; mercury > arsenic > cadmium > lead.

In NGWW after 25 days, up to 45.45% of 1,2-DCA was degraded in the presence of 0.3 mM Pb^{2+} (Figure 2.1 D), which is 7.63% higher than 1,2-DCA degradation observed in the presence of 0.3 mM of Hg^{2+}, where 37.91% 1,2-DCA was degraded (Figure 2.1 C). Microcosms containing 0.3 mM As^{3+} exhibited 42.06% (Figure 2.1 A) 1,2-DCA degradation. This is 3.47% lower than 1,2-DCA degradation observed for microcosms co-contaminated with 0.3 mM Pb^{2+}; however it is 4.15% higher than the degradation in microcosms co-contaminated with Hg^{2+} (Figure 2.1 C). Microcosms containing 0.3 mM Cd^{2+} exhibited 42.22% (Figure 2.1 B) 1,2-DCA degradation, which is 3.31% lower than that for microcosms co-contaminated with 0.3 mm Pb^{2+}. However it is 4.35% higher than the degradation in microcosms co-contaminated with Hg^{2+} (Figure 2.1 C). A similar trend was found for NWW (Figure 2.2 A-D), such that mercury inhibited degradation the most, followed by arsenic, cadmium and lead.

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

The biodegradation rate constants (k_1) of 1,2-DCA in the presence of the heavy metals range between 0.020-0.16 day^{-1} in NGWW, which is higher compared to values obtained for NWW which ranged from 0.0134-0.142 day^{-1} (Table 2.3). For both wastewater types, as the heavy metal concentration increased from 0.01 mM to 0.3 mM, the degradation rate constant decreased. The $t_{1/2}$ and relative $t_{1/2}$ for NWW and NGWW are shown in Tables 2.4 and 2.5, respectively. Mercury increased the $t_{1/2}$ of 1,2-DCA the most, followed by arsenic, cadmium and then lead. An increasing $t_{1/2}$, as the heavy metal concentration increased from 0.01-0.3 mM, was exhibited. In NWW, 0.01 mM Hg^{2+} increased the $t_{1/2}$ by 1.67-fold; whereas in the presence of 0.3 mM Hg^{2+} a 21.60-fold increase was observed. In NGWW, 0.01 mM Hg^{2+} increased the $t_{1/2}$ by 1.63-fold and 0.3 mM Hg^{2+} caused a 34.11-fold increase. Lead did not increase the $t_{1/2}$ of 1,2-DCA as substantially as the other metals. In the presence of 0.01 mM Pb^{2+} in NWW, the $t_{1/2}$ was increased by 1.12-fold and 12.60-fold when 0.3 mM was used. In NGWW, 0.01 mM Pb^{2+} did not decrease the $t_{½}$; whereas in the presence of 0.3 mM Pb^{2+}, a 14.91-fold increase was observed. It is evident that, an increase in heavy metal concentration is accompanied by an increase in the $t_{½}$

Table 2.3: Biodegradation rate constants (day^{-1}) of 1,2-DCA in wastewater microcosms co-contaminated with different concentrations of heavy metals.

Metal cation	Concentration (mM)	NGWW	NWW
None	Autoclaved control	0.015	0.01
None	Unautoclaved control	0.15	0.10 (As^{3+} and Hg^{2+}) 0.16 (Cd^{2+} and Pb^{2+})
As^{3+}			
	0.01	0.11 ± 0.0096	0.071 ± 0.011
	0.05	0.061 ± 0.012	0.041 ± 0.0054
	0.15	0.037 ± 0.006	0.021 ± 0.0060
	0.3	0.022 ± 0.0032	0.014 ± 0.0024
Cd^{2+}			
	0.01	0.12 ± 0.0037	0.12 ± 0.0054
	0.05	0.060 ± 0.0037	0.055 ± 0.0070
	0.15	0.040 ± 0.0021	0.035 ± 0.0068
	0.3	0.022 ± 0.0021	0.020 ± 0.0038
Hg^{2+}			
	0.01	0.093 ± 0.0073	0.066 ± 0.0046
	0.05	0.054 ± 0.0079	0.034 ± 0.0042
	0.15	0.025 ± 0.0047	0.015 ± 0.0024
	0.3	0.020 ± 0.0024	0.013 ± 0.0030
Pb^{2+}			
	0.01	0.16 ± 0.0055	0.14 ± 0.029
	0.05	0.063 ± 0.0024	0.065 ± 0.020
	0.15	0.043 ± 0.0018	0.038 ± 0.0087
	0.3	0.024 ± 0.0027	0.021 ± 0.0076

Values indicate the average of eight replicates ± standard deviation

and relative $t_{½}$. For both wastewater types, lead increased the relative $t_{½}$ the most, and arsenic the least. Using these criteria as a measure of heavy metal effects, 1,2-DCA biodegradation was inhibited more in NWW than in NGWW microcosms. A plot of the percentage decreases in k_1 as a function of the logarithms of metal concentrations resulted in a linear relationship for the range of metal concentrations used. Percentage decreases in k_1 values plotted as a function of the logarithms of metal concentrations were subjected to linear regression analysis to estimate MICs and HLDs. The MICs and HLDs of the heavy metals for both wastewater types are listed in Table 2.6. The MIC of the heavy metals was found to vary depending on the metals and the wastewater type. In NWW, lead had the highest MIC of 7.06 µM, wich was 3.76-fold higher than that of cadmium, 3.41-fold higher than that of arsenic and, 8.02-fold higher than that of mercury which had the lowest MIC. In NGWW, the MIC values were higher compared to NWW but the same pattern followed, with lead having the highest MIC of 10.95 µM, which is 3.25-fold higher than that of cadmium, 4.12-fold higher than that of arsenic and 9.95-fold higher than that of mercury, which again exhibited the lowest MIC. HLD determinations further confirmed that the various metals caused different levels of inhibitory effects on 1,2-DCA biodegradation rates depending on the metal and wastewater source. Mercury was the heavy metal required in the lowest concentration to cause HLD of $k_{1.}$ With only 15.44 µM required in NWW, a value which is 1.61-,1.84- and 3.10- fold lower than arsenic, cadmium and lead, respectively. These values were lower when compared to NGWW but the same pattern followed: where mercury was required in the lowest concentration to cause HLD of k_1, followed by arsenic, mercury and lead.

Table 2.4 : Effect of heavy metals on the half-life of 1,2-DCA in the NWW sample.

Metal cation	Cation concentration (mM)	$t_{1/2}$ (days) (mean ± SD)		[a] Relative $t_{½}$
		Positive control (No heavy metal added)	Wastewater (Heavy metal added)	
As^{3+}		7.51 ± 0 .54		
	0.01		11.66 ± 2.14	1.55
	0.05		22.84 ± 4.72	3.04
	0.15		101.76 ± 33.53	13.55
	0.3		130.08 ± 26.08	17.33
Cd^{2+}		4.65 ± 0.55		
	0.01		6.42 ± 0.32	1.38
	0.05		16.05 ± 2.54	3.46
	0.15		31.83 ± 9.45	6.85
	0.3		75.37 ± 25.45	14.19
Hg^{2+}		7.51 ± 0 .54		
	0.01		12.56 ± 1.02	1.67
	0.05		30.02 ± 6.14	4.00
	0.15		149.67 ± 43.56	19.94
	0.3		162.25 ± 51.33	21.62
Pb^{2+}		4.64 ± 0.55		
	0.01		5.578 ± 1.46	1.20
	0.05		14.57 ± 5.49	3.14
	0.15		29.20 ± 12.91	6.28
	0.3		58.45 ± 11.17	9.43

[a] Value for metal-treated wastewater divided by value for positive control. Values indicate the average of eight replicates ± standard deviation

Table 2.5: Effect of heavy metals on the half-life of 1,2-DCA in the NGWW sample.

Metal cation	Cation concentration (mM)	$t_{1/2}$ (days) (mean ± SD)		[a] Relative $t_{½}$
		Positive control (No heavy metal added)	Wastewater (Heavy metal added)	
As^{3+}		5.52 ± 0.6029		
	0.01		7.54 ± 0.85	1.34
	0.05		15.9 ± 3.82	2.88
	0.15		35.31 ± 13.37	6.39
	0.3		127.44 ± 32.62	23.07
Cd^{2+}		5.52 ± 0.6029		
	0.01		6.85 ± 0.24	1.24
	0.05		15.71 ± 1.41	2.84
	0.15		27.62 ± 2.38	4.99
	0.3		112.21 ± 27.48	20.31
Hg^{2+}		5.52 ± 0.6029		
	0.01		8.99 ± 0.87	1.63
	0.05		18.8 ± 4.53	3.40
	0.15		94.1 ± 32.12	17.04
	0.3		188.28 ± 55.85	34.09
Pb^{2+}		5.52 ± 0.6029		
	0.01		4.93 ± 0.19	0.890
	0.05		14.47 ± 0.73	2.620
	0.15		25.17 ± 1.51	4.560
	0.3		82.29 ± 20.83	14.90

[a] Value for metal-treated wastewater divided by value for untreated wastewater. Values indicate the average of eight replicates ± standard deviation

Table 2.6: Concentrations of metal cations inhibiting 1,2-DCA biodegradation in NWW and NGWW samples.

Cation	[a]MIC (µM) (Mean ± SD)		[b]HLD (µM) (Mean ± SD)	
	NWW	NGWW	NWW	NGWW
As^{3+}	2.07 ± 1.38	2.66 ± 1.19	24.38 ± 8.95	30.08 ± 8.98
Cd^{2+}	1.88 ± 0.51	3.37 ± 0.37	22.88 ± 8.99	34.49 ± 1.51
Hg^{2+}	0.88 ± 0.49	1.10 ± 0.46	15.44 ± 3.13	18.71 ± 4.82
Pb^{2+}	7.06 ± 0.517	10.95 ± 1.02	40.4 ± 11.54	57.21 ± 2.63

[a]MICs of the heavy metals that caused no reduction in k_1 values. Numerically, these are abscissa intercepts; [b] HLD, concentration of heavy metal that caused half-life doubling. Values indicate the average of eight replicates ± standard deviation

2.4 Discussion

This study was undertaken to investigate the biodegradation of 1,2-DCA by indigenous microorganisms in the presence of heavy metals. Although 1,2-DCA was degraded by indigenous microorganisms present in the wastewater samples, the addition of heavy metals (arsenic, cadmium, lead and mercury) retarded the biodegradation. This is because that metals are known to inhibit organic biodegradation through their effects on both the physiology and ecology of organic-degrading microorganisms (Sandrin and Maier, 2003); these include extended acclimation periods, reduced biodegradation rates and failure of target compound biodegradation (Kuo and Genthner, 1996). Additionally, organic compounds and inorganic elements may react with each other resulting in organic-inorganic complex formation and precipitation reactions (Ramakrishnan *et al*., 2011). The metal–organic complexation can alter the bioavailability of organic pollutants and may also modulate the toxicity of heavy metals rendering them bio-unavailable (White and Knowles**,** 2000).

The presence and bioavailability of nutrients that are essential for growth of microorganisms have been shown to increase microbial activities and subsequently biodehalogenation (Mohn and Tiedje, 1992). Hence, the higher concentrations of nutrients important for microbial activities in NGWW than in NWW could possibly account for the higher degradation rate of 1,2-DCA observed in microcosms constructed with NGWW. Previous reports by Huges and Poole (1991) and Poulson *et al*. (1997) have indicated that phosphates reduce the solution phase metal concentrations by acting as a metal precipitating agent. Consequently, the higher phosphate concentration in NGWW may have resulted in metal binding, causing a decrease in the concentration of bioavailable metals and an increase in the 1,2-DCA biodegradation rate. Phosphate readily sequesters metals and diminishes their bioavailability *via* the configuration of insoluble metal phosphate species. In fact, phosphate is so efficient at metal sequestration that it has been used as a metal-complexing agent in some studies to reduce free ionic metal concentrations (White and Knowles, 2000).

A wide range of metal concentrations that are inhibitory to biodegradation has been reported in the literature. Sandrin and Maier (2003) present an extensive range of reported metal concentrations that cause inhibition of aerobic and anaerobic biodegradation of organic contaminants. The variability in the inhibitory heavy metal concentrations could be due to heavy metal resistant bacterial inoculum used. Some studies were performed using a single strain of

bacteria, while others used an indigenous community of microbes for bioremediation of co-contaminated sites; therefore the way in which heavy metals impacted biodegradation OF these organisms would be different and lead to different concentration being inhibitory to biodegradation. Ecological conditions affect metal toxicity by varying metal speciation and bioavailability. Some organisms were cultured in soil or sediment microcosms, while others were cultured in minimal salts media resulting in varying inhibitory concentrations of heavy metals.

In this study, heavy metal concentrations ranging from 0.01- 0.3 mM were examined for their inhibitory effects on 1,2-DCA biodegradation. The four different heavy metals in both wastewater types were found to inhibit 1,2-DCA biodegradation in a dose dependent manner, with 0.01 mM exhibiting the least effect and 0.3 mM displaying the greatest effect. Mercury was found to cause the highest inhibition followed, in decreasing order, by arsenic, cadmium and lead. 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, this is due to the increased solubility of Hg^{2+} when bound to thiol groups (Nies, 1999). In a study by Said and Lewis (1991), five heavy metals tested (copper, cadmium, mercury, zinc, and chromium) in samples of aufwuchs, mercury was found to be the most inhibitory to the biodegradation of 2,4-dichlorophenoxyacetic acid methyl ester, with an MIC of 0.01 mM total mercury. This value is higher than the MIC of mercury inhibiting 1.2-DCA degradation in this study. Hong *et al*. (2007) also reported that out of four heavy metals investigated (arsenic, cadmium, copper and mercury), mercury was the most toxic, with a concentration of 4.9 mM strongly inhibiting the growth of *Sphingomonas wittichi* RW1 and the degradation of dibenzofuran. This value is also higher than the MIC of mercury inhibiting 1.2-DCA degradation in this study. The disparity in MICs between findings could be due to the different mediums the experiments were performed in and due to the different microoraganisms used.

The effects of arsenic on a pentachlorophenol (PCP)-degrading *Flavobacterium* sp. were examined in a liquid growth medium by Wall and Stratton, (1994). Arsernic was found to cause an increase in the time required to completely degrade PCP. Van Zwieten *et al*. (2003) investigated the impact of arsenic co-contamination on the natural breakdown of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane to 1,1-dichloro-2,2-bis(4chlorophenyl)ethane (DDD) and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene in soil. Arsenic at a concentration of 2000 $mg.kg^{-1}$ was shown to effect a 50% reduction in the concentration of DDD compared to arsenic at 5 $mg.kg^{-1}$. Degraffenreid and Shreve (1998) reported that Cd, at a concentration of 5 ppm, decreased TCE

degradation activity of *Burkholderia picketti PK01*, causing a 69% decrease in the degradation rate constant. Atagana (2009) investigated the degradation of polycyclic aromatic hydrocarbons (PAH) in sterlized soil inoculated with nonligninolytic and ligninolytic fungi in the presence of cadmum, at 300 and 500 $mg.kg^{-1}$ of soil. The degradation of the PAHs by the fungi was impaired and the severity of the impairments increased with the increasing concentrations of Cd.

The results obtained for the biodegradation of lead complexed 2,6-pyridine dicarboxylic acid (PDA) at metal chelator ratio of 1:2 in a study by Banerji and Regmi (1998) indicated that the degradation rates at different PDA concentrations did not change significantly from that for uncomplexed PDA. This suggests that the presence of lead did not cause any metabolic retardation. They deduced that it could be possible that the released lead ions were precipitated from the solution that would eliminate its toxic property. Similar results were obtained by Hong *et al*. (2007), where lead did not affect degradation of dibenzofuran by *S. wittichii* RW1, even at a very high concentration of 100 $mg.l^{-1}$ in phosphate buffered conditions. Similarly, results of the present study showed that lead had the least inhibitory effect on 1,2-DCA biodegradation. This could also be due to the fact that lead has a low solublity and can readily form precipitates such as lead phosphate (Hughes and Poole, 1991), thus rendering the metal less toxic because it is not bioavailable. In the NGWW microcosms (which had a 10-fold higher phosphate concentration than NWW), lead at a concentration of 0.01 mM was shown to stimulate biodegradation, exceeding that of the positive control. This correlates with the findings of Huang *et al*. (2008) who showed that a low concentration of lead enhanced the capability of selective lignin biodegradation by *Phanerochaete chrysosporium* and concluded that *Phanerochaete chrysosporium* could have developed an active defense mechanism to alleviate the lead toxicity. In the present study, lead was the heavy metal with the highest MIC, and caused the least reduction in k_1 and the least increase in the half-life of 1,2-DCA, thereby emphasizing its mild toxicity.

In conclusion this study has clearly demonstrated that the presence of heavy metals do inhibit 1,2-DCA biodegradation in a dose-dependent manner. The inhibition was found to be dependent on the type of heavy metal as well as the nutritional composition of the contaminated sample. Overall, the heavy metals in order of decreasing effects were: $Hg^{2+} > As^{3+} > Cd^{2+} > Pb^{2+}$. This observation was based on the 1,2-DCA degradation pattern, the degradation rate constant, the MICs of the the heavy metals and the concentration of the heavy metals that caused a HLD of 1,2-DCA.

CHAPTER THREE

EFFECTS OF BIOSTIMULATION AND BIOAUGMENTATION ON HEAVY METAL TOXICITY AND 1,2-DCA BIODEGRADATION IN CO-CONTAMINATED WASTEWATER

3.1 Introduction

The continuous increase in population and industrial development have led to the addition of an array of man-made chemicals in the environment, leading to tremendous deterioration in environmental quality (Bhatt *et al*., 2007). Chlorinated organic pollutants are widespread groundwater contaminants (Eguchi *et al*., 2001) and 1,2-DCA is of particular concern since it is the most important quantitatively (De Wildeman and Verstraete, 2003). An excess of 17.5 million tons are produced per annum in the United States, Western Europe and Japan (Field and Sierra-Alvarez, 2004). The Environmental Protection Agency and the International Agency for Cancer Research have classified 1.2-DCA to be a probable human carcinogen (Williams *et al*., 2001). In addition to pollution by 1,2-DCA, there is a continuous influx of heavy metals into the biosphere from both natural and anthropogenic sources (Perelomov and Prinsky, 2003). A high proportion of hazardous waste sites have been reported to be co-contaminated with organic and metal pollutants (Sandrin and Maier, 2003). Although some heavy metals are essential trace elements, most can be toxic to microorganisms at high concentrations, by forming complex compounds within the cell (Soboley and Begonia, 2008).

The presence of heavy metals may inhibit biodegradation by either inhibiting enzymes involved in biodegradation or the enzymes involved in microbial metabolism, thus compounding the problem of chlorinated organic contamination. The effects include extended acclimation periods, reduced biodegradation rates, and failure of target compound biodegradation (Kuo and Genthner, 1996). Remediation of sites co-contaminated with organic and metal pollutants is a complex problem, as the two components often must be treated differently (Sandrin and Maier, 2003). Conventional technologies for treating groundwater such as pump-and-treat and air-stripping with activated carbon adsorption require the construction of surface treatment facilities and produce secondary waste streams. Furthermore, these systems are not as efficient as once thought and cannot decompose the contaminants (O' Connell *et al*., 2008). Previous attempts to remediate co-contaminated environments include, the addition of metal-chelating adsorbents (Malakul *et al*., 1998), the use of treatment additives, pH adjustments and the addition of

divalent cations (Sandrin and Maier, 2002; Sandrin and Maier, 2003). These methods only target heavy metal removal and do not target degradation of the organic pollutant. In addition, they are expensive and can produce other waste problems, which have limited their industrial applications (Srivastava and Majumder, 2008). Bioremediation can therefore be an environmentally and economically feasible alternative.

Methods of bioremediation can include; bioattenuation, biostimulation and bioaugmentation. Bioattenuation is a natural process of degradation to decrease pollutant concentration over a period of time (Iwamoto and Nasu, 2001). This is an accepted option for bioremediation as it is cost effective, however; it is time consuming (Farhadian *et al*., 2008). If natural degradation does not occur or is too slow, the most widely used bioremediation technique is biostimulation, which involves manipulation of the environment to increase biodegradation and the reaction rates by stimulating the indigenous pollutant degraders (Nikolopoulou and Kalogerakis, 2009). This manipulation involves the addition of nutrients, such as input of large quantities of carbon source to stimulate the indigenous microorganism. This could also include the addition of nitrogen, phosphorus, and trace minerals while also making appropriate pH adjustments for the proliferation of indigenous microorganisms, hence speeding up the bioremediation process (Salanitro *et al*., 1997; Venosa *et al*., 1996). Another option is bioaugmentation, which involves the enhancement of the biodegradative capacities of contaminated sites by the inoculation of a non-native population with the desired catalytic capabilities into the microbial community (Wang *et al*., 2010). Bioremediation can be sped up by utilizing a combination of bioaugmentation and biostimulation. By using this technique both indigenous and exogenous microorganisms could benefit from biostimulation by the addition of energy sources (El Fantroussi and Agathos, 2005).

Microorganisms can convert 1,2-DCA rapidly into innocuous end products by the use of different pathways. Biodegradation of 1,2-DCA using *X. autotrophicus* and *A. aquaticus* strains has been previously described (Janssen *et al*., 1989; Van Den Wijngaard *et al*., 1992). Microbes may also contain mechanisms to resist metal toxicity (Bruins *et al*., 2000) and sites contaminated with heavy metals can be bioaugmented by using these heavy metal resistant strains. Roane *et al*. (2001) and Fernandes *et al*. (2009) reported success in remediating heavy metal and organic pollutant co-contaminated sites when bioaugmented with heavy metal resistant and organic pollutant degrading strains.

Although bacteria have the ability to remediate polluted sites, investigating the diversity and composition of microbial communities involved in the degradation process is complicated. Most environmental bacteria cannot be cultured by conventional laboratory techniques, therefore culture-independent approaches should be implemented (Iwamoto and Nasu, 2001). Advanced molecular techniques have been applied to ecosystems and have been found useful to study microbial diversity at the molecular level (Amann *et al*., 1995; Borneman *et al*., 1996). PCR-denaturing gradient gel electrophoresis (DGGE), allows for elucidation of the bacterial community structure and assessment of the changing composition of bacterial communities in contaminated aquifers subjected to bioremediation (Zhuang *et al*., 2005). DGGE is a technique that separates DNA fragments according to their melting behavior. 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) generates a profile, which indicates the composition of microbial communities in environmental samples (Li *et al*., 2006). The application of DGGE for analysis of biotreatment systems has provided insight into the dynamics of microorganisms involved in the remediation processes. Additionally, it is a powerful technique which provides an indication of community complexity and dynamics over time (Baptista *et al*., 2006).

Despite the frequent occurrences of co-contaminated sites, limited remediation studies have been carried out to address this problem, which continue to pose serious environmental health risk as the result of many industries, refineries and mines which release chlorinated organic compounds and heavy metals into the environment (Basak and Gokcay, 2005). Heavy metals also enter the water supply from acid rain which breaks down soils and rocks, releasing heavy metals into streams, lakes and ground water (Alluri *et al*., 2007). Chemical mixtures are generally more complicated to remove from contaminated sites than individual chemicals. To date no study has focused on the bioremediation of 1,2-DCA in the presence of heavy metal co-contaminated water. The present study therefore investigated various bioremediation options (biostimulation, bioaugmentation and dual bioaugmentation) in order to determine the best way of improving bioremediation of co-contaminated sites. Microbial diversity was also profiled at the different stages of the degradation process to determine the combined effect of 1,2-DCA and heavy metal contamination on the indigenous microorganisms in microcosms receiving different treatments and which treatment influenced diversity the most.

3.2 Material and methods

3.2.1 Selection of heavy metal resistant bacteria

3.2.1.1 Isolation and screening

Eighty bacterial isolates previously obtained from soil co-contaminated with 1,2-DCA and heavy metals (arsenic, cadmium, mercury or lead) were screened for heavy metal resistance. The isolates were grown on plate count agar (Merck Biolabs) supplemented with different concentrations (1, 1.5 and 2 mM) of As^{3+}, Cd^{2+} Hg^{2+} and Pb^{2+} separately. The heavy metals were used as the following salts: arsenic oxide (Fluka), cadmium chloride (Sigma), mercuric chloride (Sigma) and lead nitrate (Saarchem). Plates were then incubated at 30 °C for 72 h. Four isolates able to grow at the highest concentration of the heavy metals were further purified, identified and used for further study.

3.2.1.2 Identification of heavy metal-resistant bacterial strains

The four selected heavy metal-resistant bacterial isolates were grown in nutrient broth, at 30 °C for 17 h. DNA was isolated using the GENERELEASER® (Bioventures) as per manufacturer's instructions using the microwave protocol and used as template for the PCR-amplification of the 16S rRNA gene, using the universal bacterial primers set 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'- GGCGGWGTGTACAAGGC-3') (Marchesi *et al.,* 1998). The PCR amplification mix (25 µl) contained: 2.5 µl of template DNA, 1× PCR buffer, 1 mM of $MgCl_2$, 0.4 µM each of both the forward and reverse primers, 0.04 mM of deoxynucleoside triphosphate (dNTPs), and 0.5 U of SuperTherm *Taq* DNA polymerase (Southern Cross Biotech). 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 denaturation, 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 (Biorad) solution and visualized by UV transillumination (Chemi-Genius^2 BioImaging System, Syngene). The PCR products were sequenced (Inqaba Biotech) and the sequence chromatogram visualized and edited using Chromaslite201 software. Sequences were

compared to those in the GenBank database and relevant identities were determined using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

3.2.2 Standardization of bacterial cultures

The 1,2-DCA degrading strain, *Xanthobacter autotrophicus* GJ10, used in bioaugmentation studies, was kindly supplied by Prof. D. B. Janssen: Biochemical Laboratory, Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands. The four heavy metal resistant strains were used in conjunction with *Xanthobacter autotrophicus* GJ10 in dual-bioaugmentation studies. All isolates were stored on nutrient agar (Merck, Biolab) plates at 4 °C as a working stock culture. Pure cultures of the heavy metal resistant strains and *X. autotrophicus* GJ10 were separately inoculated into 50 ml sterile nutrient broth (Merck, Biolab) 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 sodium phosphate buffer and re-suspended in the same solution. The cultures were then standardized to an optical density value of 1.0 at a wavelength of 600 nm using the Biochrom, Libra S12 UV-Visible Spectrophotometer.

3.2.3 Sample collection and microcosm set-up

Wastewater samples were collected and characterised as described in Chapter two. Co-contaminated water microcosms were set up with 150 ml of wastewater from NWW and NGWW treatment plants, contaminated with 2.5 mM of 1,2-DCA and each of the heavy metals (arsenic, cadmium, mercury or lead) were added separately to the final concentration that would theoretically cause an 80% reduction in k_1 (0.19 mM As^{3+}, 0.20 mM Cd^{2+}, 0.15 mM Hg^{2+} or 0.26 mM Pb^{2+}). Biostimulation was conducted by adding glucose (Merck, Saarchem), to each microcosm at a final concentration of 1% (w/v). For bioaugmented microcosms, 1.5 ml of standardized culture of the 1,2 DCA degrading strain, *X. autotrophicus* GJ10, was added to the co-contaminated microcosm. Dual bioaugmentation was carried out by inoculating the co-contaminated microcosm with 1.5 ml of a mixed culture inoculum prepared from the standardized culture of *X. autotrophicus* GJ10 and the respective standardized heavy metal resistant strain. Water microcosms fortified with 1,2-DCA and no heavy metals were used as a positive control while autoclaved wastewater fortified with 1,2 DCA and no heavy metals served as a negative (biologically inactive) control. 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.

3.2.4 Analytical procedures

1,2-DCA concentrations were determined by periodically withdrawing 1 ml of headspace samples from the microcosms using a gas tight syringe (Hamilton) and injected into a gas chromatograph (GC) (Agilent 6820) equipped with a flame ionization detector. The samples were analyzed, with the injector at 240 °C and detector at 250 °C and a capillary column (DB1) with an initial temperature of 55 °C which was held for 10 s and then ramped up to 60 °C at a rate of 4 °C/min. Ultra high purity nitrogen was used as the carrier gas. 1,2-DCA concentrations were quantified by comparison to a standard curve derived from known quantities of 1,2-DCA 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.

3.2.5 Assessment of microbial population and diversity

3.2.5.1 1,2-DCA degrading bacterial population and identification

Samples taken from the microcosms were serially diluted using sterile distilled water and 0.1 ml was spread plated onto mineral salts medium. Five microlitres of 1,2-DCA was placed onto filter paper on the lid of the petri dish to serve as the sole carbon source for growth. Plates were incubated at 30 °C for 48 h before counting the emerging colonies, which were then expressed as colony forming units (CFU) per ml. Representative bacteria based on colonial characteristics on plates, were purified and identified as described in section 3.2.1.2.

3.2.5.2 Bacterial community profiling

The diversity of the bacterial populations in the different co-contaminated microcosms was determined *via* PCR-DGGE according to Muyzer *et al.* (1997) as follows:

3.2.5.2.1 DNA extraction and PCR amplification

DNA isolation from the co-contaminated microcosms was carried out using the GENERELEASER® (Bioventures) as per manufacturer's instructions using the microwave protocol. DNA concentration was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific) and appropriately diluted thereafter to standardize the DNA to a concentration range of 26-30 ng/µl prior to PCR. DNA samples were stored at -20 ºC until required. The PCR amplification of the 16S rRNA gene region was performed using the primers F341-GC (CCTACGGGAGGCAGCAG) with a 5' GC-clamp: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG and R907 (CCGTCAATTCMTTTGAGTTT) (Casamayor *et al*., 2002). A GC-clamp was attached to the forward primer in order to prevent the complete separation of the DNA strands during DGGE (Muyzer *et al*., 1993). The PCR was performed using 2 µl of DNA template and 48 µl of a PCR mix [1× PCR buffer, 1.25 mM $MgCl_2$, 0.5 µM each of both the forward and reverse primers, 0.2 mM dNTPs and 0.5 U of SuperTherm *Taq* DNA polymerase (Southern Cross Biotech)]. The PCR was performed using the GeneAmp PCR System (Version 2.25, Perkin Elmer) using a modified (Muyzer *et al*., 1993) touchdown thermal profile technique (Watanabe *et al*., 1998) consisting of: an initial denaturation (94 °C for 5 min), followed by annealing *via* 10 cycles of 94 °C for 1 min; 65 °C for 1 min with a decrease in temperature of 1°C per cycle; and 72 °C for 3 min. This was followed by 20 cycles of 94 °C for 1 min; 55 °C for 1 min; 72 °C for 3 min and a final 5 min extension step at 72 °C. The PCR products (585 bp) were confirmed by electrophoresis on a 2% agarose gel (w/v) in 1 × TAE running buffer with an applied voltage of 90 V for 90 min.

3.2.5.2.2 Denaturing gradient gel electrophoresis (DGGE)

The PCR products were separated by DGGE using the D-Code Universal Mutation Detection System (BioRad) (Muyzer *et al.*, 1997). Firstly, 0% and 100 % denaturing solutions solutions (100% denaturant was defined as 7 M urea and 40% [v/v] formamide) 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 (30%) and high (60%) density. The density solutions also contained 20 µl TEMED and 200 µl of 10% (w/v) ammonium persulphate. The density solutions were applied through 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 1 h to facilitate sample migration out of the wells during the electrophoretic run. Following the pre-run, samples were loaded into the gel (3 µl gel loading buffer : 10 µl PCR products) and DGGE was conducted at a constant voltage of 60 V in 1 × TAE buffer at 60 °C for 16 h. After electrophoresis, the gel was stained in 0.5 µg/ml ethidium bromide (BioRad) in 1 x TAE buffer for 30 min, destained in 1 × TAE buffer for a further 20 min and thereafter visualized by UV transillumination (Chemi-Genius^2 BioImaging System, Syngene). The DGGE gel profiles were visually analyzed based on the presence and absence of as well as intensity of the bands. The individual DGGE profiles were compared to each other, by using the Sorenson's index pair wise similarity coefficient C_s, which was determined as follows: $C_s = 2j/(a + b)$ x 100, where a was the number of DGGE bands in lane 1, b was the number of DGGE bands in lane 2, and j was the number of common DGGE bands. Two identical DGGE profiles had a *Cs* value of 100%, and two completely different profiles had a *Cs* value of 0% (Murray *et al*., 1996). Dominant DGGE bands were sequenced after excision from the gel and reamplification. Briefly, bands were excised, resuspended in l00 µl of MilliQ water and stored at 4 °C overnight. An aliquot of supernatant was used for PCR reamplification using the F341 (without the GC-clamp) and R907 primer sets with the PCR conditions as described in section 3.2.5.2.1. The reamplified PCR product was used for a sequencing reaction (with the corresponding reverse primer, R907) at Inqaba Biotech. Resulting sequences were then subjected to a BLAST search to determine identity.

3.3 Results

3.3.1 Identification of heavy metal resistant strains

The identities of heavy metal resistant strains used in dual bioaugmentation study are shown in Table 3.1. The strain resistant to arsenic was found to be closely related to *Delftia* sp., while those resistant to cadmium, mercury, and lead were *Pseudomonas* sp., *Cupriavidus* sp. and *Stenotrophomonas* sp., respectively

Table 3.1: Identity of heavy metal resistant bacterial isolates used in dual-bioaugmentation.

Resistant to	Identification	Accession number	% identity
Arsenic	*Delftia* sp.	FJ594443.1	93
Cadmium	*Pseudomonas* sp.	FN600406.1	99
Mercury	*Cupriavidus* sp.	GU338038.1	99
Lead	*Stenotrophomonas* sp.	FJ267572.1	99

3.3.2 Wastewater characterization

The physico-chemical properties of both wastewater types used in microcosm set-up are listed in Table 3.2. Wastewater obtained from NGWW demonstrated higher levels of certain elements, with calcium, magnesium, sodium, and iron being 86.13, 4.19, 15.93 and 416.67% respectively higher than the levels in NWW. Potassium however was 11.80% higher in NWW than in NGWW. The phosphate, nitrate, sulphate, total Kjeldahl nitrogen and total organic carbon levels in NGWW was 177.74, 217, 45.18, 130.77 and 9.82% higher than the concentrations in NWW. However, nitrite levels were 12.29% higher in NWW than NGWW.

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

Although heavy metals exhibited a negative impact on 1,2-DCA biodegradation, the use of biostimulation and bio-augmentation for bioremediation of heavy metal and 1,2-DCA

Table 3.2: Physico-chemical properties of the wastewater samples used in this study.

Determinant	NGWW	NWW
Calcium (mg $Ca.l^{-1}$)	26.30	14.13
Magnesium (mg $Mg.l^{-1}$)	5.72	5.49
Sodium (mg $Na.l^{-1}$)	84.6	72.98
Potassium (mg $K.l^{-1}$)	15.08	16.86
Iron (mg $Fe.l^{-1}$)	0.093	0.018
Nitrite (soluble) (mg $N.l^{-1}$)	0.13	0.15
Nitrate (soluble) (mg $N.l^{-1}$)	22.00	6.94
Sulphate (mg $SO_{4.l}{}^{-1}$)	42.48	29.26
Total organic carbon (mg $C.l^{-1}$)	12.3	11.2
Orthophosphate (PO_4) $mg.l^{-1}$ as P	5.02	1.81
Total kjeldahl nitrogen (mg $N.l^{-1}$)	3	1.3

co-contaminated water proved to be effective. In the autoclaved sample (negative control), abiotic loss of 1,2-DCA was observed, but to a lesser extent. This value was 31% for NWW and 37% for NGWW. In the unautoclaved samples not contaminated with heavy metals (positive control), 80% and 88% degradation of 1,2-DCA was observed in NWW and NGWW, respectively. In microcosms constructed with NWW (Figure 3.1 a-d), arsenic, cadmium mercury and lead inhibited degradation by 18.65, 16.51, 23.97 and 15.76%, respectively after 28 days. These values were generally (1.24, 2.73, 2.81 and 4.83% respectively) higher when compared to microcosms constructed with NGWW. In NGWW (Figure 3.2 a-d) after 28 days, degradation was inhibited by 17.41, 13.78, 21.16 and 10.94% in the presence of arsenic, cadmium mercury and lead, respectively. Biostimulation (Figure 3.1 a-d) improved biodegradation by 11.43, 13.92, 6.53 and 22.33% in NWW microcosms co-contaminated with arsenic, cadmium mercury and lead, respectively and 7.62, 8.75, 5.01 and 12.48%, respectively for microcosms constructed with NGWW (Figure 3.2 a-b). Bioaugmentation with *Xanthobacter autotrophicus* GJ10 resulted in higher degradation of 1,2-DCA compared to biostimulated microcosms. After 28 days, 1,2-DCA biodegradation in NWW (Figure 3.1 a-b) was improved by 18.60, 20.47, 12.64 and 26.07% respectively, compared to NGWW (Figure 3.2 a-b) where bioaugmented microcosms exhibited 11.66, 12.79, 10.53 and 16.33% increases in 1,2-DCA degradation in arsenic, cadmium, mercury and lead co-contaminated microcosms, respectively. However, dual bioaugmentation, using

Xanthobacter autotrophicus GJ10 and heavy metal resistant strains, demonstrated the most degradation when compared to biostimulated or bioaugmented microcosms, exhibiting 22.43, 26.54, 19.58 and 30.49% increase in 1,2-DCA degradation after 28 days in NWW (Figure 3.1 a-b) microcosms contaminated with arsenic, cadmium, mercury and lead, respectively. Similarly, Microcosms constructed with NGWW (Figure 3.2 a-b) exhibited 16.04, 19.82, 13.58 and 20.36% increase after 28 days under the same conditions.

The degradation rate constants (k_1) of 1,2-DCA (Table 3.3) ranged from 0.014-0.93 day^{-1} and 0.017-0.106 day^{-1} in microcosms constructed with NWW and NGWW, respectively. Microcosms supplemented with 1,2-DCA and heavy metals and no treatment did exhibit a higher k_1 than the negative controls, however, all microcosms undergoing biostimulation and/or bioaugmentation displayed higher k_1 than the microcosms receiving no bioremediation treatment. For both wastewater types, dual-bioaugmented microcosms exhibited the highest increase in k_1 compared to untreated microcosms, followed by bioaugmentation and then biostimulation. In microcosms constructed with NWW, arsenic did cause a 63.64% decrease in k_1 when compared to the positive control, however dual bioaugmentation, bioaugmentation and biostimulation did counteract this inhibition by causing an increase in k_1 compared to the untreated samples. Dual bioaugmentation, bioaugmentation and biostimulation of arsenic co-contaminated water resulted in 1.76-, 1.6- and 1.42-fold increase in k_1 respectively. Cadmium did cause a 45.95% decrease in k_1 when compared to the positive control. Dual bioaugmentation, bioaugmentation and biostimulation of cadmium co-contaminated water resulted in 2-, 1.65- and 1.35-fold increase in k_1 respectively. Mercury reduced the k_1 by 2-folds and dual bioaugmentation, bioaugmentation and biostimulation increased the k_1 by 1.7-, 1.44- and 1.22-fold respectively compared to the microcosm receiving no treatment. Lead caused the least (20%) reduction in k_1 while dual bioaugmentation, bioaugmentation and biostimulation increased the k_1 by 2.1-, 1.71- and 1.5-fold respectively. A similar trend was observed for microcosms constructed with NGWW.

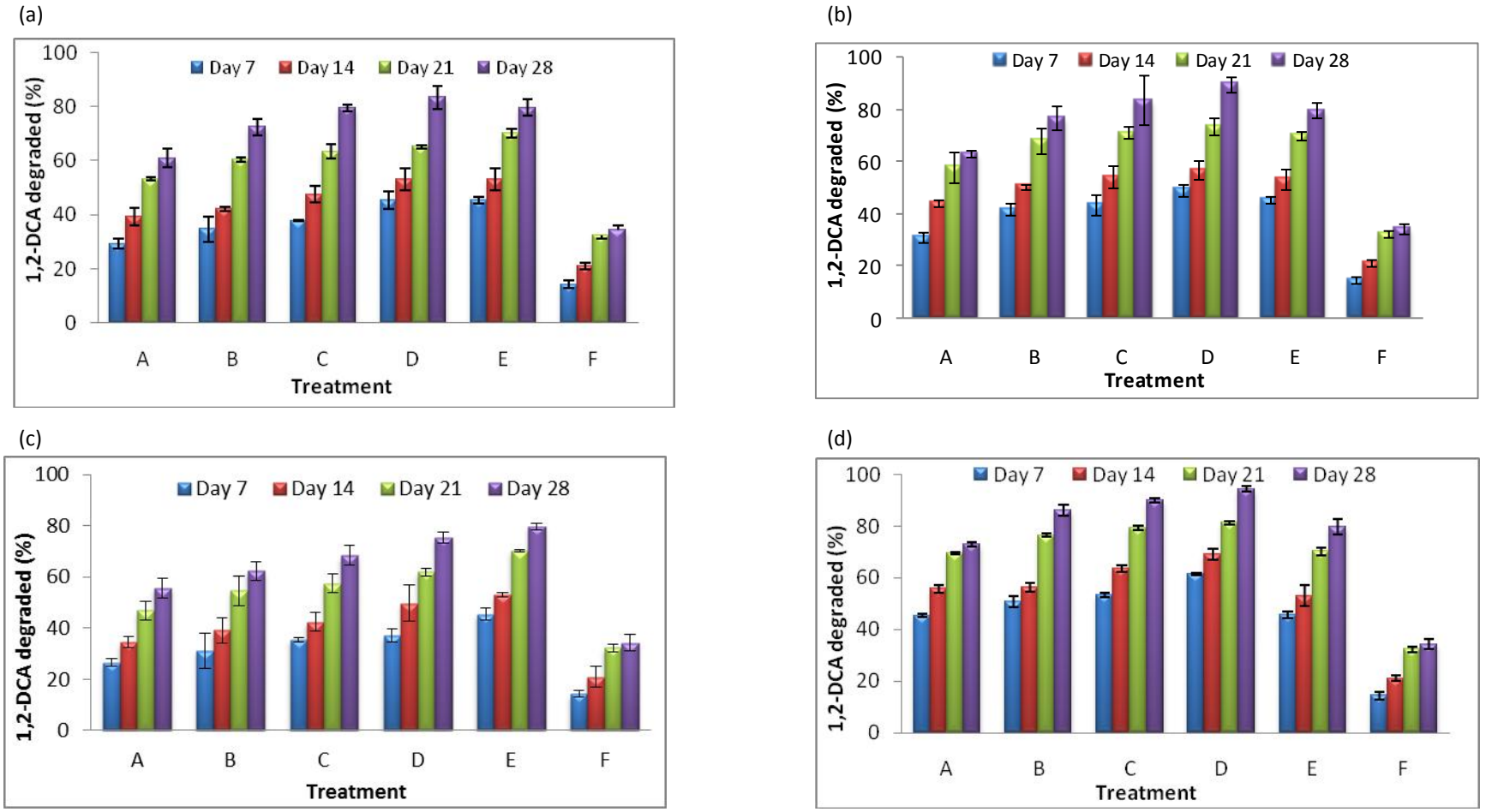


Figure 3.1: Effects of biostimulation, bioaugmentation and dual-bioaugmentation on the biodegradation of 1,2-DCA in Northern wastwater co- contaminated with (a) As^{3+}, (b) Cd^{2+}, (c) Hg^{2+} and (d) Pb^{2+}. A = wastewater + 1,2-DCA + heavy metal; B= wastewwater + 1,2-DCA + heavy metal + glucose; C = wastewater + 1,2-DCA + heavy metal + *X. autotrophicus* GJ10 ; D = wastewater + 1,2-DCA + heavy metal + *X. autotrophicus* GJ10 + heavy metal resistant strain; E = wastewater + 1,2- DCA; F= autoclaved wastewater + 1,2-DCA. Values indicate the average of triplicate samples while the error bars show the standard deviation.

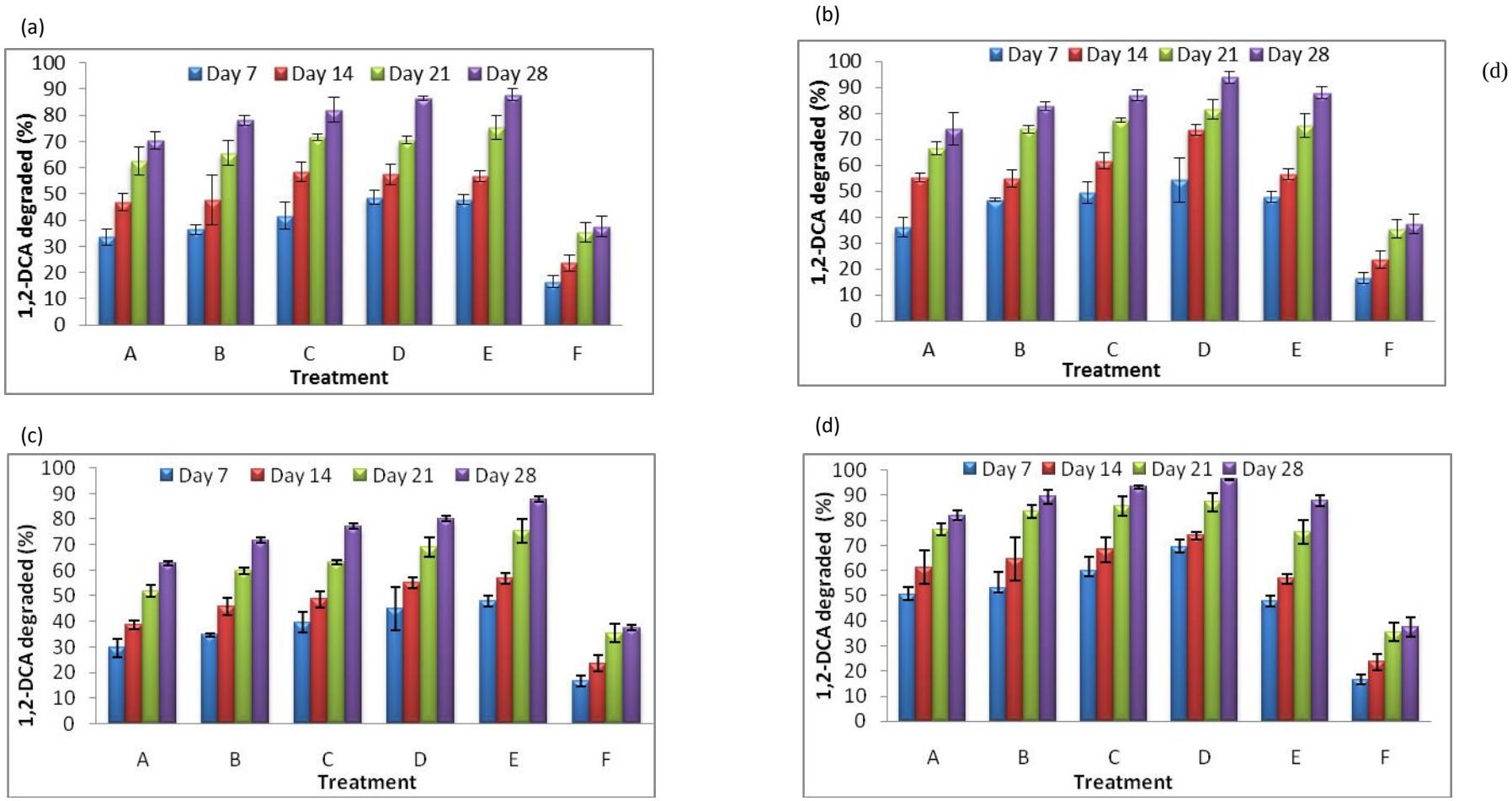


Figure 3.2: Effects of biostimulation, bioaugmentation and dual-bioaugmentation on the biodegradation of 1,2-DCA in New Germany wastewater co-contaminated with (a) As^{3+}, (b) Cd^{2+}, (c) Hg^{2+} and (d) Pb^{2+}. A = wastewater + 1,2-DCA + heavy metal; B= wastewater + 1,2-DCA + heavy metal + glucose; C = wastewater + 1,2-DCA + heavy metal + *X. autotrophicus* GJ10 ; D = wastewater + 1,2-DCA + heavy metal + *X. autotrophicus* GJ10 + heavy metal resistant strain; E = wastewater + 1,2- DCA; F= autoclaved wastewater + 1,2-DCA. Values indicate the average of triplicate samples while the error bars show the standard deviation.

Table 3.3: Biodegradation rate constants (day^{-1}) of 1,2-DCA in wastewater co-contaminated with heavy metals, under different treatment conditions.

