BIOREMEDIATION OF SOIL CONTAMINATED WITH A MIXTURE OF CHLORINATED ALIPHATIC HYDROCARBONS

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Submitted in fulfilment of the academic requirements for the degree of Master of Science in the Discipline of Microbiology, School of Biochemistry, Genetics, Microbiology and Plant Pathology, Faculty of Science and Agriculture at the University of KwaZulu-Natal, Durban.

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

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Abstract

Chlorinated aliphatic hydrocarbons (CAH's) are a diverse group of industrial chemicals that play a significant role as pollutants of soil and groundwater. They are recalcitrant and resist degradation in most waste treatment systems. Furthermore, physical removal techniques used for CAHs are often very expensive, labour intensive and time consuming. Microbial communities native to contaminated areas are known to participate in biodegradation of these CAHs to an extent. The main focus of this study was therefore to investigate the bioremediation of soil contaminated with a mixture of CAHs, namely carbon tetrachloride (CCl_4), dichloromethane (DCM) and 1, 2 dichloroethane (1, 2-DCA). Two different laboratory-scale microcosm types, a stationary microcosm (Type S) and microcosms that received a continuous circulation of groundwater (Type C) were used to determine the effects of 3 different bioremediation approaches, viz, biostimulation, bioaugmentation and a combination of biostimulation and bioaugmentation on the degradation process. For both microcosm types, gas chromatography analysis revealed that the greatest decreases in CAH concentrations occurred in soil that was biostimulated. 1, 2-DCA was rapidly biodegraded in Type C microcosms that contained glucose, with a 57% net degradation in 15 days. Consortia comprising of aerobic *Bacillus* and *Alcaligenes sp.* were used for bioaugmenting contaminated soil. However, this approach did not promote biodegradation as significantly as biostimulation experiments. A combination of biostimulation and bioaugmentation revealed that the addition of nutrients was still unable to induce the degradative ability of the introduced microorganisms to produce degradation values comparable to those of biostimulated soil microcosms. Common intermediates of CAH metabolism viz., chloroform, dichloromethane and carbon dioxide were detected by gas chromatography/mass spectrometry. The detection of chloroform and dichloromethane is sufficient evidence to assume that anaerobic conditions had developed, and that biodegradation was occurring under oxygen-limiting or oxygen-free conditions. An aerobic environment was initially created, but soil microbial respiration had probably led to the rapid development of anaerobic conditions and in all likelihood, enhanced degradation. The prevalence of anaerobic conditions can also account for the lack of appreciable degradation by the bacterial consortium used during bioaugmentation. Phospholipid phosphate analysis was conducted and used as an indicator of microbial biomass. It was noted that phospholipid phosphates did not always correlate with the degradation of CAHs in some microcosms. In this regard, different patterns were noted for Type S and Type C microcosms. Microbial biomass patterns

for Type C biostimulated and bioaugmented soil microcosms increased within the first 5 days of sampling. This could have been as a result of the larger volume of groundwater required for the circulating microcosm possibly concealing actual CAH concentrations. In contrast, in Type S microcosms, for most treatments, a sharp decline in biomass within the first week was observed. This study clearly demonstrates that the bioremediation of certain chlorinated solvents can be a function of their water solubility. It must also be emphasized that the biodegradation of some CAHs in a mixture can affect the concentrations of others present in the mixture as well, warranting further study with mixtures of CAHs. Furthermore, the development and use of bioreactors, similar to the Type C microcosm can provide novel, simple ways to hasten remediation of chlorinated solvents like 1, 2-DCA.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

".....It confirmed his suspicion that the natural world had gone badly wrong. Everything that mankind was doing on the planet had upset the delicate balance of nature. The pollution, the rampant industrialization, the loss of habitat....."

This is an excerpt from a science-fiction novel by renowned author Michael Crichton (2006). Unfortunately, man's endless quest for innovation and the need to find ways to improve life, has allowed this scenario to become a reality. The reality is that we have inaugurated an ailing, polluted and seemingly dismal planet.