Treatment	NWW	NGWW
Positive control	0.054 ± 0.0052	0.071 ± 0.0075
Negative control	0.014 ± 0.0012	0.017 ± 0.002
As^{3+}		
Biostimulation	0.047 ± 0.0026	0.052 ± 0.001
Bioaugmentation	0.053 ± 0.0023	0.061 ± 0.0065
Dual bioaugmentation	0.058 ± 0.007	0.065 ± 0.0015
No treatment	0.033 ± 0.002	0.039 ± 0.0045
Cd^{2+}		
Biostimulation	0.05 ± 0.0046	0.06 ± 0.0035
Bioaugmentation	0.061 ± 0.0026	0.069 ± 0.0044
Dual bioaugmentation	0.074 ± 0.008	0.093 ± 0.011
No treatment	0.037 ± 0.003	0.048 ± 0.0092
Hg^{2+}		
Biostimulation	0.033 ± 0.003	0.043 ± 0.0049
Bioaugmentation	0.039 ± 0.0045	0.005± 0.008
Dual bioaugmentation	0.047 ± 0.0015	0.054 ± 0.003
No treatment	0.027 ± 0.0032	0.033 ± 0.0026
Pb^{2+}		
Biostimulation	0.067 ± 0.0045	0.079 ± 0.0068
Bioaugmentation	0.077 ± 0.0015	0.091 ± 0.004
Dual bioaugmentation	0.093 ± 0.0064	0.106 ± 0.0025
No treatment	0.045 ± 0.001	0.059 ± 0.002

Values are averages of triplicate results ± standard deviation

3.3.4 Culturable 1,2-DCA-degrading bacterial population in wastewater under different bioremediation conditions

The culturable 1,2-DCA degrading bacterial population seemed to vary based on the type of wastewater, heavy metal utilized and the bioremediation strategy exploited. Seven bacterial isolates were isolated from the co-contaminated microcosms, based on color and colony morphology. Isolate A1 was identified as a *Burkholderia* sp., isolates A2 and A5 as *Pseudomonas* sp., isolates A3 and A7 as *Bacillus* sp, isolate A4 as *Enterobacter* sp. and isolate A6 was identified as *Bradyrhizobiaceae* bacteria as shown in Table 3.4

Table 3.4: Identity of 1,2-DCA degrading bacteria isolated from co-contaminated water microcosms.

	Organism	Accession	% identity	Cultural characteristics
A1	*Burkholderia* sp.	HQ441255.1	99	White to cream
A2	*Pseudomonas* sp.	DQ226203.1	98	Cream
A3	*Bacillus* sp.	HQ331104.1	99	Cream to yellow
A4	*Enterobacter* sp.	EU196755.1	99	White
A5	*Pseudomonas* sp.	HM468082.1	99	Cream
A6	*Bradyrhizobiaceae* bacterium	EU177519.1	96	Pink
A7	*Bacillus* sp.	FR727718.1	96	Cream to yellow

In microcosms constructed with NWW and co-contaminated with lead, the bacterial population seemed to be the highest when compared to other heavy metals utilized. After 28 days, the bacterial population for lead co-contaminated microcosms undergoing dual bioaugmentation, bioaugmentation and biostimulation were 49.69, 28.10 and 20.23 ($\times 10^5$ cfu/ml) respectively. These values were 8.07-, 4.57- and 3.28-fold higher when compared to lead co-contaminated microcosms not receiving treatment (with a population of 6.15×10^5 cfu/ml) (Figure 3.3 D). Lead co-contaminated microcosms undergoing dual-bioaugmentation exhibited a 1,2-DCA degrading population that is 76.62% higher than those microcosms bioaugmented and 145.63% than those biostimulated. Microcosms co-contaminated with cadmium contained bacterial populations of 36.60, 28.46 and 12.46 ($\times 10^5$ cfu/ml) in microcosms receiving dual-bioaugmentation, bioaugmentation and biostimulation, respectively after 28 days. These values were 6.58-, 5.12- and 2.24-fold higher when compared to microcosms not receiving treatment, which had a population of 5.56×10^5 cfu/ml (Figure 3.3 B). The bacterial population in arsenic co-contaminated wastewater was 30.00, 17.46, 12.00 ($\times 10^5$ cfu/ml) when microcosms were dual-

bioaugmented, bioaugmented and biostimulated respectively. This was 6.25-, 3.64- and 2.5-fold more than the untreated arsenic co-contaminated microcosms (Figure 3.3 A) which had a population of 4.8×10^5 cfu/ml. Dual bioaugmented microcosms exhibited a 1,2-DCA degrading population that was 71.82% higher than those microcosms bioaugmented and 150% than those biostimulated. The bacterial population in mercury co-contaminated microcosms was the lowest. In microcosms undergoing dual bioaugmentation, bioaugmentation and biostimulation, bacterial population was as little as 25.37, 14.46 and 9.73 ($\times10^5$ cfu/ml) respectively. This was 5.6-, 3.2- and 2.1-fold higher than the population (4.5×10^5 cfu/ml) in the untreated microcosms (Figure 3.3 C) . Dual bioaugmented microcosms exhibited a 1,2-DCA degrading population that was 75.45% higher than those microcosms bioaugmented and 160.74% higher than those biostimulated.

The same trend of 1,2-DCA degrading bacterial population dynamics follows for microcosms constructed with water from NGWW; however population was higher in NGWW than in NWW. NGWW constructed microcosms co-contaminated with lead were found to have bacterial counts of 56.32, 36.35, 30.34 ($\times10^5$ cfu/ml) for dual-bioaugmented, bioaugmented and biostimulated microcosms after 28 days, respectively. This was 4.54-, 2.93- and 2.45-fold higher than the lead co-contaminated microcosm that received no treatment which had a population of 12.4×10^5 cfu/ml (Figure 3.4 D). Dual bioaugmented microcosms exhibited a 1,2-DCA degrading population that was 54.94% higher than those microcosms bioaugmented and 85.63% higher than those biostimulated. Microcosms co-contaminated with cadmium contained bacterial populations of 44.34, 34.23 and 26.44 ($\times10^5$ cfu/ml) in microcosms receiving dual-bioaugmentation, bioaugmentation and biostimulation respectively after 28 days. This was 4.59-, 3.55- and 2.74-fold higher than the microcosms not receiving treatment which had a bacterial population of 9.65×10^5 cfu/ml (Figure 3.4 B). Cadmium co-contaminated microcosms undergoing dual-bioaugmentation exhibited a 1,2-DCA degrading population that was 29.54% higher than those microcosms bioaugmented and 67.70% than those biostimulated. The bacterial population in arsenic co-contaminated wastewater was 39.35, 32.56, 24.34 ($\times10^5$ cfu/ml) when microcosms were dual-bioaugmented, bioaugmented and biostimulated, respectively. This was 6.12-, 5.06- and 3.79-fold higher compared to the microcosm that received no treatment which had a population of 6.43×10^5 cfu/ml (Figure 3.4 A). The bacterial population in mercury co-contaminated microcosms was again the lowest. In microcosms undergoing dual bioaugmentation, bioaugmentation and biostimulation the bacterial population was as little as

32.13, 26.23, 19.23 ($\times 10^5$ cfu/ml) respectively. This was 5.92-, 4.83- and 3.54-fold higher than population (5.43×10^5 cfu/ml) in the microcosms receiving no treatment (Figure 3.4 C). Dual bioaugmented microcosms exhibited a 1,2-DCA degrading population that was 22.49% higher than those microcosms bioaugmented and 67.08% higher than those biostimulated.

3.3.5 PCR-DGGE analysis of bacterial community diversity

Microcosms constructed with NWW were chosen for DGGE analysis due to the precipitates in NGWW hindering DNA isolation using the GENERELEASER®. In addition, only arsenic, cadmium and lead co-contaminated microcosms could be investigated as mercury inhibited the PCR of the isolated DNA from the water samples. Figure 3.5 (a-c) represents DGGE profiles of the biostimulated microcosms, showing different banding patterns based on the type of heavy metal used. In arsenic co-contaminated wastewater at day 0 the bands were bright; however the brightness of the bands fade over day 7 to day 14 (Figure 3.5 a). The bands brightness intensified again from day 21 to day 28. Band A1 was constant throughout the 28 days, bands A5 and A6 was absent at day 0 but present from day 7 to day 28 with increasing intensity. The presence of a very faint band (A4) was observed at day 0, and this band disappeared from day 7-21 but was present at day 28. Bands A2 and A3 were present at day 0 but not at days 7 and 14, it reappears again at day 21 and was present at day 28. Band A7 was present at day 0 and not at any of the other days. The DGGE profile at day 0 exhibited a 16.67 % similarity to days 7 and 14, a 14.29 % similarity to day 21 and a 26.67 % similarity to day 28. Days 7 and 14 displayed a 100 % similarity to each other and days 21 and 28 displayed a 90% similarity to each other based on the Sorsen index of similarity. For microcosms co-contaminated with cadmium (Figure 3.5 b), bands B1 and B5 were bright at day 0, with a decrease in the intensity in the brightness at days 7 and 14; however the brightness increased from day 21 to day 28. Bands B2, B3 and B4 were present at low concentrations at day 0 and not present at all at days 7, 14 and 21, but present at day 28. Band B6 was only present at day 0. The DGGE profile at day 0 showed a 16.67% similarity to day 7, 15.38% to days 14 and 21, and 40% to day 28. Days 14 and 21 displayed 100% similarity to each other, while day 28 shows 80% to these two days.

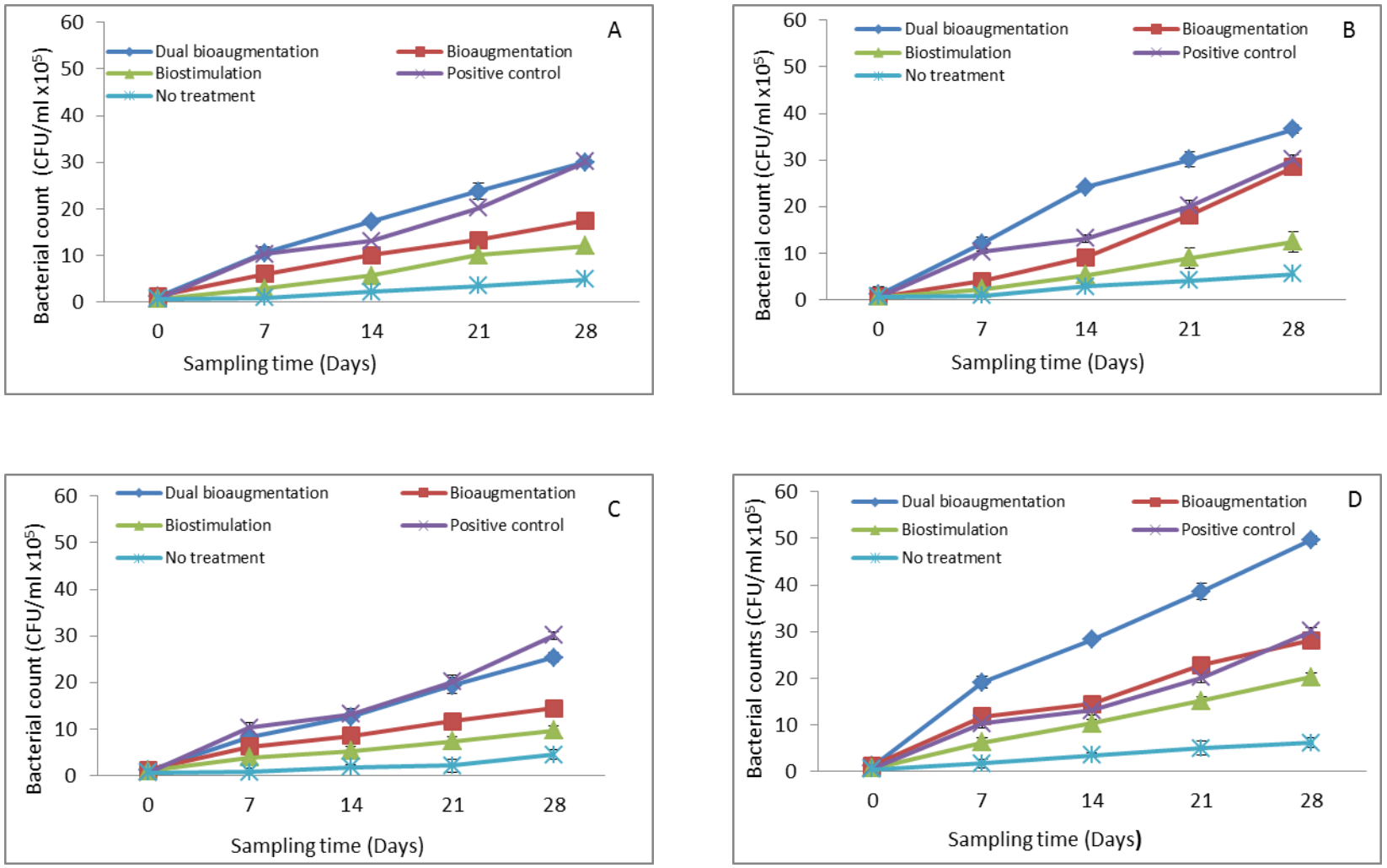


Figure 3.3: 1,2-DCA degrading bacterial population in arsenic (A), cadmium (B), mercury (C) and lead (D) co-contaminated NWW under different treatment conditions. Values indicate the average of triplicate samples while the error bars show the standard deviation.

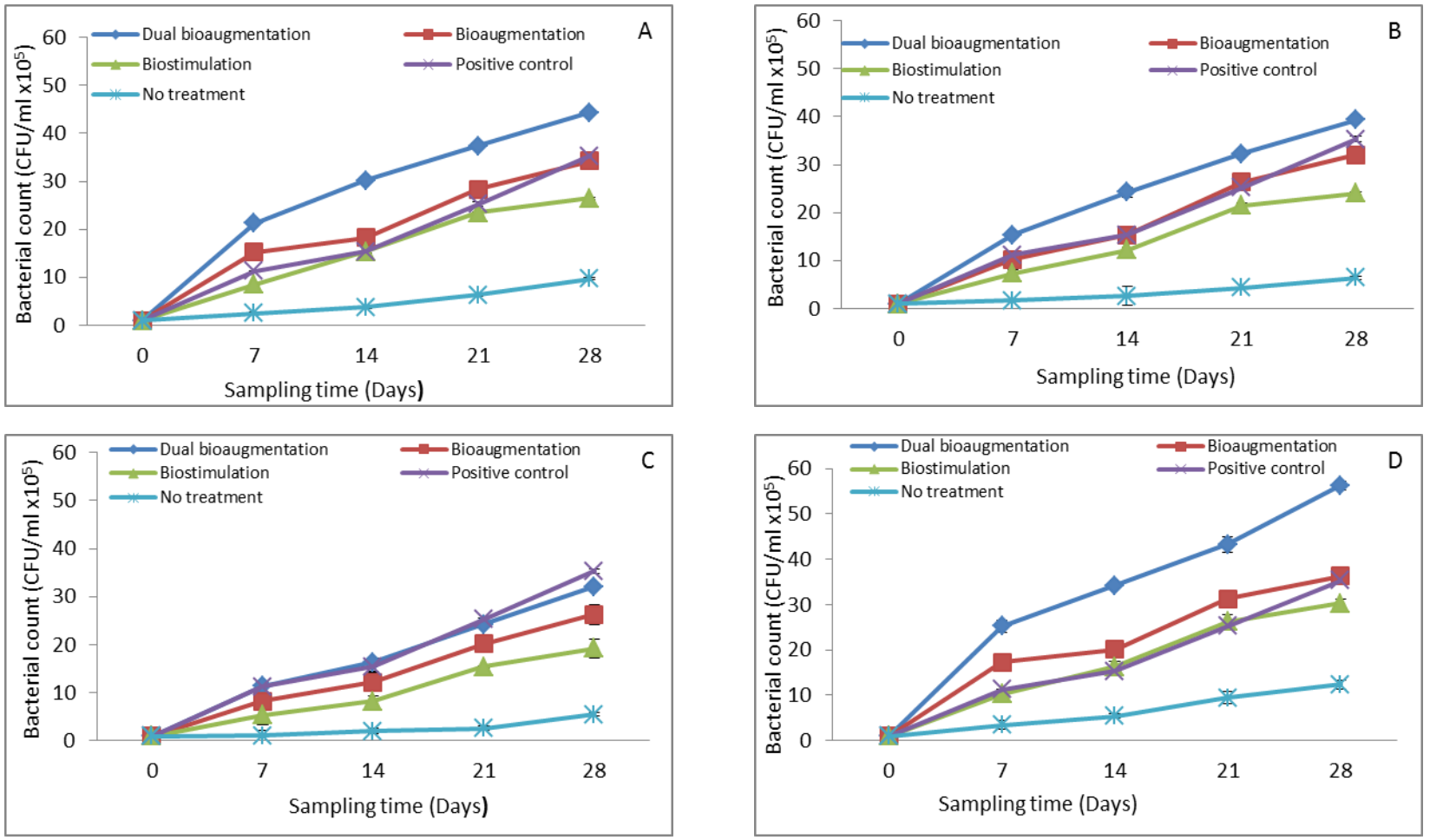


Figure 3.4: 1,2-DCA degrading bacterial population in arsenic (A), cadmium (B), mercury (C) and lead (D) co-contaminated NGWW under different treatment conditions. Values indicate the average of triplicate samples while the error bars show the standard deviation.

For lead co-contaminated microcosms (Figure 3.5 c), band C1 was brightly present at day 0, and faintly present at days 7, 14, and 21, with an increase in brightness at day 28. The presence of dominant bands (C2, C3 and C4) was observed in the latter stage of the biostimulation process. Days 7, 14, 21 and 28 show 100% similarity to each other; however day 0 displays 50% similarity to these three days.

Figure 3.6 (a-c) represents DGGE profiles of bioaugmented microcosms. In arsenic co-contaminated wastewater band D1 is absent at day 0 and only faintly present at day 7 but brightly present from days 14-28 (Figure 3.6 a). Bands D2 and D3 were faintly present at day 0, absent at day 7 and brightly present from day 14-28. In arsenic co-contaminated water the DGGE profiles at days 7, 14, 21 and 28 were identical and exhibited a Cs value of 100%. These profiles were only 25% identical to the profile at day 0. In cadmium co-contaminated wastewater (Figure 3.6 b), bands E1 and E5 were present from day 0 through day 28 at the same intensity. Bands E3 and E4 were not present at day 0; however these bands were present at a high intensity from day 14 until day 28. Band E2 was not present at day 0, and only faintly present at day 7 and 14. The brightness of this band did intensify at days 21 and 28. For cadmium bioaugmented water days 7, 14, 21 and 28 exhibited 100 % similarity to each other, however they exhibited only 40% Cs to day 0. Microcosms co-contaminated with lead is represented in Figure 3.6 (c), bands F1, F2, F3 were not present at day 0, but present from day 7 until day 28. Band F4 was only present at day 0 and not observed throughout in the latter stage of the bioaugmentation process. For lead bioaugmented microcosms days 7, 14, 21 and 28 exhibited 100 % similarity to each other, however they exhibited only 22.2% Cs to day 0.

Figure 3.7 (a-c) represents DGGE profiles of dual-bioaugmented microcosms. In arsenic co-contaminated wastewater bands G1, G4, G5 and G6 were not present at day 0, but present from days 7 to 28 at the same brightness intensity (Figure 3.7 a). Bands G2 and G3 are also not present at day 0, however these bands are present at day 7 and 21 but faintly at day 28. The arsenic dual-bioaugmented microcosms DGGE profiles at days 7 had a Cs value of 100% however day 0 only exhibited a 44.44% similarity to these days. The DGGE profiles for cadmium co-contaminated microcosms are represented in figure 3.7 (b). The bands H1-H7 were present at a brightness of constant intensity from day 7 up until day 28; however; these bands were not present at day 0. For cadmium bioaugmented water days 7, 14, 21 and 28 exhibited 100 % similarity to each other, however they exhibited only 36.36% similarity to day 0. For

microcosms co-contaminated with lead [Figure 3.7 (c)], the intensity of band I1 decreases slightly at day 7, but increases from day 21 until day 28. Bands I2, I3, and I5 are absent at day 0, but present from day 7 until day 28 at a relatively constant brightness. In lead bioaugmented water microcosms, days 7, 14, 21 and 28 exhibited 100% similarity to each other, however they exhibited only 66.66% similarity to day 0.

The identities of the dominant bands excised are listed in table 3.5 below; the organisms represented by these bands belong to the genera; *Clostridium, Cupriavidus, Dechloromonas, Enterobacter* and *Klebsiella.*

Table 3.5: Identity of bacterial isolates represented by the excised bands from DGGE gels.

Band	**Identification**	**Accession number**	**% identity**
B1	*Clostridium pasteurianum* strain CH7	EF140983.1	98
F1	*Klebsiella pneumonia*	JN545035.1	99
I5	*Dechloromonas* sp. PC1	AY126452.1	100
E4	*Cupriavidus* sp. DE7	JN226398.1	95
H4	*Enterobacter* sp. ICB551	HM748088.1	83

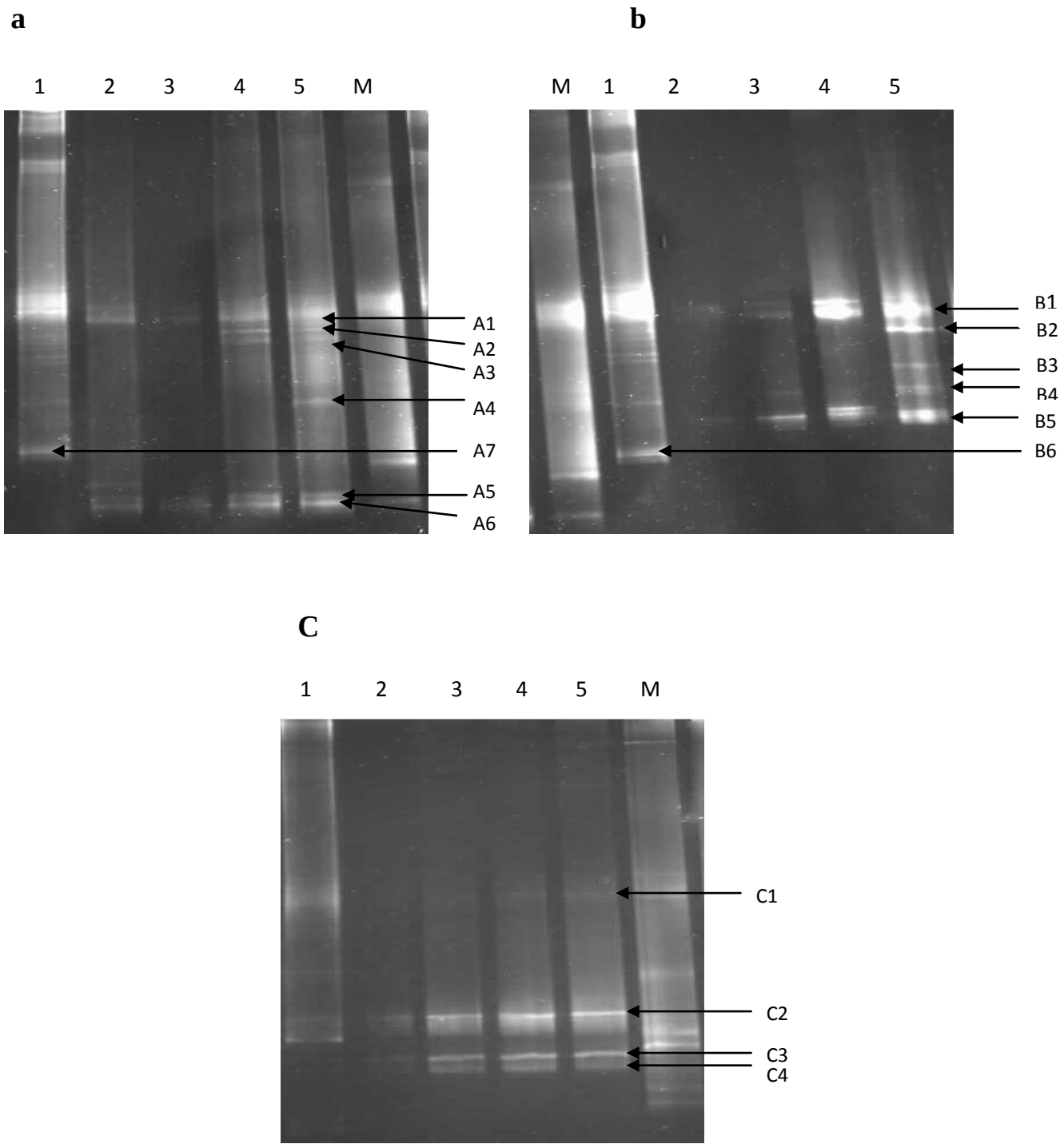


Figure 3.5: DGGE profiles of 16S rRNA fragments of NWW co-contaminated with 1,2-DCA and As^{3+} (a), Cd^{2+} (b) and Pb^{2+} (c) and biostimulated with glucose. Lanes 1, 2, 3, 4 and 5 represents day 0, 7, 14, 21, and 28, while M represents the marker.

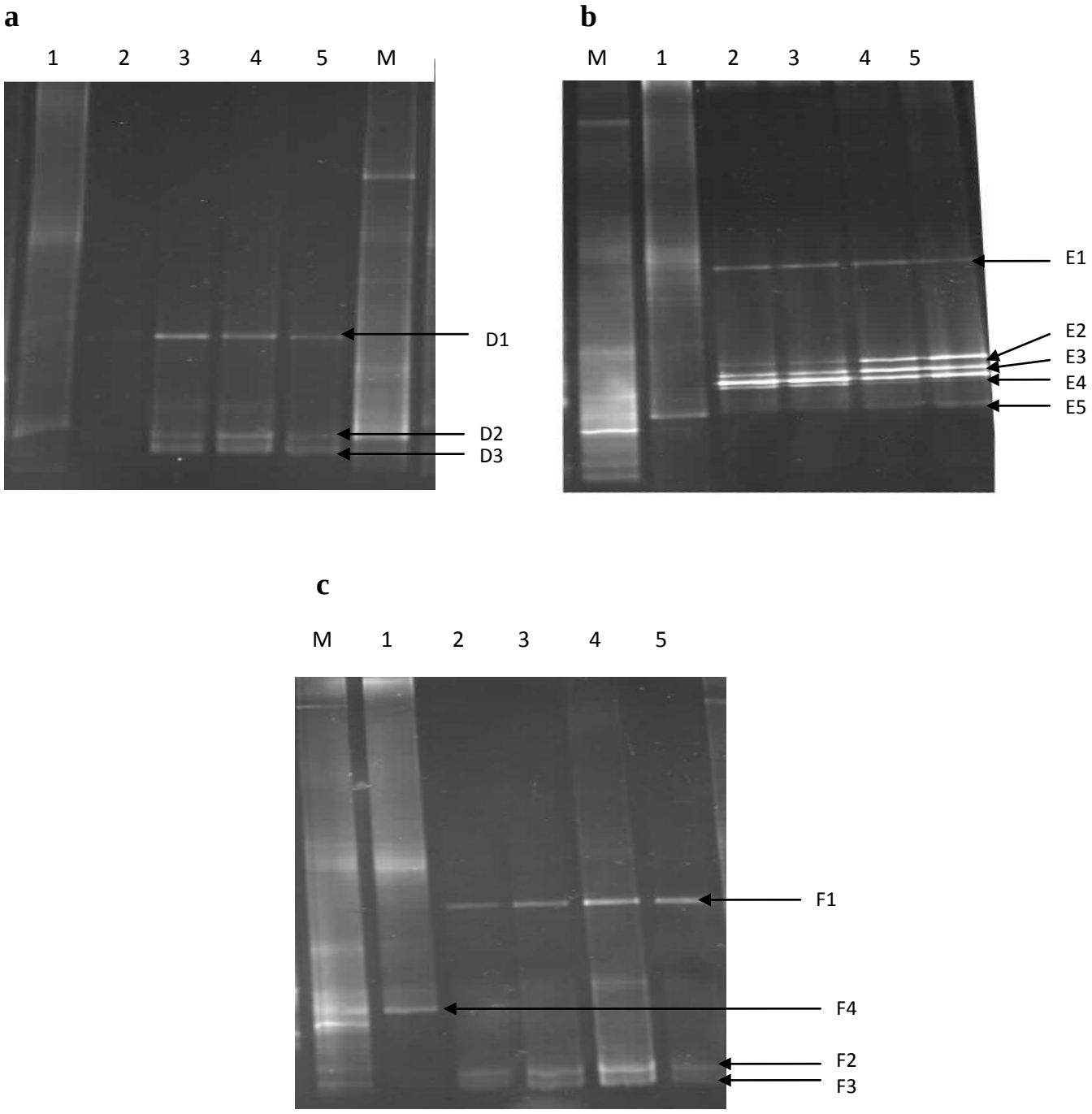


Figure 3.6: DGGE profiles of 16S rRNA fragments of NWW co-contaminated with 1,2-DCA and As^{3+} (a), Cd^{2+} (b) and Pb^{2+} (c) and bioaugmented with *X. autotrophicus*. Lanes 1, 2, 3, 4 and 5 represents day 0, 7, 14, 21, and 28, while M represents the marker.

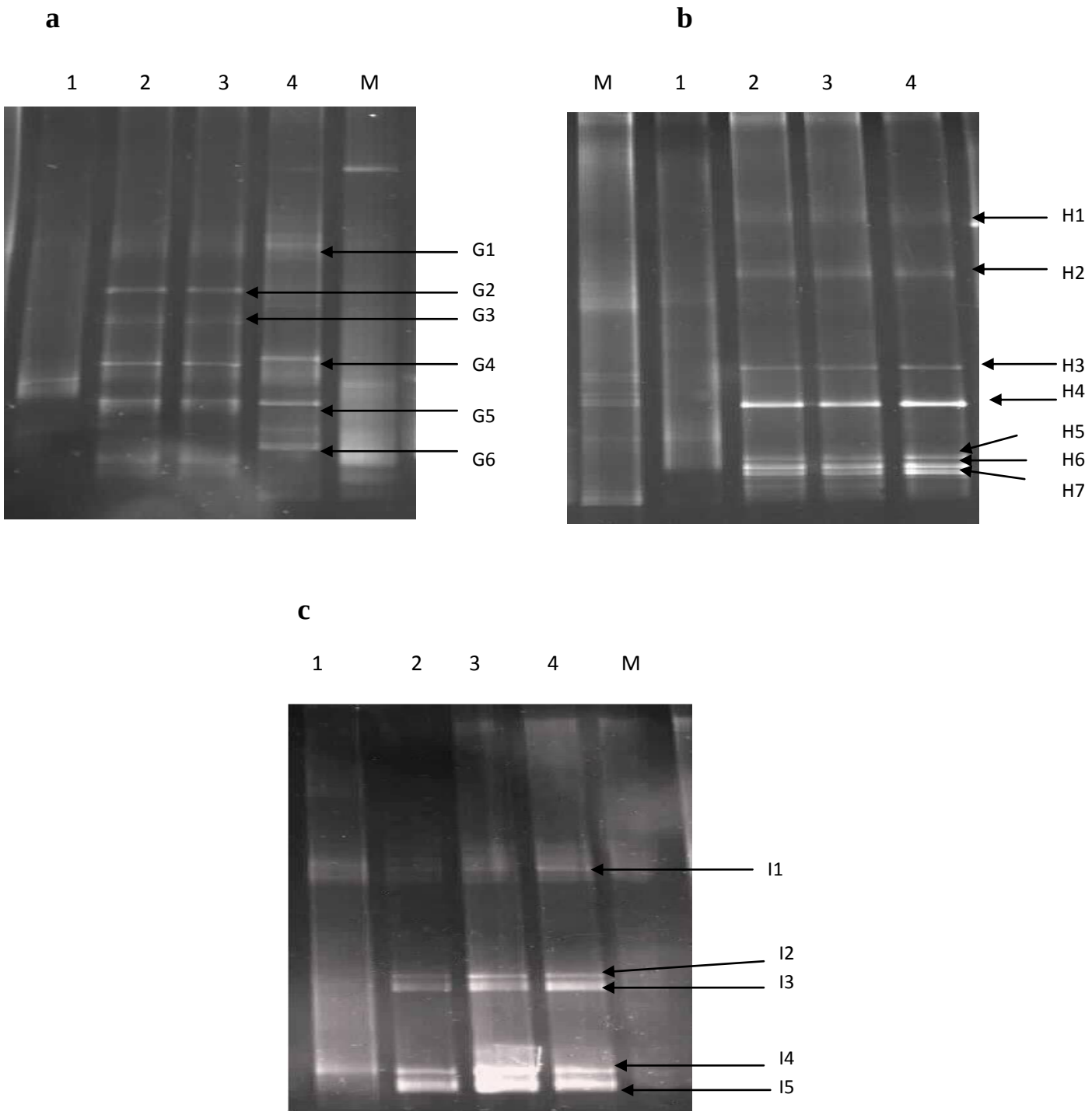


Figure 3.7: DGGE profiles of 16S rRNA fragments of NWW co-contaminated with 1,2-DCA and As^{3+} (a), Cd^{2+} (b) and Pb^{2+} (c) and dual-bioaugmented with *X. autotrophicus* and heavy metal resistant strains. Lanes 1, 2, 3, and 4 represents day 0, 7, 21, and 28, while M represents the marker.

3.4 Discussion

Biostimulation, bioaugmentation and dual-bioaugmentation can stimulate the capability of indigenous microbial populations to remove 1,2-DCA from co-contaminated wastewater. However, the observed degradation profiles in this study, varied based on the type of heavy metal used and the wastewater composition.

Biostimulation of microcosms with glucose can enhance 1,2-DCA degradation to varying degrees in the presence of heavy metals, depending on heavy metal contaminants and wastewater types. In a study by Olaniran *et al*. (2008), a mixture of chlorinated aliphatic hydrocarbons (CAH), including 1,2-DCA, were biostimulated with three different carbon sources. Of the nutrients added, glucose seemed to have the most effect in enhancing CAH degradation. In a similar investigation, biostimulation with glucose resulted in the highest overall degradation of 1,2-DCA, in heavy metal co-contaminated soil, compared to other biostimulants tested (Olaniran *et al*., 2009). Glucose has also been shown to have a significant effect on the biodegradation of *cis*- and *trans*- dichloroethene (Olaniran *et al*., 2006b). It is thus feasible to biostimulate co-contaminated sites the addition of glucose because it has several advantages over other co-metabolic electron donors due to its high water solubility and it is neither an amiable gas nor a toxic compound.

Bioaugmentation with *X. autotrophicus* GJ10 proved to be more efficient than biostimulation with glucose. This is possibly because glucose addition will stimulate the entire indigenous population and is not specific for the 1,2-DCA degrading population, whereas addition of *X. autotrophicus* GJ10 specifically targets the degradation of 1,2-DCA because it harbors a large plasmid, pXAU1, involved in 1,2-DCA degradation. At least two of the genes, *dhlA* and *ald*, which code for haloalkane dehalogenase and chloroethanol dehydrogenase respectively, are plasmid encoded. pXAU1 also carries a mercury resistance operon (Tardif *et al*., 1991), therefore making *X. autotrophicus* GJ10 the ideal strain for 1,2-DCA degradation especially in heavy metal co-contaminated environments. However, dual-bioaugmentation with *X. autotrophicus* GJ10 and a heavy metal resistant strain was shown to be the most efficient remediation method in this study.

Microorganisms are known to influence arsenic geochemistry by their metabolism, i.e., reduction, oxidation, and methylation (Muller *et al*., 2007). The isolated arsenic resistant bacteria that was used in dual bioaugmentation studies was identified as *Delftia* sp. Cai *et al*. (2009) also

identified *Delftia* sp as one of the major genera of arsenic resistant bacteria from four arsenic-contaminated soils tested. *Delftia acidovorans* is known to contain an integrative conjugative element, which contains an arsenate resistance system (Ryan *et al*., 2009). This system has the genes; *arsH, arsC, arsB* and *arsA* in the operon of this bacteria which are responsible for arsenic resistance (Ryan *et al*., 2009). The cadmium resistant isolate used in this study was identified as a *Pseudomonas* sp. Cadmium ions are toxic to bacteria, and when incorporated into sensitive cells, cause a cessation of respiration by binding to sulfhydryl groups in proteins (Foster, 1983). Microbial resistance to cadmium is usually based on energy-dependent efflux mechanisms (Silver, 1996). A fluorescent Pseudomonad (strain CW-96-1) tolerated cadmium concentrations up to 5 mM and was able to remove >99% of cadmium from solution after 140 h (Wang *et al*., 1997). Rani *et al*. (2009) concluded that *Pseudomonas* is an ideal candidate for bioremediation of cadmium in the environment. A chromosomal fragment encoding cadmium resistance was cloned from *Pseudomonas putida* 06909, and sequence analysis revealed two divergently transcribed genes, *cadA* and *cadR* (Lee *et al.,* 2001). Zeng *et al*. (2009) made *P. aeruginosa* E1 into a biosorbent for cadmium ion uptake from aqueous solution. In the present study, the microorganism used to remediate mercury co-contaminated microcosms was identified as *Cupriavidus* (formally *Ralstonia*) sp. Metal responsive MerR family transcriptional regulators are widespread in bacteria and activate the transcription of genes involved in metal ion detoxification, efflux, or homeostasis, in response to the presence of cognate metal species in the cytoplasm. MerR regulators, involved in regulating the mercury resistance genes in *Cupriavidus metallidurans* CH34 have been identified (Julian *et al.,* 2009). Mercury resistance in *Cupriavidus* sp is based on reduction of Hg^{2+} to volatile metallic mercury. The highly toxic Hg^{2+} cation is bound in the periplasm by MerP, imported into the cytoplasm by MerT, and reduced to metallic mercury by the NADPH-dependent flavoprotein MerA (Silver and Phung 1996). *Stenotrophomonas* sp. CD02 was found to tolerate high concentrations (above 2 mM) of lead (Chien *et al*., 2007). Pages *et al*. (2008) also demonstrated that *S. maltophilia* Sm777 was able to grow to a high density (10^9 CFU.ml^{-1}) in the presence of 5 mM $Pb(NO_3)_2$ and that this bacteria could tolerate high levels (0.1 to 50 mM) of various toxic metals, such as Cd, Co, Zn, Hg, Ag, selenite, tellurite and uranyl. It is therefore not surprising that the *Stenotrophomonas* sp used to remediate lead co-contaminated microcosms was found to be effective.

Heavy metal resistant strains protect the metal-sensitive organic-degrading population from metal toxicity, the primary mode of action being metal detoxification, such that organic degradation was no longer inhibited. It is therefore evident that *X. autotrophicus* GJ10 and the respective heavy metal resistant strains cooperatively functioned to remediate both metal and organic pollutants in the co-contaminated water microcosms. Roane *et al*. (2001) reported that dual-bioaugmentation, involving inoculation of soil with both metal-detoxifying (*Ralstonia eutropha* JMP134) and organic-degrading (*Pseudomonas* H1) bacteria, facilitated the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) in the presence of cadmium co-contaminant. This technique was also demonstrated by Fernandes *et al*. (2009) when bacterial strains capable of withstanding considerable concentrations of Cd^{2+} or Hg^{2+} or Pb^{2+} were coupled with strains that showed good performance at degrading the pollutants methyl tertiary butyl ether or trichloroethane. Stephen *et al*. (1999) used metal-resistant bacteria to protect indigenous ammonia oxidizing β-subgroup Proteobacterium. One reason for enhanced degradation in co-contaminated microcosms is that high metal concentrations can form selective pressure for the inoculated heavy metal resistant and organic degrading strains. This selective pressure may have reduced competition from metal-sensitive, non-degrading microorganisms, thus increasing biodegradation at higher metal concentrations (Sandrin and Maier, 2003).

Despite the limits of bioaugmentation, such as 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), biougmentation proved to be a better remediation option in the present study. The increase in the total number of 1,2-DCA degrading bacterial populations was higher in the dual-bioaugmented bioaugmented and bioaugmentated microcosms compared to those biostimulated. The increases in total 1,2-DCA degrading populations also correspond to an increase in 1,2-DCA degradation in the bioaugmentated microcosms mainly because microbial communities are without a doubt the key driving force behind the biological treatment of pollutants. The 1,2-DCA degrading bacterial isolates (*Burkholderia* sp., *Pseudomonas* sp., *Bacillus* sp., *Enterobacter* sp., *Bradyrhizobiaceae*) identified from the co-contaminated microcosms, in this study, have previously been implicated in 1,2-DCA degradation and heavy metal resistance and detoxification. For example *Pseudomonas* sp. isolated from sewage of industrial effluents from a wastewater treatment plant were shown to exhibit high resistance to heavy metals with minimum inhibitory concentration

(MIC) ranging from 50 µg/ml to 350 µg/ml (Singh *et al*., 2010). Choudhary and Sar (2009) also characterized heavy metal sequestration by a multi-metal resistant *Pseudomonas* strain for its potential application in metal bioremediation. Carboxyl and phosphoryl groups along with a possible ion exchange mechanism played a major role in cation binding. Metal binding affinity occurred in the order of $Cu^{2+} > Ni^{2+} > Co^{2+} > Cd^{2+}$. *Pseudomonas* sp. could also be used as a biosorbent to remove heavy metals (Hussein *et al.*, 2004). In addition to heavy metal resistance, *Pseudomonas* could be used for the degradation of 1,2-DCA. *Pseudomonas* sp. strain DCA1 was reported to be capable of growing on 1,2-DCA as the sole carbon and energy source. The first step in 1,2-DCA metabolism in this strain is a monooxygenase-mediated oxidation (Hage and Hartmans 1999). Furthermore, Hage *et al*. (2004) demonstrated the use of a biofilm of *Pseudomonas* sp. strain DCA1 on a membrane-aerated biofilm reactor for the removal of 1,2-DCA

Furthermore, *Bacillus* sp. has been previously implicated in the degradation of many chlorinated compounds, including 1,2-DCA. Olaniran *et al*. (2007) demonstrated that *Bacillus* sp. was one of the three 1,2-DCA degrading bacteria isolated from a pulp mill wastewater effluent in South Africa. In addition to possessing degradative capabilities, *Bacillus* sp. can be used in the removal of heavy metals. Kumar *et al*. (2010) demonstrated that *Bacillus* sp. could reduce 56% Cu and 48% Ni respectively, while Rani *et al*. (2009) also suggested that immobilized *Bacillus* sp. have potential application for the removal of Cu, Cd and Pb from industrial wastewater. These bacterial species are notorious for their ability to survive even in the presence of contaminants that are not commonly encountered (Tondo *et al*., 1998). This could be because they often occur as spores, but will germinate upon the availability of readily decomposable organic matter (Vilain *et al*., 2006). Also, *Enterobacter aerogene*s was partially able to degrade and dechlorinate four chlorobenzoic acid compounds (2-chlorobenzoic acid , 3-chlorobenzoic acid, 4-chlorobenzoic acid 4, and 3,4-dichlorobenzoic acid) in minimal salt medium at a concentration of 3.5 mM within 72 h of incubation (Tarawneh *et al*., 2010). *Enterobacter* sp. J1 could be used as a biosorbent and was able to uptake over 50 mg of Pb per gram of dry cell, *Enterobacter* was not only tolerant to heavy metals, but also bound considerable amount of heavy metals from the growth medium (El-Deeb, 2009). Sato *et al*. (2005) revealed that *Bradyrhizobium japonicum* possess genes encoding the haloalkane dehalogenases on an open reading frame *blr1087,* and confirmed that the rhizobial strain and

dehalogenase converted 11 halogenated substrates, including 1,2-DCA. Intrinsic heavy metal resistance among *Bradyrhizobium* sp. has been reported (Ahmad *et al*., 2001) and *Bradyrhizobium* sp. could accumulate cadmium using glutathione (GSH) dependant mechanisms (Bianucci *et al*., 2011).

Compared to the plate count method, PCR-DGGE can provide more detailed information about the population shift and diversity of microorganisms. The PCR-DGGE analysis of samples is a widespread method used to assess the impact of contamination and allows for monitoring reclamation processes (Li *et al*., 2006). From the DGGE profiles of biostimulated microcosms, the bands at day 0 are of a bright intensity, this intensity however decrease at day 7 and 14, only to increase again over days 21 and 28 as evident in the microcosms co-contaminated with arsenic and cadmium. For microcosms co-contaminated with lead the brightness only decreased at day 7 and increased again at day 14, probably due to the lower toxicity of lead. This decrease in brightness could be due to the fact that microcosms undergoing biostimulation have no protection from metal stress, therefore allowing the heavy metal to negatively affect the microbial population during the first 14 days. However, once the glucose is metabolized, the population acclimatizes to the heavy metal. This is indicated by the brightness of DGGE bands. However, in microcosms that were dual-bioaugmented and bioaugmented, there was no decrease from day 0, except for bioaugmented arsenic microcosms. Additional bands not present at day 0 were later observed throughout the experiment, possibly due to the heavy metal resistant strain offering protection to the organic degrading population. Biostimulated microcosms at day 0 displayed a low Cs value when compared to subsequent days. It was however found that the Cs value between day 0 and day 28 was higher when comparing day 0 to any other day. This was true for arsenic and cadmium and could be due to the microorganisms being biostimulated by the glucose and flourishing. For lead biostimulated microcosms the Sorenson's index displayed 100% similarity between the DGGE profiles from day 7 to day 28 indicating no change in diversity. There was a change in diversity from day 0 to day 7, with the profiles exhibiting a lower Sorenson index between these two days. For bioaugmentation and dual bioaugmentation the Sorenson's index displayed 100% similarity between the DGGE profiles from day 7 to day 28 indicating no change in diversity. There was a change in diversity from day 0 to day 7, with the profiles exhibiting a lower Sorenson index between these two days. This indicated that there

is a shift in community diversity from day 0 to day 7, however once the microcosms acclimatises to the treatment, no change was observed.

In general, PCR-DGGE profiles did exhibit a change in community structure during the time course of the degradation process, whereby the number of bands was reduced, the intensity of certain bands increased, and new bands appeared. Microorganisms identified from bands excised from DGGE gels were identified as strains of the genera; *Clostridium*, *Klebesilla*, *Dechloromonas, Cupriavidus* and *Enterobacter*. These organisms except for *Enterobacteriae* were not detected by plate count assay. This difference in isolates obtained in pure culture on plates and the isolates identified by culture independent methods further highlight the bias known as the ‘‘great plate anomaly’’ (Staley and Konopka, 1985). Previous studies have shown that there are variances between the bacteria isolated in the laboratory and the bacteria involved in biodegradation *in situ* on a biological level (Watanabe *et al*., 1998). Microorganisms from the genera stated above have been previously implicated in the anaerobic biodegradation of chlorinated compounds. This could be an indication that as time elapsed, the oxygen in the co-contaminated microcosms became depleted resulting in an anaerobic environment. 1,2-DCA is utilized as primary growth substrates by aerobic microorganisms but only co-metabolized by anaerobic microorganisms (Field and Sierra-Alvarez, 2004). Strains of *K. oxytoca* and *K. pneumonia* have been previously involved in the degradation of chlorinated compounds under both aerobic and anaerobic conditions (Kerr and Marchesi, 2006). *Klebsiella* strains have been previously implicated in the facultative anaerobic degradation of tetrachlorinated phenols (Hakulinen *et al*., 1985). In more recent studies, Mileva and Beschkov (2006) have found that *K. oxytoca* VA 8391 was capable of complete mineralization of 1,2- DCA and Mileva *et al*. (2008) found that immobilizing this strain onto granulated activated carbon increased the degradation rate of 1,2-DCA. Other chlorinated compounds that *Klebsiella* can degrade are 4-chloroaniline (Vangnai and Petchkroh, 2007), monochlorinated dibenzofuran and dibenzo-p-dioxin (Fukuda *et al*., 2002). The diversity of hydrocarbon-degrading *Klebsiella* sp. is further described by Rodrigues *et al*. (2009).

Numerous bacterial species that are able to reductively dechlorinate compounds utilise hydrogen as a primary hydrogen donor and for many of these organisms fermentative microorganisms are a source of hydrogen (Maymo-Gatell *et al*., 1999). Bowman *et al*. (2009) found that in the presence of chlorinated compounds such as 1,2- DCA, TCA, and PCE,

Clostridium sp. are capable of producing hydrogen. Although none of the strains biotransformed 1,2-DCA, *Clostridium* could indirectly facilitate biodegradation by producing hydrogen even in the presence of high concentrations of 1.2-DCA (29.7 mM). *Cupriavidus necator* JMP134 has been deemed a model for chloroaromatics biodegradation by P´erez-Pantoja *et al*. (2008) because of its ability to mineralize 2,4-D, halobenzoates, chlorophenols and nitrophenols, among other aromatic compounds. *Dechloromonas* is considered a facultative anaerobe (Salinero *et al*., 2009) due to its ability to anaerobically degrade chlorobenzoate (Coates *et al*., 2001) and reduce perchlorate (Chakraborty *et al*., 2005).

Overall, results from this study suggest that biostimulation and bioaugmentation are important for effective remediation of water co-contaminated with 1,2-DCA and heavy metals. In particular, dual-bioaugmentation seemed to be the most promising bioremediation technique. However, it should be taken into consideration that any bioremediation approach is site specific and the ecology of the site and the local physico-chemical constraints should be taken into consideration.