The colossal chemical industry is a major contributor to the demise of the environment. A plethora of chemicals are newly synthesized for commercial applications annually. The chlorinated hydrocarbons are an excellent example of such chemicals. These chemicals are produced in large quantities to meet the demand of various industrialized countries (Holliger *et al*., 1997). While several of these chlorinated hydrocarbons are consumed or destroyed during industrial processes, a large percentage is released into air, water and soil (Annachhatre and Gheewala, 1996). These synthetic chemicals eventually appear in many areas such as those used for food and food production and in environments that support diverse populations of animals and plants (Alexander, 1994). Besides improper disposal, accidents and spills, leakages from underground storage tanks, pipelines, production wells, refineries and distribution terminals, have caused the chlorinated hydrocarbons to become subsurface contaminants (Chang and Alvarez-Cohen, 1996).

The chlorinated hydrocarbons are widely publicized, mainly due to their potential to impact human health (Chaudhry and Chapalamadugu, 1991). These chlorinated hydrocarbons can also seriously threaten the sustainability of various species of flora and fauna (Holliger *et al*., 1997).

Comprehensive research has indicated that decontamination strategies involving microorganisms is an encouraging technique to recover environments polluted by chlorinated hydrocarbons (Leisinger, 1996). Several different bacterial genera have already been identified as appreciable degraders of chlorinated hydrocarbons (Chaudhry and Chapalmagudu, 1991). The biodegradation of most chemicals in the environment occurs naturally, but not at the desired capacity (Holliger *et al*., 1997). Microbial communities native to contaminated sites are known to participate in biodegradation to varying degrees. In certain instances however, it can be necessary to implement further engineering steps to improve this process. Nutrients, electron acceptors or donors and specific microbial consortia capable of degrading contaminants have been shown to increase the biodegradation of contaminants in the subsurface with resounding success (Holliger *et al.*, 1997). Determining the conditions and requirements needed to improve the microbial degradation of these synthetic contaminants is warranted.

1.1 CHLORINATED HYDROCARBONS

Chlorinated hydrocarbons are amongst the most significant pollutants in the world (Furukawa, 2003). They are used much more frequently than their fluorinated or brominated counterparts. These chemicals are the subject of intensive research and are

amongst the most widely studied group of compounds. Due to their extensive use in industry and agriculture, chlorinated organic hydrocarbons have become very prominent (Aulenta *et al*., 2005).

While most chlorinated hydrocarbons are borne from industrial origins, chlorinated hydrocarbons can also originate from the chlorination of water. Chlorination has been used successfully to disinfect drinking water for many years. However, several decades ago it was realized that this process formed trace amounts of trihalomethanes. Furthermore, some of the compounds that were identified after chlorination were recognized as known or suspected carcinogens (Philp *et al*., 2005). In the case of wastewater, aquifers that have been used to transport the chlorination by-products may impose certain restrictions on the possible use of the aquifer. For instance, such aquifers may not be used for drinking water purposes (Diamadopoulous *et al*., 1998). Consequently, many environmental protection agencies have now stipulated maximium concentration levels of many chlorinated hydrocarbons in groundwater and are regulating and monitoring their concentrations closely (Aulenta *et al*., 2005).

Contamination of soil by chlorinated hydrocarbons, often results in the contamination of groundwater. The interactions that occur between these compounds and soil particles play an important role in predicting their migration within a soil matrix. In general, the adsorption of the volatile chlorinated compounds by most soils is only slight to moderate, since plumes of such compounds can be detected quite far away from the initial sites of contamination. Interaction of these volatile organic compounds with the soil is often

found to be quite important in predicting their fate (Diamadopoulous *et al*., 1998).

1.2 IMPORTANT CHLORINATED HYDROCARBONS

1.2.1 DDT (1, 1, 1-trichloro-2, 2 bis (*p*-chlorophenyl) ethane)

An excellent example of the toxicity and environmental problems associated with chlorinated hydrocarbons is provided by the case of 1, 1, 1-trichloro-2, 2 bis (*p*-chlorophenyl) ethane; better known as dichlorodiphenyltrichloroethane or DDT (Hornback, 2006).

1.2.1.1 Commercial uses

Developed in 1930, DDT was the first organic synthetic pesticide. DDT was initially found to be extremely effective in controlling mosquitoes and flies and was subsequently used with much success during World War II for the control of body lice that transmit typhus in Italy and against mosquitoes that transmit malaria in the Pacific. DDT gained much popularity in the United States as the agricultural community used it enthusiastically because of its ideal properties as an insecticide. It was also considered non-toxic to mammals, persistent and most importantly; very cheap. By 1961, approximately 160 million pounds of DDT was used in the United States (Hornback, 2006).

1.2.1.2 Environmental properties

The characteristics that made it valuable as an insecticide, also led to it becoming an environmental hazard. DDT began to accumulate in the environment. Due to its strong

stability qualities, its biodegradation proved to be very slow. The rates of decomposition and transformation of most chemicals vary significantly, depending on the prevailing conditions in the environment such as temperature, types and density of organisms. It was calculated that the average half-life of DDT was estimated at about ten years, which proved to be unacceptable (Fellenberg, 2000).

1.2.1.3 Health implications

DDT is very hydrophobic, insoluble in water and quite soluble in non-polar compounds. Upon ingestion of this chlorinated hydrocarbon, an organisms' excretion became significantly delayed, due to the chemicals water insolubility. Thus accumulation of the compound occurs in the organism, specifically in the non-polar regions e.g. fats/lipids (Hornback, 2006).

Large decreases in wildlife populations, especially birds were attributed to the large concentrations of DDT that was found in them. By 1973, the United States Environmental Protection Agency (USEPA) banned the use of DDT in the United States. Supporters of the use of pesticides claim there is no substantiated evidence that DDT causes adverse effects in humans, and that it is one of the least toxic and safest. Due to its low cost and effectiveness, DDT is still widely used in some poorer countries where malaria is endemic (Hornback, 2006).

1.2.2 Polychlorinated biphenyls (PCBs)

Since its detection in 1966 by Jensen, PCBs have become widespread environmental

contaminants and can be found in various environments, including polar regions like the Arctic and Antarctic (Mackova´*et al*., 2007).

1.2.2.1 Commercial uses

PCBs have 209 possible isomers in theory, but about a 100 exist in commercial formulations that are nearly water insoluble, nonpolar, lipid soluble and inert (Chaudhry and Chapalamadugu, 1991). In early 1930's, PCBs were widely used as non-flammable heat resistant oils in heat transfer systems, as hydraulic fluids and lubricants, as transformer fluids in capacitors, as plasticizers in food packaging materials and as petroleum derivatives. They are also useful constituents of insulators, herbicides, medicines and antimicrobial agents (Chaudhry and Chapalamadugu, 1991; Tiirola *et al*., 2002). Different countries used PCBs as mixtures of various chlorinated isomers and marketed it under different trade names e.g. Arochlor (United States), Clophen (West Germany), Phenochlor (Italy), Pyralne (France) and Soval (USSR). In the United States, sales of Arochlor 1221–1268 (the last two numbers indicate the percentage of chlorination) rocketed from 32 000 000 pounds in 1957 to 80 000 000 pounds in 1970 (Maier, 2000).

1.2.2.2 Environmental properties

Any compound that is persistent and hydrophobic, lipophilic and fat soluble will be subject to the processes of biomagnification and may present special problems (Hornback, 2006). There are several ways in which PCBs can enter the environment. One of these is through run-off from industrial waste-dumps and spills. Other sources include

points of PCB manufacture and processing into other products. Because PCB input into fresh water has been high in the past, PCBs have thus accumulated in sediments. Even if PCB input was stopped completely, previously contaminated sediments could continue to release PCBs into freshwater systems for years to come and as such represent potential health hazards (Hornback, 2006). They are now suspected carcinogens and as a result are no longer manufactured in the United States. Their residues however, are still widely distributed in the environment (Maier, 2000).

1.2.2.3 Health implications

PCBs gained international infamy due to a rice oil factory accident that occurred in Japan in 1968, where the solvent was used as a heat exchange fluid. It leaked into a batch of rice oil as a result of a broken heat exchanger pipe. Obviously unnoticed, rice oil was packaged and consumed by the local population. It was estimated that over a thousand people were poisoned by the contaminated rice, producing a spectrum of symptoms including chloroacne, gum and nail bed discolouration, joint swelling, emission of a waxy secretion from the eye-lid glands and lethargy. Subsequently, the United States Food and Drug Administration (FDA) issued tolerance levels for PCBs in food and packaging products and the EPA issued strict guidelines governing the use of PCBs. These actions drastically reduced the production as well as the use of PCBs in the United States (Maier, 2000)

1.2.3. Pentachlorophenol (PCP)

Pentachlorophenol (PCP), a weak acid, is the highest chlorine substituted species of the

chlorophenol group (Antonai *et al*., 2007).