CHAPTER FOUR

BIOSORPTION OF HEAVY METALS USING AGRICULTURAL WASTES FOR ENHANCED 1,2-DCA DEGRADATION IN CO-CONTAMINATED WASTEWATER

4.1 Introduction

Present day industries are, to a large extent, liable for environmental pollution, as lakes, rivers and oceans are being beleaguered with detrimental pollutants (Regine and Volesky, 2000). Amongst toxic substances escalating to hazardous levels are heavy metals. Raw wastewater contains high concentrations of heavy metals, which are impervious to degradation by orthodox processes of wastewater treatment (Rajbanshi, 2008). Many organic pollutants are vulnerable to biodegradation; whereas heavy metal ions cannot undergo biodegradation into innocuous end-products (Igwe and Abia, 2007). Despite being a priority pollutant, heavy metals are still being disseminated into the environment, owing to their use in the manufacturing of a myriad of industrial products. For instance, arsenic is employed in the manufacturing of insecticides, doping agents in semiconductor manufacturing and in lead based alloys to enhance hardening, while lead and its salts and oxides are used in paints and pigments, battery industries and lead smelter (Özcan *et al*., 2009). In the environment, lead binds strongly to particles such as oil, sediments and sewage sludge so its removal is of great concern (Sud *et al*., 2008). Cadmium is introduced into water from metal mines mining, melting, plating, cadmium nickel batteries, pesticide, oil paint, pigments, alloy and sewage sludge (Li *et al*., 2007). Mercury is one of the most toxic elements in the environment and its levels in water bodies and soils have been raised by metal mining, fossil combustion and the chloralkali and acetaldehyde industries (Rojas *et al*., 2011).

In addition to heavy metals being detrimental to the environment, metals such as arsenic, cadmium, chromium, copper, lead, mercury, nickel, and zinc have been found to be noxious to many microorganisms involved in biodegradation of a wide array of organic compunds (Sandrin and Maier, 2003; Chatain *et al*., 2004; Fernandes *et al*. 2009). The presence of heavy metals may impede biodegradation by either inhibiting enzymes involved in biodegradation or the enzymes involved in microbial metabolism, thus compounding the problem of organic contamination (Kuo and Genthner, 1996). Hence, efficient bioremediation-based strategies for metal and organic co-contaminated environments must focus on two key issues: (1) limited organic

pollutant solubility and bioavailability and (2) toxic metals that inhibit biodegrading microorganisms (Hoffman *et al*., 2010).

The conventional methods for heavy metal removal from wastewater incorporates reduction, precipitation, ion exchange, reaction with silica, electrochemical reduction, evaporation, reverse osmosis and direct precipitation (Iqbal *et al*., 2009). The most frequent problems faced with these conventional treatment methods include high initial capital costs, high maintenance and generation of huge quantities of toxic chemical sludge (O'Connell *et al*., 2008). This has warranted research into the possible use of non-conventional agricultural by-products and adsorbents of biological origin. Biosorption is a process in which solids of natural origin are employed for binding the heavy metals (Igwe *et al*., 2008). The major interaction mechanisms are based on ionic interactions and complex formation between metal cations and ligands contained in biomaterials (Stefan *et al*., 2006). The use of biological materials as biosorbent has been thought-provoking due to the diversity of sorption sites available for metallic ions uptake; high selectivity for metallic ions, and their different chemical forms (Gonzalez *et al*., 2008). In addition, they are biodegradable and renewable and require little processing. Agriculture, forestry and fisheries have been generating large quantities of various biomass wastes and some of them contain various natural materials with interesting functional groups for heavy metal binding, such as carboxyl, hydroxyl and amidocyanogen (Li *et al*., 2007).

Orange peel (OP) is an agricultural byproduct and can be freely obtained from soft drink industries where it is treated as unsalvageable waste. Coconut fibre (CNF) is an abundant natural material, which basically contains cellulose and lignin. After the separation of the fibre from the coconut husk, the CNF has no use and is recognized as waste in the agricultural or industrial sector. Any attempt to reuse the CNF will be worthwhile (Anirudhan *et al*., 2008). Maize is a staple cereal in South Africa and therefore produces large volumes of waste [corncobs (CC)]. CCs are rich in cellulose and can be converted into metal ion adsorbents for wastewater treatment (Stefan *et al*., 2006). The aim of this chapter was to assess OP, CC and CNF, as biosorption agents for the removal of arsenic, cadmium, mercury and lead from wastewater. The impact of biosorption of the heavy metals by these agricultural wastes on the biodegradation of 1,2-DCA in the co-contaminated water was also evaluated.

4.2 Materials and Methods

4.2.1 Preparation of biosorbents from agricultural waste

4.2.1.1 Orange peel

The OP was washed three times with double distilled water to remove extraneous materials and then dried at 37 °C in an incubator for 16 h. This material was then ground to a particle size of 0.8 and 1 mm (Schiewer and Patil, 2008). The ground particles were then dried in an incubator at 37 °C for 16 h and were used as the biosorbent.

4.2.1.2 Corncobs

Corncobs (CC) were washed with double distilled water, dried at 37 °C in an incubator for 16 h and milled to a 0.8-1 mm particle size. The ground particles were then soaked in 2% (v/v) dilute nitric acid for 18 h. Thereafter, the CC was rinsed twice with doubled distilled water and dried in an incubator at 37 °C for 16 h (Abia and Igwe, 2005). The dried CC was used as the biosorbent.

4.2.1.3 Coconut fibre

The fibrous part of the coconut, i.e. the coconut fibre (CNF), was removed washed with deionized water, cut into small pieces, air dried and thereafter milled to a particle size of 0.8-1 mm. The ground particles were then soaked in 2% (v/v) dilute nitric acid for 18 h. Thereafter the CNF was rinsed twice with double distilled water and dried for 16 h in an incubator at 37 °C (Igwe and Abia, 2007). The dried CNF was used as the biosorbent.

4.2.2 Determination of the optimum biosorbent concentration for heavy metal removal

Each of the three biosorbents (OP, CNF and CC) was separately added to a 100 ml solution containing 2 mM of each respective heavy metal (arsenic, cadmium, mercury or lead) at the following concentrations (w/v): 0.5, 1, 1.5, 2 and 2.5%. Initial heavy metal concentrations and concentrations after 2 days were obtained by filtering the samples rapidly and determining the metal content of the filtrates using ICP-OES as described in chapter 2 (section 2.2.5). The

final metal uptake (qt) in mg per gram of biosorbent material was determined by employing the mass balance (Benaïssa, 2005) according to the equation:

$$qt = \frac{V(C_0 - C_t)}{M}$$

where C_0 and C_t are the initial and final metal concentration ($mg.l^{-1}$) of heavy metal respectively, V is the suspension volume (l) and m is the mass (g) of biosorbent material used.

4.2.3 Biosorption of heavy metals and 1,2-DCA biodegradation

Wastewater was collected from NWW and NGWW treatment plants and characterized as described in chapters 2 and 3. Microcosms were set up with 150 ml of wastewater, 1,2-DCA at a concentration of 2.5 mM and 1 mM of heavy metal (arsenic, cadmium, mercury or lead). Biosorbents chosen based on the metal uptake results (section 4.2.2) were OP and CNF. The two biosorbents were added separately to the microcosms at a pre-determined optimum concentration of 2% (w/v). Microcosms consisting of unautoclaved wastewater and 1,2-DCA served as the positive control and microcosms containing autoclaved wastewater and 1,2-DCA served as the negative control. Microcosms consisting of unautoclaved wastewater, 1,2-DCA and the heavy metal served as the untreated control. 1,2-DCA concentrations was monitored weekly as described in chapter three using gas chromatographic analysis of headspace samples. Heavy metal concentrations were monitored at days 1, 4, 14 and 28 using ICP-OES.

4.2.4. Visualization of heavy metal adsorption onto the biosorbents using a scanning electron microscope (SEM)

To assess the attachment of the heavy metals to the biosorbents, the SEM images of the biosorbents before and after heavy metal uptake were obtained following a modified protocol of Oliveira *et al*. (2008). The samples (OP or CNF) were dispersed on double-faced conducting tape fixed on a graphite support. A sputter coater then covered the sample with a conductive film of carbon. A LEO SEM electronic microscope was used to capture the images using both the SE1 and RSBD detectors.

4.3 Results

4.3.1 Biosorption potential of agricultural waste

The profiles of heavy metal removal by the different concentrations of biosorbent are represented by Figure 4.1. The concentration of each biosorbent was varied from 0-2.5%, while heavy metal concentration was fixed at 2 mM. An increase in the heavy metal removal was observed as the biosorbent (OP, CNF and CC) concentration increased from 0 to 2.5%, with the highest heavy metal uptake obtained at a biosorbent concentration of 2.5%. The maximum adsorption capacity was found to be the highest for Pb^{2+} followed by Cd^{2+}, Hg^{2+} and As^{3+} for CC and OP, while the heavy metal that was adsorbed the most by CNF was Pb^{2+}, followed by Hg^{2+} , Cd^{2+}, then As^{3+} (Figure 4.1 A-C). OP was found to be the most efficient biosorbent removing up to 97.45% lead, compared to CNF (67%) and CC (27.63%) at the same biosorption concentration. The amount of heavy metal adsorbed per unit biomass (Qt) was determined and the values presented in Table 4.1. Generally, as the concentration of biosorbent increased, the amount of heavy metal adsorbed per unit biomass decreased. This trend was evident for Cd^{2+}, Hg^{2+} and Pb^{2+} adsorbed by CNF and OP and cadmium adsorbed by CC. However, this trend was not consistent when low concentrations of heavy metals were adsorbed. The Qt did not decrease with increase in concentration of biosorbent, when Cd^{2+}, Hg^{2+} and Pb^{2+} were adsorbed by CC, and also when As^{3+} was adsorbed by all biosorbents. Overall, OP displayed the highest Qt, with values ranging from 0.96–36.77 mg of heavy metal per gram of biosorbent, followed by CNF and CC with Qt values ranging from 0.39 -21.48 $mg.g^{-1}$ to 0.54 – 5.22 $mg.g^{-1}$, respectively.

A clear trend follows such that percentage heavy metal removal increased with an increase in biosorbent dosage and Qt decreased with increasing concentration of biomass. Since CC proved to be the least efficient in removing all four heavy metals, it was not used in further experiments, hence only OP and CNF was utilized in further biodegradation experiments.

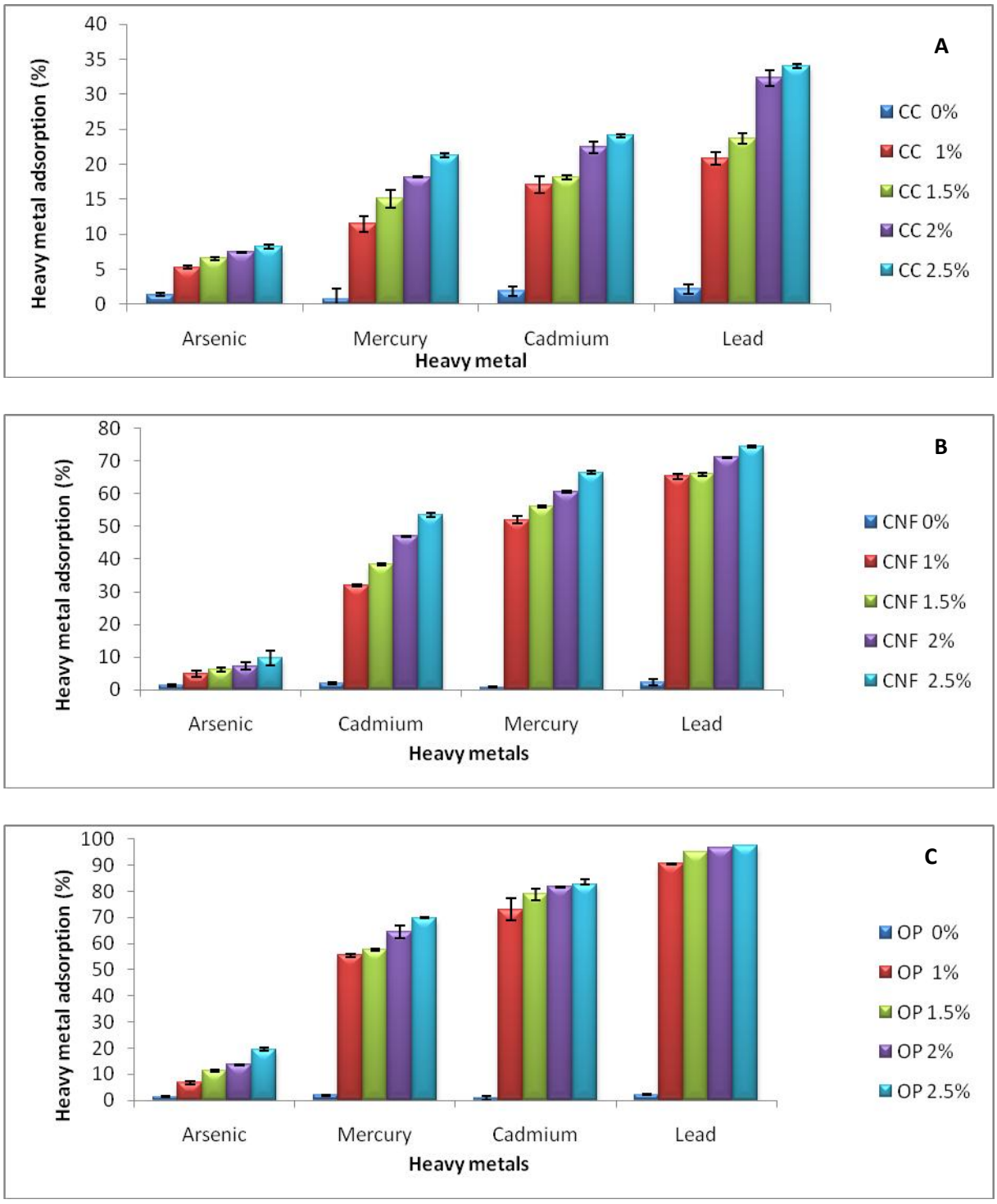


Figure 4.1: Biosorption of heavy metals by varying concentrations of CC (A), CNF (B) and OP (C). Values represent the average of triplicate samples while error bars represent the standard deviation.

Table 4.1: Heavy metal uptake ($mg.g^{-1}$ of biosorbent) at varying biosorbent concentrations.

Heavy metal	Concentration of biosorbent (%)	CNF[a]	OP[b]	CC[c]
Arsenic	1	0.39 ± 0.31	0.96 ± 0.18	0.54 ± 0.07
	1.5	0.56 ± 0.13	1.58 ± 0.09	0.60 ± 0.04
	2	0.56 ± 0.15	1.52 ± 0.23	0.59 ± 0.01
	2.5	0.74 ± 0.27	1.88 ± 0.09	0.57 ± 0.03
Cadmium	1	4.94 ± 0.21	14.21 ± 0.12	1.62 ± 0.26
	1.5	3.76 ± 0.14	10.33 ± 0.05	1.24 ± 0.05
	2	3.07 ± 0.01	8.06 ± 0.01	1.13 ± 0.03
	2.5	2.56 ± 0.04	6.53 ± 0.04	1.04 ± 0.02
Mercury	1	14.17 ± 0.15	10.73 ± 1.74	2.83 ± 0.24
	1.5	9.67 ± 0.12	7.71 ± 0.61	2.64 ± 0.20
	2	8.32 ± 0.02	7.17 ± 0.49	2.36 ± 0.01
	2.5	7.19 ± 0.11	6.58 ± 0.31	2.45 ± 0.04
Lead	1	21.48 ± 0.43	36.77 ± 0.12	5.22 ± 0.45
	1.5	15.37 ± 0.08	25.79 ± 0.01	4.44 ± 0.32
	2	12.45 ± 0.08	19.65 ± 0.05	3.95 ± 0.02
	2.5	10.92 ± 0.07	15.87 ± 0.01	4.09 ± 0.20

Values are averages of triplicate data ± standard deviation. a= coconut fibre; b= orange peel; c=corncobs

4.3.2 Wastewater characterization

The physico-chemical properties of both wastewater types used in this study are listed in Table 4.2. The elements calcium, magnesium, potassium were 52.53, 20.31 and 8.44% higher in NGWW compared to NWW, however, sodium and iron were 42.65 and 74.47% higher in NWW than in NGWW. The phosphate, nitrate, sulphate, total Kjeldahl nitrogen and total organic carbon levels in NGWW was 25.27, 32.03, 56.35, 50 and 40.70% higher than in NWW, with only nitrite levels being the same in both wastewater types.

Table 4.2: Physico-chemical properties of the wastewater samples used in this study.

Determinant	**NGWW**	**NWW**
Calcium (mg Ca.l^{-1})	22.59	14.81
Magnesium (mg Mg.l^{-1})	6.16	5.12
Sodium (mg Na.l^{-1})	79.86	139.25
Potassium (mg K.l^{-1})	16.44	15.16
Iron (mg Fe.l^{-1})	0.017	0.03
Nitrite (soluble) (mg.l^{-1}) as N	0.15	0.15
Nitrate (soluble) (mg.l^{-1}) as N	7.79	5.90
Sulphate (mg SO_4.l^{-1})	69.95	30.68
Total organic carbon (mg.l^{-1})	12.1	8.6
Orthophosphate (PO_4) mg.l^{-1} as P	4.56	3.64
Total kjeldahl nitrogen (mg.l^{-1})	2.1	1.4

4.3.3 Effect of agricultural biosorbents on the biodegradation of 1,2-DCA in co-contaminated wastewater

The degradation profiles of 1,2-DCA in heavy metal co-contaminated NWW, in the presence and absence of biosorbents (OP and CNF), are represented in Figure 4.2 (A-D). In the absence of heavy metals (positive control), the indigenous microorganisms present in the wastewater readily degraded 1,2-DCA, by up to 83.29% after 28 days. When compared to the positive control, As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} did inhibit 1,2-DCA degradation by

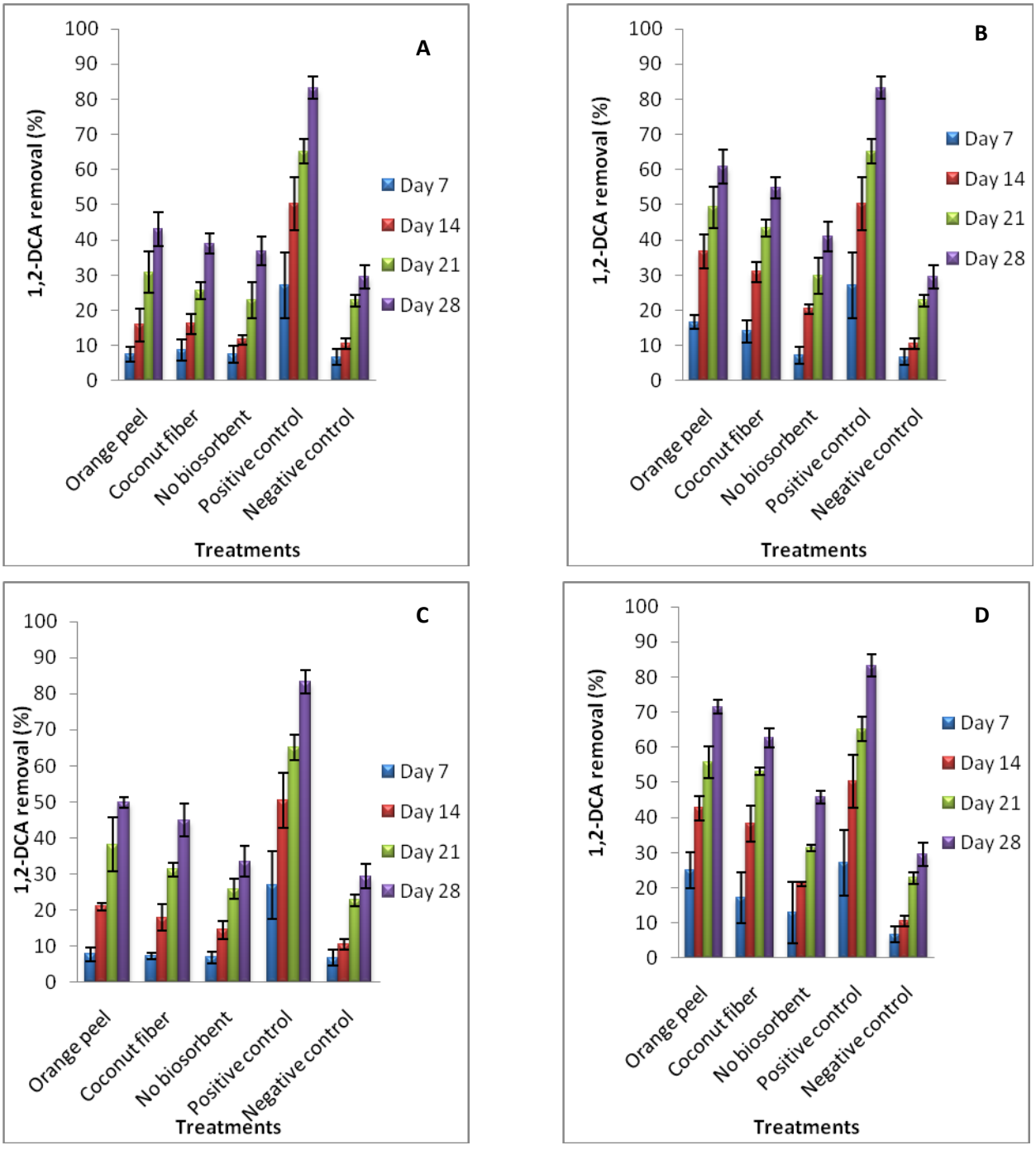


Figure 4.2: The removal profile of 1,2-DCA in NWW, co-contaminated with As^{3+} (A), Cd^{2+} (B), Hg^{2+} (C) and Pb^{2+} (D) in the presence of biosorbents (OP and CNF). Values indicate the average of triplicate values while the error bars show the standard deviation.

46.43, 42.31, 49.75 and 37.48% respectively after 28 days. However, addition of biosorbents did result in an increase in 1,2-DCA removal. In As^{3+} co-contaminated wastewater, there was a 6.85% and 2.11% increase in 1,2-DCA degradation (compared to untreated controls) in the presence of OP and CNF, respectively (Figure 4.2 A). Furthermore, an increased biodegradation of 19.90% and 13.47%, respectively, with OP and CNF as biosorbents in Cd^{2+} co-contaminated wastewater was observed (Figure 4.2 B). For Hg^{2+} co-contaminated wastewater, a 17.29% increase in 1,2-DCA degradation was observed for OP supplemented microcosms, compared to a 10.39% increase when CNF was utilized (Figure 4.2 C). In Pb^{2+} co-contaminated microcosms, up to 25.66% increase in 1,2-DCA degradation was observed in OP supplemented microcosms compared to CNF which displayed a 16.82% increase (Figure 4.2 D). Microcosms supplemented with OP were observed to exhibit a higher 1,2-DCA degradation compared to CNF as evident for all four heavy metals. Microcosms supplemented with OP exhibited up to 4.45, 6.42, 6.9 and 8.84% higher degradation in As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} co-contaminated water respectively when compared to CNF. The heavy metal inhibitory pattern, in the presence of biosorbents, seemed to follow the following pattern in order of decreasing 1,2-DCA degradation was: As^{3+}, Hg^{2+}, Cd^{2+} and Pb^{2+}. The same trend followed for microcosms constructed with NGWW (Figure 4.3 A-D); however degradation was higher when compared to NWW. In the absence of heavy metals (positive control), 88.50% degradation of 1,2-DCA was observed after 28 days, which is 5.21% higher than that in NWW. When compared to the positive control As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} did inhibit 1,2-DCA degradation with up to 44.62, 41.74, 49.75 and 34.42% inhibition respectively after 28 days. Microcosms supplemented with OP were observed to exhibit a higher 1,2-DCA degradation compared to CNF, with up to 5.99, 6.80, 5.92 and 7.69% higher degradation in As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} co-contaminated water, respectively.

As with percentage degradation, the biodegradation rate constant was higher for NGWW compared to NWW. The positive controls for both wastewater treatment plants displayed the highest biodegradation rate constant (k_1) of 0.062 day^{-1} (for NWW) and 0.074 day^{-1} (for NGWW) as shown in Table 4.3. However, addition of heavy metals did result in a decrease in k_1 when compared to the positive control. Nevertheless when comparing the untreated positive control to microcosms supplemented with biosorbents, there was an increase in k_1 for all treatments. The addition of OP did result in a higher k_1 compared to the addition of CNF. The k_1 ranged from

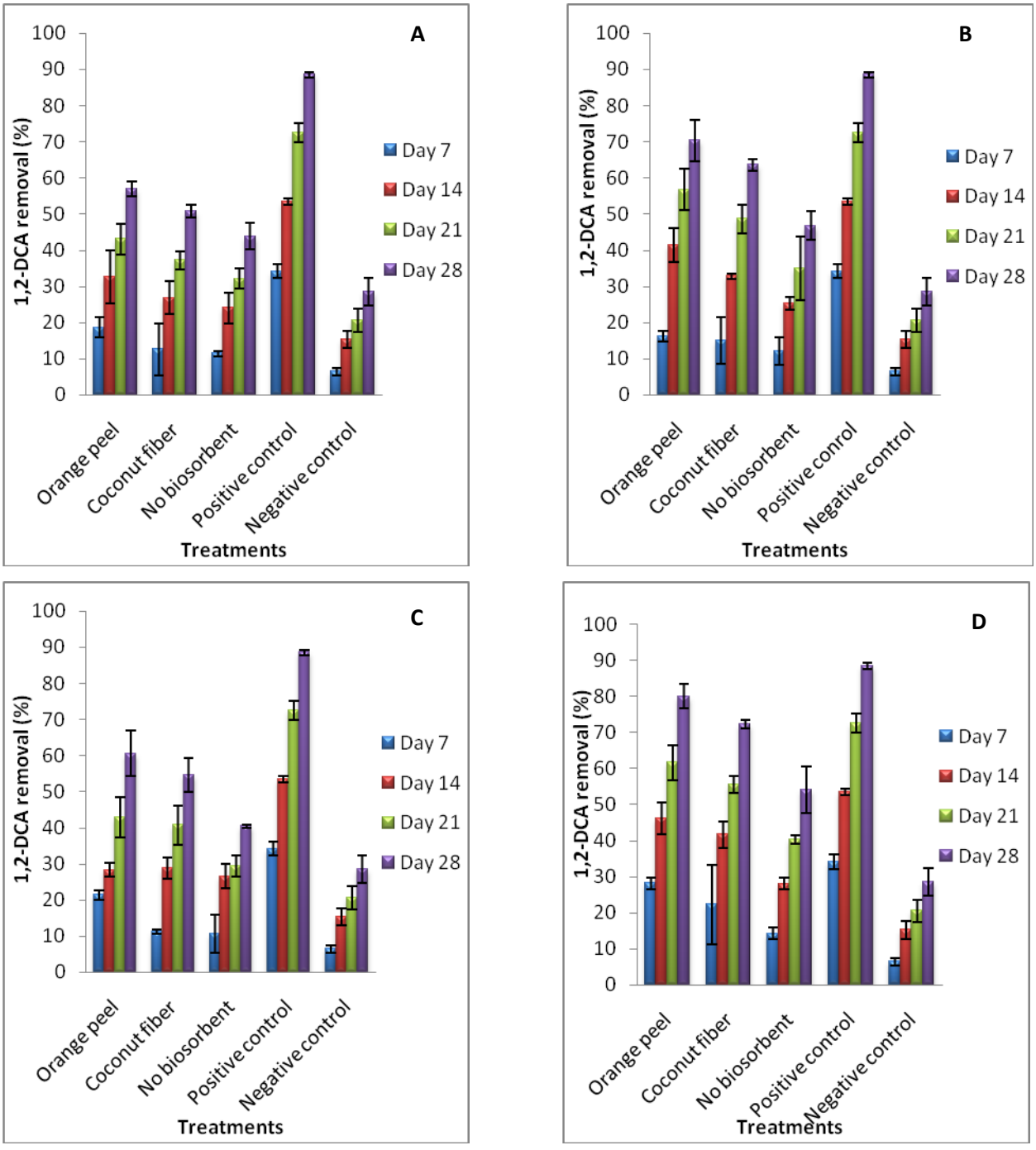


Figure 4.3: The removal profile of 1,2-DCA in NGWW, co-contaminated with As^{3+} (A), Cd^{2+} (B), Hg^{2+} (C) and Pb^{2+} (D) in the presence of biosorbents (orange peel and coconut fibre). Values indicate the average of triplicate values while the error bars show the standard deviation.

Table 4.3: Biodegradation rate constants (day^{-1}) of 1,2-DCA in wastewater co-contaminated with heavy metals, in the presence and absence of biosorbents.

Treatment	Northern wastewater	New Germany wastewater
Positive control	0.062 ± 0.004	0.074 ± 0.0046
Negative control	0.012 ± 0.0021	0.012 ± 0.0057
Orange peel		
As^{3+}	0.020 ± 0.0032	0.029 ± 0.005
Cd^{2+}	0.034 ± 0.0017	0.044 ± 0.0023
Hg^{2+}	0.026 ± 0.003	0.031 ± 0.0021
Pb^{2+}	0.043 ± 0.002	0.055 ± 0.0026
Coconut fibre		
As^{3+}	0.016 ± 0	0.025 ± 0.0025
Cd^{2+}	0.028 ± 0.011	0.036 ± 0.014
Hg^{2+}	0.021 ± 0.0023	0.027 ± 0.0061
Pb^{2+}	0.036 ± 0.0021	0.044 ± 0.0046
No biosorbent		
As^{3+}	0.015 ± 0.0031	0.019 ± 0.002
Cd^{2+}	0.019 ± 0.0036	0.022 ± 0.006
Hg^{2+}	0.014 ± 0.00058	0.018 ± 0.0031
Pb^{2+}	0.020 ± 0.00058	0.027 ± 0.0042

Values are averages of triplicate data ± standard deviation

0.020 - 0.043 day^{-1} for NWW and 0.029-0.055 day^{-1} for NGWW when OP was added, however the k_1 ranged from 0.016 – 0.036 $day^{-1}$for NWW and 0.025 – 0.044 day^{-1} in NGWW, when CNF was added. This further indicates that degradation occurred at a faster rate when OP was added, compared to CNF and also that degradation did occur at a faster rate in NGWW than NWW. For both wastewater types (NWW and NGWW), both treatment additives (OP and CNF) effects were more pronounced in the Pb^{2+} co-contaminated water, with the highest 1,2-DCA degradation rate constant obtained followed by Cd^{2+}, Hg^{2+} and As^{3+}. However, when no biosorbents were added, microcosms co-contaminated with Pb^{2+}, exhibited highest 1,2-DCA degradation rate constant followed by Cd^{2+}, As^{3+} and lastly Hg^{2+}.

4.3.4 Removal of heavy metals from co-contaminated microcosms

The heavy metal uptake by OP (Figure 4.4 A) and CNF (Figure 4.4 B) in NWW revealed that most of the heavy metals was adsorbed by day 1, with minor concentrations adsorbed subsequently. At day 28, 14.59, 74.79, 60.79 and 87.53% of As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+}, respectively was adsorbed by OP (Figure 4.4 A) whereas 10.03, 40.29, 68.47 and 70.00% As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} respectively was adsorbed by CNF (Figure 4.4 B). These values correspond to only a 6.89, 5.85, 3.15 and 3.81% increase in As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} removal respectively by OP and a 6.93, 11.43, 3.27 and 4.04% increase in As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} removal respectively by CNF when compared to day 1. Similarly in NGWW, at day 1, 19.59, 82.47, 69.73 and 97.28% of As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} respectively was adsorbed by OP (A) whereas 17.75, 49.29, 75.22 and 77.36% of As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+}, respectively was adsorbed by CNF (A). At day 28, however, there was only a 6.18, 3.60, 4.35 and 2.38% increase from day 1 in As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} removal respectively by OP and a 8.48, 7.45, 5.43, 5.30% As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} removal compared to day 1 by CNF. The heavy metal that was removed the most by coconut fibre was Pb^{2+} followed by Hg^{2+}, Cd^{2+}, and As^{3+}. The order of heavy metal removal by orange peel is as follows Pb^{2+}> Cd^{2+}> Hg^{2+}> As^{3+}.

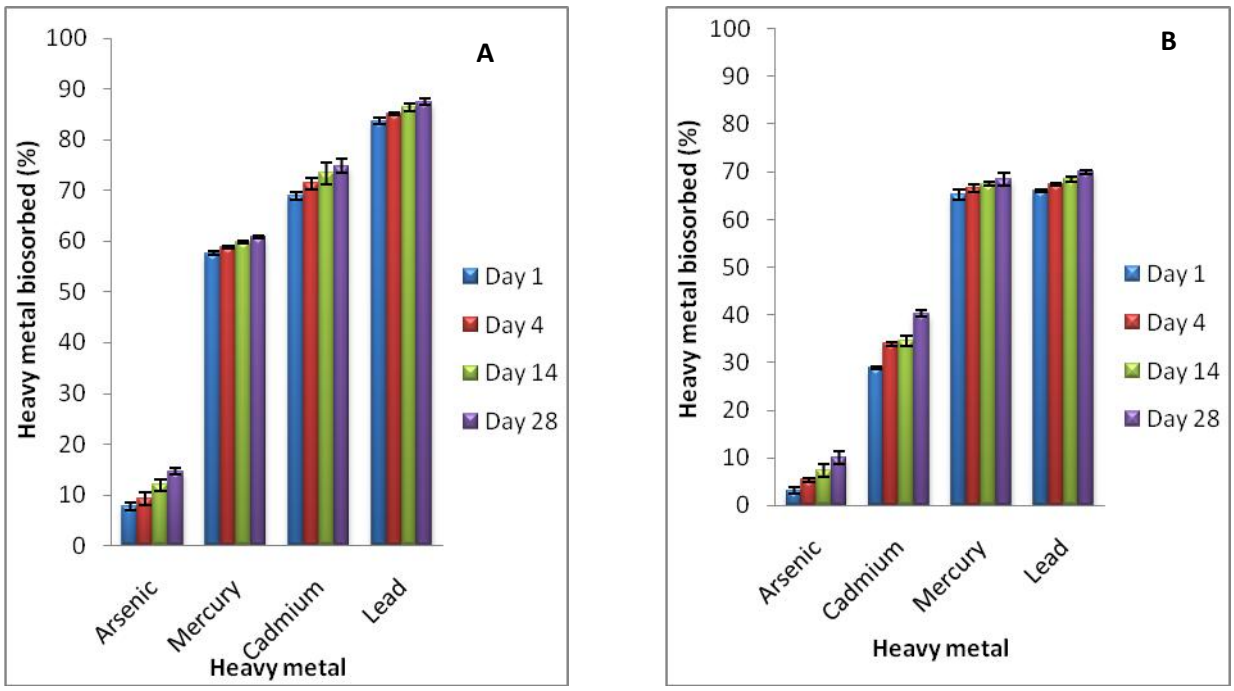


Figure 4.4: Time course heavy metal removal by OP (A) and CNF (B) in NWW. Values represent the average of triplicate samples while error bars represents the standard deviation.

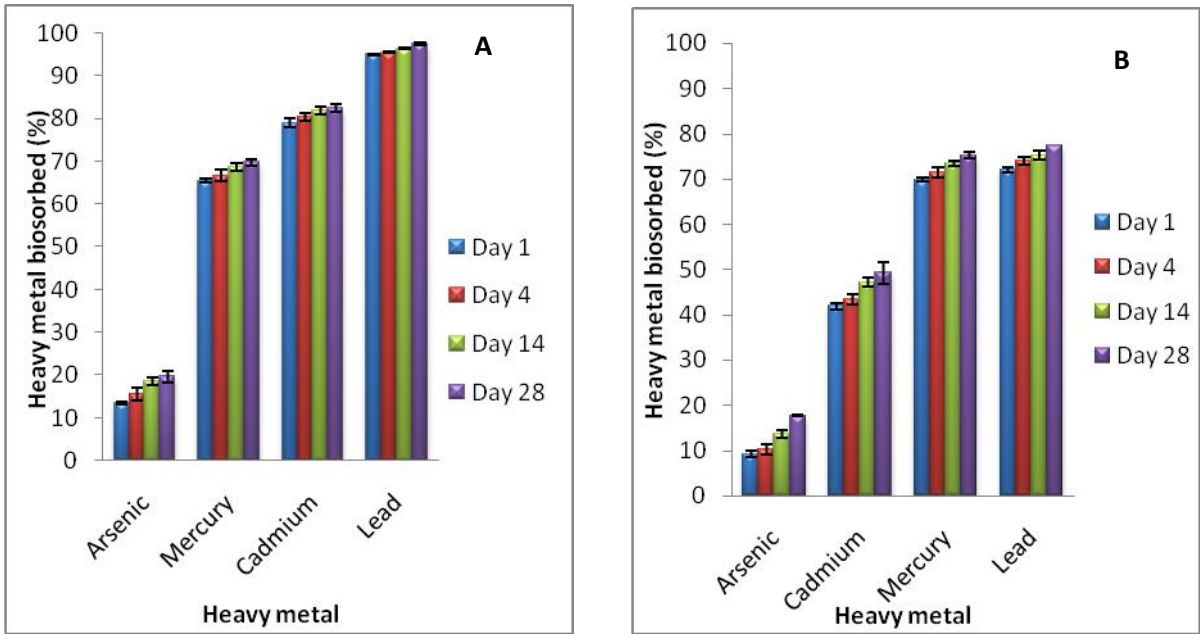
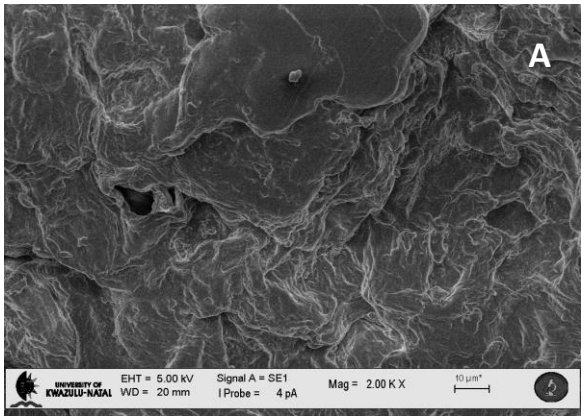
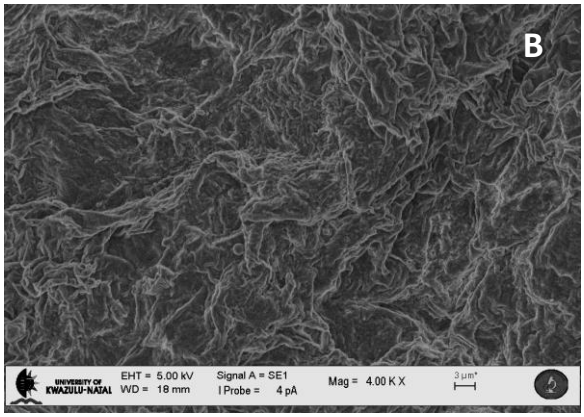


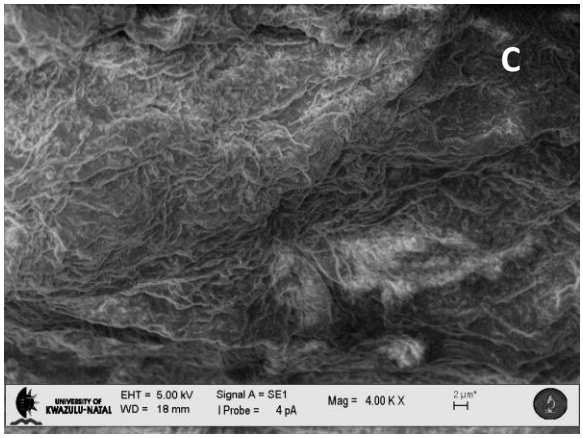
Figure 4.5: Time course heavy metal removal by OP (A) and CNF (B) in NGWW. Values represent the average of triplicate samples while error bars represent the standard deviation.

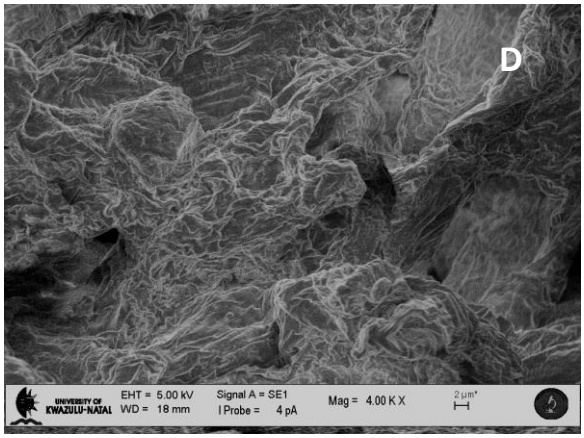
4.3.5 Visualization of heavy metal adsorption onto the biosorbents using a scanning electron microscope

Scanning electron microscopy (SEM) was used to examine the surface of the two adsorbents before and after biosorption as illustrated in Figures 4.6-4.9. The scanning electron micrographs of the biosorbents clearly revealed the surface texture and morphology of both biosorbents. Two different types of detectors, SEI and backscatter (RBSD) were utilized. Using the SE1 detector, the surface of OP in its native state (i.e. before biosorption) is represented in Figure 4.6 (A). The surface morphology of OP was found to be irregular and porous. After biosorption of heavy metals, the surface of the biosorbent seemed to resemble a network of interconnected veins. This was found to occur for all heavy metals adsorbed on OP. Figure 4.7 illustrates the surface of CNF before and after adsorption of heavy metals; Figure 4.7 (A) represents CNF in its native state. It can be seen that the surface of the native CNF is smooth. This however is altered after complexation with heavy metals as seen in Figure 4.7 (B-E). Again as for heavy metal complexed OP, an interconnected network of veins (highlighted by white boxes) is formed. The backscatter detector (RBSD) was used to show the uptake of heavy metals on the biosorbents. Only low magnifications (100x) could be used with the RBSD detector so as not to compromise resolution. Figure 4.8 (A) demonstrates the OP in its native state since no heavy metals are added and there is no backscatter, while Figure 4.8 (B-C) illustrates the OP with bright areas. A similar result was observed for the CNF. Figure 4.9 (A) shows no backscatter since no heavy metals was added and Figure 4.9 (B-E) showed areas of light (heavy metal) against a dark background (CNF). For both biosorbents, it was observed that As^{3+} and Cd^{2+} appear to be adsorbed towards the center of the biosorbent (Figure 4.8: B-C; Figure 4.9: B-C), Hg^{2+} seemed to be adsorbed towards the center as well but also in a scattered pattern on the biosorbent (Figures 4.8D and 4.9D) and Pb^{2+} seemed to be adsorbed toward the edges of the biosorbent (Figures 4.8E and 4.9E). The RSBD also allowed for the visualization of the irregular structure of the biosorbent. The surface texture of both the biosorbents seemed to be porous with holes and small openings on the surface.









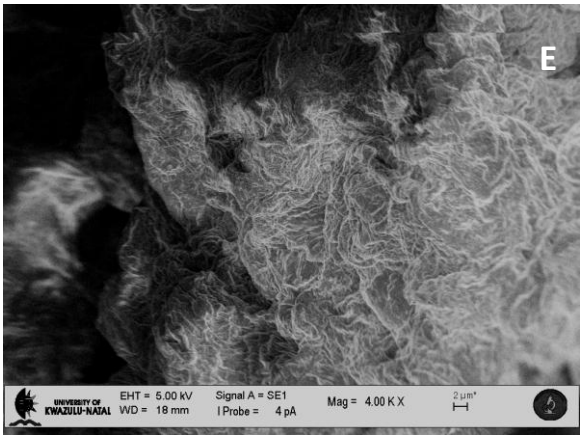


Figure 4.6: Scanning Electron Micrographs of orange peel before heavy metal adsorption (A) (2000x magnification) and after adsorption of arsenic (B), cadmium (C), mercury (D) and lead (E) at 4000x magnification.

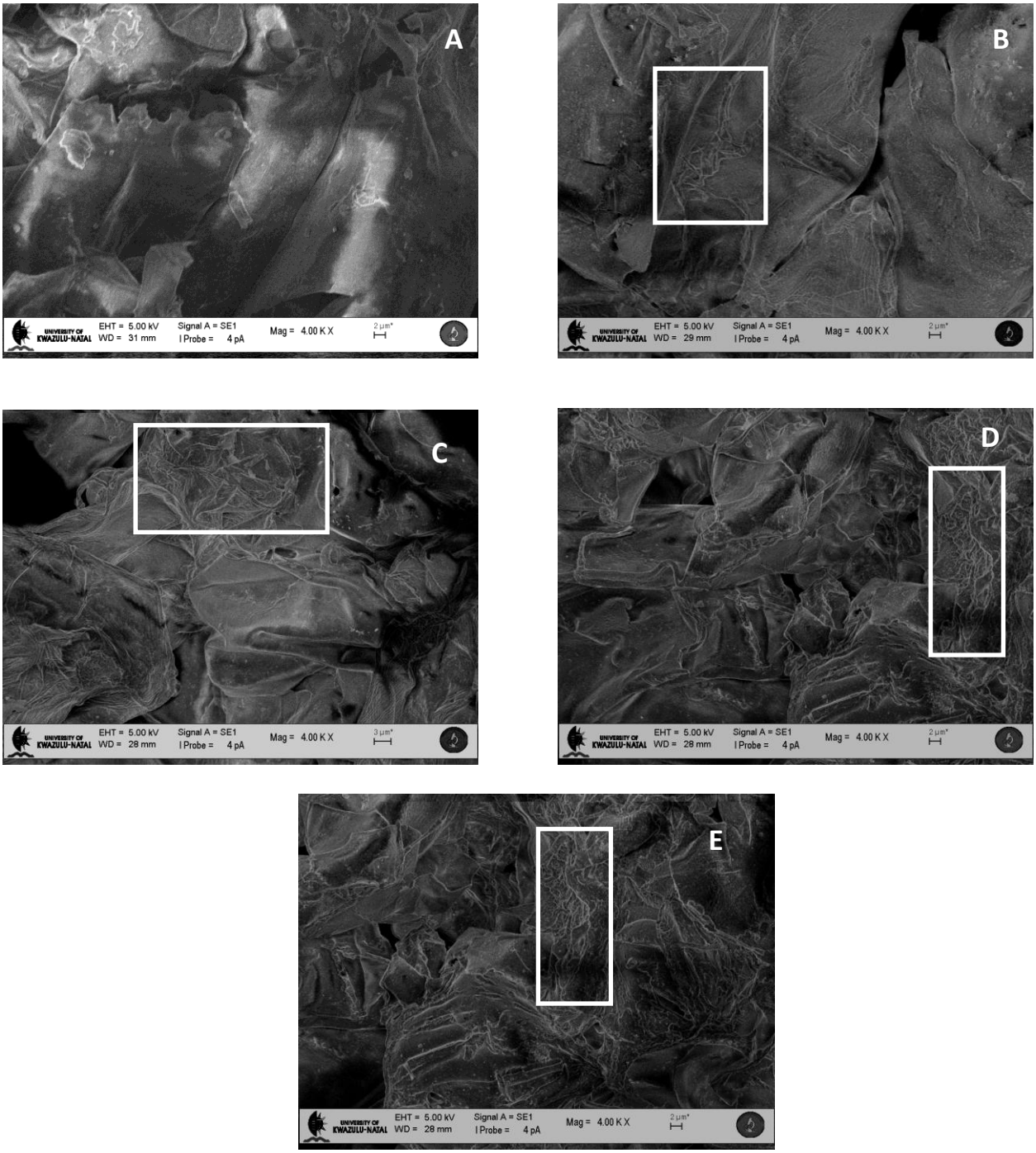


Figure 4.7: Scanning Electron Micrographs of CNF before heavy metal adsorption (A) and after adsorption of arsenic (B), cadmium (C), mercury (D) and lead (E) at 4000x magnification. White outlined blocks highlights the heavy metal network.

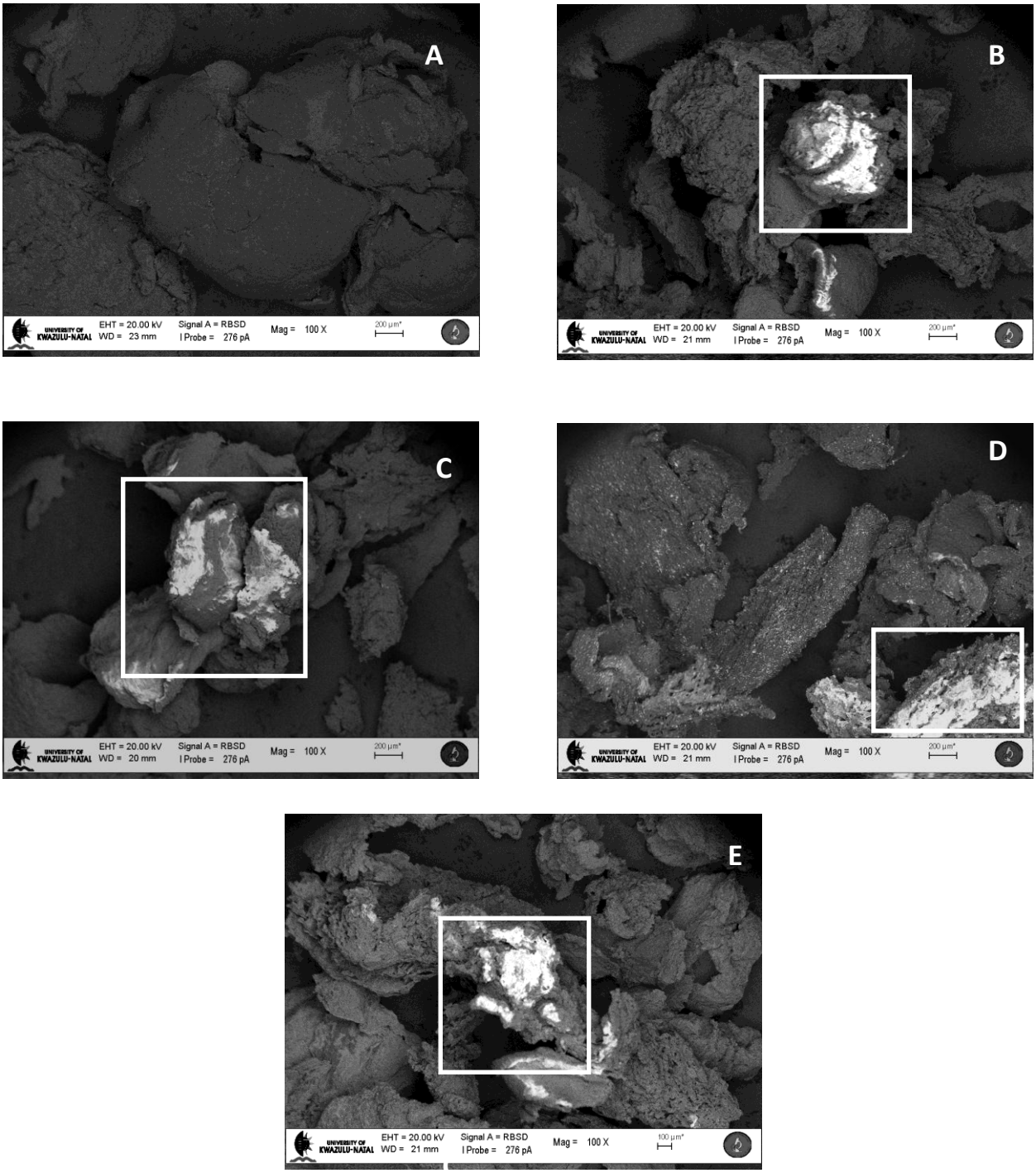


Figure 4.8: Scanning Electron Micrographs (using a backscatter detector, RSBD) of OP before heavy metal adsorption (A) and after the adsorption of arsenic (B), cadmium(C), mercury (D) and lead (E) at 100x magnification. Heavy metal backscatter is highlighted by white outlined boxes.

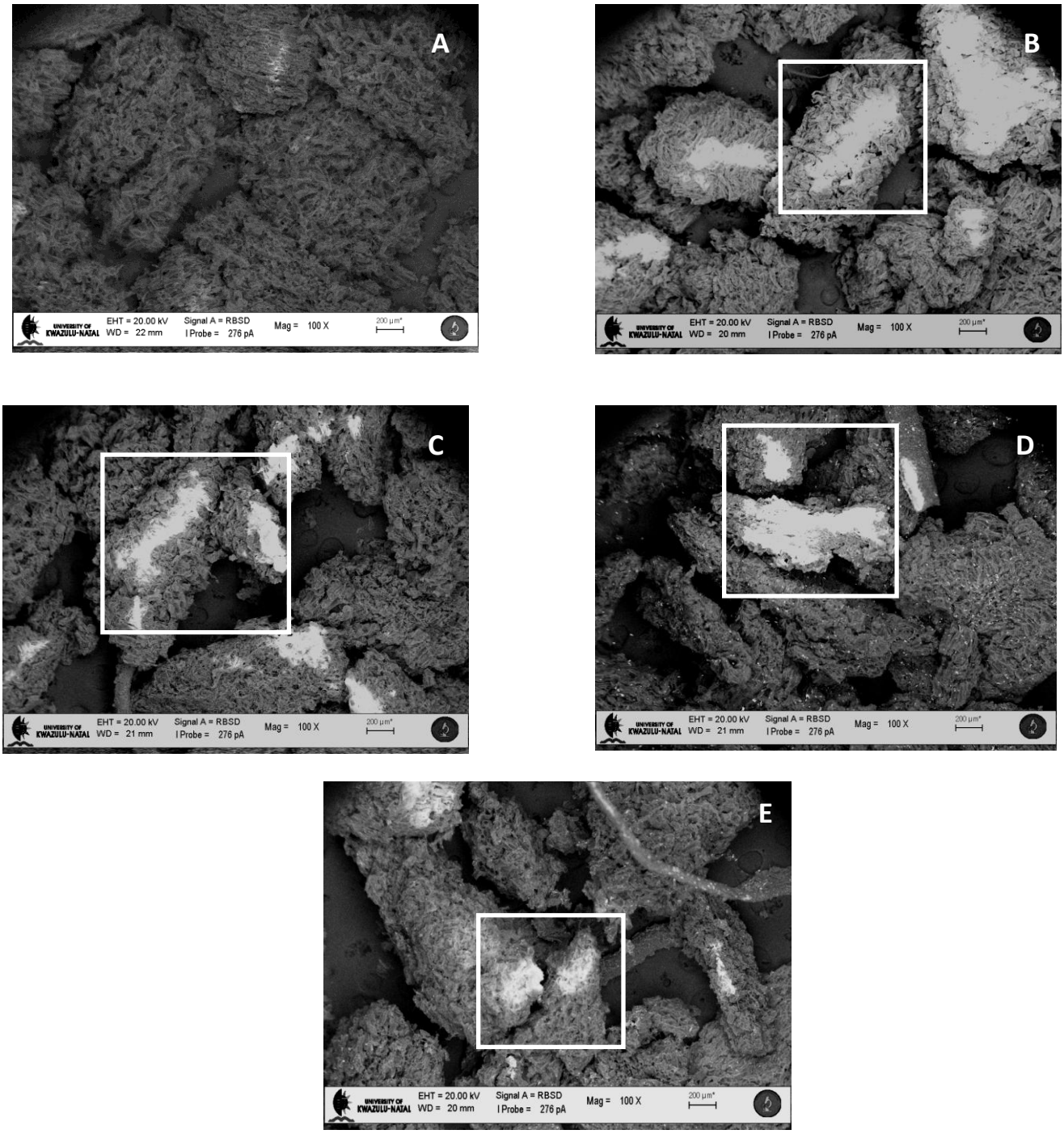


Figure 4.9: Scanning Electron Micrographs (using a backscatter detector, RSBD) of CNF before heavy metal adsorption (A) and after the adsorption of arsenic (B), cadmium(C), mercury (D) and lead (E) at 100x magnification. Heavy metal backscatter is highlighted by white outlined boxes.

4.4 Discussion

When choosing the most efficient biosorbent, many factors such as the size and the concentration of the biosorbent, as well as the contact time between the biosorbent and the heavy metal need to be considered. The particle size of 0.8-1 mm of biosorbents used in this study is similar to that previously used for CC to adsorb cadmium, zinc and lead by Abia and Igwe (2005) and was found to be efficient. Biosorbent concentration of 2% (w/v) used in this study proved to be effective for the removal of heavy metals. For example 2% (w/v) of OP, CNF and CC were able to remove 96.53, 71.19 and 32.36% of Pb^{2+} respectively. Remarkable increases in heavy metal adsorbtion were obtained when the biosorbent concentration was increased from 1 to 2%. The ensuing effect can be easily explained by an increase in surface area, allowing for more availability of active adsorption sites with the increase in biosorbent mass (Oliveira *et al*., 2008). Slight increases in heavy metal removal of 0.95, 3.24 and 1.71% for OP, CNF and CC, respectively, were obtained at 2.5% concentration when compared to 2% (Figure 4.1). This suggests that after a certain dose of adsorbent, the maximum adsorption is accomplished and hence the amount of ions bound to the adsorbent and the amount of free ions remains constant even with further addition of the adsorbent (Abdel-Ghani *et al*., 2007). Biosorbent concentration of 2% has previously been reported for effective remediation of heavy metals from aqueous solutions. Igwe and Abia (2007) reported the use of 2% of CNF for the removal of arsenic, similarly 2% of coconut fibre was used to remove lead, mercury and arsenic from an aqueous solution (Igwe *et al*., 2008). Abia and Igwe (2005) also used a 2% concentration of corncobs to remove cadmium, zinc and lead from an aqueous solution. The amount of heavy metal adsorbed per unit mass of the biosorbents decreased with increasing bisorbent mass, probably due to the reduction in effective surface area. However, this pattern was not followed when heavy metal removal was low.

Based on the results from this study, OP was the best biosorbent for the heavy metals tested, followed by CNF and then CC. The inefficiency of CC for heavy metal sorption observed in this study corroborates the findings of Abia and Igwe (2005) who studied the removal of Pb^{2+} from wastewater by different low-cost abundant adsorbents; rice husks, maize cobs and sawdust and found rice husk to be the most effective and CC the least. Overall, 1,2-DCA biodegradation was higher in NGWW than NWW, probably due to the composition of the wastewater. When comparing the composition of wastewater it was observed that the composition of NGWW is

more suitable for degradation to occur, since it contained higher levels of nutrients than NWW. In addition the phosphate levels in NGWW are higher than in NWW and phosphate can lead to increased microbial activities and subsequent biodehalogenation as well as reduce the bioavailable concentrations of heavy metals.

In this study, the presence of bioavailable heavy metals drastically reduced 1,2-DCA biodegradation. However, biosorbents, OP and CNF drastically reduce the bioavailable concentrations of heavy metals by adsorbing the heavy metals and thereby counteracting degradation inhibition. Coconut fibre can be classified as a lignocellulosic material, composed of water solubles (5.25%), pectin and related compounds (3.00%), hemi-cellulose (0.25%); lignin (45.84%), cellulose (43.44%) and ash (2.22%) (Igwe and Abia, 2007). Acid–base potentiometric titration indicated that about 73% of sorption sites on CNF were from phenolic compounds, probably lignin (Gonzalez *et al*., 2008). Ewecharoen *et al*. (2008) showed that the main components that play a role in heavy metal adsorpotion were lignin and holo-cellulose and that the mechanism by which CNF adsorbed heavy metals were chemisorptions by which hydroxyl and methoxyl are the main functional groups involved in heavy metal adsorption. Worldwide, oranges make up 75% of the total citrus fruits (Li *et al*., 2008) and OP principally consists of cellulose, hemi-cellulose, pectin substances, chlorophyll pigments and other low molecular weight compounds like limonene (Ajmal *et al*., 2000). The exact composition of OP was given by Ajmal *et al*. (2000) to be 42.2% carbon, 5.4% hydrogen, 51.4% oxygen and 1.0% nitrogen. The OP porosity is located in the albedo zone, the white and spongy part of the peel, which consists of enlarged parenchymatous cells with great intercellular spaces. This particular cell arrangement of albedo zone explains the ability of the OP for the uptake of the heavy metals (Lugo-Lugo *et al*., 2009).

Overall, microcosms supplemented with OP exhibited higher 1,2-DCA degradation as well as higher degradation rate constant when compared to microcosms supplemented with CNF. This is due to OP adsorbing a higher concentration of heavy metals. The order in which OP adsorbs heavy metals is $Pb^{2+} > Cd^{2+,} > Hg^{2+} > As^{3+}$. This was also the order in which the highest degradation occurred for microcosms supplemented with OP, with Pb^{2+} co-contaminated wastewater displaying the most degradation, followed by cadmium, mercury and Arsenic supplemented microcosms, the least. The order in which heavy metals were removed by CNF is $Pb^{2+} > Hg^{2+} > Cd^{2+} > As^{3+}$. Igwe *et al*. (2008) also showed maximum adsorption capacity for

CNF to be the highest for Pb^{2+} followed by Hg^{2+} and As^{3+}. For microcosms supplemented with CNF, the most degradation occurred in the Pb^{2+} co-contaminated microcosm, followed by the microcosms containing Cd^{2+}, Hg^{2+} and lastly As^{3+}. Cd^{2+} co-contaminated microcosms exhibited higher degradation than Hg^{2+} co-contaminated microcosms, even though a higher percentage of Hg^{2+} was removed. This could be due to the fact that Hg^{2+} is more toxic than cadmium, even at a lower concentration (Nies, 1999). As^{3+} resulted in the most inhibition of 1,2-DCA degradation probably because it was the least biosorbed heavy metal by both biosorbents, making it mostly bioavailable. It has been reported that As^{3+} does not bind strongly to solid phases; rather its oxidized form As^{5+} does (Wang and Mulligan, 2006). 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). It seems clear that the increased 1,2-DCA biodegradation by the indigenous microorganism in the wastewater was related to the decrease of the bioavailable fraction of metals.

Scanning electron micrographs of the OP and CNF after heavy metal sorption revealed the presence of a net-like structure of interconnected veins on the surface on both OP and CNF veins using the SE1 detector. Oliveira *et al*. (2008) also observed chromium adsorbed to coffee husks to resemble a network of interconnected veins. The net-like structure can be due to the strong cross linking of heavy metal and negatively charged groups on the biosorbent (Arief *et al*., 2008). Since the net–like structure is on the surface of the biosorbent, it can be hypothesized that the primary heavy metal sequestering sites are on the surface of the biosorbent instead of the intracellular sites (Han *et al*., 2006). The second detector, the backscatter detector records not surface morphologies but chemical compositions, as structures composed of heavier elements appear brighter than those of lighter ones. When no heavy metals were added the biosorbents remained dull, with no backscatter, however in the presence of heavy metals, areas of brightness was observed. This is because the main component of the biosorbents are carbon which is a relatively light element compared to the heavy metals which are heavier elements, therefore the areas of the biosorbent which has taken up the heavy metal appeared brighter than the biosorbent itself. Elements with high atomic number can be visualized on account of the strong electron dispersion therefore appearing as white spots (Iqbal *et al*., 2009). The RSBD also indicates that the biosorbents have irregular structure, thus making it possible for the adsorption of heavy metal ions on different parts of the adsorbent. The surface texture and porosity of adsorbent with holes

and small openings on the surface increases the contact area, leading to pores diffusions during adsorption. Surface area is related to the adsorption capacity of an adsorbent. As the surface area increases, more binding sites are available for the adsorbate to be adsorbed (Rozaini *et al*., 2010).

As demonstrated in this study, the bioavailability of heavy metals can be reduced by biosorbents and this can consequently lead to higher 1,2-DCA biodegradation rate because of the reduced metal toxicity. Heavy metal biosorption was found to be dependent on type and concentration of biosorbent, OP being most efficient biosorbent, when compared to CNF and CC. SEM was found to be a valuable tool to characterize the biosorbents, providing information on the surface texture and morphology of the biosorbents. Since these biosorbents are of an agricultural nature and because they are waste products, it is highly feasible to commercialize their production or establish a wastewater treatment system which integrates the use of these biosorbents.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1 The research in perspective

Halogenated organic compounds represent one of the principal groups of volatile organic compounds (Kwok *et al.,* 2007). A large volume of chlorinated compounds, in particular, are produced yearly for a variety of industrial, agricultural and commercial uses, including the production of solvents, lubricants, intermediates in chemical industry, pesticides, pharmaceuticals, and medical equipment (Hileman, 1993). One such chlorinated compound, 1,2-DCA, is mainly used as an intermediate in the synthesis of vinyl chloride and used in the production of chlorinated solvents such as trichloroethene, tetrachloroethene, and 1,1,1-trichloroethane. 1,2-Dichloroethane released into rivers and lakes breaks down very slowly and most of it will evaporate into the air (Hage and Hartmans, 1999). Improper disposal practices and/or accidental spills of these compounds can result in environmental and human health problems due to their persistence and toxicity (Squillace *et al.*, 1999). Since the first study of the degradation of chlorinated compounds about 100 years ago, the use of microbes to clean up polluted environments, bioremediation, has become a rapidly changing and expanding area of environmental biotechnology (Kerr and Marchesi, 2006). However bioremediation can be hindered if these sites are further contaminated with heavy metals.

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 (Sandrin and Maier, 2003; Sandrin *et al*., 2000). Co-contamination by heavy metals and organic compounds has been detected in many industrial areas. 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). Heavy metals increase the lag phases of degrading organisms, reduce degradation rates, and cause complete inhibition of dechlorination of chlorinated compounds. The presence of heavy metals may inhibit biodegradation by either inhibiting enzymes involved in biodegradation or the enzymes involved in microbial metabolism, thus compounding the problem of chlorinated organic contamination (Kuo and Genthner, 1996). The effects of metals on biodegradation have not been well-characterized; with a large range of reported inhibitory

concentrations due to differences in experimental protocols (Sandrin and Maier, 2003). Majority of results from co-contaminated studies thus far suggests that as the concentration of heavy metal increases, inhibition of biodegradation will increase; however different inhibition patterns such as no inhibition, dose-dependent inhibition, and non dose-dependent inhibition have also been reported (Hoffman *et al*., 2005).

Since the metal toxicity on organic pollutant biodegradation have not been adequately defined, quantitatively or qualitatively, the first aim of this study was to quantitatively assess the effects of heavy metals on the biodegradation of 1,2-DCA. Heavy metals were found to inhibit 1,2-DCA degradation in a dose-dependent manner, i.e as the concentration of heavy metal increased from 0.01 – 0.3 mM, the inhibition of 1,2-DCA degradation also increased. The order of toxicity was as follows: $Hg^{2+} > As^{3+} > Cd^{2+} > Pb^{2+}$. Heavy metals were also found to reduce the biodegradation rate constant (k_1), and increase the half lives of 1,2-DCA in the same dose-dependent manner, confirming the toxic nature of these heavy metals on indigenous 1,2-DCA degrading microorganisms present in the contaminated water. Lead displayed the highest MIC, followed by cadmium, arsenic and mercury; thus implying that a very low concentration of mercury is required to cause inhibition of 1,2-DCA, compared to lead. HLD determinations further confirmed various levels of inhibitory effects on 1,2-DCA biodegradation rates depending on the metal and wastewater source. Mercury was the heavy metal needed in the least quantity to cause HLD of $k_{1,}$ with only 15.44 µM required in NWW and 18.71 µM in NGWW. Subsequently, the effects of biostimulation, bioaugmentation and biosorption on the 1,2-DCA degradation process were investigated in co-contaminated water microcosms. Microcosms can be created to mimic environmental conditions and can offer a degree of control. Intermittently, microcosm testing measures may be more definitive than those of field monitoring (Findlay and Fogel, 2000) thereby the use of microcosms did serve as practical testing systems for bioremediation.

Sandrin and Maier (2003) suggested that the main remediation methods for co-contaminated sites are bioaugmentation (with metal resistant strains) and the addition of treatment additives (such as calcium carbonate, phosphate, cement, manganese oxide, and magnesium hydroxide), clay minerals and chelating agents which all act to reduce metal bioavailability and therefore toxicity. Other possible approaches mentioned by Sandrin and Maier (2003) include pH adjustment and addition of divalent cations. Some of these remediation

approaches are typically only appropriate for soil co-contaminated sites, however, remediation approaches such as biostimulation, bioaugmentation and biosorption can be used for co-contaminated water. When deciding on the most appropriate remediation strategy for co-contaminated environments, considerations should be given to the characteristics of the water and contaminant. Therefore, the physico-chemical properties of the wastewater used in biostimulation experiments were determined. Addition of glucose as a carbon source was exploited as a biostimulation approach in this study to stimulate growth of indigenous 1,2-DCA degrading microorganisms and consequently increasing degradation. Gao and Skeen (1999) suggested that glucose can induce biological transformations of cis-DCE in sediments under aerobic conditions by aerobic co-metabolism. Biostimulation has been proven to be a highly promising technology for remediating contaminated sites by stimulating the decontaminating capability of the indigenous microorganisms. In the current study, biostimulation enhanced biodegradation by 11.43, 13.92, 6.53 and 22.33% in NWW microcosms co-contaminated with arsenic, cadmium mercury and lead, respectively and by 7.62, 8.75, 5.01 and 12.48%, respectively for microcosms constructed with NGWW. However it may not be the best remediation option for sites co-contaminated with 1,2-DCA and heavy metals, as it was the least effective out of all the bioremediation approaches investigated.

Bioaugmentation by a known 1,2-DCA degrader *X. autotrophicus* GJ10 did exhibit a higher 1,2-DCA degradation when compared to biostimulation. Bioaugmented microcosms demonstrated improved biodegradation by 18.6, 20.47, 12.64 and 26.07% respectively in NWW co-contaminated with arsenic, cadmium, mercury and lead and 11.66, 12.79, 10.53 and 16.33% increased 1,2-DCA degradation in NGWW co-contaminated with arsenic, cadmium, mercury and lead, respectively. However, dual-bioaugmented microcosms using *X. autotrophicus* GJ10 and a heavy metal resistant strain exhibited the most 1,2-DCA degradation. Dual-bioaugmented microcosms, increased 1.2-DCA degradation in arsenic, cadmim, mercury and lead co-contaminated microcosms by 22.43, 26.54, 19.58 and 30.49%, respectively in NWW. Similarly, microcosms constructed with NGWW exhibited 16.04, 19.82, 13.58 and 20.36% increased 1,2-DCA degradation after 28 days under the same conditions. The heavy metal resistant strains used in this study belong to the genera: *Delftia, Pseudomonas, Cupriavidus and Stenotrophomonas*, which are known to exhibit resistance towards heavy metals. Increased degradation did result in a concomitant proliferation of the 1,2-DCA degrading population, with highest population

obtained in dual-bioaugmented microcosms compared to bioaugmentated and biostimulated microcosms. Seven dominant strains were obtained from the co-contaminated microcosms and identified to belong to the genera, *Burkholderia*, *Bacillus*, *Enterobacter*, *Pseudomonas and Bradyrhizobiaceae*, which have been previously reported to have the potential to degrade 1,2-DCA and/or other chlorinated compounds. These strains also belong to genera that have been shown to have resistance to heavy metals. This could explain the high 1,2-DCA degradation observed in dual-bioaugmentated microcosms, because in addition to inoculation with 1,2-DCA degrading and heavy metal resistant strains, the indigenous population already consisted of strains with both this properties. However, the success of dual bioaugmentation relies on the survival of the inoculated as well as the indigenous strains.

Although plate counts may provide an indication of the microbial population, molecular techniques may give a better overview of the community diversity. In this study, DGGE profiles revealed that in dual bioaugmentation, heavy metal resistant strains offered protection to the indigenous microbial population from metal stress, since there was no decrease in band intensity over time; however in microcosms that were biostimulated, the DGGE profiles revealed a decrease in intensity and also disappearance of certain bands that could be due to the toxic nature of the heavy metals. The Sorenson similarity index did provide an indication of the community change over time by comparing the similarity between two DGGE profiles. Excision and sequencing of the important bands from the DGGE gels revealed the following genera: *Clostridium*, *Klebesilla*, *Dechloromonas*, *Cupriavidus* and *Enterobacteriae*. Except for *Enterobacteriae*, these genera were not detected by the standard plate count, further emphasizing the need to incorporate culture independent techniques to monitor microbial population and diversity in bioremediation treatments. However, successful application of bioaugmentation techniques is dependent on the identification and isolation of appropriate microbial strains, and their subsequent survival and activity, once released into the target habitat.

In addition to using traditional bioremediation options, a biosorption approach was employed whereby three agricultural wastes (coconut fibre, orange peels and corncobs) were used to adsorb the heavy metals and rendering them unavailable to hinder biodegradation process, thus enhancing 1,2-DCA degradation. Biosorption efficiency was found to be dependent on the type of agricultural biosorbent used as well on the type of heavy metal. In this study, orange peel (OP) was most effective biosorbent than coconut fibre (CNF) and corncobs (CC) in

removing heavy metals from water, removing up to 97.45% lead, compared to CNF (67%) and CC (27.63%) at the same biosorption concentration.. The maximum adsorption capacity was found to be the highest for Pb^{2+} followed by Cd^{2+}, Hg^{2+} and As^{3+} for CC and OP, while Pb^{2+} was adsorbed the most by CNF, followed by Hg^{2+} , Cd^{2+}, and As^{3+}. Since OP removed the highest amount of heavy metals, 1,2-DCA biodegradation was higher in microcosms remediated with OP than CNF. For example, NWW microcosms supplemented with OP exhibited up to 4.45, 6.42, 6.9 and 8.84% higher degradation in As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} co-contaminated water, respectively when compared to CNF. Electron microscopic analysis of the biosorbents revealed the presence of a porous surface that can facilitate the uptake of heavy metals. Using a backscatter probe, the heavy metal uptake was visualized as areas of brightness against the biosorbent background. A web of interconnected veins on the suface of the biosorbent revealed that the sorption occurs at the surface.

Inhibition of 1,2-DCA by heavy metals and bioremediation trends were found to be similar in both wastewater used. Results obtained in this study are promising for the treatment of co-contaminated sites and may also provide the foundation for new and innovative treatment strategies. In this regard, the bioremediation strategies investigated in this study may be adopted 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.

5.2 Potential for future development of the study

This study has established that biodegradation of 1,2-DCA was inhibited by mercury, arsenic, cadmium and lead, in that order. It also revealed that bioremediation options such as dual-bioaugmentation shows promise in remediating co-contaminated water. However, this approach requires inoculating co-contaminated sites with two different bacterial isolates. Recombinant strains can be used as an alternative, whereby a single strain could be used if the bacteria could be engineered to be resistant to heavy metals but still retain activity against the organic contaminant in heavy metal-contaminated environments, thus increasing degradation in the presence of heavy metals. These recombinant strains have previously been shown to exhibit promising results for the degradation of trichloroethene in the presence of cadmium (Lee *et al*.,

2006) and also for 2,4-dichlorophenoxyacetic acid degradation in the presence of nickel or zinc (Springael *et al*., 1993). However, the release of such engineered organisms into the environment is still of concern. Bioremediation can also be sped up by utilizing a combination of biostimulation and bioaugmentation, since both indigenous and exogenous microorganisms could benefit from biostimulation by the addition of energy sources or electron acceptors (El Fantroussi and Agathos. 2005). It would be essential to determine the end products of 1,2-DCA degradation in these microcosms, to elucidate the pathways involved in the degradation process as well as ascertain if the end products are toxic or innocuous.

Agricultural biosorbents did prove to be efficient in removing heavy metals in this study, and consequently increasing biodegradation rates; however the application of biosorbents in co-contaminated areas still need to be developed as no other trials in co-contaminated environments have been carried out. Only two biosorbents were tested in this study; therefore various biosorbents available which would normally be regarded as agricultural waste could be exploited in future studies. The choice of biosorbent would lie in the ease of obtaining it, the labor involved in processing it and the heavy metal uptake ability. Even though biosorbents evaluated in this study showed excellent results for heavy metal removal, the main disadvantages of these materials are their low resistance to abrasive forces in batch or column applications (Anirudhan *et al*., 2008); however reinforcement by chemical processing may improve the physical and chemical properties and can also increase the adsorption potential. Modifications such as esterification, graft copolymerization, cross-linking and quarternisation as well as incorporation of different functional groups onto chemically modified lignocellulosic materials, including OP and CNF, could be made (Li *et al*., 2008; Liang *et al*., 2009; Igwe and Abia, 2007; Anirudhan *et al*., 2008). Reuse of the biosorbent is also a very important factor to consider and additional studies are needed to optimize the system from the regeneration point of view. Since ion exchange is generally reversible (Anirudhan *et al*., 2008), the heavy metals can be desorbed from the biosorbent and both the biosorbent and heavy metal can be re-used. Regeneration and reuse of a number of the modified cellulose adsorbents has been demonstrated in a number of papers reviewed by O'Connell *et al*. (2008). Agricultural biosorbents should definitely be considered for remediation of co-contaminated sites because it does not only cut costs for the clean-up of wastewater, but also adds value to what would have been regarded as waste.

Other interesting options for co-contaminated site remediation include the use of zero-

valent ions and cyclcodextrin (CD). Chatain *et al*. (2004) found that CD could be used as an innovative means to facilitate the removal of both organic and inorganic contaminants by dissolving them to facilitate easy removal from the porous media. While metals can be chelated by the oxygen atoms on the rime of the CD, hydrophobic organic pollutants can also be included within the nonpolar cavity of CD. Zerovalent ions mechanism takes place in a number of steps resulting in reducing halogenated organic compounds *via* intermediates, to non-hazardous products such as ethylene, ethane and acetylene. Detoxification mechanisms for heavy metals are founded on transformation from hazardous to non- hazardous forms or adsorption on the iron surface depending on the type of heavy metals (Junyapoon, 2005). This should be the subject of future investigation.

In addition to determining the best bioremediation method, various options to determining population dynamics should be investigated. In this study, DGGE was employed; however various other molecular techniques exist. One of such efficient methods that can be used is terminal restriction fragment length polymorphism, where the 16S rRNA genes from the community can be amplified with fluorescence-labeled primers. The PCR product can then be restricted with one or more restriction enzymes and the fluorescence-labeled terminal restriction fragment measured by an automated DNA sequencer. Microarrays can also be utilized to compare gene expression between different microcosms, between normal and polluted, or between a treated and control microcosm. A better picture of how metal toxicity affects gene expression can also be obtained using microarrays. These bioremediation methods for the cleanup of heavy metal and chlorinated organic co-contaminated sites are still in its infancy and need to be further evaluated and compared against each other to determine the best technology for treatment application.

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APPENDIX A: REAGENTS

Chloride release assay reagents

- Reagent one : 0.25 M Fe (NH_4) $(S04)_2$ Ferric ammonium sulphate in 9 M nitric acid (HN03)
  Ferric ammonium sulphate (60.2725 g) dissolved in 30 ml doubled distilled water conc HNO_3 286.635 ml
  Double Distilled water (bring up) 500 ml

- Reagent two
  2.5 g of Hg(SCN2) in 250ml of 95 % ethanol
  Dilute to 500 ml in 95 % ethanol
  Mix and filter through filter paper
  Cover with foil

Table 1: Composition of minimal salts medium (MSM medium) per liter of deionized water.

Reagent	**Quantity**
KH_2PO_4	1.36 g
$Na_2HPO_4.12H_2O$	5.37 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4.7H_2O$	0.2 g
Trace element solution	5 ml
Bacteriological agar	12 g

Table 2: Composition of trace element solution per liter 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

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)	pH 8	

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%) (6% gel)

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

Denaturing solution (100%) (6% gel)

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: Triplicate absorbance (λ_{460nm}) values for construction of standard curve for estimating the concentration of free chlorides released during 1,2-DCA degradation (Chapter 2).

Concentration of Cl^- (mM)	**Absorbance (λ_{460nm})**		
	Sample 1	Sample 2	Sample 3
0.56	0.988	0.982	0.979
1.12	1.185	1.186	1.194
1.68	1.503	1.521	1.538
2.24	1.733	1.724	1.724
2.8	1.931	1.924	1.924

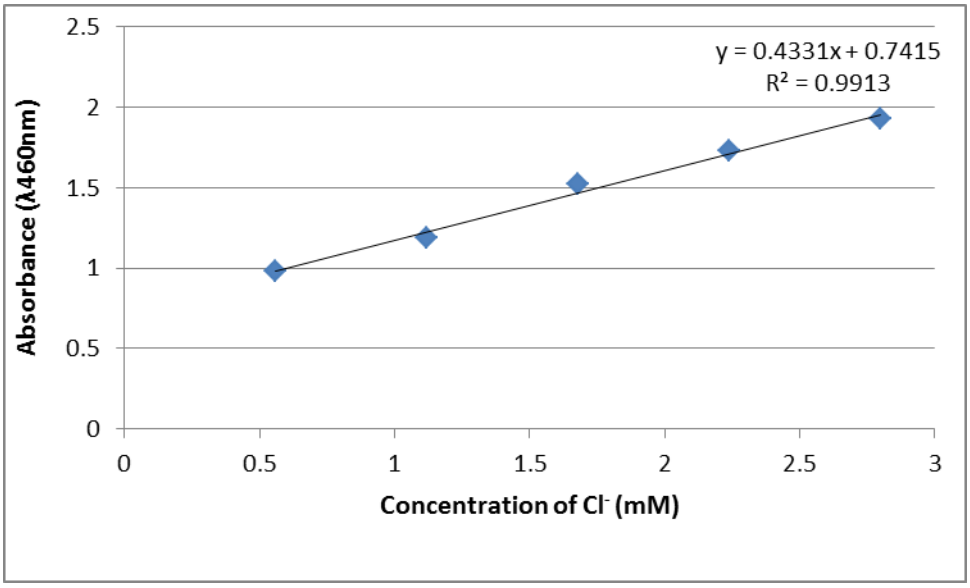


Figure 1: Standard curve for estimating the concentration of free chlorides released during 1,2-DCA degradation in chapter 2.

Table 2: Absorbance values (λ_{460nm}) for NWW samples co-contaminated with various concentrations of arsenic (Chapter 2).

	Day 5									Day 10							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.324	1.331	1.353	1.346	1.310	1.375	1.412	1.405	**0.01**	1.573	1.610	1.559	1.639	1.493	1.646	1.683	1.595
0.05	1.280	1.288	1.310	1.288	1.273	1.317	1.310	1.302	**0.05**	1.500	1.427	1.493	1.573	1.420	1.508	1.566	1.544
0.15	1.229	1.229	1.236	1.266	1.185	1.244	1.288	1.273	**0.15**	1.434	1.413	1.420	1.478	1.361	1.449	1.456	1.420
0.3	1.156	1.193	1.163	1.185	1.149	1.134	1.200	1.149	**0.3**	1.354	1.405	1.339	1.398	1.332	1.310	1.405	1.325
	Day 15									Day 20							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.708	1.722	1.678	1.766	1.620	1.773	1.810	1.722	**0.01**	1.866	2.012	1.946	1.873	1.749	2.048	2.070	1.851
0.05	1.605	1.539	1.597	1.686	1.547	1.613	1.693	1.662	**0.05**	1.763	1.719	1.727	1.829	1.646	1.814	1.895	1.800
0.15	1.539	1.517	1.545	1.497	1.466	1.459	1.620	1.474	**0.15**	1.588	1.583	1.602	1.653	1.583	1.661	1.675	1.668
0.3	1.466	1.499	1.459	1.415	1.408	1.400	1.539	1.422	**0.3**	1.507	1.544	1.588	1.558	1.478	1.522	1.668	1.551
	Day 25									Day 30							
mM	A1	A1	A2	A2	B1	B1	B2	B2	Mm	A1	A1	A2	A2	B1	B1	B2	B2
0.01	2.119	2.067	1.987	2.089	1.855	2.009	2.119	2.104	**0.01**	2.31	2.26	2.25	2.33	2.30	2.27	2.31	2.30
0.05	1.870	1.899	1.833	1.936	1.826	1.848	1.929	1.980	**0.05**	2.13	2.24	2.19	2.20	2.24	2.11	2.19	2.17
0.15	1.695	1.658	1.782	1.746	1.629	1.768	1.855	1.782	**0.15**	1.96	1.89	2.05	2.01	1.89	2.03	2.12	2.05
0.3	1.621	1.643	1.695	1.665	1.585	1.629	1.775	1.658	**0.3**	1.94	1.80	1.91	1.92	1.89	1.89	1.89	1.92

Table 3: Absorbance values (λ_{460nm}) for NWW samples co-contaminated with various concentrations of cadmium (Chapter2)

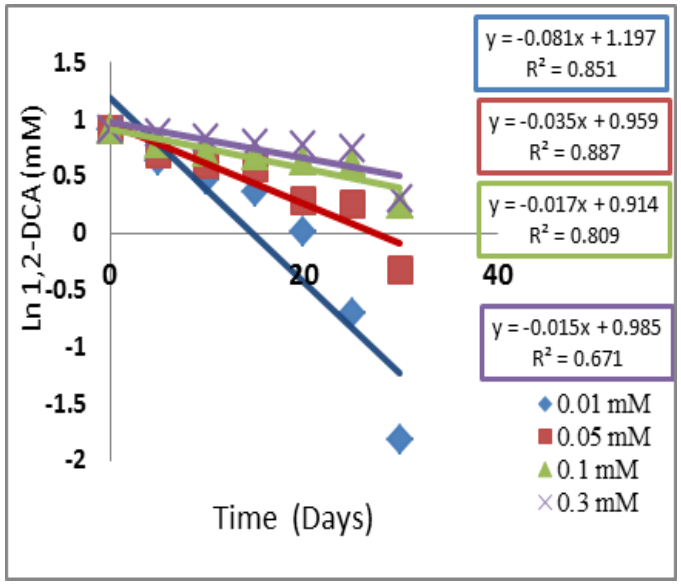
	Day 5									Day 10							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.340	1.297	1.355	1.355	1.377	1.304	1.384	1.311	**0.01**	1.623	1.542	1.564	1.557	1.557	1.535	1.549	1.615
0.05	1.311	1.333	1.333	1.355	1.245	1.326	1.311	1.384	**0.05**	1.535	1.535	1.511	1.528	1.469	1.517	1.535	1.601
0.15	1.201	1.209	1.201	1.194	1.194	1.209	1.216	1.151	**0.15**	1.484	1.403	1.418	1.387	1.444	1.389	1.400	1.381
0.3	1.150	1.136	1.143	1.158	1.172	1.165	1.180	1.143	**0.3**	1.408	1.352	1.345	1.356	1.421	1.345	1.345	1.367
	Day 15									Day 20							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.807	1.851	1.792	1.843	1.807	1.880	1.865	1.865	**0.01**	1.930	1.901	1.927	1.911	2.026	1.941	1.978	1.976
0.05	1.705	1.712	1.661	1.683	1.734	1.705	1.697	1.699	**0.05**	1.880	1.895	1.891	1.879	1.925	1.898	1.940	1.881
0.15	1.593	1.609	1.588	1.617	1.588	1.500	1.595	1.485	**0.15**	1.719	1.734	1.736	1.765	1.739	1.734	1.721	1.734
0.3	1.529	1.478	1.471	1.471	1.530	1.471	1.471	1.471	**0.3**	1.591	1.555	1.539	1.555	1.584	1.583	1.534	1.555
	Day 25									Day 30							
mM	A1	A1	A2	A2	B1	B1	B2	B2	$mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	2.000	2.109	2.044	2.066	2.124	2.051	2.109	2.051	**0.01**	2.30	2.30	2.29	2.30	2.29	2.29	2.30	2.31
0.05	1.949	1.963	1.941	1.963	2.000	1.963	2.022	2.007	**0.05**	2.17	2.18	2.10	2.16	2.20	2.13	2.18	2.18
0.15	1.780	1.797	1.800	1.816	1.854	1.841	1.917	1.930	**0.15**	1.95	1.95	1.98	2.00	2.07	2.05	2.09	2.08
0.3	1.671	1.641	1.671	1.671	1.691	1.656	1.641	1.634	**0.3**	1.89	1.79	1.88	1.85	1.95	1.97	2.00	2.01

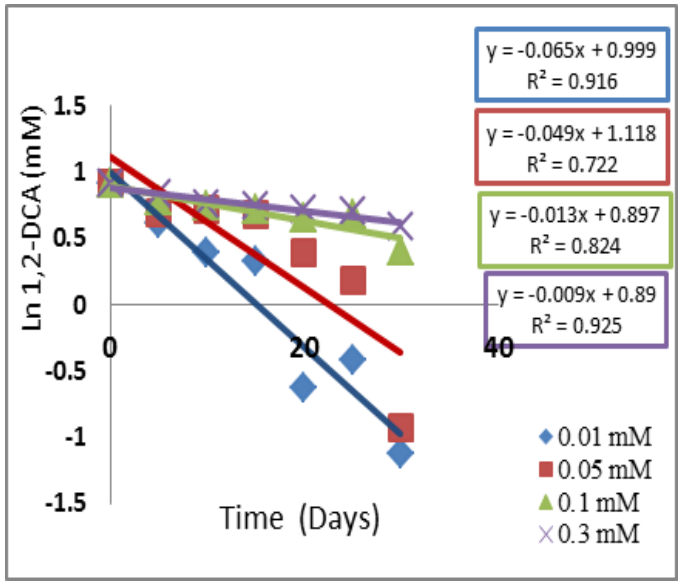
Table 4: Absorbance values (λ_{460nm}) for NWW samples co-contaminated with various concentrations of mercury (Chapter 2).

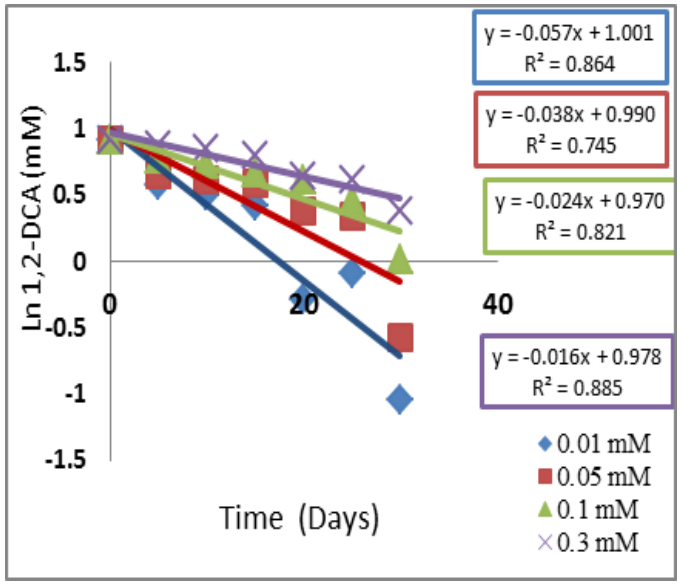
	Day 5									Day 10							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.346	1.361	1.376	1.390	1.383	1.354	1.362	1.381	**0.01**	1.582	1.536	1.521	1.518	1.552	1.531	1.557	1.586
0.05	1.288	1.295	1.303	1.332	1.310	1.281	1.320	1.310	**0.05**	1.461	1.453	1.415	1.425	1.416	1.459	1.485	1.459
0.15	1.266	1.266	1.281	1.222	1.273	1.259	1.288	1.251	**0.15**	1.409	1.410	1.384	1.399	1.353	1.394	1.418	1.384
0.3	1.164	1.149	1.156	1.171	1.173	1.173	1.198	1.156	**0.3**	1.322	1.322	1.321	1.339	1.336	1.334	1.341	1.326
	Day 15									Day 20							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.528	1.551	1.566	1.580	1.573	1.544	1.558	1.566	**0.01**	1.924	1.924	1.931	1.953	1.939	1.946	1.946	1.866
0.05	1.478	1.485	1.493	1.522	1.500	1.471	1.507	1.500	**0.05**	1.624	1.632	1.785	1.873	1.866	1.851	1.785	1.792
0.15	1.456	1.456	1.471	1.412	1.463	1.449	1.478	1.442	**0.15**	1.602	1.602	1.675	1.690	1.705	1.712	1.683	1.690
0.3	1.432	1.376	1.368	1.381	1.445	1.368	1.368	1.390	**0.3**	1.577	1.573	1.629	1.621	1.623	1.594	1.594	1.594
	Day 25									Day 30							
(mM)	A1	A1	A2	A2	B1	B1	B2	B2	(mM)	A1	A1	A2	A2	B1	B1	B2	B2
0.01	2.119	2.097	2.163	2.141	2.053	2.061	2.105	2.141	**0.01**	2.237	2.247	2.281	2.259	2.260	2.267	2.238	2.259
0.05	2.002	2.002	1.914	1.988	1.914	1.980	1.914	1.973	**0.05**	2.120	2.120	2.032	2.105	2.032	2.098	2.032	2.091
0.15	1.783	1.841	1.789	1.739	1.761	1.812	1.768	1.768	**0.15**	1.901	1.959	1.805	1.857	1.879	1.930	1.886	1.886
0.3	1.739	1.768	1.696	1.688	1.696	1.754	1.739	1.732	**0.3**	1.836	1.925	1.764	1.771	1.813	1.897	1.826	1.847

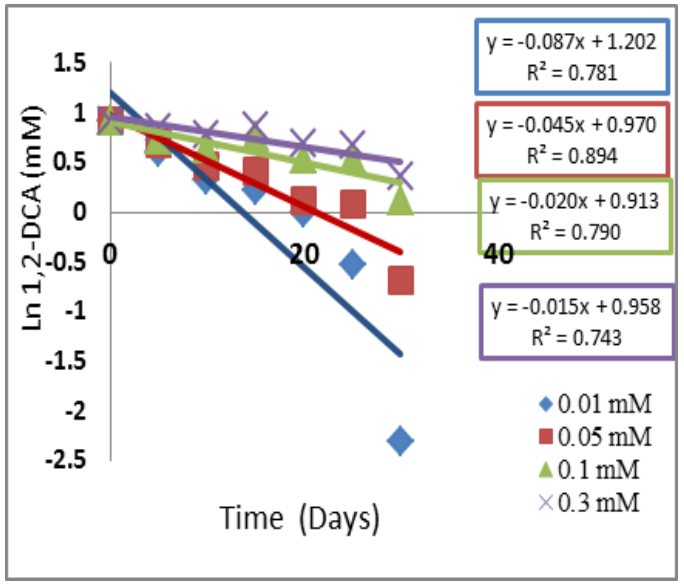
Table 5: Absorbance values (λ_{460nm}) for NWW samples co-contaminated with various concentrations of lead (Chapter 2).