1.2.3.1 Commercial uses

It is an extremely important chlorinated solvent, used widely in agriculture, as it is the main component of wood preservatives. This solvent is also applied as a herbicide, algaecide and molluskcide (Anotai *et al*., 2007; Ge *et al*., 2007). As a component of wood treatment mixtures, it is active against blue staining and soft-rot fungi (Tirrola *et al*., 2002). Approximately 100 000 tonnes of PCP have been produced in China; since 1997. This alone accounted for 20% of the global PCP production, the large volumes easily proving its value. It was used worldwide for numerous years. However, concern regarding its deposition in the environment has led to its production and applications, seriously declining. Several countries such as Austria, India, Indonesia, New Zealand, Sweden, Switzerland and Germany had ceased the use of PCP at various times between 1978 and 1991. In the United States of America, PCP can only be used as a wood preservative and is registered as a 'restricted use' pesticide (Ge *et al*., 2007).

1.2.3.2 Environmental properties

This chemical exhibits good molecular stability and sorption characteristics. As a result, it is used extensively, eventually resulting in widespread contamination of the environment (He *et al*., 2005). The highest concentrations of PCP are typically found in soil and aquatic systems. The biodegradation of PCP in soil is relatively slow, and this compound can therefore present certain toxicity risks in contaminated soil, for prolonged periods of time (He *et al*., 2007). The presence of PCP in soil and water is important

because this solvent has the potential to bioaccumulate, thus retaining the ability to exert toxic effects. Its ability to bioaccumulate explains why it is still an environmental hazard at many sites

1.2.3.3 Health implications

The USEPA currently regards PCP as a probable human carcinogen. Studies have revealed that PCP is readily absorbed into the lungs, gastrointestinal tract lining and skin (Fellenberg, 2000). PCP has also shown to be toxic to reproductive systems, embryonic and fetal development and neonatal survival in rats (Antonai *et al.*, 2007).

1.3 CHLORINATED ALIPHATIC HYDROCARBONS (CAHs)

Chlorinated aliphatic hydrocarbons (CAHs) are a diverse collection of industrial chemicals whose representatives play a considerable role as environmental pollutants. They have become prominent with respect to industrial use, environmental persistence, toxicity and potential carcinogenicity (Leisinger, 1996). They are primarily used as intermediates in the chemical industry, as solvents for metal degreasing, paint stripping, in the dry cleaning industry and in various household cleaning products. In some cases, they are also used as pesticides (Bejankiwar *et al*., 2005). In the United States, it was found that almost 20 chlorinated aliphatic hydrocarbons (e.g., trichloroethylene, 1, 1, 1-trichloroethane, perchloroethylene, 1, 2-dichloroethylene and 1, 1-dichloroethylene) frequently appeared in contaminated systems. This information was revealed after a survey of 945 finished water supply systems. Nearly half of the contaminated systems showed the presence of multiple contaminants. The presence of such CAHs is therefore

alarming, because they are not readily reduced in municipal treatment processes to safe concentrations (Chang and Alvarez-Cohen, 1996).

Similar to other chlorinated hydrocarbons, these compounds have become widely distributed environmental contaminants through discharge of industrial wastewaters, seepage from landfills, and leakage from underground storage tanks. CAHs are able to form dense non-aqueous phase liquids, once released. They physically and chemically interact with the soil, and are able to sorb strongly with soil organics and minerals, and ultimately dissolve into, and contaminate groundwater (Ferguson and Pietari, 2000).

Many of these halogenated organic compounds are not very soluble and tend to be highly lipophilic. These properties permit them to bioaccumulate in some food chains, possibly endangering many species of wildlife and aquatic organisms. Their relatively low boiling points; and higher degree of water solubility compared to polycyclic aromatic hydrocarbons afford these solvents a great capacity to spread. Their volatile components are able to penetrate the cement walls of water conduits and as such reach the groundwater (Fellenberg, 2000). Some volatile chlorinated aliphatics can travel into certain atmospheric compartments. Investigations have proved some long-lived members of this family can journey into the stratosphere. Here, it is possible that they have the potential to exert detrimental effects on the global environment (Fischli, 1996). Adverse health effects and ecosystem perturbations can easily be expected when considering the chemical properties and inherent toxicity of CAHs (Chang and Alvarez-Cohen, 1996; Oldenhuis *et al*., 1991).

Under natural conditions, CAHs have proved to be persistent in groundwater, mainly due to their poor rates of biodegradability. When present in solution, this group of chlorinated hydrocarbons has the ability to be absorbed through the digestive tract and the skin. In mammals, the openings for the Na^+ inflow can no longer be closed, once they have successfully managed to lodge in the membranes of nerve cells. Thus, under the influence of such materials, the original potential may not be restored, or may only be partially restored after an instance of excitation. Such chlorinated hydrocarbons increase the excitability of nerve cells and the motor nerves are first affected. At higher concentrations however, the sensory neurons are also affected. Humans do not undergo these effects if they ingest chlorinated hydrocarbons that may occur in food e.g. as in the case with pesticides as it must be in much larger quantities. Trace amounts of chlorinated hydrocarbons can also be significant, because they have the potential to accumulate in the body and to react with other synthetic materials (Fellenberg, 2000).

Apart from the lesser chlorinated compounds, such as dichloromethane, vinyl chloride and 1, 2-dichloroethane; they do not generally serve as growth substrates, and resist treatment in biological and wastewater treatment systems. The chlorinated aliphatic hydrocarbons carbon tetrachloride, also known as tetrachloromethane (CCl_4); dichloromethane, also known as methylene chloride (CH_2Cl_2); and 1, 2-dichloroethane ($ClCH_2CH_2Cl$), also referred to as ethylene dichloride are amongst the 33 synthetic organic contaminants frequently detected in groundwater (Table 1). These CAHs are also notorious soil pollutants.

Table 1.1: The 33 synthetic organic contaminants reported to be most frequently found in drinking water wells*.

AROMATIC AND RELATED COMPOUNDS	BROMINATED	CHLORINATED
Xylene	Dibromochloropropane	Trichloroethene
Benzene	Dibromochloromethane	Carbon tetrachloride
Parathion	Bromoform	1, 1-Dichloroethane
Cyclohexane	Ethylene dibromide	1, 2-Dichloroethene
Butyl benzyl-phthalate		Chloroform
Toulene		Tetrachloroethene
Isopropyl benzene		Trichloroethene
Dioxane		1, 1-Dichloroethene
Ethyl benzene		1, 2-Dichloroethane
Acetone		Methylene chloride
Di-n-butyl-phthalate- benzene		1, 1, 1-Trichloroethane
Bis(2-ethylhexyl) Phthalate		Dibromochloropropane
		Trifluorotrichloroethane
		Dibromochloromethane
		Vinyl chloride
		Chloromethane
		1, 1, 2-Trichloroethane
		Lindane
		Alpha-BHC
		Delta-BHC

* Adapted from Rittman and McCarty, 2001.

1.3.1 Carbon Tetrachloride (CCl_4)

This solvent has been characterised as one of the strongest hepatotoxic chlorinated hydrocarbons (Table 2) known to man (Fellenberg, 2000). Similar to chloroform, carbon tetrachloride (CCl_4) is a heavy, colourless and organic liquid with a sweet aromatic odour (Table 3). Common trade names include perchloromethane, methane tetrachloride or tetrachloromethane. The United States produced approximately 143 000 tonnes of CCl_4 in 1991, which was when it was listed in the top 45 organic chemicals produced in the US

(van Eekert *et al*., 1998). It is assumed that about 5-10% of all CCl_4 produced, enters the environment. This huge amount has led to the USEPA setting the maximum contaminant level at 5 parts per billion.

Table 1.2: Examples of widespread chlorinated alkanes and alkenes, classified according to hepatotoxicity (Fellenberg, 2000).