	Day 5									Day 10							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.285	1.270	1.402	1.416	1.250	1.282	1.294	1.264	**0.01**	1.469	1.454	1.615	1.651	1.564	1.578	1.622	1.673
0.05	1.226	1.226	1.285	1.277	1.226	1.255	1.255	1.241	**0.05**	1.417	1.479	1.483	1.549	1.461	1.454	1.433	1.644
0.15	1.212	1.212	1.233	1.241	1.197	1.241	1.219	1.212	**0.15**	1.403	1.403	1.395	1.402	1.403	1.418	1.410	1.439
0.3	1.160	1.168	1.182	1.182	1.146	1.182	1.160	1.160	**0.3**	1.359	1.359	1.359	1.337	1.359	1.359	1.381	1.359
	Day 15									Day 20							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.796	1.950	1.950	1.789	1.825	2.038	2.030	1.774	**0.01**	2.078	2.063	2.011	1.895	2.012	2.092	2.074	2.085
0.05	1.635	1.613	1.804	1.673	1.701	1.782	1.760	1.736	**0.05**	1.734	1.816	1.885	1.790	1.919	1.909	1.990	1.949
0.15	1.548	1.562	1.474	1.533	1.526	1.665	1.687	1.540	**0.15**	1.624	1.631	1.785	1.675	1.774	1.860	1.758	1.698
0.3	1.431	1.394	1.438	1.438	1.511	1.584	1.555	1.460	**0.3**	1.522	1.478	1.551	1.493	1.675	1.690	1.631	1.631
	Day 25																
mM	A1	A1	A2	A2	B1	B1	B2	B2									
0.01	2.151	2.150	2.124	2.115	2.122	2.143	2.134	2.148									
0.05	1.917	1.950	2.002	1.909	2.036	2.035	2.100	2.038									
0.15	1.797	1.752	1.884	1.900	1.875	1.890	1.946	1.935									
0.3	1.599	1.610	1.744	1.767	1.797	1.812	1.723	1.682									


y = -0.081x + 1.197
R² = 0.851
y = -0.035x + 0.959
R² = 0.887
y = -0.017x + 0.914
R² = 0.809
y = -0.015x + 0.985
R² = 0.671
0.01 mM
0.05 mM
0.1 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
0
20
40


y = -0.065x + 0.999
R² = 0.916
y = -0.049x + 1.118
R² = 0.722
y = -0.013x + 0.897
R² = 0.824
y = -0.009x + 0.89
R² = 0.925
0.01 mM
0.05 mM
0.1 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)
1.5
1
0.5
0
-0.5
-1
-1.5
0
20
40


y = -0.057x + 1.001
R² = 0.864
y = -0.038x + 0.990
R² = 0.745
y = -0.024x + 0.970
R² = 0.821
y = -0.016x + 0.978
R² = 0.885
0.01 mM
0.05 mM
0.1 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)
1.5
1
0.5
0
-0.5
-1
-1.5
0
20
40


y = -0.087x + 1.202
R² = 0.781
y = -0.045x + 0.970
R² = 0.894
y = -0.020x + 0.913
R² = 0.790
y = -0.015x + 0.958
R² = 0.743
0.01 mM
0.05 mM
0.1 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
0
20
40

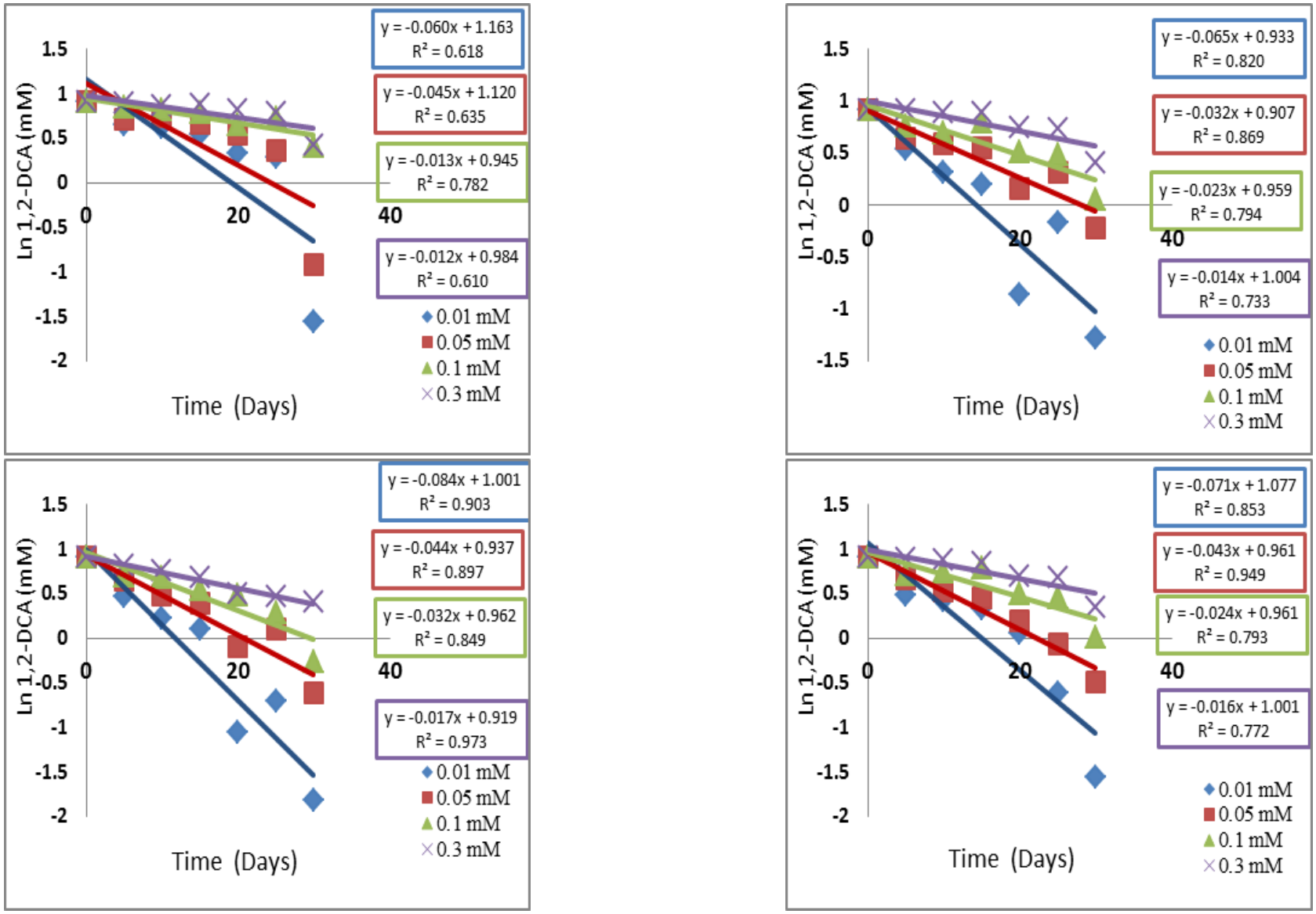
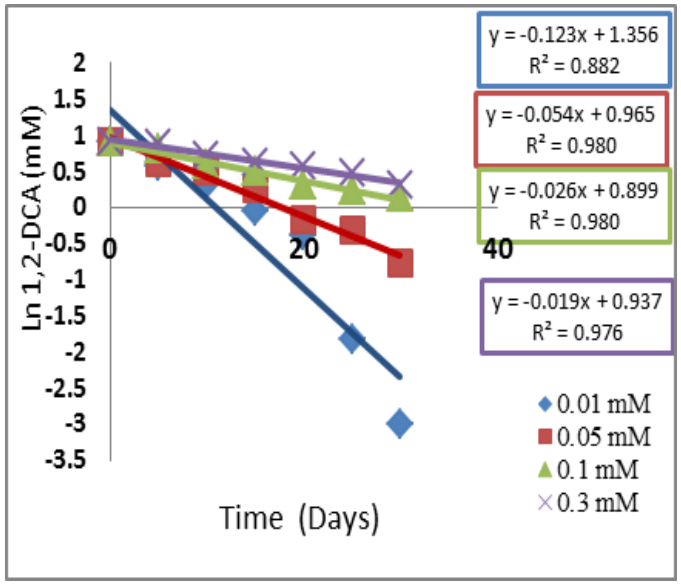
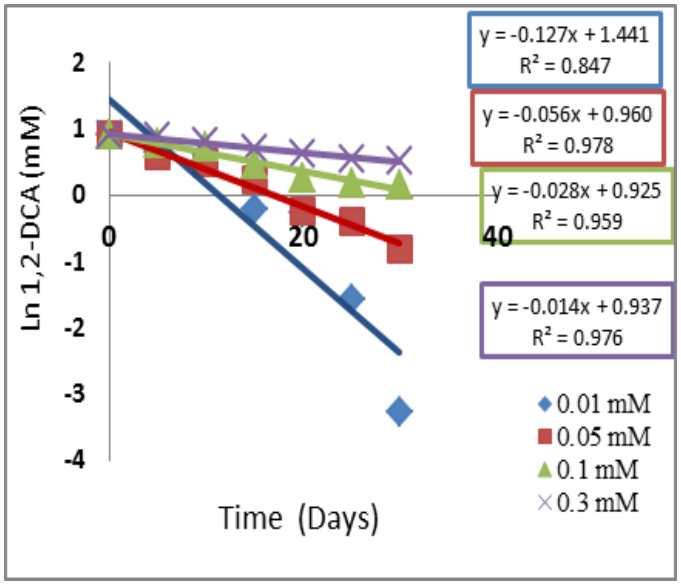
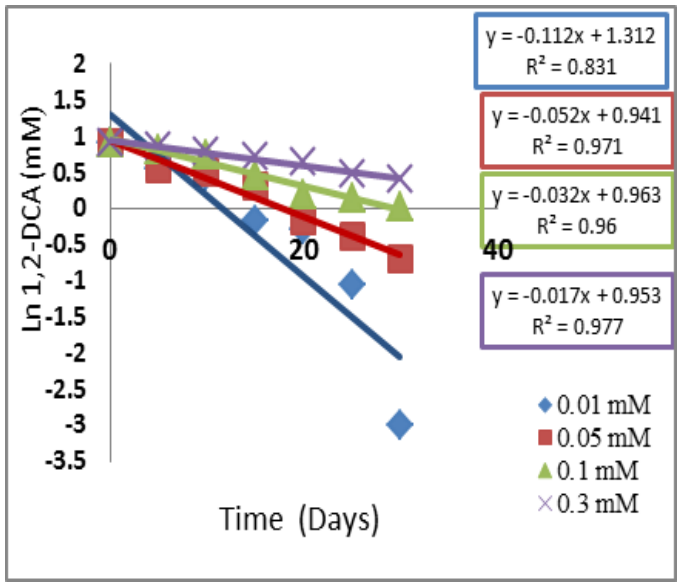
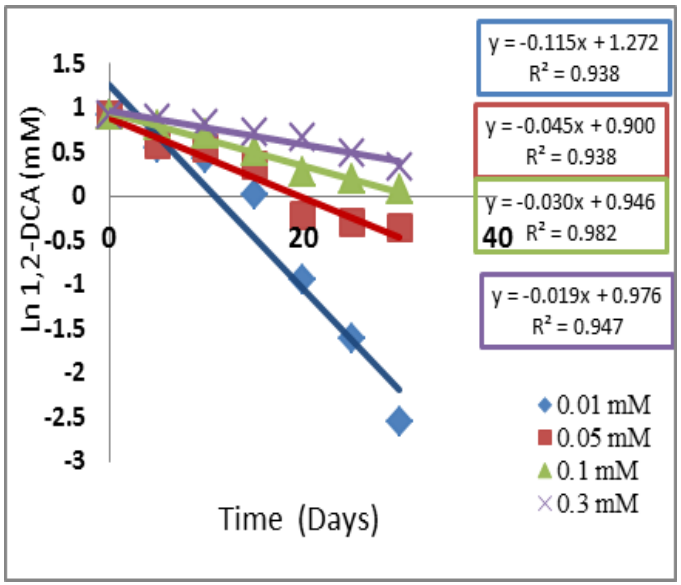


Figure 2: Inhibitory effects of As^{3+} on pseudo-first order 1,2-DCA biodegradation rates by indigenous microorganisms in NWW (replicates of eight) (Chapter 2).


y = -0.123x + 1.356
R² = 0.882
y = -0.054x + 0.965
R² = 0.980
y = -0.026x + 0.899
R² = 0.980
y = -0.019x + 0.937
R² = 0.976
Ln 1,2-DCA (mM)
Time (Days)
0.01 mM
0.05 mM
0.1 mM
0.3 mM


y = -0.127x + 1.441
R² = 0.847
y = -0.056x + 0.960
R² = 0.978
y = -0.028x + 0.925
R² = 0.959
y = -0.014x + 0.937
R² = 0.976
Ln 1,2-DCA (mM)
Time (Days)
0.01 mM
0.05 mM
0.1 mM
0.3 mM


y = -0.112x + 1.312
R² = 0.831
y = -0.052x + 0.941
R² = 0.971
y = -0.032x + 0.963
R² = 0.96
y = -0.017x + 0.953
R² = 0.977
Ln 1,2-DCA (mM)
Time (Days)
0.01 mM
0.05 mM
0.1 mM
0.3 mM


y = -0.115x + 1.272
R² = 0.938
y = -0.045x + 0.900
R² = 0.938
y = -0.030x + 0.946
R² = 0.982
y = -0.019x + 0.976
R² = 0.947
Ln 1,2-DCA (mM)
Time (Days)
0.01 mM
0.05 mM
0.1 mM
0.3 mM

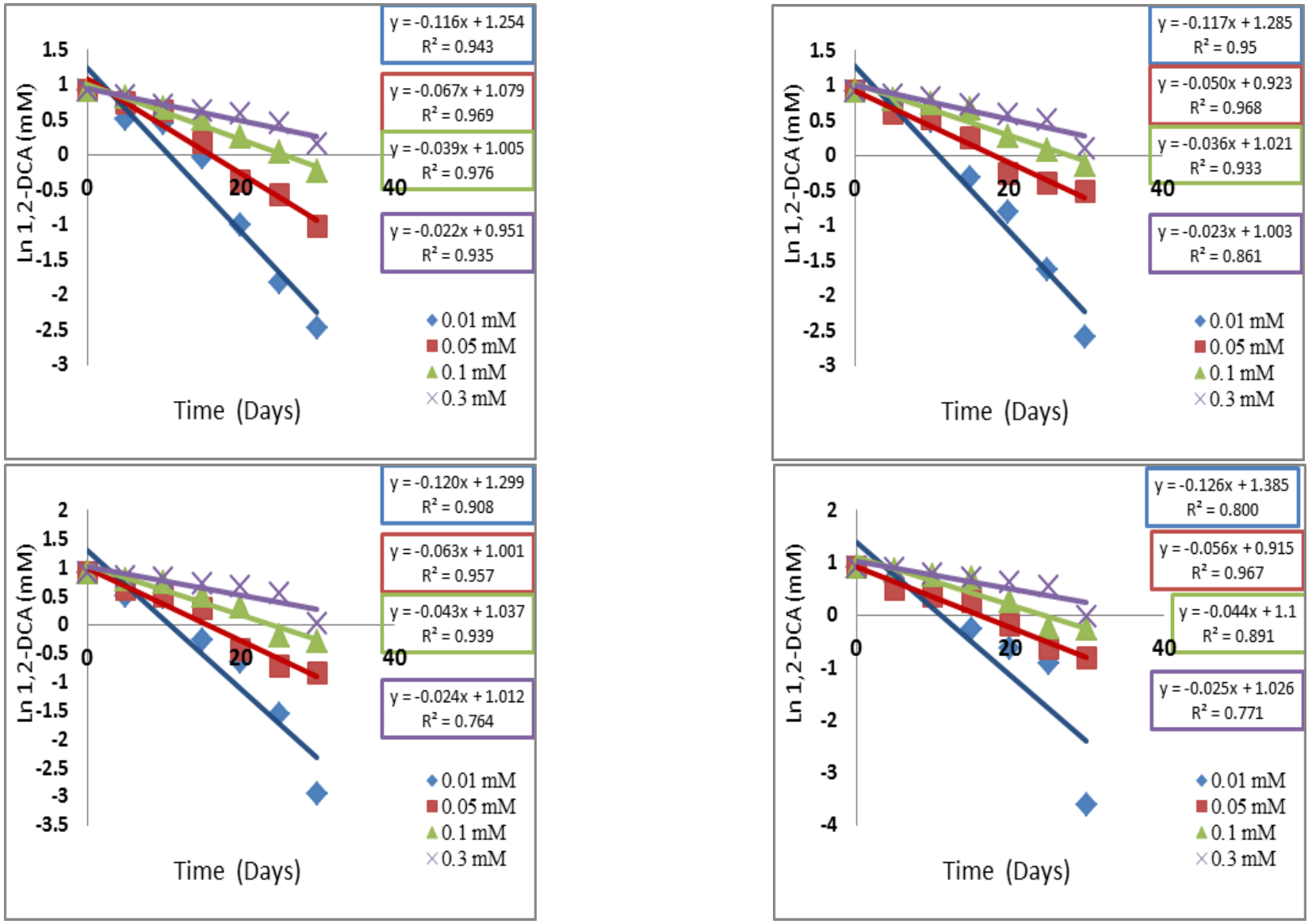
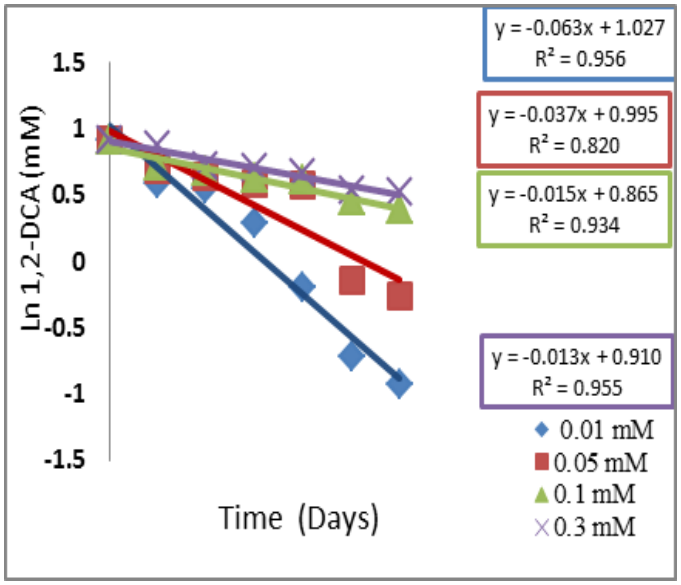
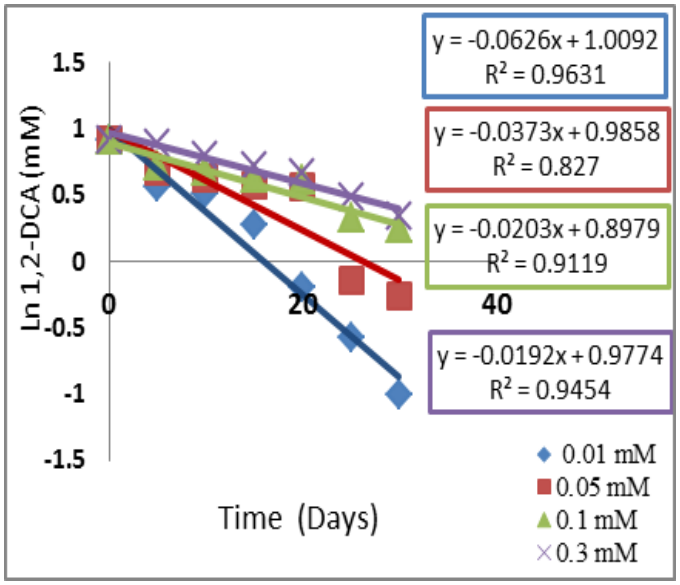
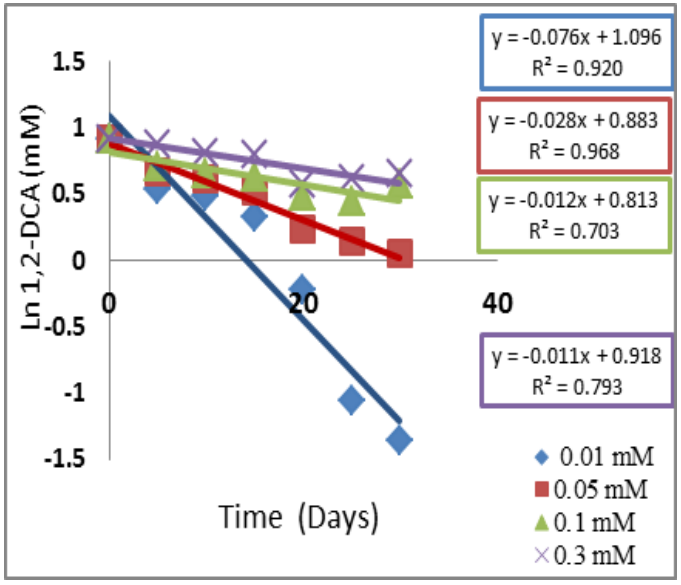
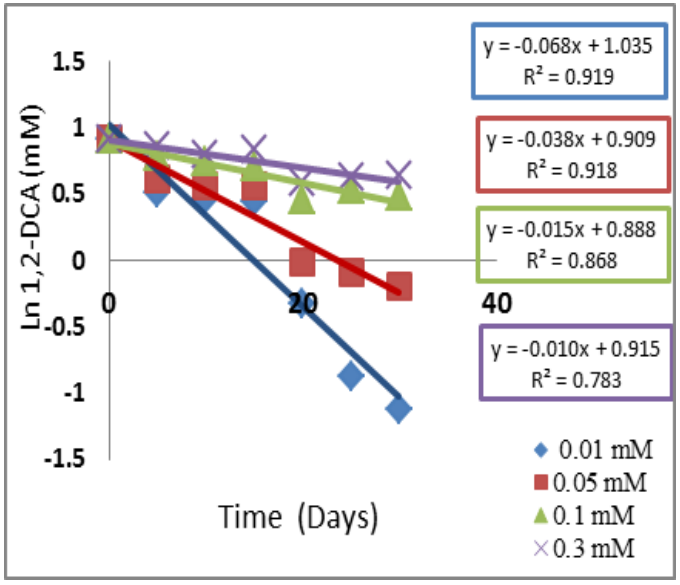


Figure 3: Inhibitory effects of Cd^{2+} on pseudo-first order 1,2-DCA biodegradation rates by indigenous microorganisms in NWW (replicates of eight) (Chapter 2).


y = -0.063x + 1.027
R² = 0.956
y = -0.037x + 0.995
R² = 0.820
y = -0.015x + 0.865
R² = 0.934
y = -0.013x + 0.910
R² = 0.955
0.01 mM
0.05 mM
0.1 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)
1.5
1
0.5
0
-0.5
-1
-1.5


y = -0.0626x + 1.0092
R² = 0.9631
y = -0.0373x + 0.9858
R² = 0.827
y = -0.0203x + 0.8979
R² = 0.9119
y = -0.0192x + 0.9774
R² = 0.9454
0.01 mM
0.05 mM
0.1 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)
1.5
1
0.5
0
-0.5
-1
-1.5
0
20
40


y = -0.076x + 1.096
R² = 0.920
y = -0.028x + 0.883
R² = 0.968
y = -0.012x + 0.813
R² = 0.703
y = -0.011x + 0.918
R² = 0.793
0.01 mM
0.05 mM
0.1 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)
1.5
1
0.5
0
-0.5
-1
-1.5
0
20
40


y = -0.068x + 1.035
R² = 0.919
y = -0.038x + 0.909
R² = 0.918
y = -0.015x + 0.888
R² = 0.868
y = -0.010x + 0.915
R² = 0.783
0.01 mM
0.05 mM
0.1 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)
1.5
1
0.5
0
-0.5
-1
-1.5
0
20
40

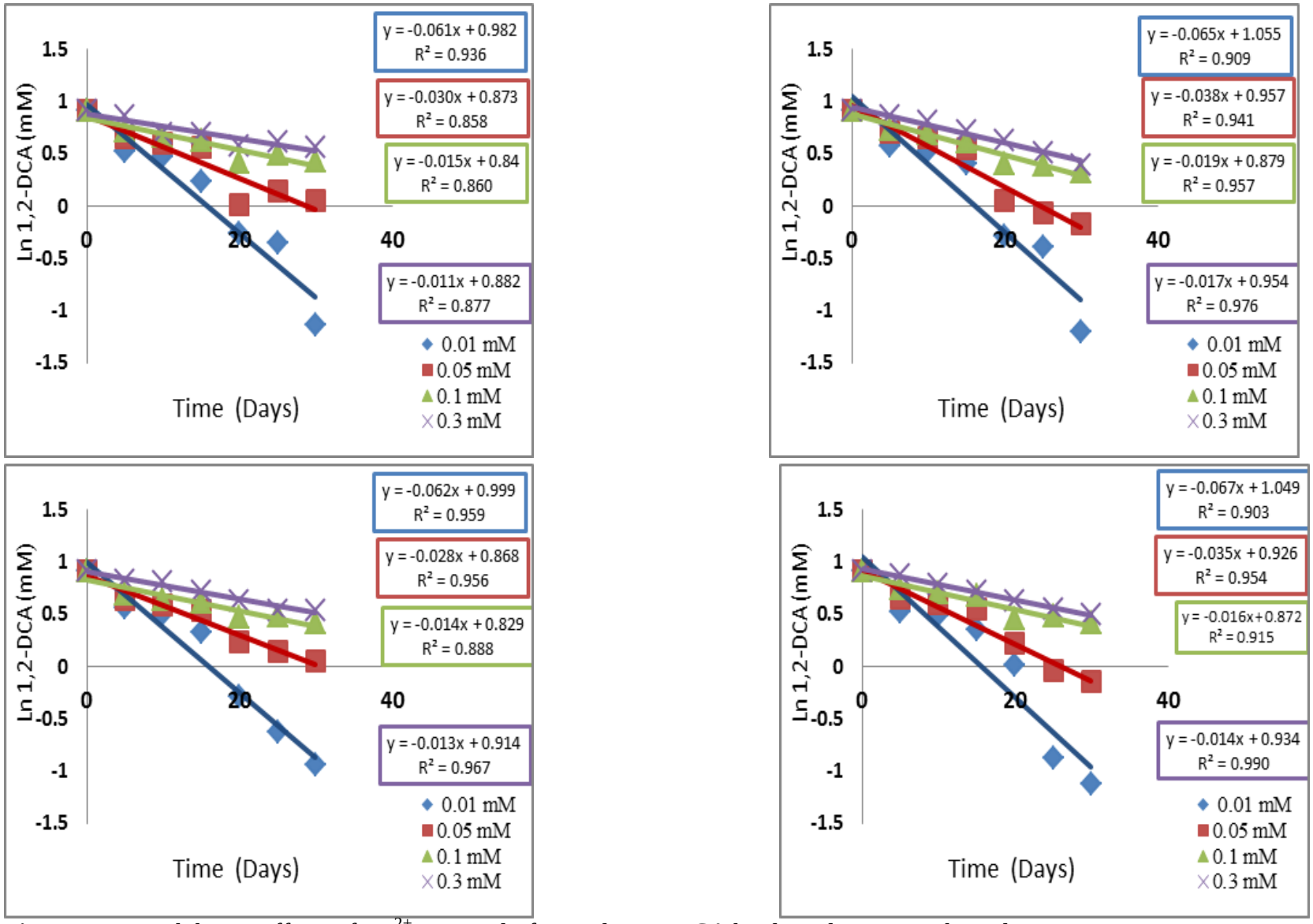
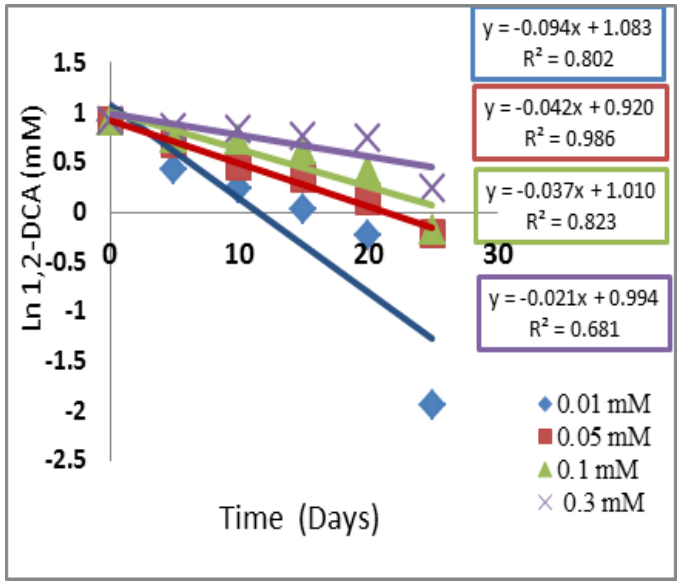
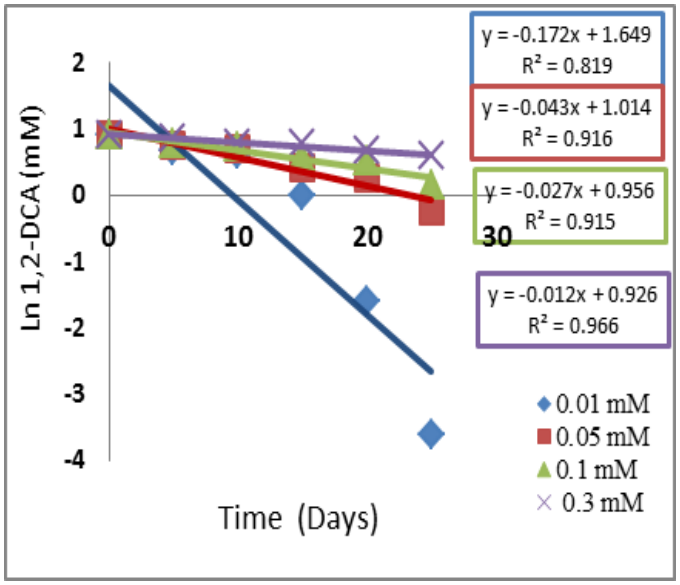
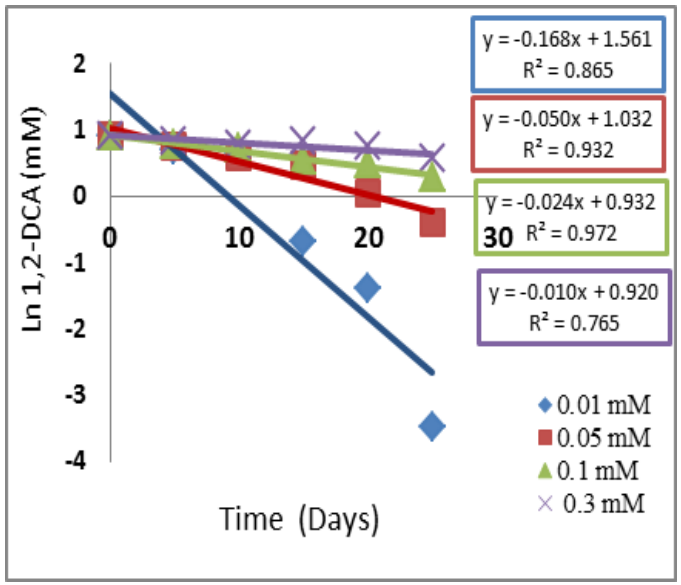
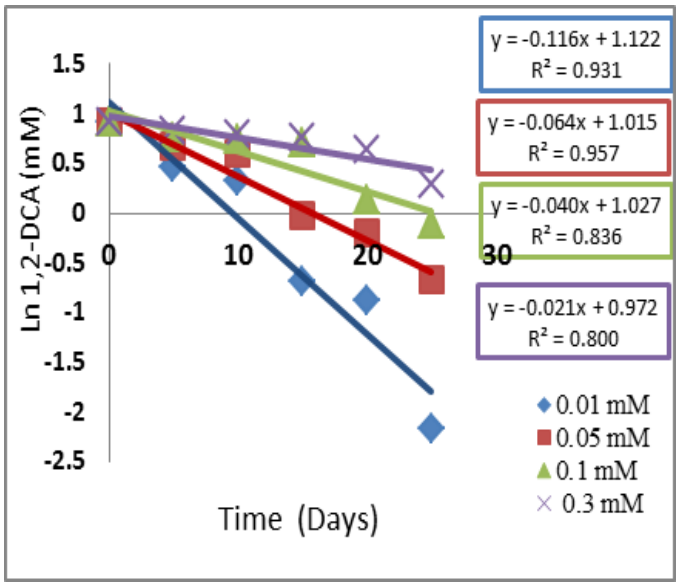


Figure 4: Inhibitory effects of Hg^{2+} on pseudo-first order 1,2-DCA biodegradation rates by indigenous microorganisms in NWW (replicates of eight) (Chapter 2)


y = -0.094x + 1.083
R² = 0.802
y = -0.042x + 0.920
R² = 0.986
y = -0.037x + 1.010
R² = 0.823
y = -0.021x + 0.994
R² = 0.681
Ln 1,2-DCA (mM)
Time (Days)
0.01 mM
0.05 mM
0.1 mM
0.3 mM


y = -0.172x + 1.649
R² = 0.819
y = -0.043x + 1.014
R² = 0.916
y = -0.027x + 0.956
R² = 0.915
y = -0.012x + 0.926
R² = 0.966
Ln 1,2-DCA (mM)
Time (Days)
0.01 mM
0.05 mM
0.1 mM
0.3 mM


y = -0.168x + 1.561
R² = 0.865
y = -0.050x + 1.032
R² = 0.932
y = -0.024x + 0.932
R² = 0.972
y = -0.010x + 0.920
R² = 0.765
Ln 1,2-DCA (mM)
Time (Days)
0.01 mM
0.05 mM
0.1 mM
0.3 mM


y = -0.116x + 1.122
R² = 0.931
y = -0.064x + 1.015
R² = 0.957
y = -0.040x + 1.027
R² = 0.836
y = -0.021x + 0.972
R² = 0.800
Ln 1,2-DCA (mM)
Time (Days)
0.01 mM
0.05 mM
0.1 mM
0.3 mM

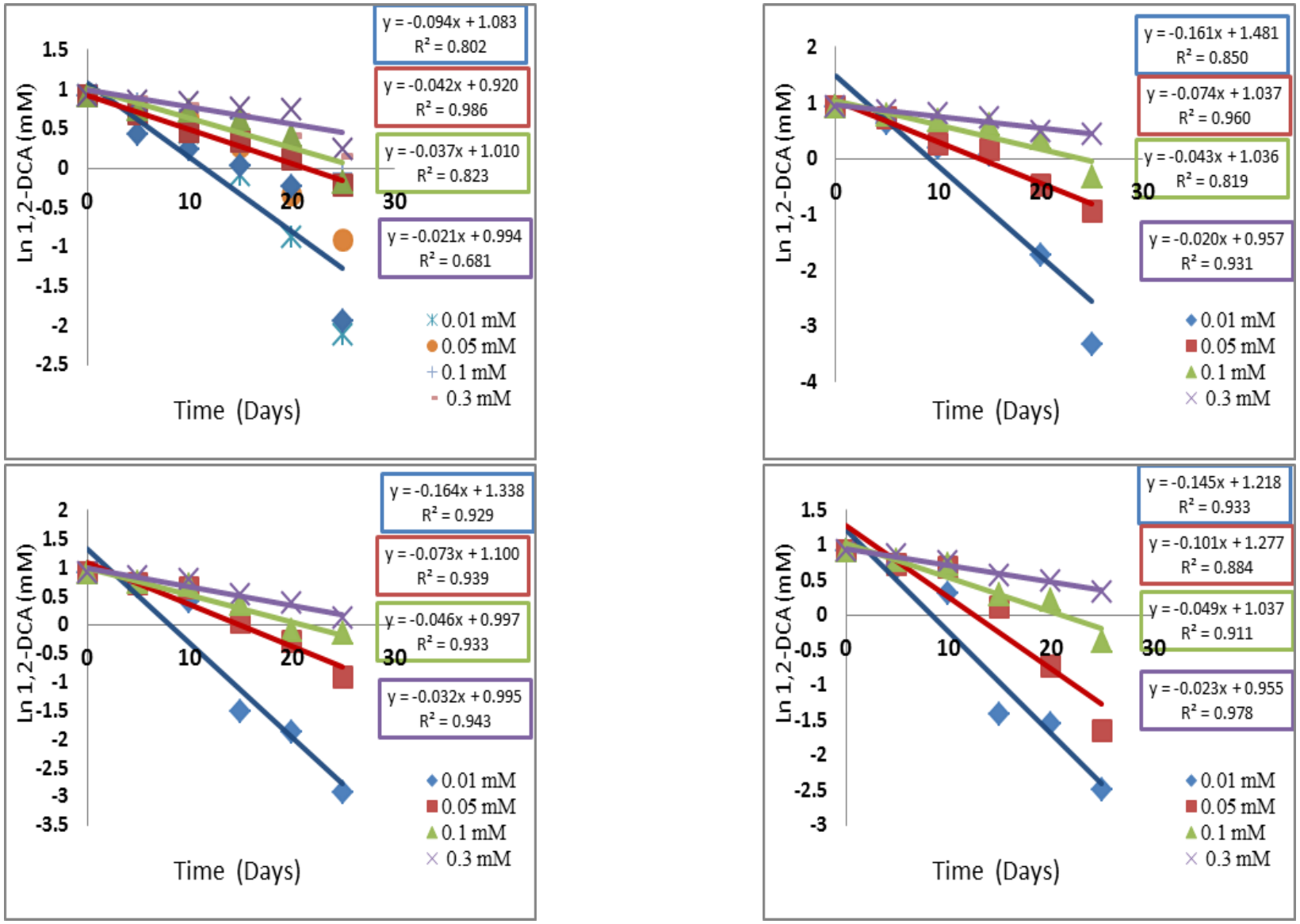
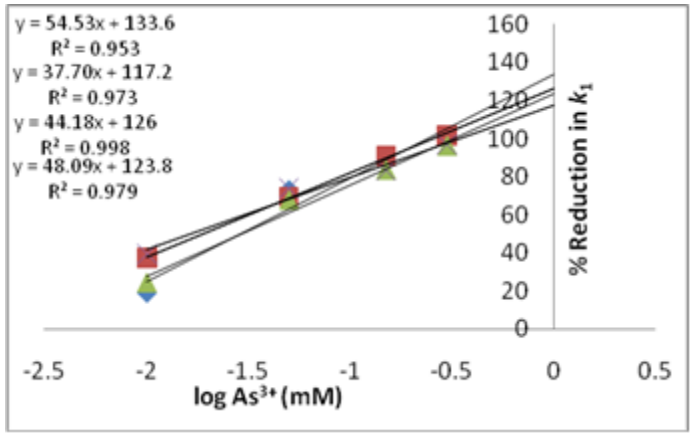


Figure 5: Inhibitory effects of Pb^{2+} on pseudo-first order 1,2-DCA biodegradation rates by indigenous microorganisms in NWW (replicates of eight) (Chapter 2)



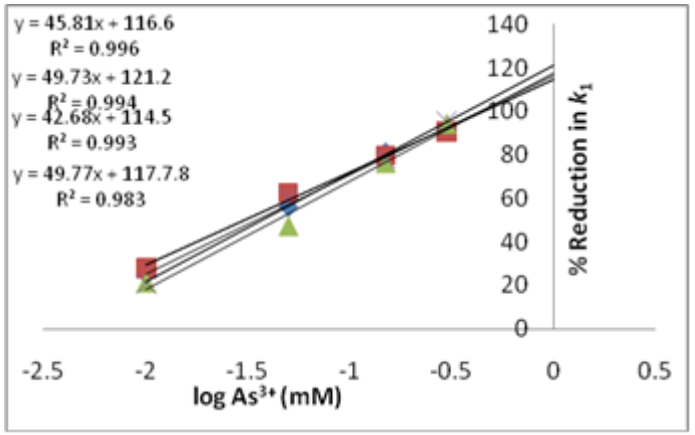
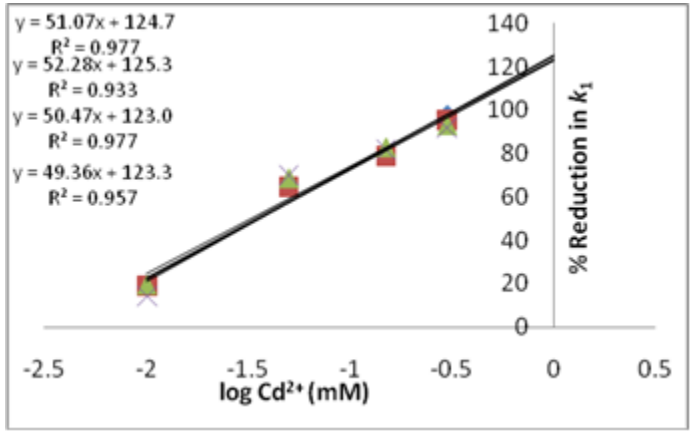


Figure 6: Inhibitory effects of a wide range of As^{3+} concentrations in NWW on pseudo-first order degradation rate coefficient k_1 (Chapter 2).



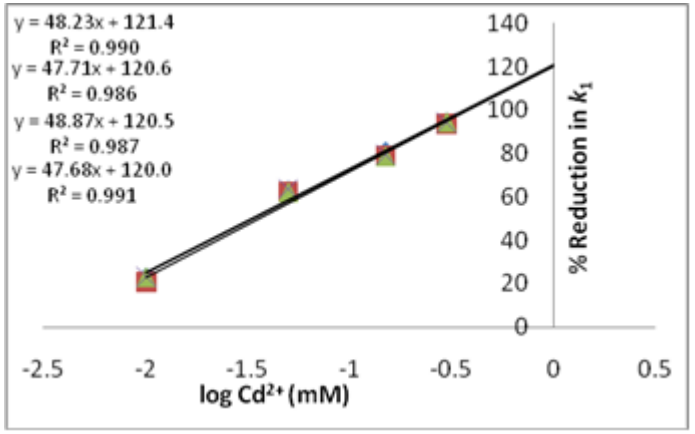
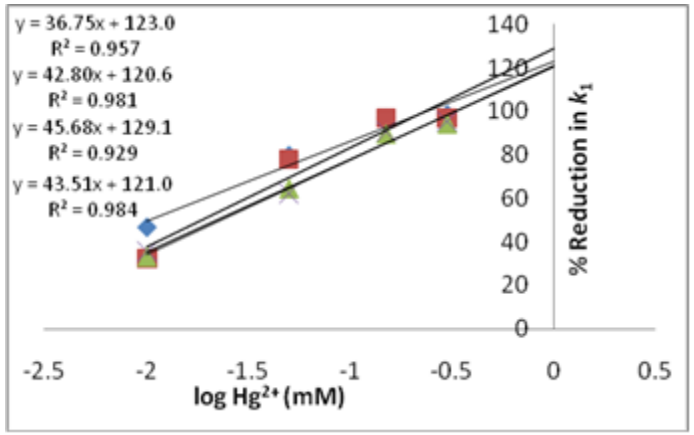


Figure 7: Inhibitory effects of a wide range of Cd^{2+} concentrations in NWW on pseudo-first order degradation rate coefficients, k_1 (Chapter 2).



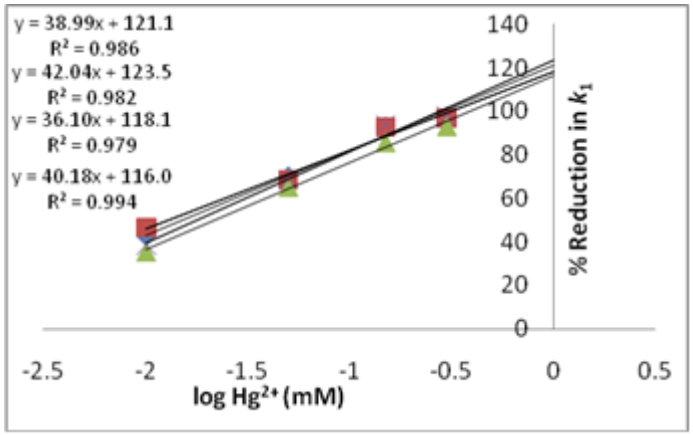
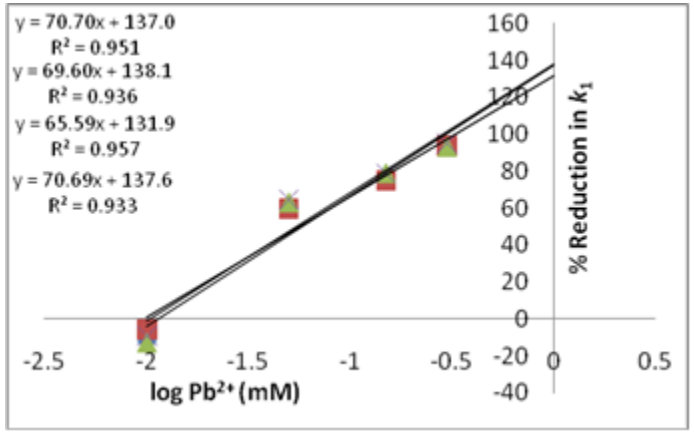


Figure 8: Inhibitory effects of a wide range of Hg^{2+} concentrations in NWW on pseudo-first order degradation rate coefficients, k_1 (Chapter 2).



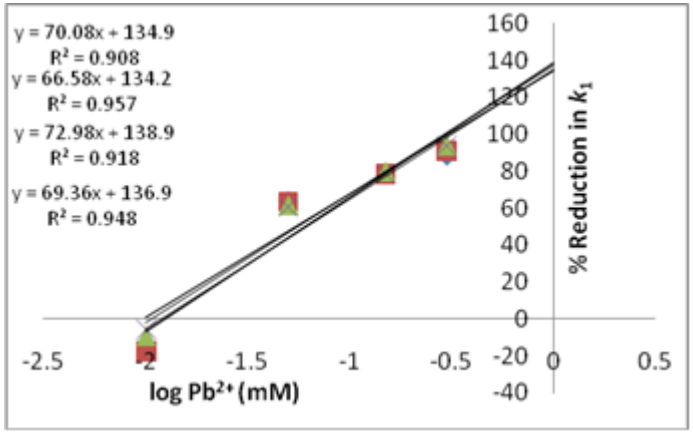


Figure 9: Inhibitory effects of a wide range of Pb^{2+} concentrations in NWW on pseudo-first order degradation rate coefficients, k_1 (Chapter 2).

Table 6: Absorbance values (λ_{460nm}) for NGWW samples co-contaminated with various concentrations of arsenic (Chapter 2).

	Day 5								Day 10								
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.299	1.270	1.365	1.372	1.301	1.280	1.325	1.299	**0.01**	1.522	1.474	1.565	1.580	1.536	1.533	1.562	1.620
0.05	1.256	1.266	1.296	1.303	1.223	1.277	1.270	1.304	**0.05**	1.452	1.483	1.473	1.514	1.441	1.462	1.460	1.598
0.15	1.193	1.197	1.204	1.204	1.182	1.212	1.204	1.174	**0.15**	1.419	1.379	1.383	1.371	1.400	1.380	1.381	1.386
0.3	1.142	1.146	1.149	1.149	1.146	1.160	1.157	1.143	**0.3**	1.360	1.332	1.328	1.323	1.366	1.328	1.339	1.339
	Day 15								Day 20								
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.754	1.759	1.752	1.750	1.768	1.748	1.754	1.750	**0.01**	1.968	1.946	1.934	1.867	1.953	1.981	1.990	1.995
0.05	1.622	1.615	1.684	1.630	1.669	1.695	1.680	1.669	**0.05**	1.772	1.820	1.852	1.799	1.887	1.868	1.930	1.880
0.15	1.522	1.538	1.483	1.527	1.509	1.534	1.593	1.465	**0.15**	1.636	1.647	1.725	1.685	1.721	1.762	1.704	1.680
0.3	1.432	1.388	1.406	1.406	1.473	1.479	1.465	1.417	**0.3**	1.521	1.481	1.510	1.488	1.594	1.601	1.547	1.558
	Day 25																
mM	A1	A1	A2	A2	B1	B1	B2	B2									
0.01	2.095	2.057	2.093	2.076	2.088	2.075	2.089	2.089									
0.05	1.914	1.936	1.936	1.911	1.994	1.966	2.027	1.991									
0.15	1.758	1.739	1.813	1.829	1.845	1.842	1.899	1.895									
0.3	1.616	1.589	1.685	1.690	1.735	1.739	1.698	1.678									

Table 7: Absorbance values (λ_{460nm}) for NGWW samples co-contaminated with various concentrations of cadmium (Chapter 2).

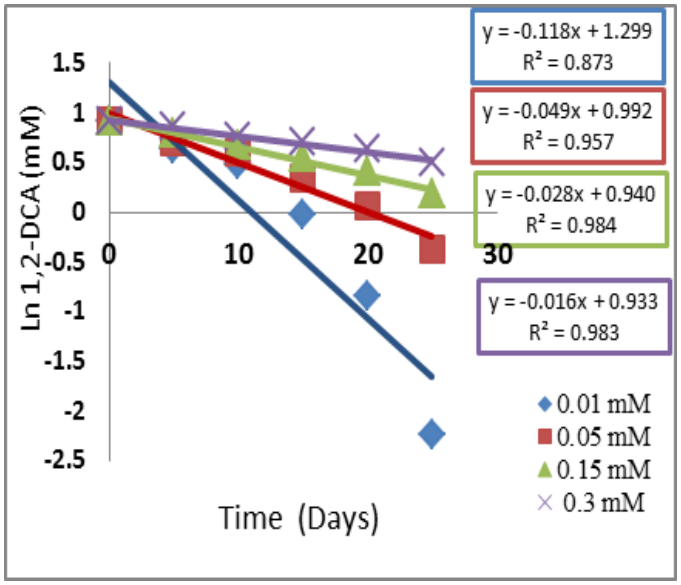
	Day 5								Day 10								
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.285	1.286	1.320	1.319	1.254	1.254	1.276	1.266	**0.01**	1.518	1.511	1.552	1.569	1.490	1.520	1.507	1.510
0.05	1.305	1.254	1.247	1.247	1.306	1.249	1.239	1.256	**0.05**	1.407	1.406	1.480	1.481	1.450	1.480	1.437	1.437
0.15	1.196	1.195	1.255	1.254	1.252	1.235	1.247	1.243	**0.15**	1.379	1.384	1.380	1.418	1.375	1.387	1.385	1.384
0.3	1.154	1.164	1.195	1.182	1.216	1.195	1.198	1.199	**0.3**	1.328	1.313	1.313	1.356	1.355	1.340	1.335	1.333
	Day 15								Day 20								
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.750	1.751	1.774	1.774	1.744	1.731	1.765	1.748	**0.01**	1.949	1.962	1.947	1.961	1.949	1.951	1.955	1.962
0.05	1.656	1.668	1.743	1.721	1.651	1.648	1.698	1.699	**0.05**	1.794	1.840	1.809	1.841	1.869	1.868	1.841	1.841
0.15	1.545	1.558	1.587	1.587	1.573	1.557	1.588	1.567	**0.15**	1.701	1.716	1.693	1.724	1.756	1.725	1.724	1.725
0.3	1.423	1.444	1.463	1.463	1.491	1.494	1.464	1.441	**0.3**	1.546	1.631	1.601	1.632	1.623	1.632	1.622	1.631
	Day 25																
mM	A1	A1	A2	A2	B1	B1	B2	B2									
0.01	2.096	2.095	2.097	2.102	2.091	2.094	2.090	2.089									
0.05	1.976	1.958	1.929	1.907	1.967	1.967	1.967	1.960									
0.15	1.875	1.867	1.844	1.844	1.835	1.875	1.875	1.866									
0.3	1.645	1.637	1.715	1.720	1.690	1.678	1.676	1.678									

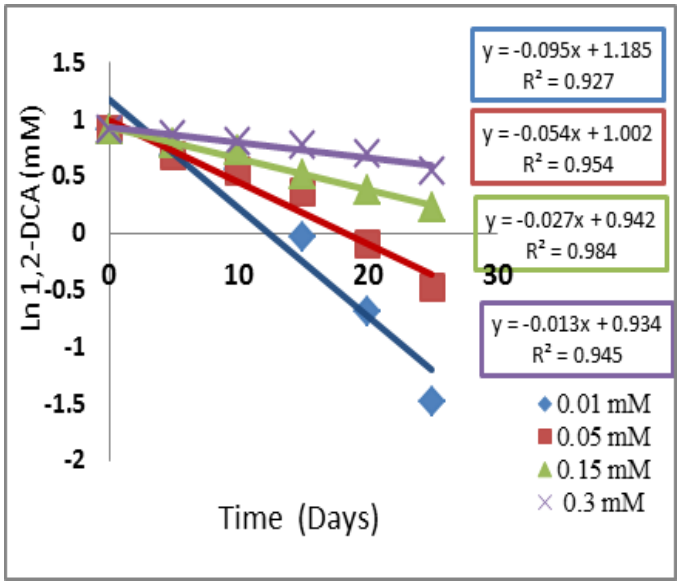
Table 8: Absorbance values (λ_{460nm}) for NGWW samples co-contaminated with various concentrations of mercury (Chapter 2).