STRONG LIVER TOXINS	WEAK LIVER TOXINS
Tetrachloromethane	Trichloroethene
1,1,2,2- tetrachloroethane	Tetrachloroethene
1,1,2- trichloroethane	1,1,1- trichloroethane
1,2- dichloroethane	Dichloroethane

1.3.1.1 Commercial uses

Vast quantities of CCl_4 were used to produce the Freon refrigerants R-11 (trichlorofluoromethane) and R-12 (dichlorodifluoromethane), prior to the Montreal Protocol. These refrigerants are now suspected to lead to ozone depletion and have, therefore, been phased out. However, CCl_4 is still used to manufacture less destructive refrigerants. It is still primarily used as a transition product in the production of chloro-fluoro-carbons (CFC's); although its use for that purpose has, also, been declining steadily. Other uses include being used as a dry cleaning agent and fire extinguisher, in making nylon, as a solvent for rubber cement, soaps and insecticides. It can also be used to dissolve fat (http://www.epa.gov/safewater/contaminants/dw_contamfs/carbonte.html; Fellenberg, 2000).

Table 1.3: Some properties of CCl_4 (Baden, 2008).

Molecular formula	CCl_4
Molar mass	153.82 g/mole
Appearance	Colourless liquid
Density	1.59 g/cm^3 (20º C)
Melting point	- 23ºC (250 K)
Boiling point	76.7ºC (350 K)
Solubility in water	0.8 g/litre at 20ºC

1.3.1.2 Environmental properties

It is unusually persistent under aerobic conditions, and when in the atmosphere and in oxygen-rich surface water, it is presumed to have a half-life of sixty to a hundred years.

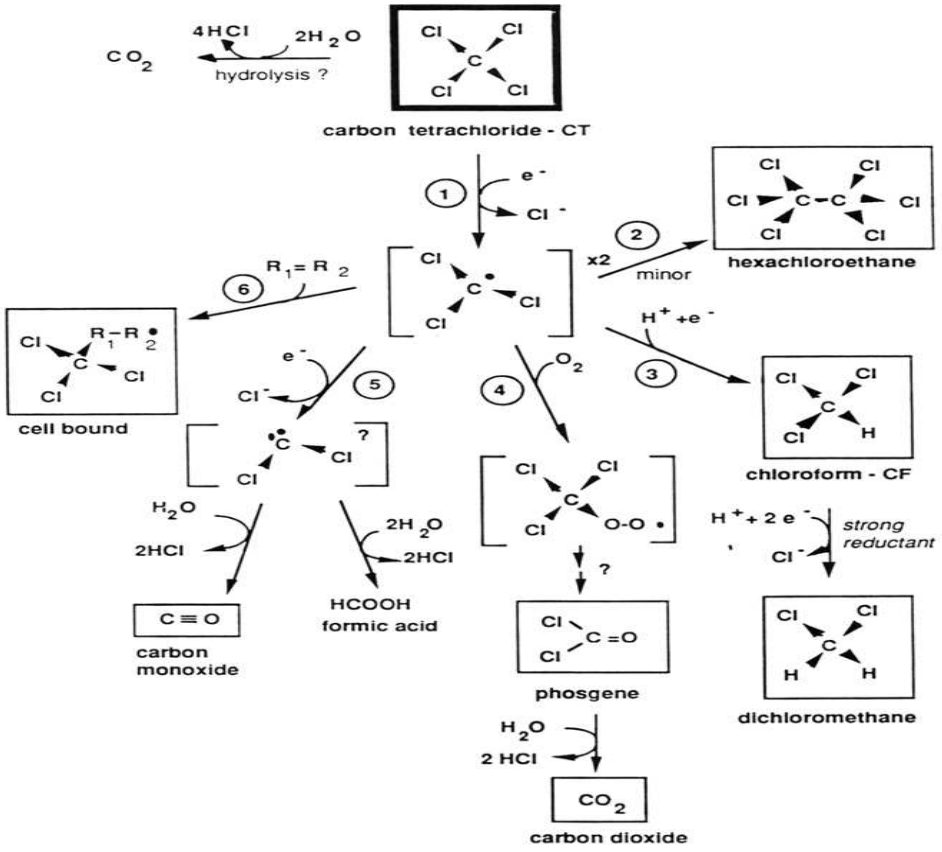

Fig. 1.1: Abiotic and biotic transformations of CCl_4 (Criddle and McCarty, 1991).

Its behaviour in anaerobic conditions such as sludge or flood waters, however, is different. In this case, metabolism (not entire decomposition) may occur within fourteen to sixteen days. However, it should not be introduced into purification plants because it hinders multiplication of microorganisms, and thus, their performance in decomposition (Fellenberg, 2000).

1.3.1.3 Health implications

The USEPA has stated that short-and long-term exposure to CCl_4 can result in liver, kidney and lung damage. Long-term exposure to levels above the maximum contaminant concentrations can ultimately result in extensive liver damage and cancer. Humans may be indirectly endangered if CCl_4 is added to waste materials, because under anoxic conditions, it can form chloroform (Fig. 1.1) which is familiar as a narcotic. Its metabolism in the liver can lead to direct danger. It can lead to the deterioration of fatty acids into several end products, after a series of reactions. Metabolism of the entire cell then becomes hindered. As a result of the deterioration of the fatty acids, the functions of the mitochondria, the golgi apparatus and other cell compartments become affected. This causes a basic alteration in the phosphate lipids that build up the cell membranes. Subsequently, various enzymes enter the blood and the electrolyte management in the body becomes uncontrollable. Damage to the central nervous system eventually results from the continual influence of this chlorinated aliphatic hydrocarbon (http://www.epa.gov/safewater/contaminants/dw_contamfs/carbonte.html; Fellenberg, 2000).

1.3.2 Dichloromethane (CH_2Cl_2)

Dichloromethane (DCM) also known as methylene chloride is a relatively stable, non-flammable solvent that is widely used. It is a colourless liquid that has an overwhelming ether-like sweet odour. It is relatively water soluble (Table 4), and is ubiquitous in natural aqueous environments. It has been estimated that treated sewage effluents can contain up to 24 µg DCM per litre (Rittmann and McCarty, 1980).

1.3.2.1 Commercial uses

DCM became popular predominantly through its use for paint stripping and degreasing operations. It is also widely used as a solvent in various chemical and polymer syntheses. Dichloromethane and chloromethane have been acknowledged as the only chlorinated hydrocarbons that are able to serve as growth substrates for aerobic and anaerobic microorganisms. Therefore, DCM has been exploited for use in bioreactors for both wastewater and groundwater treatment (de Best *et al*., 2000). In anticipation of stronger regulations for environmental protection, the production of DCM decreased considerably during the nineties (Herbst and Wiesmann, 1996). In 2004, an estimated 600 000 tonnes of DCM was used globally, illustrating that it still maintains an active role in industry (Wang and Chen, 2006).

1.3.2.2 Environmental properties

DCM has easily become one of the most significant trace pollutants of the atmosphere and natural waters. It has even been implicated in the contamination of drinking water supplies (Nikolausz *et al*., 2005).

Table 1.4: Some properties of DCM (Baden, 2008).

Molecular formula	CH_2Cl_2
Molar mass	84.93 g/mol
Appearance	Colorless liquid
Density	1.33 g/cm³ (20°C)
Melting point	-95°C (175.7 K)
Boiling point	40°C (312.8 K)
Solubility in water	20 g/litre at 20°C

Usually, DCM is disposed off by incineration. However, it is quite likely, that a large portion of DCM that is produced will travel into the environment. Its chemical properties, such as its boiling point (Table 4) and its high vapour pressure (47 kPa at 20ºC) ensure that considerable amounts of DCM enter the environment in its gaseous phase (Wang and Chen, 2006). This chemical exhibits high vapour pressure and high solubility in water. These characteristics allow it to be partially stripped in normal aeration tanks of industrial or municipal wastewater treatment plants. Oxidation of DCM in the higher levels of the atmosphere can reduce ozone concentration (Herbst and Wiesmann, 1996).

1.3.2.3 Health implications

Much interest has been focused on the microorganisms involved in microbial degradation of DCM, mainly as a result of its genotoxic and carcinogenic effects (Nikolausz *et al.*, 2005). In humans, it has the ability to cause degenerative changes in the nervous system if inhaled over long periods of time. It is particularly dangerous because it can be oxidized to phosphene ($COCl_2$) gas under an open flame. Phosphene is a very strong and effective toxin, and has the ability to cause lung oedemas (Fellenberg, 2000).