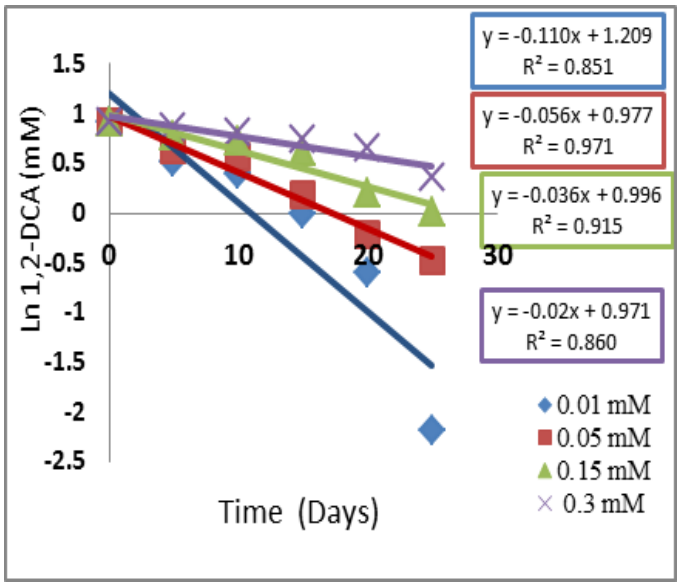
	Day 5									Day 10							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.320	1.328	1.276	1.285	1.334	1.309	1.304	1.309	**0.01**	1.582	1.536	1.521	1.518	1.552	1.531	1.557	1.586
0.05	1.261	1.252	1.252	1.235	1.228	1.251	1.284	1.255	**0.05**	1.461	1.453	1.415	1.425	1.416	1.459	1.485	1.459
0.15	1.240	1.235	1.223	1.210	1.182	1.199	1.200	1.199	**0.15**	1.409	1.410	1.384	1.399	1.353	1.394	1.418	1.384
0.3	1.137	1.120	1.119	1.134	1.130	1.168	1.172	1.168	**0.3**	1.322	1.322	1.321	1.339	1.336	1.334	1.341	1.326
	Day 15									Day 20							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.642	1.764	1.670	1.684	1.772	1.650	1.704	1.698	**0.01**	1.855	1.994	1.850	1.850	1.974	1.881	1.977	1.943
0.05	1.527	1.495	1.553	1.556	1.522	1.526	1.593	1.527	**0.05**	1.709	1.710	1.755	1.754	1.781	1.743	1.830	1.743
0.15	1.474	1.460	1.495	1.489	1.464	1.463	1.554	1.452	**0.15**	1.599	1.577	1.661	1.636	1.652	1.633	1.703	1.652
0.3	1.430	1.432	1.393	1.432	1.430	1.433	1.449	1.433	**0.3**	1.553	1.571	1.601	1.561	1.570	1.571	1.642	1.571
	Day 25																
mM	A1	A1	A2	A2	B1	B1	B2	B2									
0.01	2.058	2.062	2.086	2.082	2.036	2.051	2.061	2.062									
0.05	1.888	1.906	1.988	2.001	1.937	1.968	1.968	1.969									
0.15	1.669	1.686	1.751	1.751	1.685	1.706	1.778	1.689									
0.3	1.603	1.627	1.671	1.672	1.627	1.658	1.687	1.627									

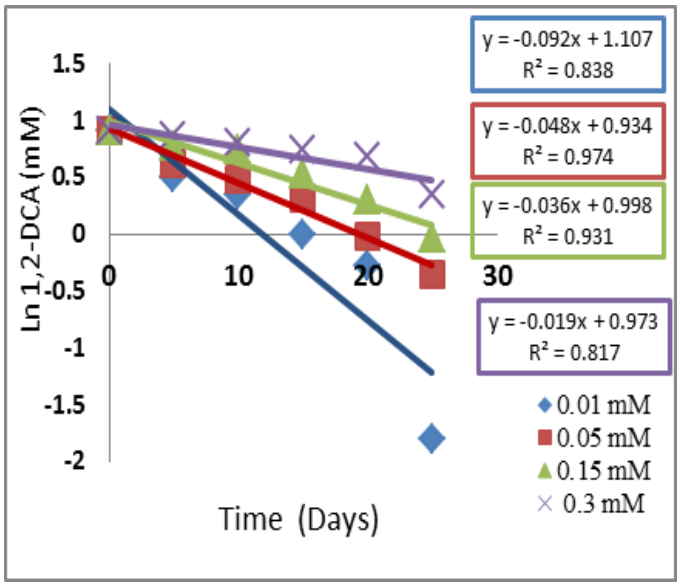
Table 9: Absorbance values (λ_{460nm}) for NGWW samples co-contaminated with various concentrations of lead (Chapter 2).

	Day 5									Day 10							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.301	1.316	1.312	1.300	1.313	1.267	1.301	1.316	**0.01**	1.580	1.575	1.534	1.536	1.644	1.559	1.498	1.575
0.05	1.287	1.289	1.259	1.265	1.261	1.258	1.303	1.320	**0.05**	1.499	1.502	1.473	1.493	1.474	1.487	1.487	1.479
0.15	1.237	1.258	1.234	1.231	1.236	1.255	1.241	1.258	**0.15**	1.394	1.382	1.399	1.395	1.392	1.394	1.402	1.402
0.3	1.201	1.203	1.200	1.199	1.196	1.203	1.170	1.197	**0.3**	1.335	1.337	1.336	1.346	1.337	1.360	1.338	1.389
	Day 15									Day 20							
mM	A1	A1	A2	A2	B1	B1	B2	B2	mM	A1	A1	A2	A2	B1	B1	B2	B2
0.01	1.748	1.779	1.779	1.740	1.794	1.792	1.733	1.655	**0.01**	1.955	1.970	1.967	1.941	1.908	1.908	1.960	1.960
0.05	1.699	1.713	1.655	1.709	1.716	1.715	1.681	1.683	**0.05**	1.882	1.878	1.823	1.819	1.849	1.828	1.885	1.878
0.15	1.589	1.582	1.593	1.592	1.553	1.560	1.590	1.591	**0.15**	1.767	1.763	1.709	1.712	1.754	1.740	1.699	1.639
0.3	1.443	1.468	1.497	1.496	1.496	1.470	1.450	1.441	**0.3**	1.692	1.690	1.614	1.579	1.611	1.583	1.645	1.627
	Day 25																
mM	A1	A1	A2	A2	B1	B1	B2	B2									
0.01	2.121	2.121	2.119	2.118	2.119	2.121	2.120	2.119									
0.05	1.949	1.955	1.988	1.992	1.986	1.994	1.957	1.953									
0.15	1.864	1.856	1.866	1.874	1.904	1.883	1.883	1.908									
0.3	1.752	1.702	1.699	1.719	1.699	1.722	1.681	1.671									


y = -0.118x + 1.299
R² = 0.873
y = -0.049x + 0.992
R² = 0.957
y = -0.028x + 0.940
R² = 0.984
y = -0.016x + 0.933
R² = 0.983
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)


y = -0.095x + 1.185
R² = 0.927
y = -0.054x + 1.002
R² = 0.954
y = -0.027x + 0.942
R² = 0.984
y = -0.013x + 0.934
R² = 0.945
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)


y = -0.110x + 1.209
R² = 0.851
y = -0.056x + 0.977
R² = 0.971
y = -0.036x + 0.996
R² = 0.915
y = -0.02x + 0.971
R² = 0.860
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)


y = -0.092x + 1.107
R² = 0.838
y = -0.048x + 0.934
R² = 0.974
y = -0.036x + 0.998
R² = 0.931
y = -0.019x + 0.973
R² = 0.817
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)

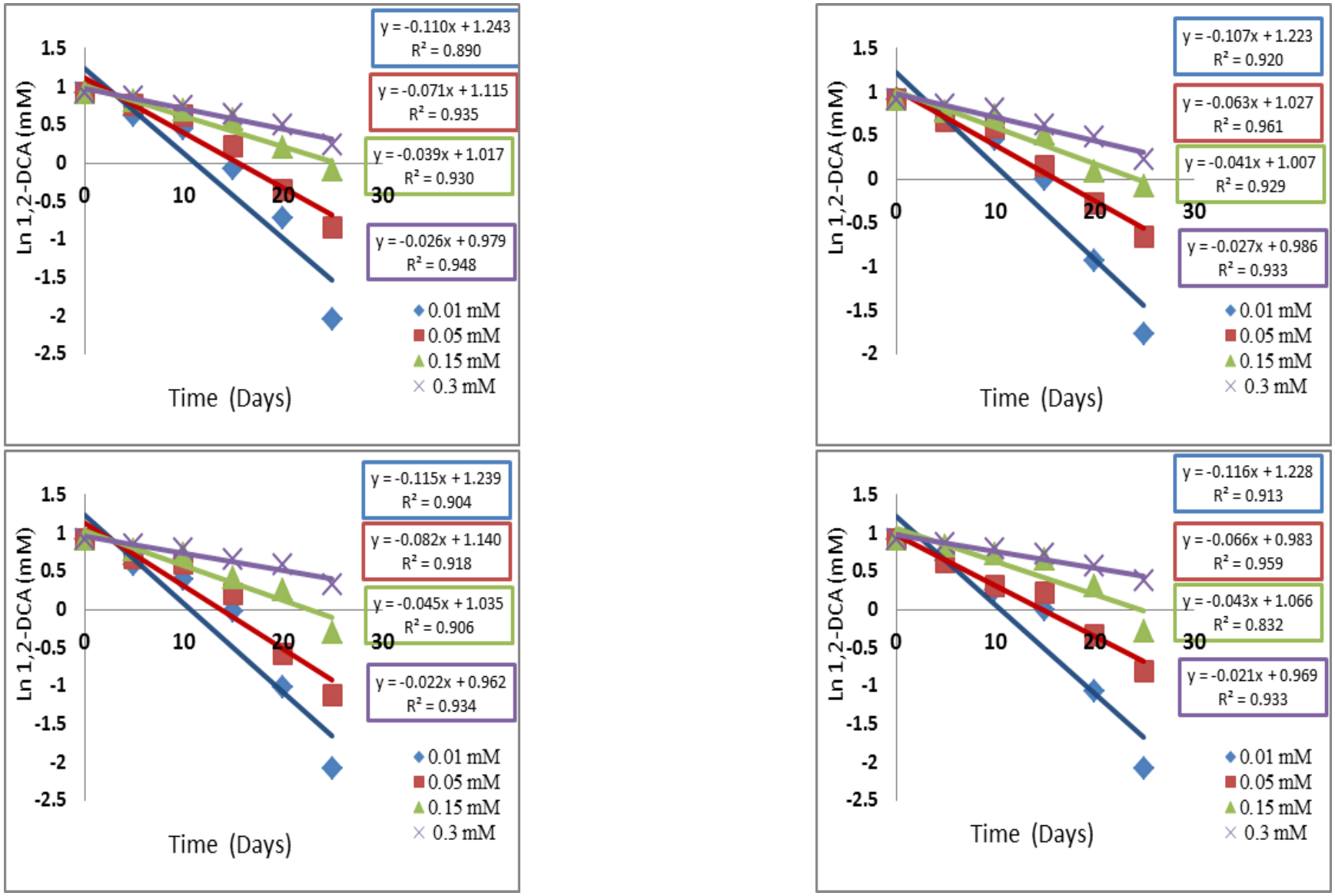
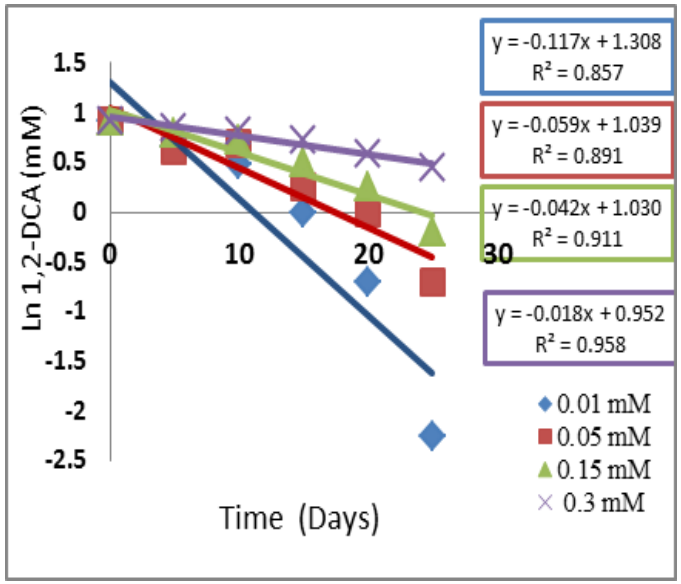
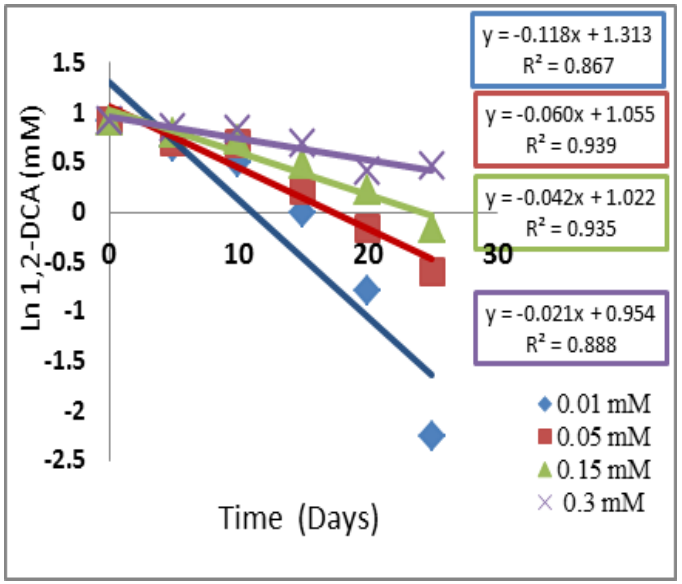
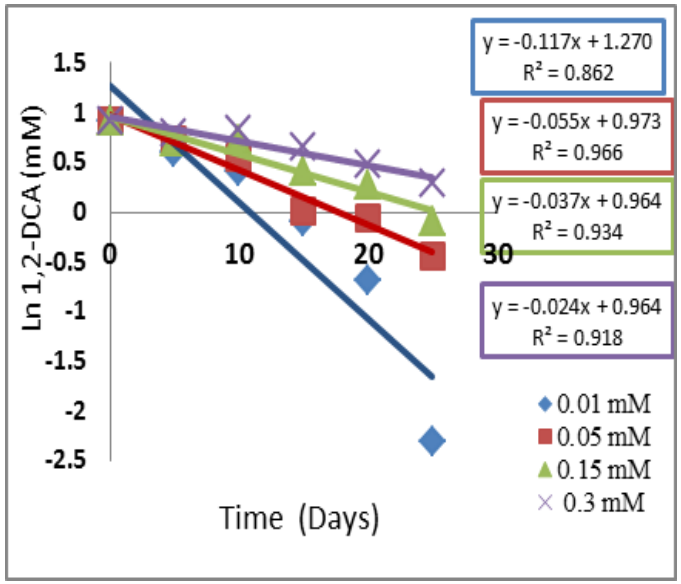
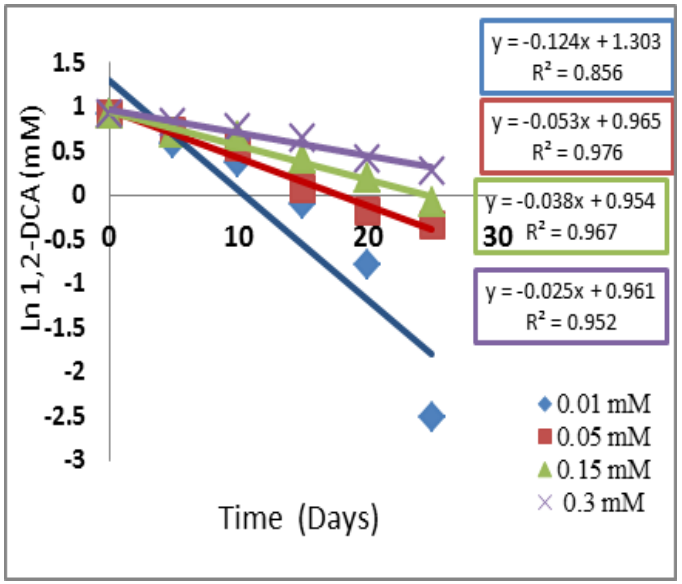


Figure 10: Inhibitory effects of As^{3+} on pseudo-first order 1,2-DCA biodegradation rates by indigenous microorganisms in NGWW (replicates of eight) (Chapter 2).


y = -0.117x + 1.308
R² = 0.857
y = -0.059x + 1.039
R² = 0.891
y = -0.042x + 1.030
R² = 0.911
y = -0.018x + 0.952
R² = 0.958
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)


y = -0.118x + 1.313
R² = 0.867
y = -0.060x + 1.055
R² = 0.939
y = -0.042x + 1.022
R² = 0.935
y = -0.021x + 0.954
R² = 0.888
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)


y = -0.117x + 1.270
R² = 0.862
y = -0.055x + 0.973
R² = 0.966
y = -0.037x + 0.964
R² = 0.934
y = -0.024x + 0.964
R² = 0.918
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)


y = -0.124x + 1.303
R² = 0.856
y = -0.053x + 0.965
R² = 0.976
y = -0.038x + 0.954
R² = 0.967
y = -0.025x + 0.961
R² = 0.952
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2-DCA (mM)
Time (Days)

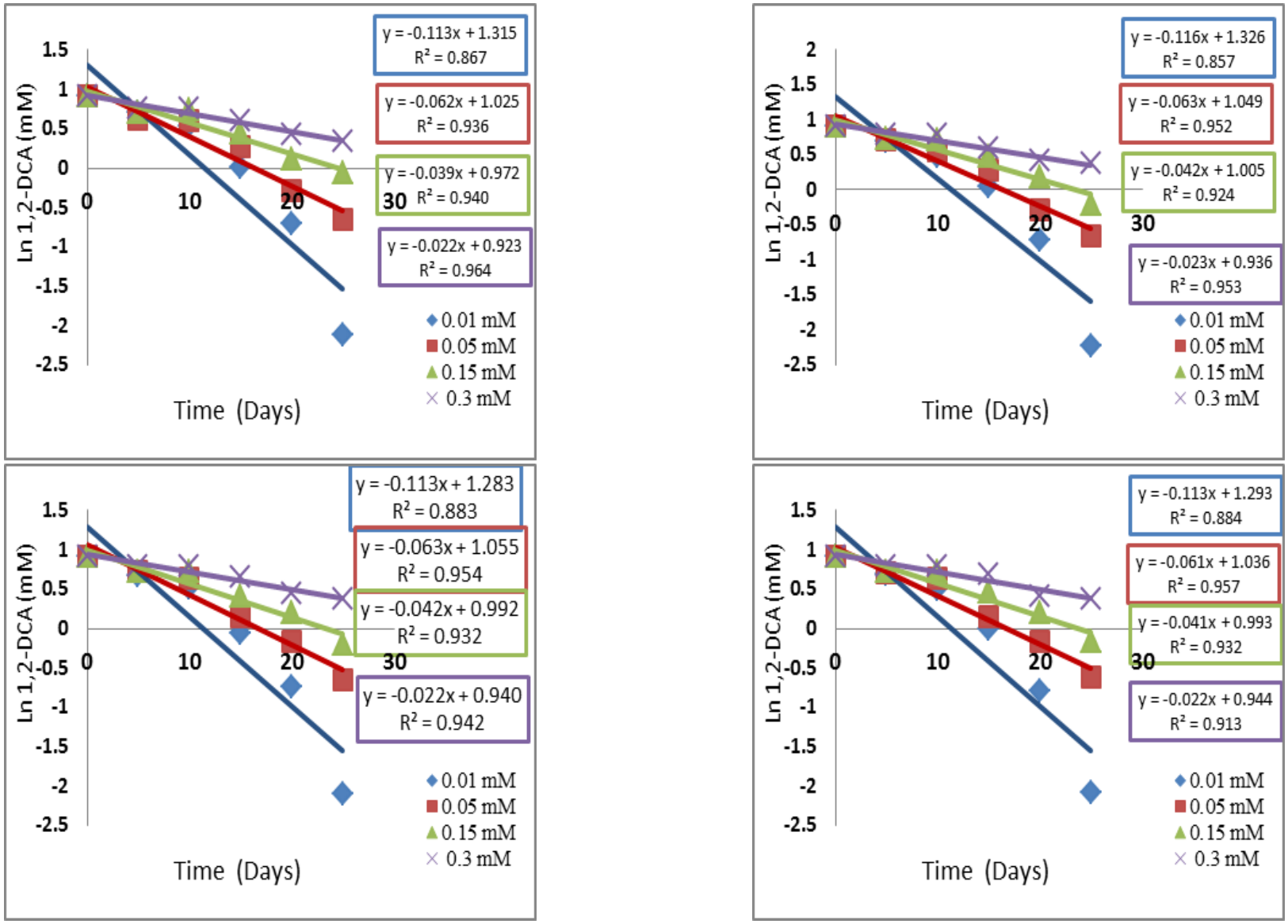
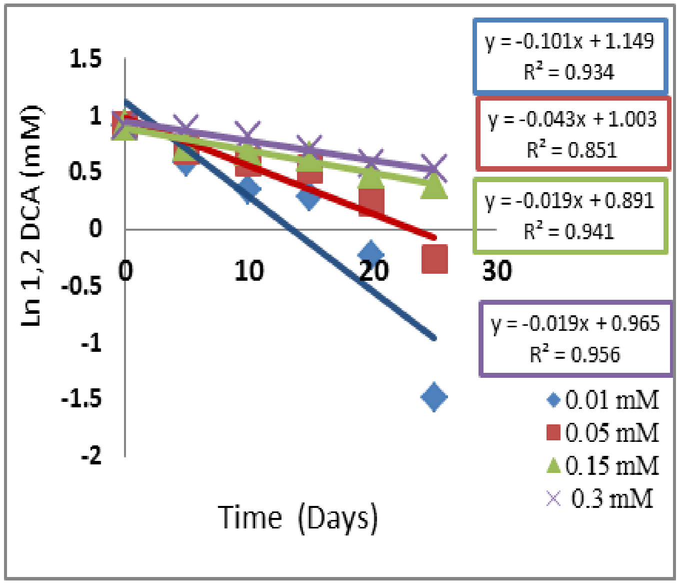
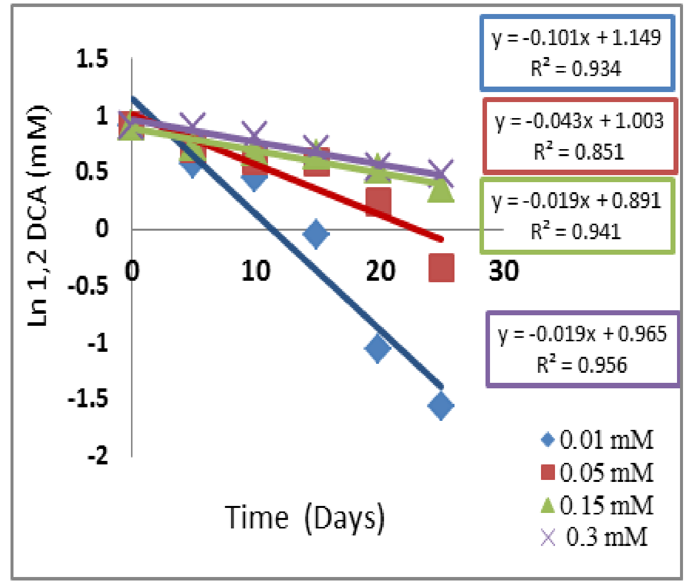
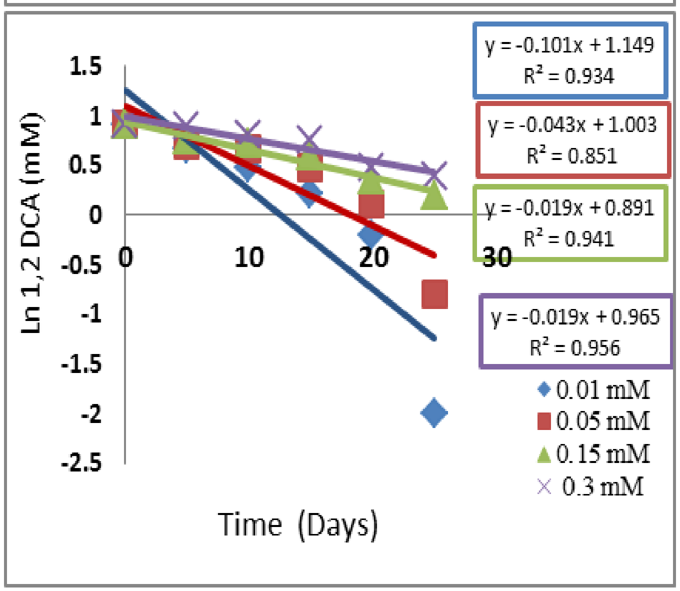
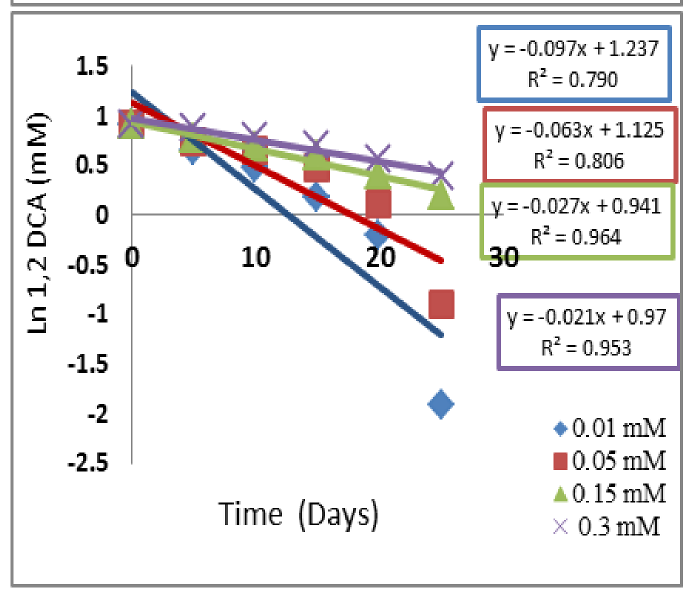


Figure 11: Inhibitory effects of Cd^{2+} on pseudo-first order 1,2-DCA biodegradation rates by indigenous microorganisms in NGWW (replicates of eight) (Chapter 2)


y = -0.101x + 1.149
R^2 = 0.934
y = -0.043x + 1.003
R^2 = 0.851
y = -0.019x + 0.891
R^2 = 0.941
y = -0.019x + 0.965
R^2 = 0.956
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2 DCA (mM)
Time (Days)


y = -0.101x + 1.149
R^2 = 0.934
y = -0.043x + 1.003
R^2 = 0.851
y = -0.019x + 0.891
R^2 = 0.941
y = -0.019x + 0.965
R^2 = 0.956
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2 DCA (mM)
Time (Days)


y = -0.101x + 1.149
R^2 = 0.934
y = -0.043x + 1.003
R^2 = 0.851
y = -0.019x + 0.891
R^2 = 0.941
y = -0.019x + 0.965
R^2 = 0.956
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2 DCA (mM)
Time (Days)


y = -0.097x + 1.237
R^2 = 0.790
y = -0.063x + 1.125
R^2 = 0.806
y = -0.027x + 0.941
R^2 = 0.964
y = -0.021x + 0.97
R^2 = 0.953
0.01 mM
0.05 mM
0.15 mM
0.3 mM
Ln 1,2 DCA (mM)
Time (Days)

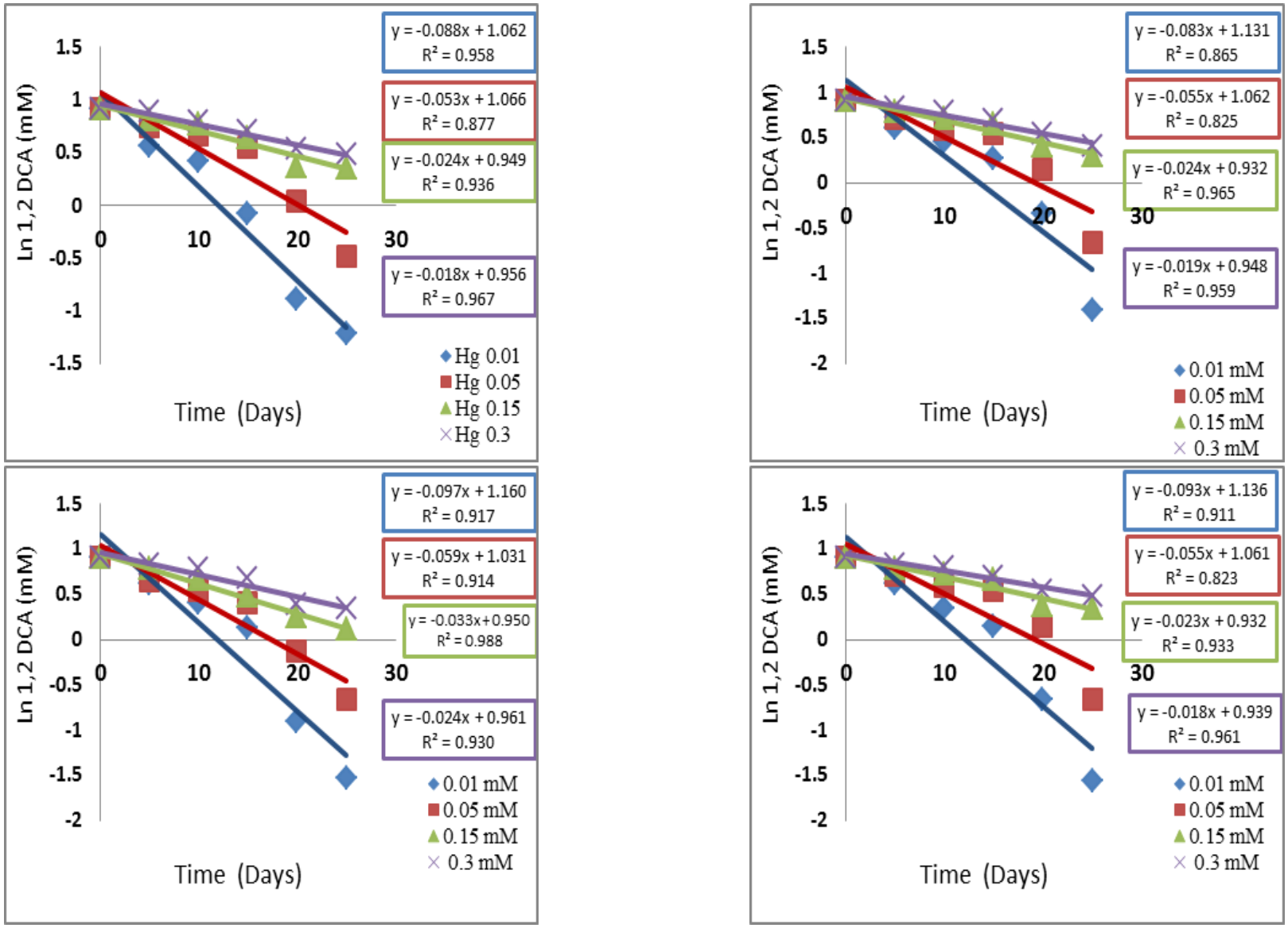
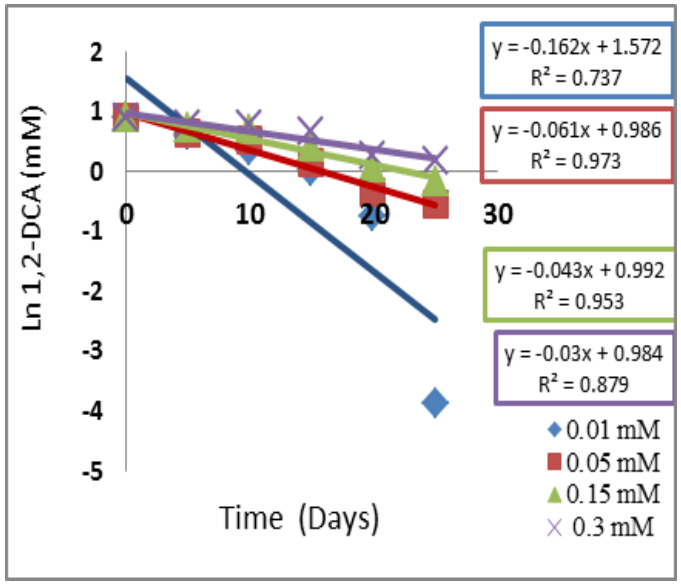
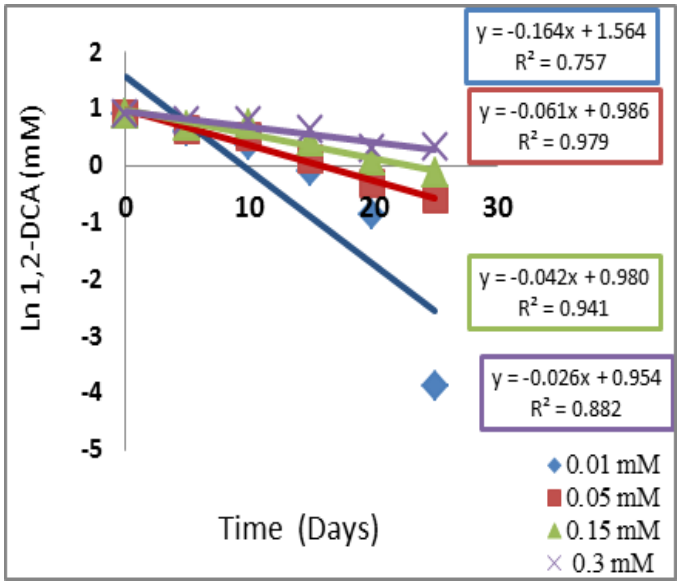
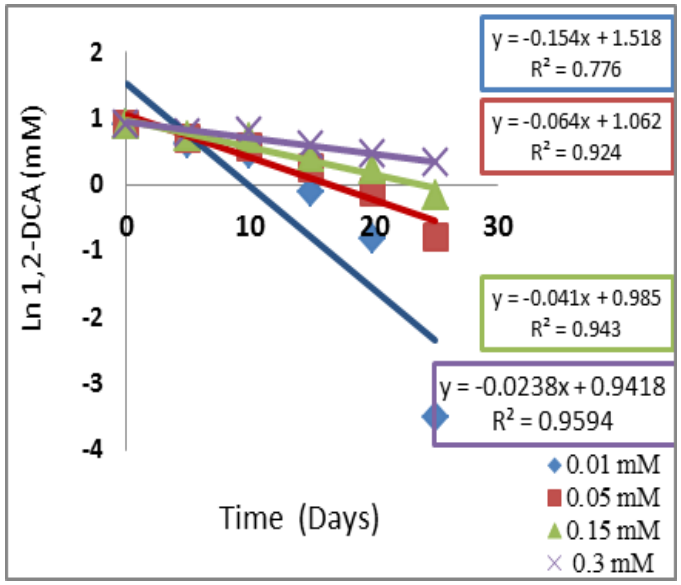
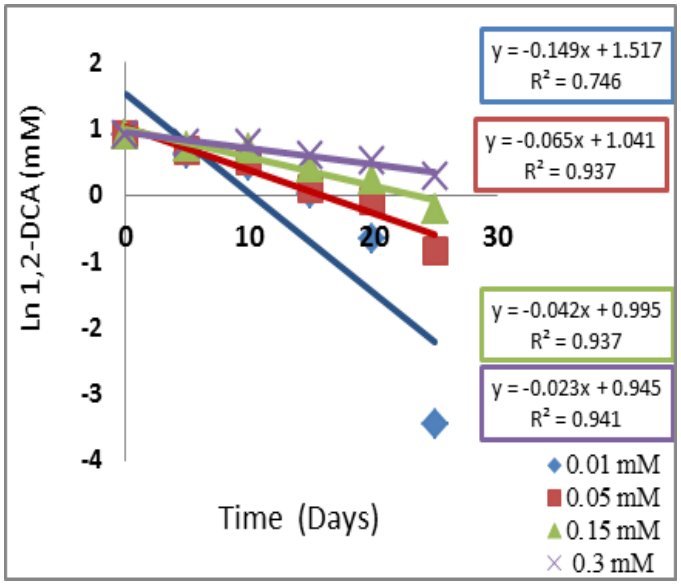


Figure 12: Inhibitory effects of Hg^{2+} on pseudo-first order 1,2-DCA biodegradation rates by indigenous microorganisms in NGWW (replicates of eight) (Chapter 2).


Ln 1,2-DCA (mM)
Time (Days)
y = -0.162x + 1.572
R² = 0.737
y = -0.061x + 0.986
R² = 0.973
y = -0.043x + 0.992
R² = 0.953
y = -0.03x + 0.984
R² = 0.879
0.01 mM
0.05 mM
0.15 mM
0.3 mM


Ln 1,2-DCA (mM)
Time (Days)
y = -0.164x + 1.564
R² = 0.757
y = -0.061x + 0.986
R² = 0.979
y = -0.042x + 0.980
R² = 0.941
y = -0.026x + 0.954
R² = 0.882
0.01 mM
0.05 mM
0.15 mM
0.3 mM


Ln 1,2-DCA (mM)
Time (Days)
y = -0.154x + 1.518
R² = 0.776
y = -0.064x + 1.062
R² = 0.924
y = -0.041x + 0.985
R² = 0.943
y = -0.0238x + 0.9418
R² = 0.9594
0.01 mM
0.05 mM
0.15 mM
0.3 mM


Ln 1,2-DCA (mM)
Time (Days)
y = -0.149x + 1.517
R² = 0.746
y = -0.065x + 1.041
R² = 0.937
y = -0.042x + 0.995
R² = 0.937
y = -0.023x + 0.945
R² = 0.941
0.01 mM
0.05 mM
0.15 mM
0.3 mM

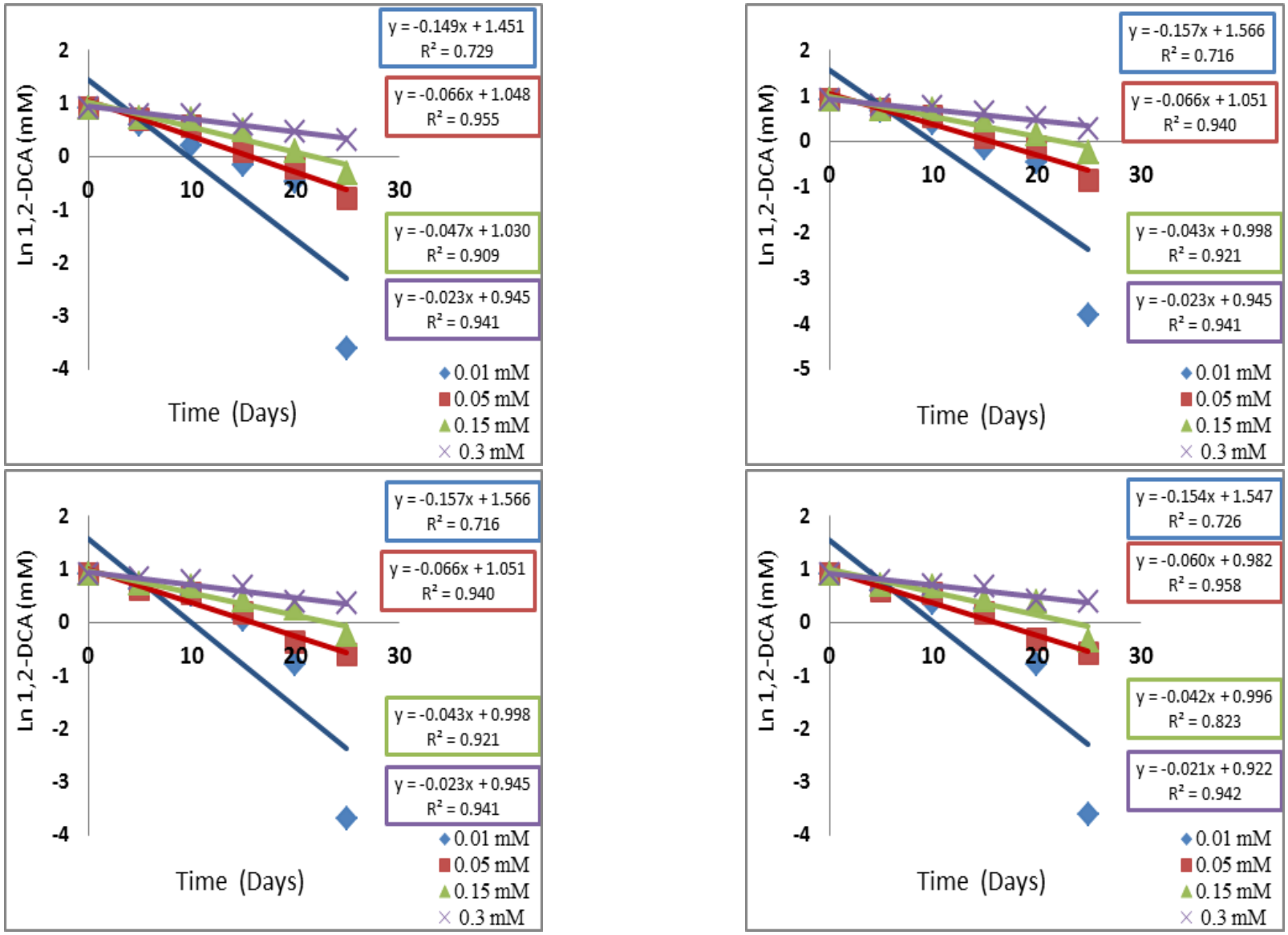
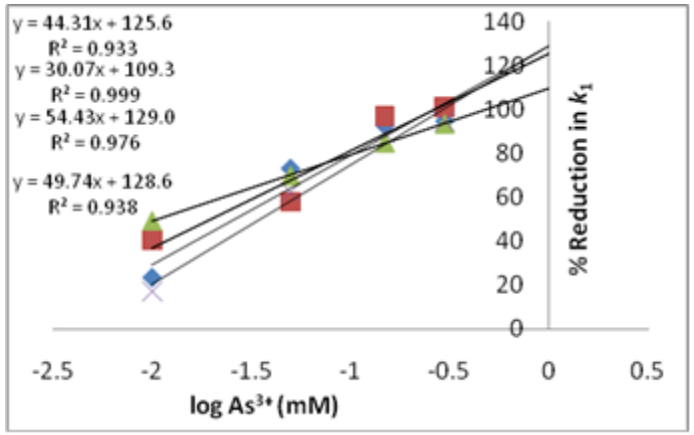


Figure 13: Inhibitory effects of $Pb2^{+}$ on pseudo-first order 1,2-DCA biodegradation rates by indigenous microorganisms in NGWW (replicates of eight) (Chapter 2)



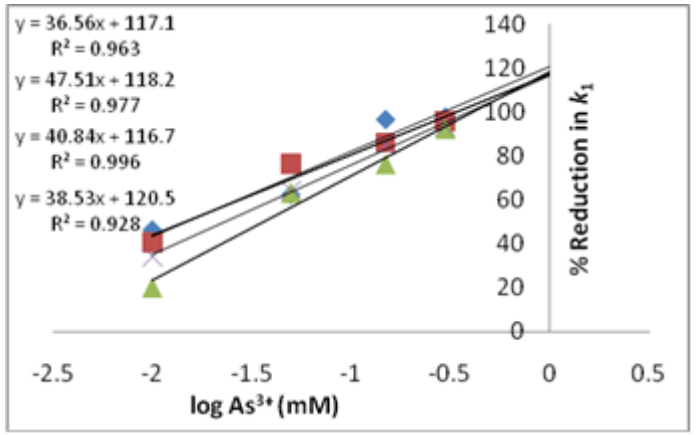
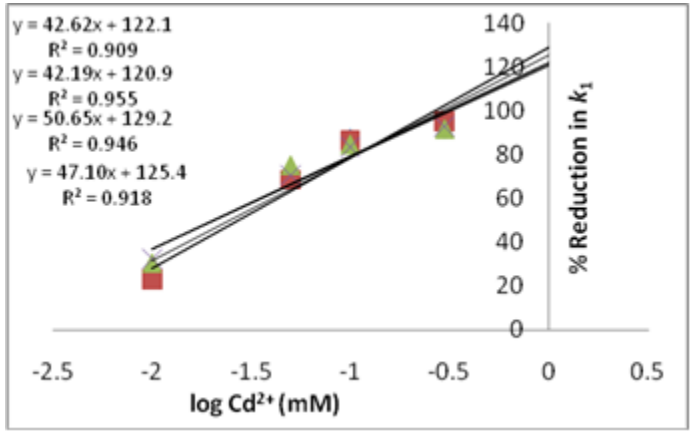


Figure 14: Inhibitory effects of a wide range of As^{3+} concentrations in NGWW on pseudo-first order degradation rate coefficients, k_1 (Chapter 2).



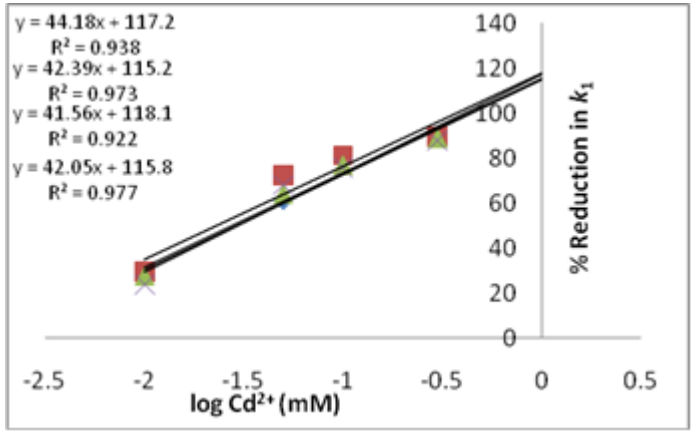
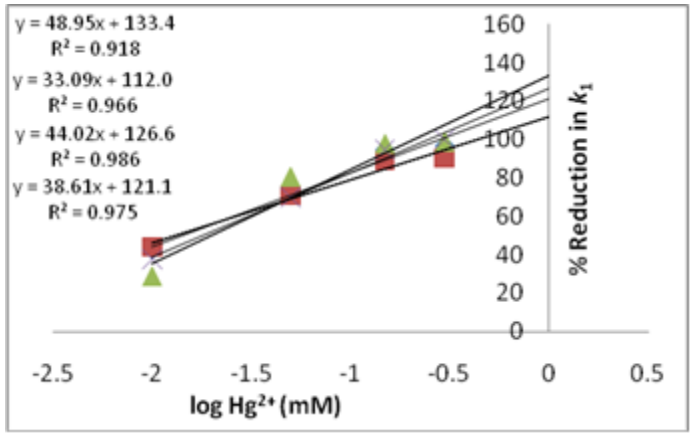


Figure 15: Inhibitory effects of a wide range of Cd^{2+} concentrations in NGWW on pseudo-first order degradation rate coefficients, k_1 (Chapter 2).



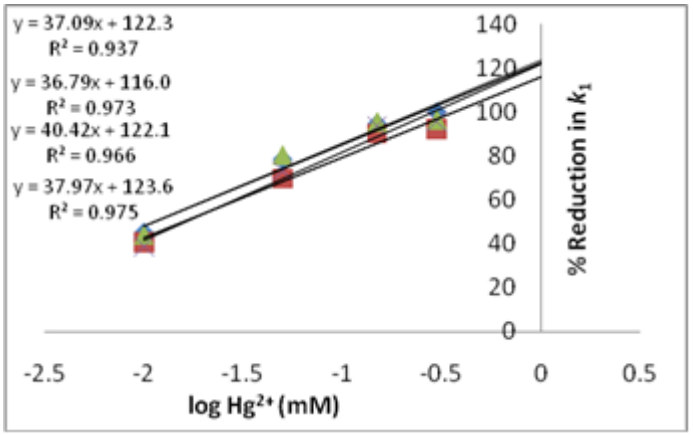
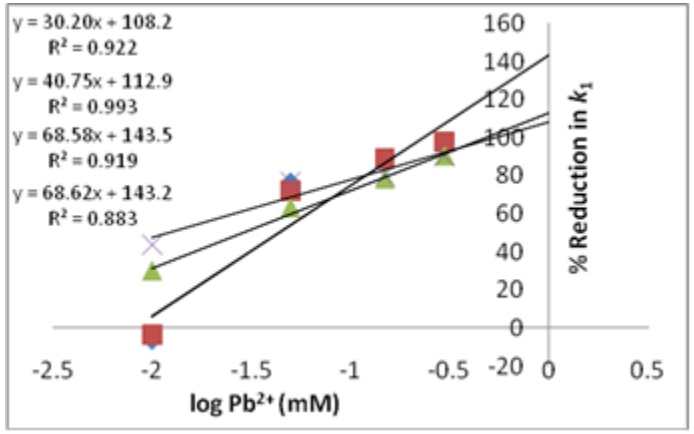


Figure 16: Inhibitory effects of a wide range of Hg^{2+} concentrations in NGWW on pseudo-first order degradation rate coefficients, k_1 (Chapter 2).



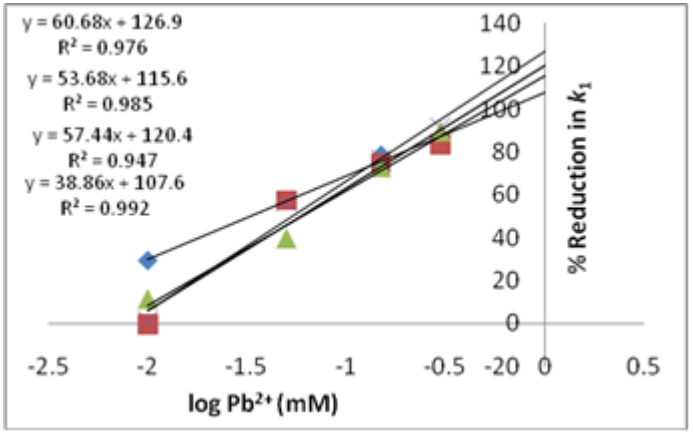


Figure 17: Inhibitory effects of a wide range of Pb^{2+} concentrations in NGWW on pseudo-first order degradation rate coefficients, k_1 (Chapter 2).

Table 10: Triplicate gas chromatography peak area values for construction of 1,2-DCA standard curve in NWW undergoing biostimulation and bioaugmentation treatments (Chapter 3).

1,2-DCA concentration (mM)	0.208	0.417	0.8	1.25	1.67	2.08	2.5
GC Peak area	599.75	905.43	1313.02	1915.02	2897.21	3948.84	4544.23
	579.18	786.13	1299.62	2155.89	3117.44	3798.42	4467.30
	589.47	815.20	1385.97	1861.35	2836.09	3775.45	4942.22

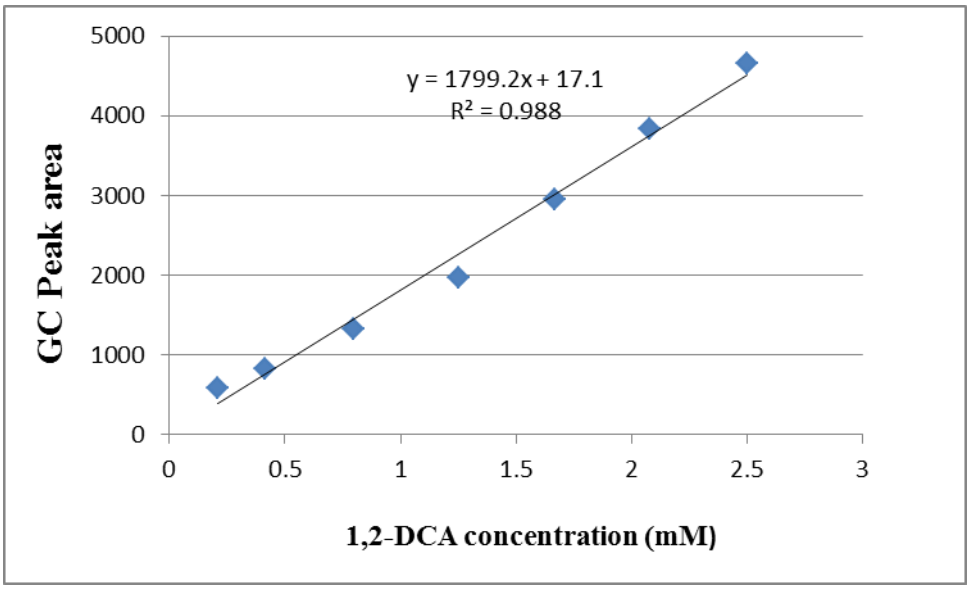
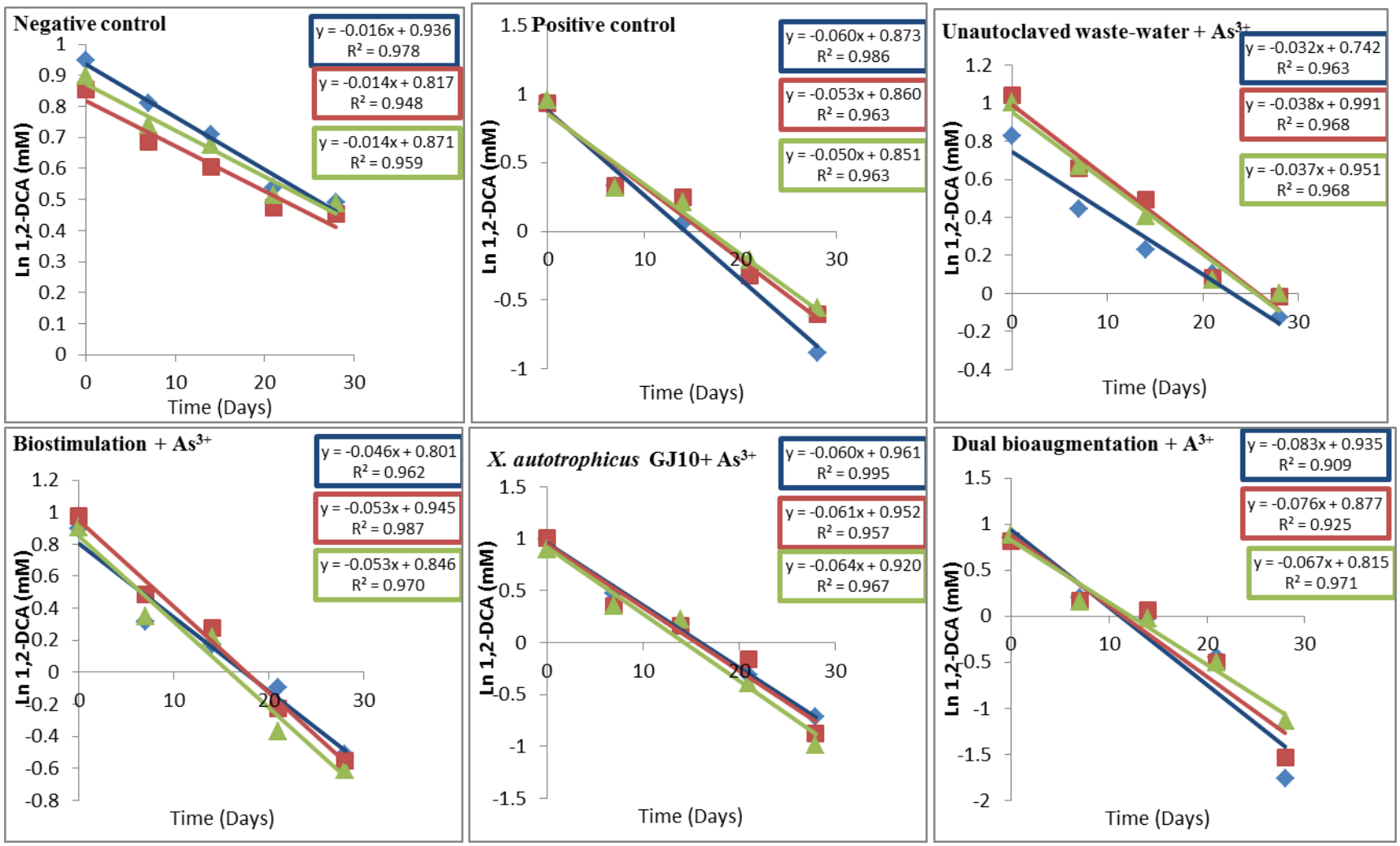
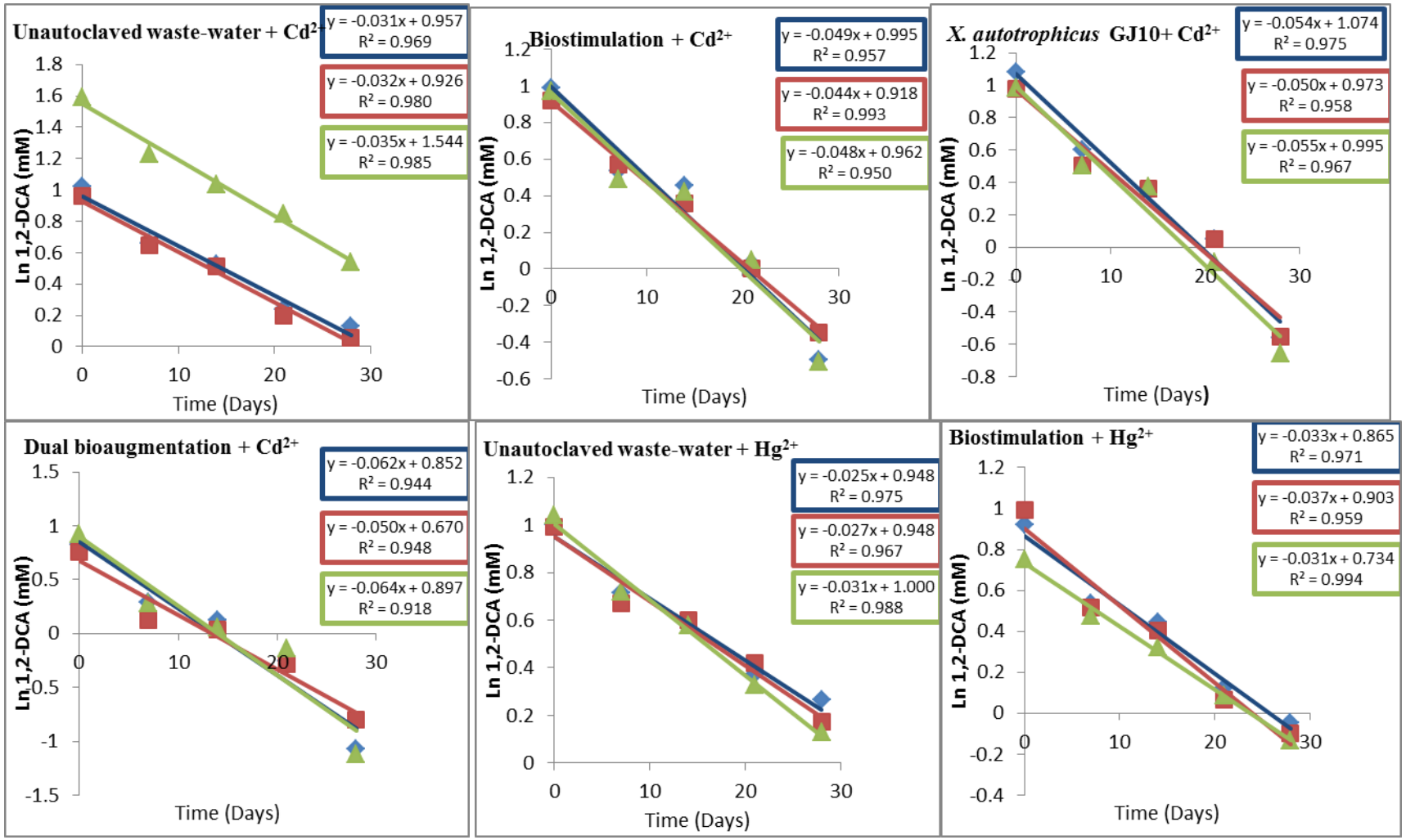


Figure 18: Standard curve for determination of 1,2-DCA concentration in NWW undergoing biostimulation and bioaugmentation treatments (Chapter 3).

Table 11: Triplicate gas chromatographic peak area values for heavy metal co-contaminated NWW microcosms undergoing biostimulation and bioaugmentation treatments (Chapter 3).

Microcosm	Day 0	Day 7	Day 14	Day 21	Day 28
Autoclaved control	4657.96	4058.88	3675.01	3096.99	2958.46
	4244.07	3587.36	3310.12	2907.08	2845.13
	4432.61	3785.52	3548.70	3026.79	2947.25
Unautoclaved control	4536.73	2523.66	1924.49	1315.38	762.31
	4587.20	2514.45	2312.07	1317.84	1000.21
	4678.35	2484.74	2239.70	1481.97	1045.35
Unautoclaved control + As^{3+}	5015.17	3506.24	3055.33	2305.64	2061.70
	4723.81	3443.75	3027.29	2207.47	1925.34
	8841.04	6159.51	5095.56	4199.73	3109.30
Glucose+ As^{3+}	4848.76	3082.87	2849.34	1876.71	1113.55
	4520.15	3188.98	2592.50	1823.05	1286.28
	4762.62	2946.26	2750.70	1905.42	1100.27
X. autotrophicus GJ10+ As^{3+}	5330.62	3296.53	2603.34	1907.62	1046.22
	4813.84	3003.69	2600.63	1910.89	1053.66
	4816.19	2995.94	2626.91	1662.14	952.91
Dual-bioaugmentation+ As^{3+}	4139.09	2421.02	2050.88	1416.12	633.64
	3857.81	2046.06	1877.75	1375.84	821.60
	4536.91	2386.33	1913.03	1581.00	602.49
Unautoclaved control + Cd^{2+}	4124.06	2815.79	2275.82	2008.60	1604.43
	5128.11	3494.86	2959.84	1967.21	1788.92
	4934.52	3524.83	2718.45	1953.40	1820.41
Glucose + Cd^{2+}	4419.40	2488.64	2160.16	1651.28	1092.65
	4796.15	2934.12	2390.28	1449.00	1052.61
	4453.23	2566.63	2249.66	1261.22	993.46
X. autotrophicus GJ10 + Cd^{2+}	4871.87	2905.92	2129.97	1331.15	893.98
	4935.59	2574.75	2131.14	1556.01	763.81
	4426.29	2575.33	2262.55	1235.79	686.02
Dual-bioaugmentation + Cd^{2+}	4225.56	2207.73	1762.15	1172.77	325.35
	4089.32	2137.70	1947.43	1107.75	403.97
	4361.80	2120.60	1777.04	1102.34	592.77
Unautoclaved control + Hg^{2+}	4904.32	3690.72	3235.50	2623.78	2364.06
	4863.63	3536.25	3290.28	2751.14	2156.40
	5098.04	3706.87	3227.69	2510.93	2063.34
Glucose+ Hg^{2+}	4531.55	3099.60	2828.21	2049.50	1734.98
	4879.02	3033.40	2707.41	1934.68	1648.92
	3839.98	2915.34	2497.16	1975.43	1588.79
X. autotrophicus GJ10+ Hg^{2+}	4554.87	2897.36	2706.44	2002.71	1644.59
	5530.27	3573.36	2960.80	2135.50	1584.48
	4205.96	2744.79	2513.68	1900.63	1278.11
Dual-bioaugmentation+ Hg^{2+}	4754.75	2936.61	2006.37	1893.04	1291.43
	4695.17	3083.51	2547.95	1746.77	1477.95
	4969.61	3047.20	2706.36	1867.53	1200.71
Unautoclaved control + Pb^{2+}	3723.72	2003.42	1653.48	1456.60	1992.89
	9823.32	5389.19	4193.77	3769.22	2860.48
	8697.61	4789.12	3987.82	3551.42	2302.82
Glucose + Pb^{2+}	4289.72	2172.13	1891.35	1035.32	596.44
	4231.55	2121.23	1770.77	990.75	670.94
	4395.17	2048.09	1982.35	1004.19	504.72
X. autotrophicus GJ10 + Pb^{2+}	4318.80	2002.12	1556.73	905.98	426.36
	4574.54	2177.73	1630.63	901.51	502.67
	4569.64	2102.28	1733.96	964.63	419.60
Dual-bioaugmentation + Pb^{2+}	4529.35	1756.22	1507.66	876.66	312.11
	4635.14	1768.53	1391.93	832.62	238.41
	4301.30	1683.55	1258.53	806.42	204.35


Negative control
y = -0.016x + 0.936
R² = 0.978
y = -0.014x + 0.817
R² = 0.948
y = -0.014x + 0.871
R² = 0.959
Ln 1,2-DCA (mM)
Time (Days)
Positive control
y = -0.060x + 0.873
R² = 0.986
y = -0.053x + 0.860
R² = 0.963
y = -0.050x + 0.851
R² = 0.963
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved waste-water + As3+
y = -0.032x + 0.742
R² = 0.963
y = -0.038x + 0.991
R² = 0.968
y = -0.037x + 0.951
R² = 0.968
Ln 1,2-DCA (mM)
Time (Days)
Biostimulation + As3+
y = -0.046x + 0.801
R² = 0.962
y = -0.053x + 0.945
R² = 0.987
y = -0.053x + 0.846
R² = 0.970
Ln 1,2-DCA (mM)
Time (Days)
X. autotrophicus GJ10+ As3+
y = -0.060x + 0.961
R² = 0.995
y = -0.061x + 0.952
R² = 0.957
y = -0.064x + 0.920
R² = 0.967
Ln 1,2-DCA (mM)
Time (Days)
Dual bioaugmentation + A3+
y = -0.083x + 0.935
R² = 0.909
y = -0.076x + 0.877
R² = 0.925
y = -0.067x + 0.815
R² = 0.971
Ln 1,2-DCA (mM)
Time (Days)


Unautoclaved waste-water + Cd2+
y = -0.031x + 0.957
R² = 0.969
y = -0.032x + 0.926
R² = 0.980
y = -0.035x + 1.544
R² = 0.985
Ln 1,2-DCA (mM)
Time (Days)
Biostimulation + Cd2+
y = -0.049x + 0.995
R² = 0.957
y = -0.044x + 0.918
R² = 0.993
y = -0.048x + 0.962
R² = 0.950
Ln 1,2-DCA (mM)
Time (Days)
X. autotrophicus GJ10+ Cd2+
y = -0.054x + 1.074
R² = 0.975
y = -0.050x + 0.973
R² = 0.958
y = -0.055x + 0.995
R² = 0.967
Ln 1,2-DCA (mM)
Time (Days)
Dual bioaugmentation + Cd2+
y = -0.062x + 0.852
R² = 0.944
y = -0.050x + 0.670
R² = 0.948
y = -0.064x + 0.897
R² = 0.918
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved waste-water + Hg2+
y = -0.025x + 0.948
R² = 0.975
y = -0.027x + 0.948
R² = 0.967
y = -0.031x + 1.000
R² = 0.988
Ln 1,2-DCA (mM)
Time (Days)
Biostimulation + Hg2+
y = -0.033x + 0.865
R² = 0.971
y = -0.037x + 0.903
R² = 0.959
y = -0.031x + 0.734
R² = 0.994
Ln 1,2-DCA (mM)
Time (Days)

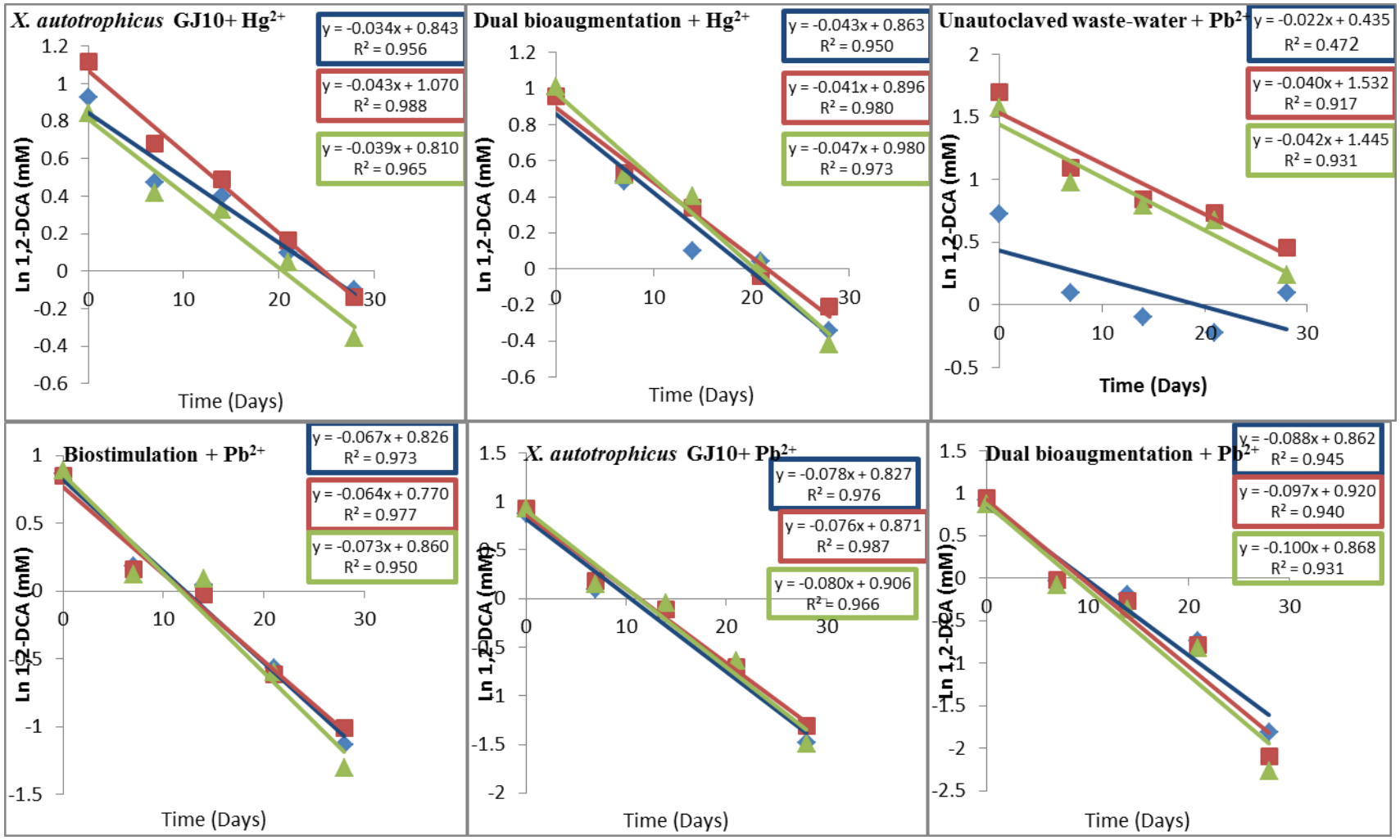


Figure 19: Graphical representation of the degradation rate constants of 1,2-DCA in arsenic, cadmium, mercury and lead co-contaminated NWW samples undergoing various bioremediation strategies. (◆)= sample 1; (■) = sample 2; (▲) = sample 3 (Chapter 3).

Table 12: The total 1,2-DCA degrading bacterial population in NWW co-contaminated with arsenic (As^{3+}) undergoing various bioremediation treatments (Chapter 3).

	No treatment						Biostimulation				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	39	86	213	343	432	10^{-2}	70	TMTC	TMTC	TMTC	TMTC
	40	87	244	321	534		65	TMTC	TMTC	TMTC	TMTC
	43	89	195	354	495		68	TMTC	TMTC	TMTC	TMTC
10^{-3}		8	22	34	45	10^{-3}		29	56	121	121
		9	23	37	50			32	60	102	134
		8	18	36	53			26	50	112	123
10^{-4}	0	0	0	0	4	10^{-4}	0	0	6	10	13
	0	0	0	0	5		0	0	5	9	12
	0	0	0	0	5		0	0	6	8	10
	Bioaugmentation						**Dual bioaugmentation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	120	TMTC	TMTC	TMTC	TMTC	10^{-2}	107	TMTC	TMTC	TMTC	TMTC
	121	TMTC	TMTC	TMTC	TMTC		110	TMTC	TMTC	TMTC	TMTC
	125	TMTC	TMTC	TMTC	TMTC		108	TMTC	TMTC	TMTC	TMTC
10^{-3}		61	101	133	178	10^{-3}		110	180	234	325
		59	124	143	183			112	174	254	301
		62	99	122	163			97	168	235	293
10^{-4}		6	10	13	18	10^{-4}	0	11	17	22	30
		7	11	14	17		0	10	18	24	31
		5	9	13	16		0	10	16	25	28
10^{-5}		0	0	0	0	10^{-5}	0	0	0	2	3
		0	0	0	0		0	0	0	3	4
		0	0	0	0		0	0	0	2	2

TMTC = Too many to count for a definitive colony count

Table 13: The total 1,2-DCA degrading bacterial population in NWW co-contaminated with cadmium (Cd^{2+}) undergoing various bioremediation treatments (Chapter 3).

	No treatment						Biostimulation				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	70	95	294	412	560	10^{-2}	70	TMTC	TMTC	TMTC	TMTC
	65	92	292	405	567		65	TMTC	TMTC	TMTC	TMTC
	68	93	290	423	576		68	TMTC	TMTC	TMTC	TMTC
10^{-3}		8	29	42	56	10^{-3}		23	55	93	124
		9	27	41	51			21	50	96	127
		9	28	39	60			22	53	91	129
10^{-4}	0	0	0	4	6	10^{-4}	0	2	6	8	11
	0	0	0	4	5		0	2	5	9	12
	0	0	0	5	5		0	3	5	9	13
	Bioaugmentation						**Dual bioaugmentation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	70	TMTC	TMTC	TMTC	TMTC	10^{-2}	107	TMTC	TMTC	TMTC	TMTC
	65	TMTC	TMTC	TMTC	TMTC		110	TMTC	TMTC	TMTC	TMTC
	68	TMTC	TMTC	TMTC	TMTC		108	TMTC	TMTC	TMTC	TMTC
10^{-3}		23	55	93	124	10^{-3}		128	243	294	395
		21	50	96	127			125	203	312	383
		22	53	91	129			130	250	293	433
10^{-4}	0	2	6	8	11	10^{-4}	0	13	24	34	34
	0	2	5	9	12		0	12	26	38	39
	0	3	5	9	13		0	10	22	30	36
10^{-5}	0	0	0	0	0	10^{-5}	0	0	3	3	3
	0	0	0	0	0		0	0	2	3	3
	0	0	0	0	0		0	0	3	2	4

TMTC = Too many to count for a definitive colony count

Table 14: The total 1,2-DCA degrading bacterial population in NWW co-contaminated with mercury (Hg^{2+}) undergoing various bioremediation treatments (Chapter 3).

	No treatment						Biostimulation				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	72	212	214	312	510	10^{-2}	104	TMTC	TMTC	TMTC	TMTC
	64	154	220	325	493		97	TMTC	TMTC	TMTC	TMTC
	69	145	210	311	512		89	TMTC	TMTC	TMTC	TMTC
10^{-3}		20	22	32	54	10^{-3}		40	64	79	120
		16	19	29	45			45	48	89	95
		13	23	31	60			53	56	75	100
10^{-4}	0	0	0	4	5	10^{-4}	0	3	6	8	10
	0	0	0	3	5		0	3	5	7	8
	0	0	0	3	4		0	4	4	6	9
	Bioaugmentation						**Dual bioaugmentation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	125	TMTC	TMTC	TMTC	TMTC	10^{-2}	107	TMTC	TMTC	TMTC	TMTC
	124	TMTC	TMTC	TMTC	TMTC		110	TMTC	TMTC	TMTC	TMTC
	122	TMTC	TMTC	TMTC	TMTC		108	TMTC	TMTC	TMTC	TMTC
10^{-3}		64	89	125	154	10^{-3}		96	136	213	250
		62	86	112	145			100	125	203	238
		63	90	108	137			85	156	190	247
10^{-4}	0	6	8	14	16	10^{-4}	0	8	12	19	25
	0	6	7	10	14		0	9	16	17	26
	0	7	8	11	12		0	8	14	18	27
10^{-5}	0	0	0	0	0	10^{-5}	0	0	1	2	3
	0	0	0	0	0		0	0	1	3	3
	0	0	0	0	0		0	0	1	1	2

TMTC = Too many to count for a definitive colony count

Table 15: The total 1,2-DCA degrading bacterial population in NWW co-contaminated with lead (Pb^{2+}) undergoing various bioremediation treatments (Chapter 3).

	No treatment						**Biostimulation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	39	167	407	534	614	10^{-2}	70	TMTC	TMTC	TMTC	TMTC
	40	156	354	512	635		65	TMTC	TMTC	TMTC	TMTC
	43	176	393	499	600		68	TMTC	TMTC	TMTC	TMTC
10^{-3}		20	38	53	65	10^{-3}		64	112	153	203
		16	39	55	63			67	99	156	210
		13	34	49	72			65	120	149	199
10^{-4}	0	0	2	5	6	10^{-4}	0	5	10	15	23
	0	0	3	4	5		0	6	11	14	20
	0	0	3	5	6		0	7	9	16	18
	Bioaugmentation						**Dual bioaugmentation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	120	TMTC	TMTC	TMTC	TMTC	10^{-2}	107	TMTC	TMTC	TMTC	TMTC
	121	TMTC	TMTC	TMTC	TMTC		110	TMTC	TMTC	TMTC	TMTC
	125	TMTC	TMTC	TMTC	TMTC		108	TMTC	TMTC	TMTC	TMTC
10^{-3}		118	142	233	285	10^{-3}		183	287	394	493
		120	153	213	312			199	316	412	489
		114	134	202	276			212	296	343	533
10^{-4}		12	14	23	28	10^{-4}	0	18	30	41	51
		13	16	24	27		0	19	26	38	47
		11	13	20	28		0	18	28	40	49
10^{-5}	0			2	3	10^{-5}	0	0	3	4	5
	0			2	3		0	0	2	3	5
	0			3	4		0	0	3	4	4

TMTC = Too many to count for a definitive colony count

Table 16: Triplicate gas chromatography peak area values for construction of 1,2-DCA standard curve in NGWW undergoing biostimulation and bioaugmentation treatments (Chapter 3).

1,2-DCA concentration (mM)	**0.208**	**0.417**	**0.8**	**1.25**	**1.67**	**2.08**	**2.5**
GC Peak area	613	910.43	1327.02	1938.27	2910.46	3962.09	4557.48
	596.43	806.13	1284.62	2169.14	3130.69	3811.67	4480.55
	599.72	827.2	1372.72	1874.6	2849.34	3788.7	4955.47

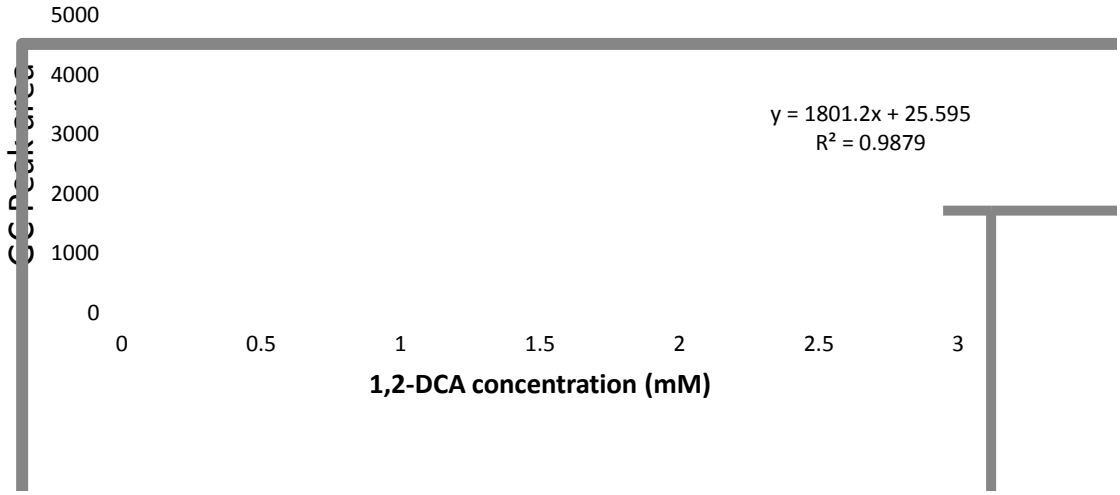
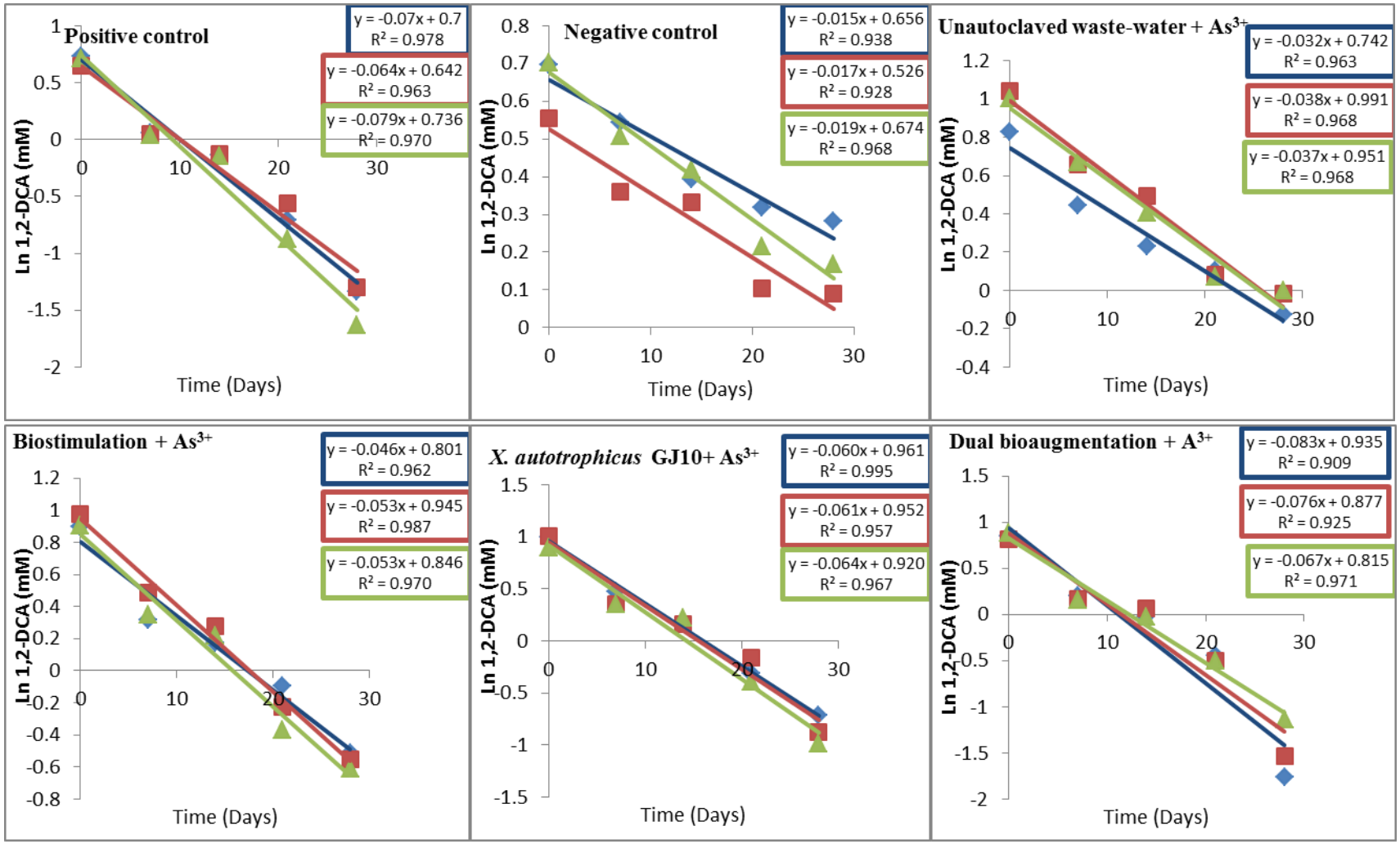
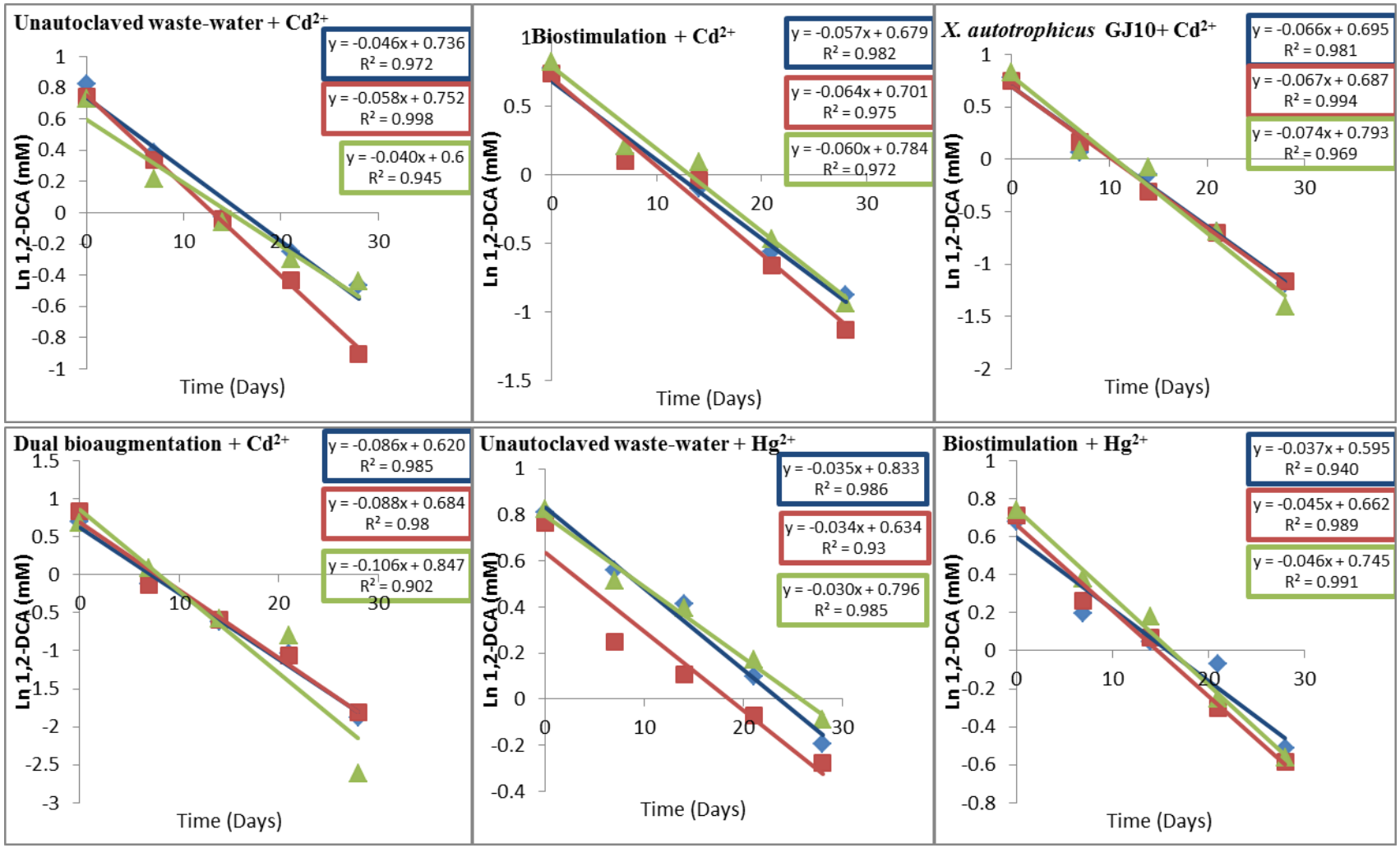


Figure 20: Standard curve for determination of 1,2-DCA concentration in NWW undergoing biostimulation and bioaugmentation treatments (Chapter 3).

Table 17: Triplicate gas chromatographic peak area values for heavy metal co-contaminated NGWW microcosms undergoing biostimulation and bioaugmentation treatments (Chapter 3).

Microcosm	Day 0	Day 7	Day 14	Day 21	Day 28
Autoclaved control	3618.19	3109.23	2673.09	2483.21	2398.34
	3147.95	2589.35	2518.58	2004.37	1976.35
	3643.30	2999.23	2745.99	2238.49	2137.39
Unautoclaved control	3752.06	1908.24	1572.14	889.58	478.35
	3465.69	1889.23	1587.25	1028.84	498.03
	3664.37	1879.24	1557.35	756.35	358.25
Unautoclaved control + As^{3+}	4095.55	2781.68	2315.13	1339.90	1258.47
	5089.79	3199.72	2546.35	1871.68	1695.07
	4025.61	2756.13	2136.15	1745.36	1487.84
Glucose+ As^{3+}	4451.39	2765.52	2146.69	1725.33	885.19
	4234.10	2669.70	1923.90	1245.81	991.74
	3674.04	2417.41	2326.31	1308.94	840.11
X. autotrophicus GJ10+ As^{3+}	4095.53	2634.56	1830.87	1101.55	903.69
	3722.22	2012.03	1392.08	1096.34	470.58
	4198.40	2382.42	1791.19	1219.22	687.61
Dual-bioaugmentation+ As^{3+}	4291.78	2299.28	1971.39	1345.50	603.89
	4107.91	1996.94	1580.73	1189.91	527.28
	3859.78	2008.67	1682.03	1095.77	542.60
Unautoclaved control + Cd^{2+}	4104.99	2660.84	1759.83	1403.94	1132.11
	3783.46	2535.56	1734.57	1174.51	734.12
	3744.21	2246.86	1698.25	1342.42	1167.88
Glucose + Cd^{2+}	3912.29	2096.57	1633.32	1033.45	751.88
	3778.15	1997.61	1735.75	937.47	585.68
	4109.31	2223.50	1987.23	1128.75	707.73
X. autotrophicus GJ10 + Cd^{2+}	3910.23	1928.44	1554.79	903.41	558.33
	3830.15	2118.16	1331.12	900.56	565.17
	4119.76	1957.65	1668.70	904.23	444.84
Dual-bioaugmentation + Cd^{2+}	3620.48	1609.79	970.00	640.32	281.42
	4135.18	1570.99	997.36	630.03	298.36
	3572.11	1955.88	1012.23	809.10	136.44
Unautoclaved control + Hg^{2+}	4066.14	3163.16	2735.53	1993.17	1488.65
	3879.81	2314.63	2011.23	1679.64	1373.00
	4111.55	3020.49	2685.36	2147.25	1654.54
Glucose+ Hg^{2+}	3546.43	2199.80	1890.36	1688.78	1084.63
	3667.07	2353.44	1933.77	1339.73	1006.72
	3788.75	2640.55	2159.65	1408.17	1031.41
X. autotrophicus GJ10+ Hg^{2+}	3587.76	2243.25	1970.86	1368.11	1075.30
	3648.77	2196.96	1967.17	1258.11	798.98
	7299.78	4284.75	3317.28	2777.65	1226.20
Dual-bioaugmentation+ Hg^{2+}	3456.57	2012.25	1651.37	1148.94	618.13
	3721.40	1968.97	1606.08	1105.35	738.29
	3920.87	2131.00	1710.75	1188.48	857.59
Unautoclaved control + Pb^{2+}	4137.74	2031.27	1634.98	875.12	790.77
	3900.98	1839.88	1246.90	974.48	627.70
	3945.11	2089.30	1783.29	987.35	767.35
Glucose + Pb^{2+}	3971.82	1583.35	1432.32	597.50	311.29
	4236.90	2134.36	1128.23	651.46	567.23
	3968.75	2012.25	1734.25	778.23	436.73
X. autotrophicus GJ10 + Pb^{2+}	4427.221	1509.852	1154.362	505.951	277.652
	4704.893	1931.453	1593.408	619.582	332.345
	3815.773	1734.446	1345.234	709.345	280.630
Dual-bioaugmentation + Pb^{2+}	3735.31	1097.52	911.42	427.93	149.77
	3754.43	1075.03	997.79	633.93	132.70
	3656.79	1264.35	1010.22	378.43	150.18


Positive control
y = -0.07x + 0.7
R² = 0.978
y = -0.064x + 0.642
R² = 0.963
y = -0.079x + 0.736
R² = 0.970
Ln 1,2-DCA (mM)
Time (Days)
Negative control
y = -0.015x + 0.656
R² = 0.938
y = -0.017x + 0.526
R² = 0.928
y = -0.019x + 0.674
R² = 0.968
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved waste-water + As3+
y = -0.032x + 0.742
R² = 0.963
y = -0.038x + 0.991
R² = 0.968
y = -0.037x + 0.951
R² = 0.968
Ln 1,2-DCA (mM)
Time (Days)
Biostimulation + As3+
y = -0.046x + 0.801
R² = 0.962
y = -0.053x + 0.945
R² = 0.987
y = -0.053x + 0.846
R² = 0.970
Ln 1,2-DCA (mM)
Time (Days)
X. autotrophicus GJ10+ As3+
y = -0.060x + 0.961
R² = 0.995
y = -0.061x + 0.952
R² = 0.957
y = -0.064x + 0.920
R² = 0.967
Ln 1,2-DCA (mM)
Time (Days)
Dual bioaugmentation + A3+
y = -0.083x + 0.935
R² = 0.909
y = -0.076x + 0.877
R² = 0.925
y = -0.067x + 0.815
R² = 0.971
Ln 1,2-DCA (mM)
Time (Days)


Unautoclaved waste-water + Cd2+
y = -0.046x + 0.736
R² = 0.972
y = -0.058x + 0.752
R² = 0.998
y = -0.040x + 0.6
R² = 0.945
Ln 1,2-DCA (mM)
Time (Days)
Biostimulation + Cd2+
y = -0.057x + 0.679
R² = 0.982
y = -0.064x + 0.701
R² = 0.975
y = -0.060x + 0.784
R² = 0.972
Ln 1,2-DCA (mM)
Time (Days)
X. autotrophicus GJ10+ Cd2+
y = -0.066x + 0.695
R² = 0.981
y = -0.067x + 0.687
R² = 0.994
y = -0.074x + 0.793
R² = 0.969
Ln 1,2-DCA (mM)
Time (Days)
Dual bioaugmentation + Cd2+
y = -0.086x + 0.620
R² = 0.985
y = -0.088x + 0.684
R² = 0.98
y = -0.106x + 0.847
R² = 0.902
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved waste-water + Hg2+
y = -0.035x + 0.833
R² = 0.986
y = -0.034x + 0.634
R² = 0.93
y = -0.030x + 0.796
R² = 0.985
Ln 1,2-DCA (mM)
Time (Days)
Biostimulation + Hg2+
y = -0.037x + 0.595
R² = 0.940
y = -0.045x + 0.662
R² = 0.989
y = -0.046x + 0.745
R² = 0.991
Ln 1,2-DCA (mM)
Time (Days)

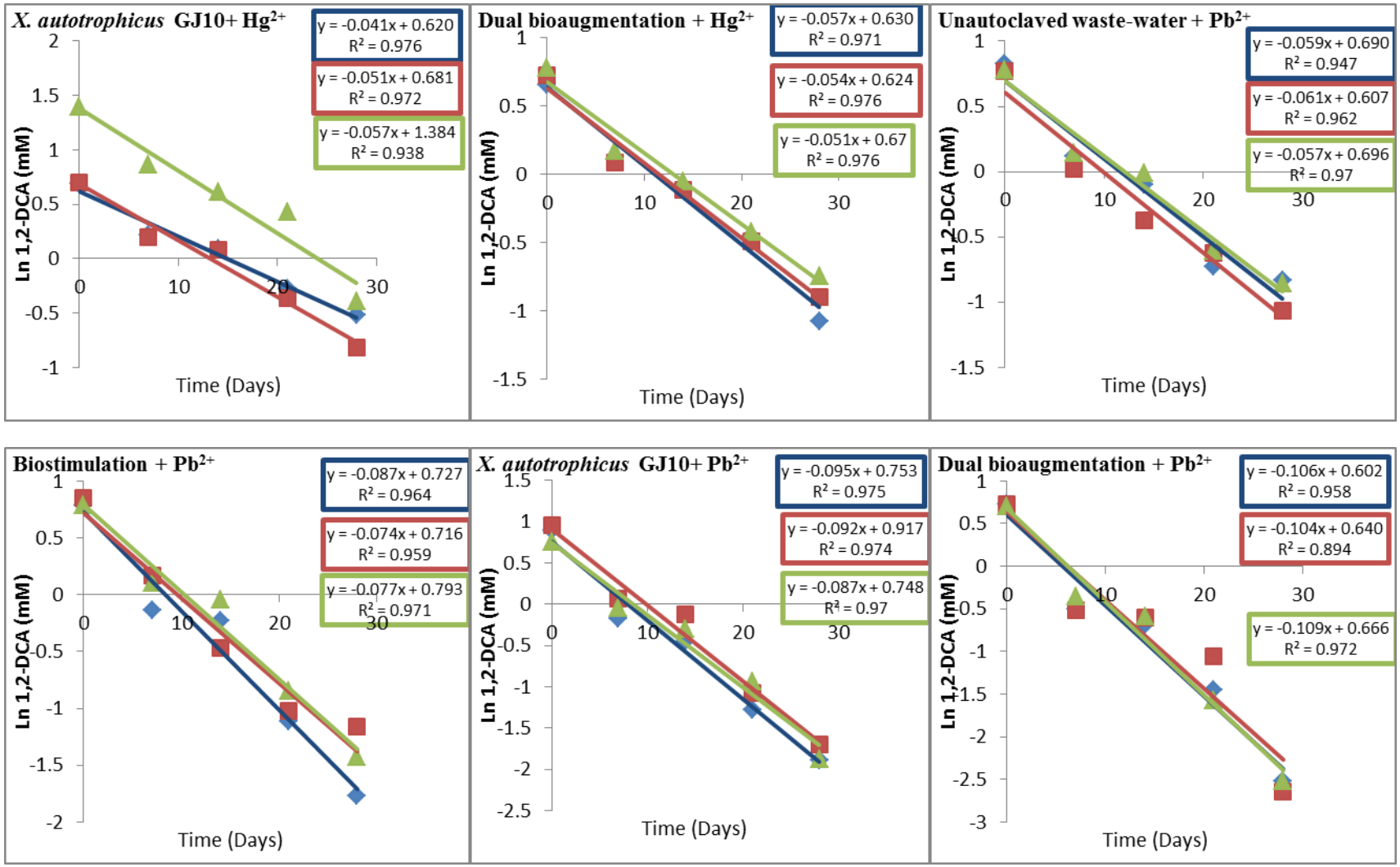


Figure 21: Graphical representation of the degradation rate constants of 1,2-DCA in arsenic, cadmium, mercury and lead co-contaminated NGWW samples undergoing various bioremediation strategies. (◆) = sample 1; (■) = sample 2; (▲) = sample 3 (Chapter 3).

Table 18: The total 1,2-DCA degrading bacterial population in NGWW co-contaminated with arsenic (As^{3+}) undergoing various bioremediation treatments (Chapter 3).

	No treatment						**Biostimulation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	39	167	407	534	614	10^{-2}	70	TMTC	TMTC	TMTC	TMTC
	40	156	354	512	635		65	TMTC	TMTC	TMTC	TMTC
	43	176	393	499	600		68	TMTC	TMTC	TMTC	TMTC
10^{-3}		20	38	53	65	10^{-3}	0	64	112	153	203
	0	16	39	55	63		0	67	99	156	210
	0	13	34	49	72		0	65	120	149	199
10^{-4}	0	0	2	5	6	10^{-4}	0	5	10	15	23
	0	0	3	4	5		0	6	11	14	20
	0	0	3	5	6		0	7	9	16	18
	Bioaugmentation						**Dual bioaugmentation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	120	TMTC	TMTC	TMTC	TMTC	10^{-2}	107	TMTC	TMTC	TMTC	TMTC
	121	TMTC	TMTC	TMTC	TMTC		110	TMTC	TMTC	TMTC	TMTC
	125	TMTC	TMTC	TMTC	TMTC		108	TMTC	TMTC	TMTC	TMTC
10^{-3}		118	142	233	285	10^{-3}		183	287	394	493
		120	153	213	312			199	316	412	489
		114	134	202	276			212	296	343	533
10^{-4}		11	13	20	28	10^{-4}		18	28	40	49
		12	14	23	28			18	30	41	51
		13	16	24	27			19	26	38	47
10^{-5}	0	0	0	3	3	10^{-5}		0	2	3	5
	0	0	0	2	4			0	3	4	4
	0	0	0	2	3			0	3	4	5

TMTC = Too many to count for a definitive colony count

Table 19: The total 1,2-DCA degrading bacterial population in NGWW co-contaminated with cadmium (Cd^{2+}) undergoing various bioremediation treatments (Chapter 3).

	No treatment						**Biostimulation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	39	167	407	534	614	10^{-2}	70	TMTC	TMTC	TMTC	TMTC
	40	156	354	512	635		65	TMTC	TMTC	TMTC	TMTC
	43	176	393	499	600		68	TMTC	TMTC	TMTC	TMTC
10^{-3}		20	38	53	65	10^{-3}		64	112	153	203
		16	39	55	63			67	99	156	210
		13	34	49	72			65	120	149	199
10^{-4}		0	2	5	6	10^{-4}		5	10	15	23
		0	3	4	5			6	11	14	20
		0	3	5	6			7	9	16	18
	Bioaugmentation						**Dual bioaugmentation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	120	TMTC	TMTC	TMTC	TMTC	10^{-2}	107	TMTC	TMTC	TMTC	TMTC
	121	TMTC	TMTC	TMTC	TMTC		110	TMTC	TMTC	TMTC	TMTC
	125	TMTC	TMTC	TMTC	TMTC		108	TMTC	TMTC	TMTC	TMTC
10^{-3}		118	142	233	285	10^{-3}		183	287	394	493
		120	153	213	312			199	316	412	489
		114	134	202	276			212	296	343	533
10^{-4}		12	14	23	28	10^{-4}		18	30	41	51
		13	16	24	27			19	26	38	47
		11	13	20	28			18	28	40	49
10^{-5}		0	0	2	3	10^{-5}		0	3	4	5
		0	0	2	3			0	2	3	5
		0	0	3	4			0	3	4	4

TMTC = Too many to count for a definitive colony count

Table 20: The total 1,2-DCA degrading bacterial population in NGWW co-contaminated with mercury (Hg^{2+}) undergoing various bioremediation treatments (Chapter 3).

	No treatment						**Biostimulation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	39	167	407	534	614	10^{-2}	70	TMTC	TMTC	TMTC	TMTC
	40	156	354	512	635		65	TMTC	TMTC	TMTC	TMTC
	43	176	393	499	600		68	TMTC	TMTC	TMTC	TMTC
10^{-3}		20	38	53	65	10^{-3}		64	112	153	203
		16	39	55	63			67	99	156	210
		13	34	49	72			65	120	149	199
10^{-4}			2	5	6	10^{-4}		5	10	15	23
			3	4	5			6	11	14	20
			3	5	6			7	9	16	18
	Bioaugmentation						**Dual bioaugmentation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	120	TMTC	TMTC	TMTC	TMTC	10^{-2}	107	TMTC	TMTC	TMTC	TMTC
	121	TMTC	TMTC	TMTC	TMTC		110	TMTC	TMTC	TMTC	TMTC
	125	TMTC	TMTC	TMTC	TMTC		108	TMTC	TMTC	TMTC	TMTC
10^{-3}		118	142	233	285	10^{-3}		183	287	394	493
		120	153	213	312			199	316	412	489
		114	134	202	276			212	296	343	533
10^{-4}		12	14	23	28	10^{-4}		18	30	41	51
		13	16	24	27			19	26	38	47
		11	13	20	28			18	28	40	49
10^{-5}				2	3	10^{-5}		0	3	4	5
				2	3			0	2	3	5
				3	4			0	3	4	4

TMTC = Too many to count for a definitive colony count

Table 21: The total 1,2-DCA degrading bacterial population in NGWW co-contaminated with lead (Pb^{2+}) undergoing various bioremediation treatments (Chapter 3).

	No treatment						**Biostimulation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	39	167	407	534	614	10^{-2}	70				
	40	156	354	512	635		65				
	43	176	393	499	600		68				
10^{-3}		20	38	53	65	10^{-3}		64	112	153	203
		16	39	55	63			67	99	156	210
		13	34	49	72			65	120	149	199
10^{-4}			2	5	6	10^{-4}		5	10	15	23
			3	4	5			6	11	14	20
			3	5	6			7	9	16	18
	Bioaugmentation						**Dual bioaugmentation**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**		**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
10^{-2}	120					10^{-2}	107				
	121						110				
	125						108				
10^{-3}		118	142	233	285	10^{-3}		183	287	394	493
		120	153	213	312			199	316	412	489
		114	134	202	276			212	296	343	533
10^{-4}						10^{-4}					
		12	14	23	28			18	30	41	51
		13	16	24	27			19	26	38	47
10^{-5}		11	13	20	28	10^{-5}		18	28	40	49
				2	3				3	4	5
				2	3				2	3	5
				3	4				3	4	4

TMTC = Too many to count for a definitive colony count

Table 22: Residual concentration of heavy metals (mM) in microcosms constructed to evaluate biosorption by varying concentration of corncobs (Chapter 4).

Heavy metal	Concentration of biosorbent (%)	Heavy metal concentration (mM) Sample 1	Heavy metal concentration (mM) Sample 2	Heavy metal concentration (mM) Sample 3
As^{3+}	0	2.00	1.95	1.97
	1	1.89	1.90	1.89
	1.5	1.87	1.87	1.87
	2	1.85	1.85	1.85
	2.5	1.83	1.84	1.84
Cd^{2+}	0	1.96	1.96	1.96
	1	1.64	1.68	1.65
	1.5	1.64	1.64	1.63
	2	1.56	1.56	1.53
	2.5	1.51	1.52	1.52
Hg^{2+}	0	1.99	1.96	2.00
	1	1.79	1.75	1.78
	1.5	1.73	1.68	1.69
	2	1.64	1.64	1.64
	2.5	1.57	1.58	1.57
Pb^{2+}	0	1.97	1.96	1.94
	1	1.57	1.58	1.60
	1.5	1.51	1.54	1.52
	2	1.33	1.35	1.38
	2.5	1.32	1.31	1.32

Table 23: Residual concentration of heavy metals (mM) in microcosms constructed to evaluate biosorption by varying concentration of coconut fiber (Chapter 4).

Heavy metal	**Concentration of biosorbent (%)**	Heavy metal concentration (mM) Sample 1	Heavy metal concentration (mM) Sample 2	Heavy metal concentration (mM) Sample 3
As^{3+}	0	2.00	1.95	1.97
	1	1.91	1.88	1.92
	1.5	1.89	1.86	1.87
	2	1.88	1.84	1.85
	2.5	1.78	1.86	1.77
Pb^{2+}	0	1.97	1.96	1.94
	1	0.69	0.70	0.69
	1.5	0.69	0.67	0.68
	2	0.58	0.57	0.58
	2.5	0.50	0.53	0.51
Hg^{2+}	0	1.99	1.96	2.00
	1	0.95	0.98	0.94
	1.5	0.89	0.88	0.88
	2	0.80	0.78	0.79
	2.5	0.68	0.66	0.67
Cd^{2+}	0	1.96	1.96	1.96
	1	1.34	1.38	1.37
	1.5	1.24	1.23	1.23
	2	1.07	1.06	1.06
	2.5	0.93	0.93	0.93

Table 24: Residual concentration of heavy metals (mM) in microcosms constructed to evaluate biosorption by varying concentration of orange peel (Chapter 4).

Heavy metal	**Concentration of biosorbent (%)**	Heavy metal concentration (mM) Sample 1	Heavy metal concentration (mM) Sample 2	Heavy metal concentration (mM) Sample 3
As^{3+}	0	2.00	1.95	1.97
	1	1.87	1.87	1.86
	1.5	1.76	1.78	1.77
	2	1.76	1.70	1.73
	2.5	1.63	1.60	1.62
Cd^{2+}	0	1.96	1.96	1.96
	1	0.54	0.53	0.55
	1.5	0.43	0.43	0.42
	2	0.37	0.37	0.37
	2.5	0.36	0.35	0.34
Hg^{2+}	0	1.99	1.96	2.00
	1	0.85	0.99	0.83
	1.5	0.80	0.90	0.84
	2	0.66	0.76	0.71
	2.5	0.56	0.64	0.60
Pb^{2+}	0	1.97	1.96	1.94
	1	0.20	0.19	0.19
	1.5	0.10	0.10	0.10
	2	0.06	0.07	0.07
	2.5	0.05	0.05	0.05

Table 25: Triplicate gas chromatography peak area values for construction of 1,2-DCA standard curve in NWW undergoing different biosorption treatments (Chapter 4).

1,2-DCA concentration (mM)	0.208	0.417	0.8	1.25	1.67	2.08	2.5
	623	903	1300.717	1923.125	2898.314	3949.94	4542.329
GC Peak area	588	823	1387.072	2056.993	3128.541	3899.517	4768.401
	574	826	1319.122	1822.453	2898.314	3736.547	5000.322

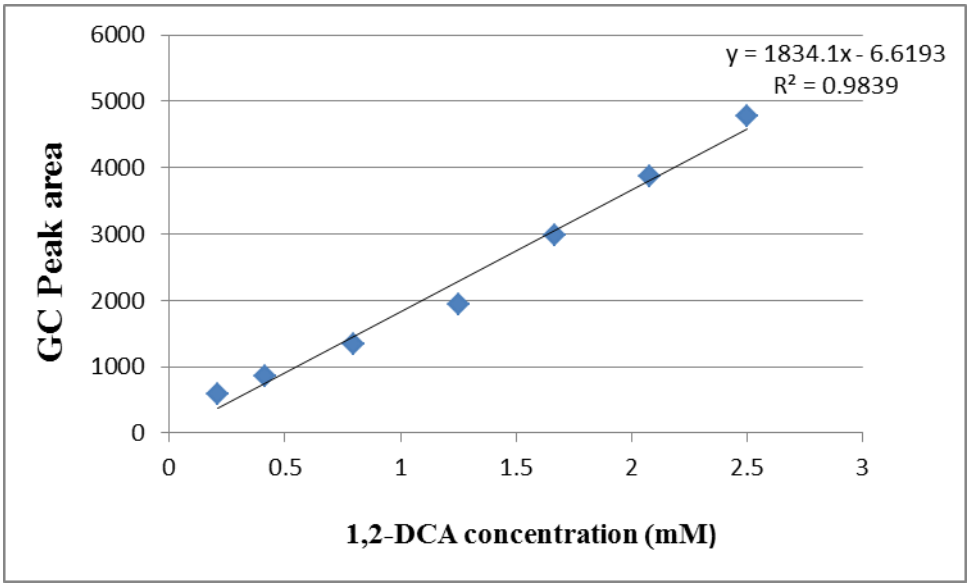
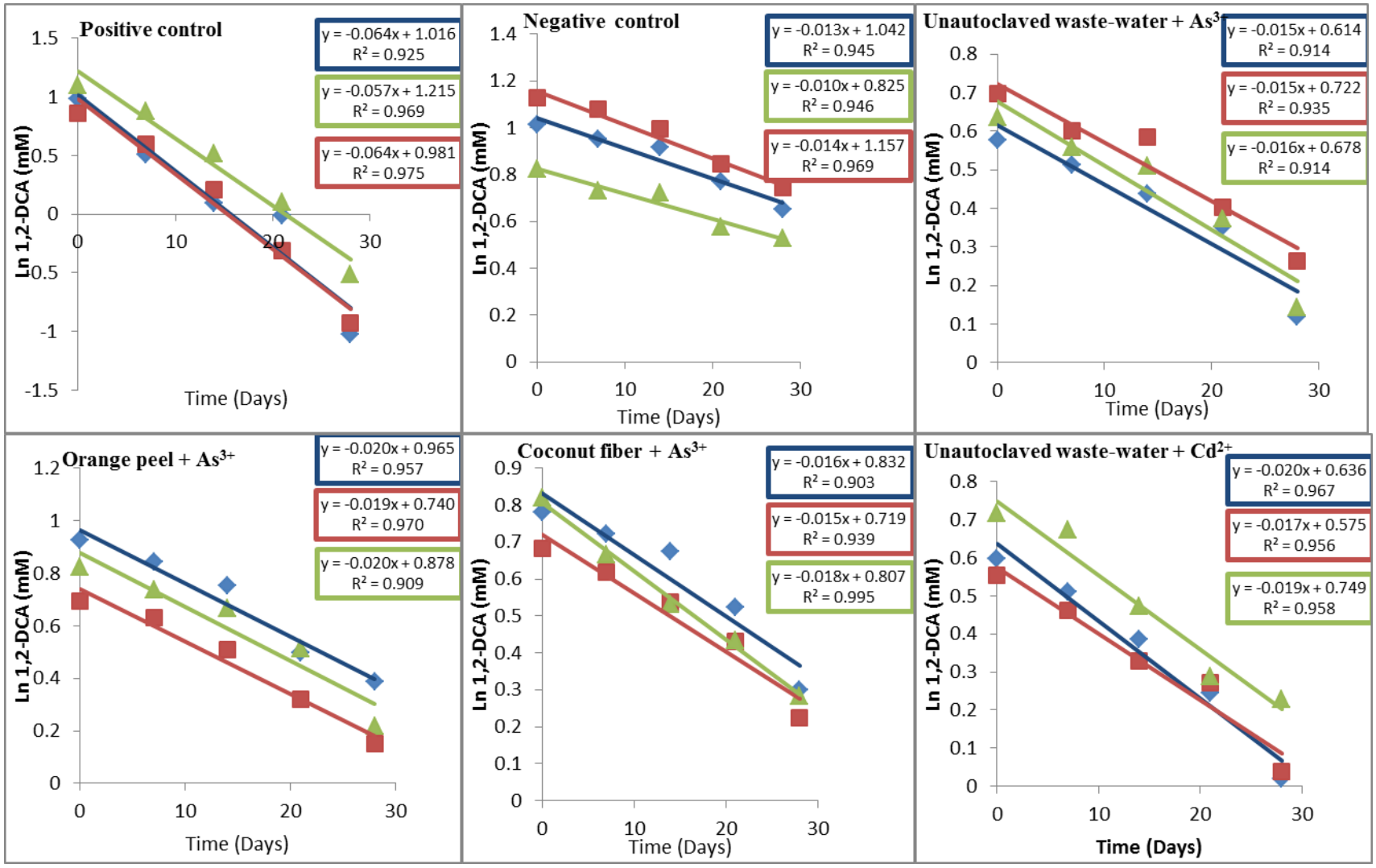
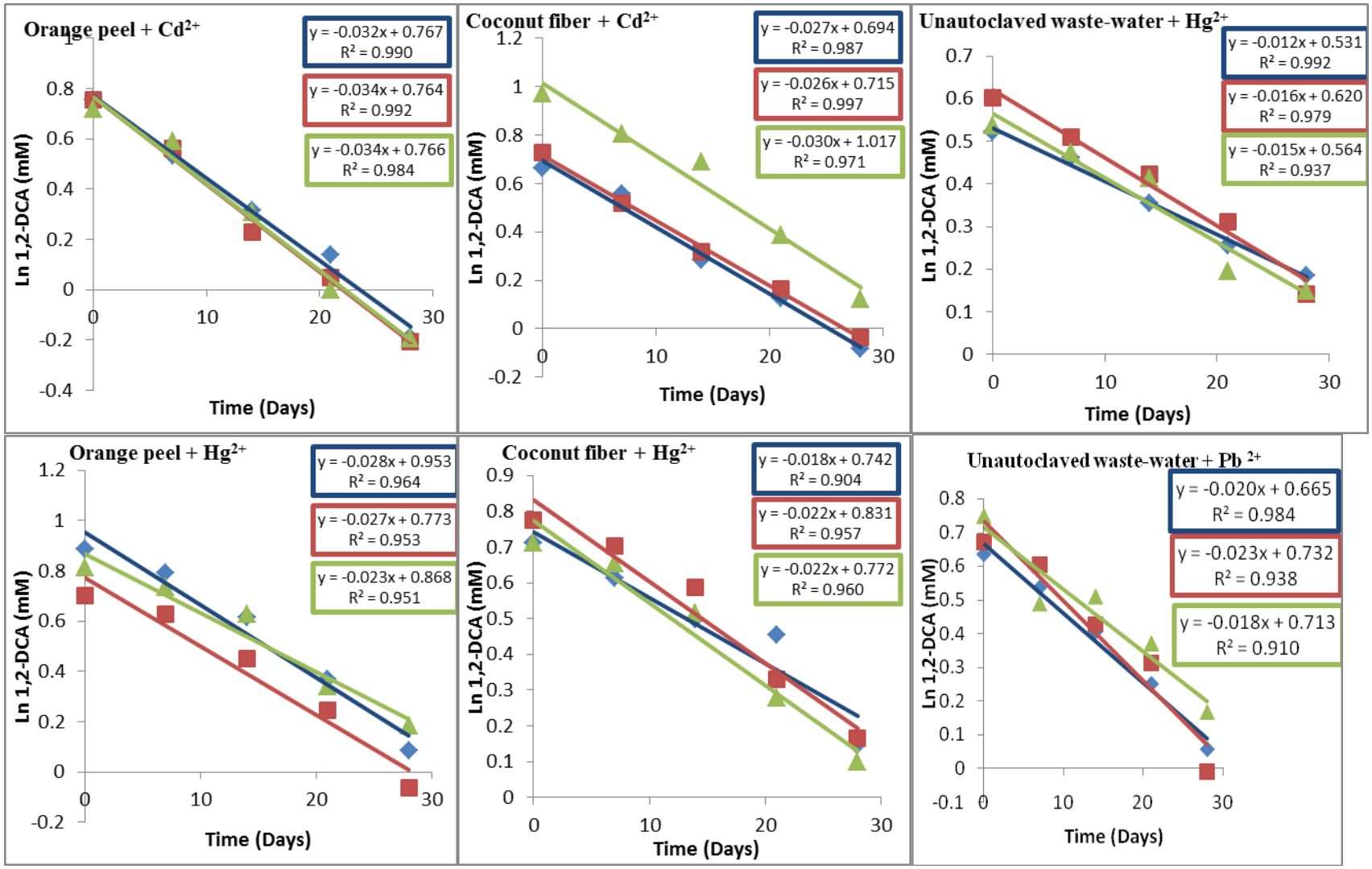


Figure 22: Standard curve for determination of 1,2-DCA concentration in NWW undergoing different biosorption treatments (Chapter 4).

Table 26: Triplicate gas chromatographic peak area values for heavy metal co-contaminated NWW microcosms undergoing biosorption via orange peel and coconut fiber (Chapter 4).

Treatments	Day 0	Day 7	Day 14	Day 21	Day 28
Autoclaved control	5052.73	4738.73	4578.85	3962.31	3505.23
	5653.10	5379.28	4954.36	4257.82	3845.33
	4178.51	3793.58	3764.31	3254.36	3102.09
Unautoclaved control	4889.09	3047.77	2007.53	1803.33	652.66
	4327.54	3321.15	2254.78	1330.58	718.86
	5504.79	4395.85	3054.15	2018.07	1091.32
Unautoclaved control + As^{3+}	3256.89	3053.97	2836.48	2600.16	2060.05
	3670.71	3341.05	3287.21	2733.23	2380.49
	3455.09	3201.02	3049.73	2657.21	2110.98
Orange peel + As^{3+}	4616.40	4255.18	3886.86	3014.13	2694.13
	3670.60	3435.03	3042.86	2521.57	2129.57
	4172.58	3825.40	3558.57	3061.74	2269.74
Coconut fiber + As^{3+}	3997.74	3767.44	3592.34	3084.61	2465.31
	3619.08	3398.61	3134.24	2812.32	2284.82
	4151.99	3565.89	3112.12	2827.22	2425.41
Unautoclaved control + Cd^{2+}	3325.49	3053.02	2691.04	2338.59	1860.86
	3187.04	2901.48	2538.59	2401.04	1899.06
	3754.13	3591.46	2938.59	2438.59	2299.90
Orange peel + Cd^{2+}	3561.50	3190.77	2423.95	2072.52	1683.02
	3787.26	3074.93	2515.50	2157.31	1759.57
	4839.59	4087.54	3641.82	2687.34	2063.57
Coconut fiber + Cd^{2+}	3898.00	3417.74	2696.70	2198.96	1639.90
	3892.90	3208.39	2798.71	2116.67	1783.42
	3753.83	3297.28	2485.04	2221.36	1800.35
Unautoclaved control + Hg^{2+}	3087.21	2900.76	2605.21	2362.34	2202.34
	3340.23	3044.05	2791.07	2496.42	2106.42
	3129.13	2940.47	2764.44	2220.67	2120.67
Orange peel + Hg^{2+}	3728.82	3378.76	3013.90	2885.61	2116.32
	3972.83	3700.53	3294.84	2541.67	2154.13
	3734.39	3516.86	3067.01	2412.62	2015.59
Coconut fiber + Hg^{2+}	4434.29	4044.40	3389.82	2647.50	1989.56
	3692.09	3424.80	2865.77	2337.85	1714.58
	4116.37	3817.32	3422.86	2567.73	2196.30
Unautoclaved control + Pb^{2+}	3453.45	3128.74	2744.68	2350.45	1935.36
	3584.46	3350.04	2806.71	2499.27	1809.29
	3873.08	2984.06	3047.41	2649.65	2160.67
Orange peel + Pb^{2+}	3853.63	3169.55	2499.37	1719.75	1324.23
	3798.85	3196.19	2220.14	1841.67	1523.23
	3815.77	3133.76	2354.42	1803.58	1423.56
Coconut fiber + Pb^{2+}	3955.94	3042.61	2502.39	1816.84	1040.85
	4063.27	2964.47	2236.58	1795.25	1220.06
	4173.17	3132.10	2237.41	1789.96	1230.75


Positive control
y = -0.064x + 1.016
R² = 0.925
y = -0.057x + 1.215
R² = 0.969
y = -0.064x + 0.981
R² = 0.975
Ln 1,2-DCA (mM)
Time (Days)
Negative control
y = -0.013x + 1.042
R² = 0.945
y = -0.010x + 0.825
R² = 0.946
y = -0.014x + 1.157
R² = 0.969
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved waste-water + As3+
y = -0.015x + 0.614
R² = 0.914
y = -0.015x + 0.722
R² = 0.935
y = -0.016x + 0.678
R² = 0.914
Ln 1,2-DCA (mM)
Time (Days)
Orange peel + As3+
y = -0.020x + 0.965
R² = 0.957
y = -0.019x + 0.740
R² = 0.970
y = -0.020x + 0.878
R² = 0.909
Ln 1,2-DCA (mM)
Time (Days)
Coconut fiber + As3+
y = -0.016x + 0.832
R² = 0.903
y = -0.015x + 0.719
R² = 0.939
y = -0.018x + 0.807
R² = 0.995
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved waste-water + Cd2+
y = -0.020x + 0.636
R² = 0.967
y = -0.017x + 0.575
R² = 0.956
y = -0.019x + 0.749
R² = 0.958
Ln 1,2-DCA (mM)
Time (Days)


Orange peel + Cd2+
y = -0.032x + 0.767
R² = 0.990
y = -0.034x + 0.764
R² = 0.992
y = -0.034x + 0.766
R² = 0.984
Ln 1,2-DCA (mM)
Time (Days)
Coconut fiber + Cd2+
y = -0.027x + 0.694
R² = 0.987
y = -0.026x + 0.715
R² = 0.997
y = -0.030x + 1.017
R² = 0.971
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved waste-water + Hg2+
y = -0.012x + 0.531
R² = 0.992
y = -0.016x + 0.620
R² = 0.979
y = -0.015x + 0.564
R² = 0.937
Ln 1,2-DCA (mM)
Time (Days)
Orange peel + Hg2+
y = -0.028x + 0.953
R² = 0.964
y = -0.027x + 0.773
R² = 0.953
y = -0.023x + 0.868
R² = 0.951
Ln 1,2-DCA (mM)
Time (Days)
Coconut fiber + Hg2+
y = -0.018x + 0.742
R² = 0.904
y = -0.022x + 0.831
R² = 0.957
y = -0.022x + 0.772
R² = 0.960
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved waste-water + Pb 2+
y = -0.020x + 0.665
R² = 0.984
y = -0.023x + 0.732
R² = 0.938
y = -0.018x + 0.713
R² = 0.910
Ln 1,2-DCA (mM)
Time (Days)

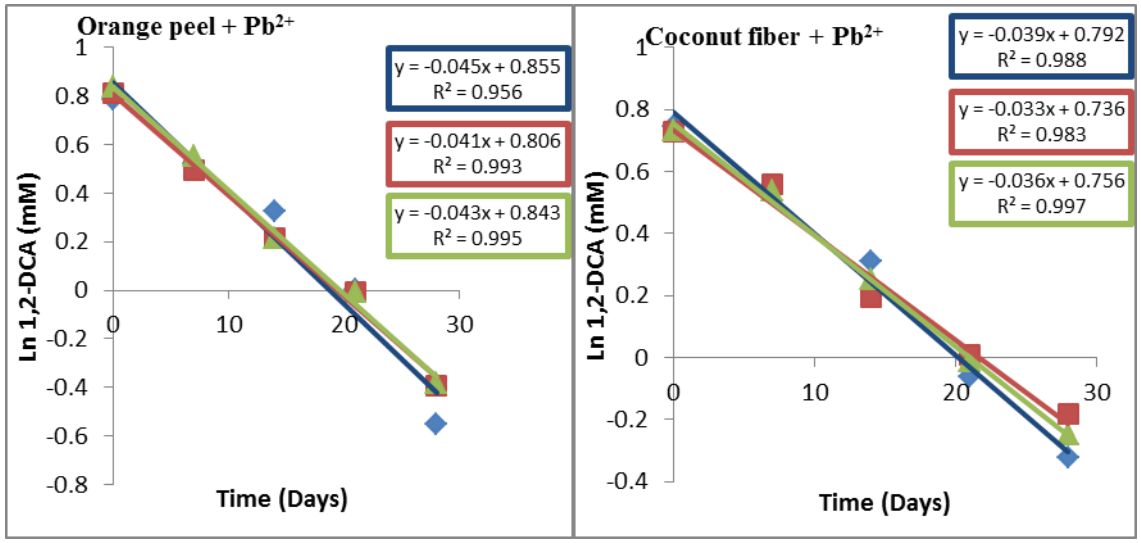


Figure 23: Graphical representation of the degradation rate constants of 1,2-DCA in arsenic, cadmium, mercury and lead co-contaminated NWW samples undergoing biosorption via coconut fiber and orange peel. (◆) = sample 1; (■) = sample 2; (▲) = sample 3 (Chapter 4).

Table 27: Triplicate gas chromatography peak area values for construction of 1,2-DCA standard curve in NGWW undergoing different biosorption treatments (Chapter 4).

1,2-DCA concentration (mM)	0.208	0.417	0.8	1.25	1.67	2.08	2.5
GC Peak area	451	832	1252	2409	2844	3716	4678
	472	814	1356	2578	2656	3589	4534
	427	826	1167	2237	2987	3876	4712

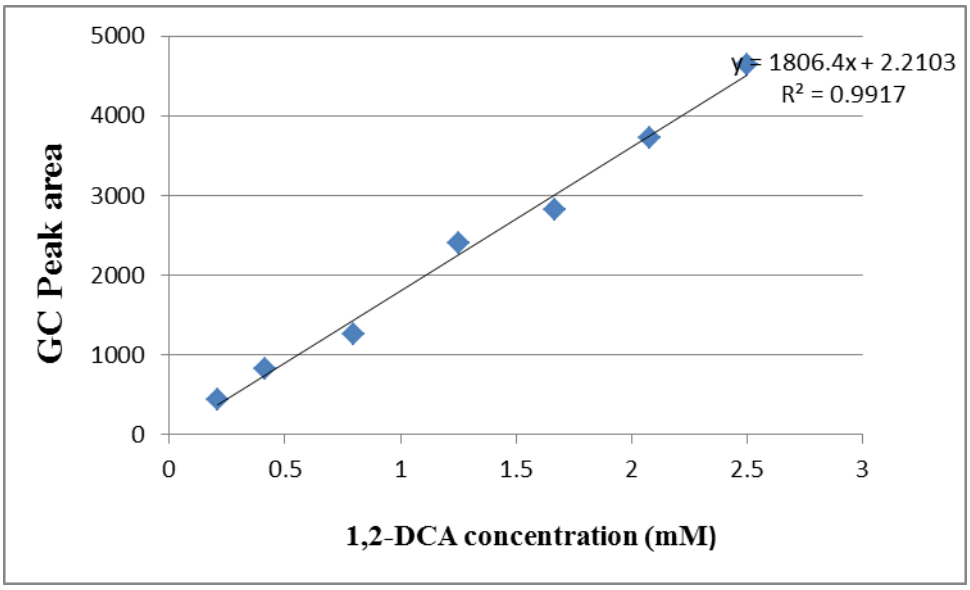
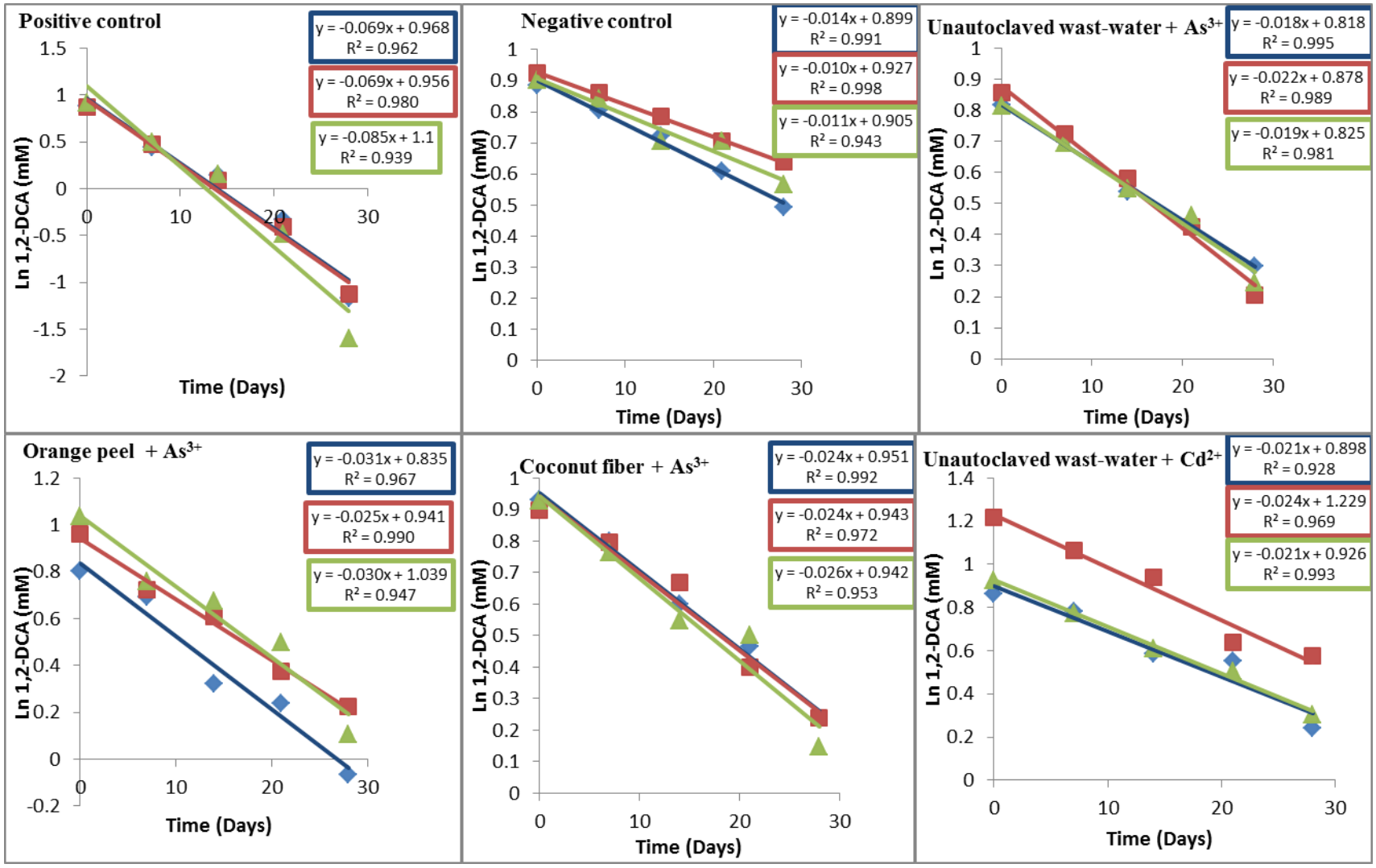
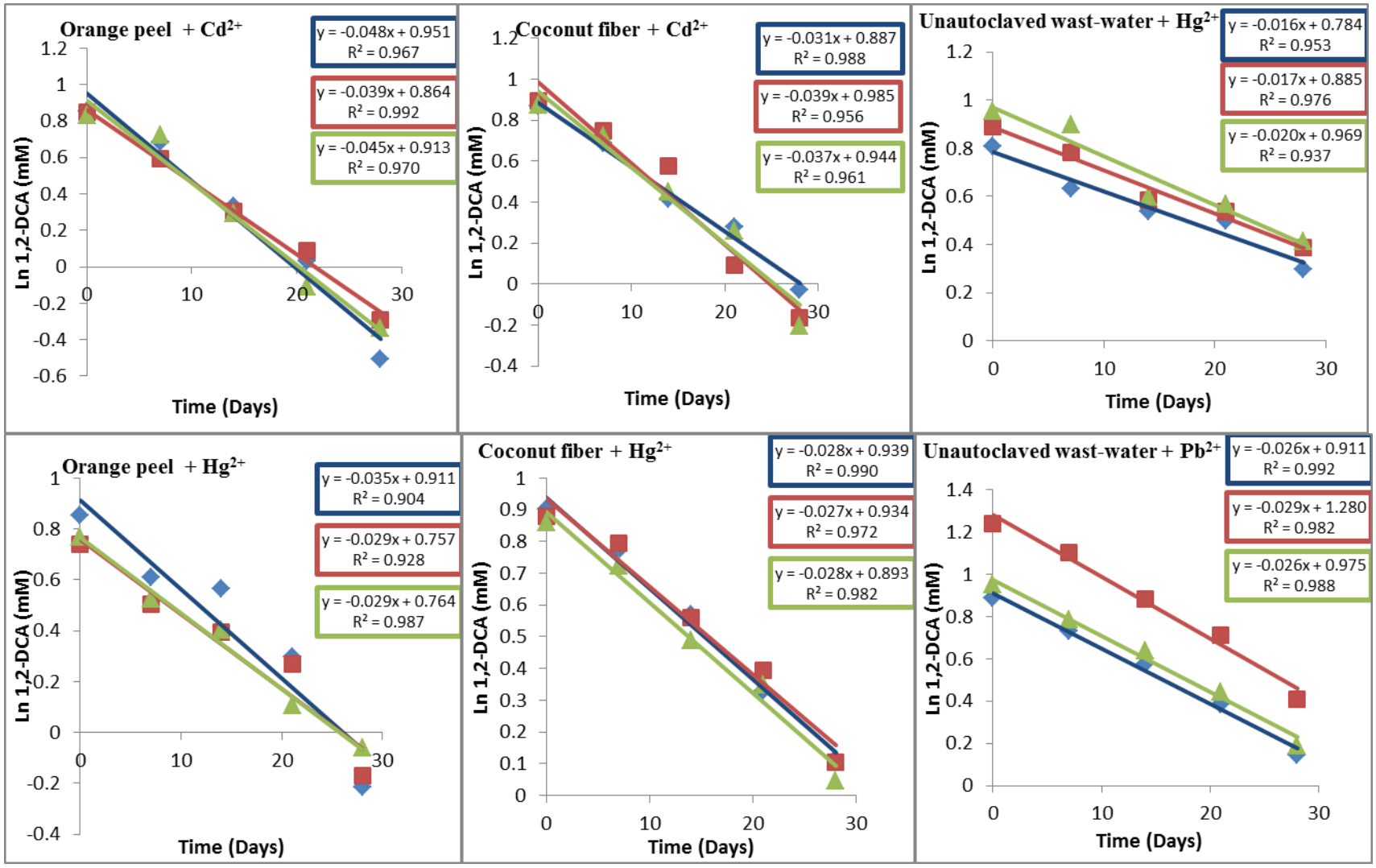


Figure 24: Standard curve for determination of 1,2-DCA concentration in NGWW undergoing different biosorption treatments (Chapter 4).

Table 28: Triplicate gas chromatographic peak area values for heavy metal co-contaminated NGWW microcosms undergoing biosorption via orange peel and coconut fiber (Chapter 4).

Treatments	Day 0	Day 7	Day 14	Day 21	Day 28
Autoclaved control	4379.23	4045.22	3726.55	3326.55	2959.25
	4550.78	4278.83	3958.61	3658.61	3426.45
	4450.99	4198.25	3658.20	3658.20	3183.30
Unautoclaved control	4385.76	2799.49	2083.19	1305.64	565.64
	4309.16	2919.21	1971.02	1205.25	586.25
	4516.58	2980.13	2107.78	1118.63	367.67
Unautoclaved control + As^{3+}	4088.85	3647.65	3091.13	2800.23	2433.41
	4250.98	3732.40	3230.73	2763.10	2220.71
	4075.50	3614.62	3123.32	2868.46	2310.15
Orange peel + As^{3+}	4027.30	3608.93	2500.03	2289.79	1696.02
	4716.06	3726.31	3320.63	2629.80	2265.95
	5098.45	3856.01	3551.32	2978.00	2008.08
Coconut fiber + As^{3+}	4585.66	4020.24	3292.03	2873.51	2293.57
	4440.25	4005.44	3524.56	2696.15	2297.15
	4575.53	3874.05	3129.01	2981.10	2094.01
Unautoclaved control + Cd^{2+}	4275.95	3947.65	3250.71	3143.12	2298.61
	6118.63	5232.40	4617.16	3427.24	3211.00
	4562.36	3914.62	3319.28	2988.99	2443.73
Orange peel+ Cd^{2+}	4235.56	3574.11	2522.47	1867.41	1091.10
	4219.83	3262.58	2447.08	1978.96	1349.07
	4138.69	3733.31	2424.26	1615.93	1295.02
Coconut fiber + Cd^{2+}	4316.31	3602.97	2739.76	2385.84	1758.52
	4428.57	3819.25	3209.89	1987.12	1531.59
	4330.79	3715.08	2843.53	2340.19	1476.60
Unautoclaved control + Hg^{2+}	4046.74	3397.30	3099.14	2978.72	2435.22
	4396.41	3947.57	3241.41	3092.18	2665.59
	4696.64	4436.42	3291.98	3191.39	2731.03
Orange peel+ Hg^{2+}	4241.07	3318.78	3185.24	2430.49	1459.57
	3778.65	2997.15	2677.21	2368.89	1525.63
	3898.97	3054.82	2708.10	2012.41	1699.45
Coconut fiber+ Hg^{2+}	4447.59	3905.50	3193.11	2511.22	2026.84
	4347.52	3993.92	3162.23	2679.35	2009.75
	4268.28	3724.03	2947.43	2557.75	1892.26
Unautoclaved control + Pb^{2+}	4400.16	3757.23	3204.35	2648.20	2094.18
	6242.84	5450.58	4378.57	3683.18	2721.93
	4686.57	3954.71	3424.63	2817.08	2185.67
Orange peel + Pb^{2+}	4022.38	2965.80	2082.80	1476.78	820.10
	4372.05	3053.13	2282.80	1777.78	908.77
	4672.28	3362.77	2684.31	1764.22	880.21
Coconut fiber + Pb^{2+}	4028.99	3172.71	2526.58	1802.57	1123.51
	4351.79	3431.56	2454.55	1988.90	1229.37
	4325.42	3270.76	2422.00	1850.40	1165.23


Positive control
y = -0.069x + 0.968
R² = 0.962
y = -0.069x + 0.956
R² = 0.980
y = -0.085x + 1.1
R² = 0.939
Ln 1,2-DCA (mM)
Time (Days)
Negative control
y = -0.014x + 0.899
R² = 0.991
y = -0.010x + 0.927
R² = 0.998
y = -0.011x + 0.905
R² = 0.943
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved wast-water + As3+
y = -0.018x + 0.818
R² = 0.995
y = -0.022x + 0.878
R² = 0.989
y = -0.019x + 0.825
R² = 0.981
Ln 1,2-DCA (mM)
Time (Days)
Orange peel + As3+
y = -0.031x + 0.835
R² = 0.967
y = -0.025x + 0.941
R² = 0.990
y = -0.030x + 1.039
R² = 0.947
Ln 1,2-DCA (mM)
Time (Days)
Coconut fiber + As3+
y = -0.024x + 0.951
R² = 0.992
y = -0.024x + 0.943
R² = 0.972
y = -0.026x + 0.942
R² = 0.953
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved wast-water + Cd2+
y = -0.021x + 0.898
R² = 0.928
y = -0.024x + 1.229
R² = 0.969
y = -0.021x + 0.926
R² = 0.993
Ln 1,2-DCA (mM)
Time (Days)


Orange peel + Cd2+
y = -0.048x + 0.951
R² = 0.967
y = -0.039x + 0.864
R² = 0.992
y = -0.045x + 0.913
R² = 0.970
Ln 1,2-DCA (mM)
Time (Days)
Coconut fiber + Cd2+
y = -0.031x + 0.887
R² = 0.988
y = -0.039x + 0.985
R² = 0.956
y = -0.037x + 0.944
R² = 0.961
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved wast-water + Hg2+
y = -0.016x + 0.784
R² = 0.953
y = -0.017x + 0.885
R² = 0.976
y = -0.020x + 0.969
R² = 0.937
Ln 1,2-DCA (mM)
Time (Days)
Orange peel + Hg2+
y = -0.035x + 0.911
R² = 0.904
y = -0.029x + 0.757
R² = 0.928
y = -0.029x + 0.764
R² = 0.987
Ln 1,2-DCA (mM)
Time (Days)
Coconut fiber + Hg2+
y = -0.028x + 0.939
R² = 0.990
y = -0.027x + 0.934
R² = 0.972
y = -0.028x + 0.893
R² = 0.982
Ln 1,2-DCA (mM)
Time (Days)
Unautoclaved wast-water + Pb2+
y = -0.026x + 0.911
R² = 0.992
y = -0.029x + 1.280
R² = 0.982
y = -0.026x + 0.975
R² = 0.988
Ln 1,2-DCA (mM)
Time (Days)

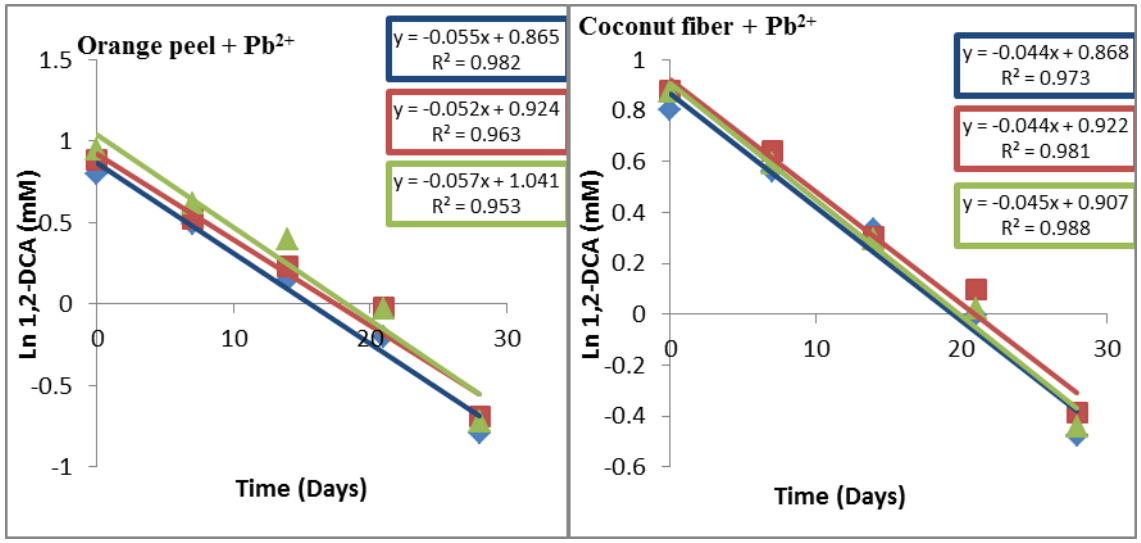


Figure 25: Graphical representation of the degradation rate constants of 1,2-DCA in arsenic, cadmium, mercury and lead co-contaminated NGWW samples undergoing biosorption via coconut fiber and orange peel. (◆) = sample 1; (■) = sample 2; (▲) = sample 3 (Chapter 4).

Table 29: Residual concentration of heavy metals (mM) in microcosms constructed with NWW undergoing various biosorption treatments (Chapter 4).

		Orange peel						Coconut fiber			
		Day 1	Day 4	Day 14	Day 28			Day 1	Day 4	Day 14	Day 28
Arsenic (mM)	Sample 1	0.90	0.90	0.86	0.82	Arsenic (mM)	Sample 1	0.97	0.96	0.93	0.91
	Sample 2	0.90	0.90	0.87	0.82		Sample 2	0.98	0.94	0.92	0.91
	Sample 3	0.92	0.88	0.86	0.82		Sample 3	0.96	0.94	0.93	0.88
Cadmium (mM)	Sample 1	0.58	0.55	0.53	0.48	Cadmium (mM)	Sample 1	0.69	0.67	0.62	0.60
	Sample 2	0.58	0.58	0.54	0.53		Sample 2	0.72	0.68	0.67	0.59
	Sample 3	0.59	0.57	0.52	0.51		Sample 3	0.71	0.66	0.65	0.60
Mercury (mM)	Sample 1	0.30	0.27	0.26	0.25	Mercury (mM)	Sample 1	0.35	0.33	0.33	0.32
	Sample 2	0.31	0.30	0.27	0.25		Sample 2	0.35	0.34	0.33	0.32
	Sample 3	0.30	0.29	0.27	0.24		Sample 3	0.35	0.33	0.32	0.31
Lead (mM)	Sample 1	0.27	0.27	0.24	0.24	Lead (mM)	Sample 1	0.34	0.32	0.32	0.30
	Sample 2	0.28	0.26	0.26	0.22		Sample 2	0.34	0.33	0.31	0.30
	Sample 3	0.29	0.25	0.24	0.23		Sample 3	0.34	0.32	0.32	0.30

Table 30: Residual concentration of heavy metals (mM) in microcosms constructed with NGWW undergoing various biosorption treatments (Chapter 4).

		Orange peel						Coconut fiber			
		Day 1	Day 4	Day 14	Day 28			Day 1	Day 4	Day 14	Day 28
Arsenic (mM)	Sample 1	0.86	0.85	0.82	0.79	Arsenic (mM)	Sample 1	0.92	0.91	0.90	0.84
	Sample 2	0.87	0.86	0.81	0.8		Sample 2	0.93	0.92	0.86	0.85
	Sample 3	0.87	0.83	0.82	0.82		Sample 3	0.91	0.90	0.89	0.87
Cadmium (mM)	Sample 1	0.22	0.21	0.17	0.17	Cadmium (mM)	Sample 1	0.32	0.30	0.27	0.25
	Sample 2	0.22	0.2	0.19	0.19		Sample 2	0.30	0.29	0.27	0.26
	Sample 3	0.2	0.19	0.18	0.17		Sample 3	0.31	0.27	0.25	0.25
Mercury (mM)	Sample 1	0.35	0.32	0.31	0.31	Mercury (mM)	Sample 1	0.42	0.41	0.40	0.39
	Sample 2	0.35	0.35	0.32	0.3		Sample 2	0.43	0.42	0.41	0.40
	Sample 3	0.34	0.34	0.31	0.3		Sample 3	0.43	0.41	0.40	0.39
Lead (mM)	Sample 1	0.05	0.04	0.04	0.03	Lead (mM)	Sample 1	0.16	0.15	0.14	0.13
	Sample 2	0.05	0.05	0.03	0.02		Sample 2	0.16	0.15	0.13	0.12
	Sample 3	0.05	0.05	0.04	0.03		Sample 3	0.17	0.15	0.14	0.13