1.3.3 1, 2-Dichloroethane ($ClCH_2CH_2Cl$)

It has been alleged that this compound is produced in larger quantities than any other chlorinated hydrocarbon. In 1994, the United States, alone, produced 16 000 000 tonnes of 1, 2-DCA (Hunkeler and Aravena, 2000).

1.3.3.1 Commercial uses

1, 2-DCA finds its principal applications as a precursor for the production of polyvinyl chloride (De Wildeman *et al*., 2004). It has also been used in organic synthesis for extraction and cleaning. It also has applications as a solvent and is used in formulations of various products e.g. varnishes, metal degreasers, soaps and scouring compounds and adhesives (http://www.epa.gov/OGWDW/contaminants/dw_contamfs/12-dichl.html).

1.3.3.2 Environmental properties

Analogous to its aforementioned chlorinated aliphatic counterparts, it is frequently detected in the environment (Hunkeler and Aravena, 2000). If released into the environment, 1, 2-DCA can easily contaminate groundwater because it has a high aqueous solubility (Table 5) and a low sorption coefficient. It is extremely hazardous, and has an environmental half-life of up to 50 years (De Wildeman *et al*., 2004).

1.3.3.3 Health implications

An efficient detoxification technology for this compound is yet to be developed. This fact remains quite alarming as 1, 2-DCA appears on many lists as the most abundant C_2 groundwater pollutant on earth, and is classified by the USEPA as a priority pollutant and

a suspected carcinogen (De Wildeman *et al*., 2004). Studies have shown that 1, 2-DCA can cause circulatory and respiratory failure associated with neurological disorders in human beings (Bejankiwar *et al*., 2005).

Table 1.5: Some properties of 1, 2-DCA (Baden, 2008).

Molecular formula	$C_2H_4Cl_2$
Molar mass	98.97 g/mole
Appearance	Colourless liquid with characteristic odour
Density	1.25 g/cm³, liquid
Melting point	-35.5°C (238 K)
Boiling point	83.5– 84.1°C (357 K)
Solubility in water	8.7 g/litre (20°C)

1.4 DEGRADATION OF CAHs

Usually, degradation of such chlorinated compounds is accomplished by first stripping them with air or steam. This is then followed by adsorption on activated carbon, desorption and incineration. These types of physico-chemical procedures have been the only way to deal with materials, contaminated with chlorinated hydrocarbons (Herbst and Wiesmann, 1996). Chlorinated hydrocarbons are generally resistant to microbial attack, however, biodegradation of such compounds, either aerobically or anerobically has been documented and reviewed (Fogel *et al*., 1986; Chaudhry and Chapalamadugu, 1991; Alexander, 1994; Zacharias *et al.,*1995; Annachhatre and Gheewala, 1996; Chang and Alvarez-Cohen, 1996; Timmis and Pieper, 1999; Wu *et al*., 2002; Duhamel *et al*., 2005; Nikolausz *et al*., 2005; Matafonova *et al*, 2006; Nair *et al*., 2007; Luo *et al*., 2008). Understanding the biodegradative capabilities of microorganisms has led to the remediation of some environments contaminated with chlorinated hydrocarbons.

1.5 BIOREMEDIATION

Environmental longevity and issues regarding the harmful effects of pollution has recently been the subject of much attention. The acknowledgement and frequent detection of polluted sites globally, has encouraged an impressive increase in research aimed at strategies to remedy polluted environments. Bioremediation can be considered as a new technology that involves the use of biological agents. These agents are able to treat contaminated or polluted environments by modifying or decomposing target pollutants. Bioremediation techniques have high public acceptance and encompasses the use of relatively low-cost, low technology techniques. In addition, it can be carried out on site (Vidali, 2001). The goal of bioremediation is to degrade organic pollutants to concentrations that are either undetectable, or if detectable, to concentrations below the limits established as safe/acceptable by regulatory agencies. Bioremediation can and is, being used for the destruction of chemicals in soils, ground and wastewater, sludges and industrial waste-systems (Alexander, 1994).

1.5.1 Conventional remediation techniques

The conventional techniques used for remediation are now being reviewed. Many remediation specialists realize that excavating contaminated soil and removing it to a landfill, or the 'cap-and-contain' method is no longer feasible. These methods have obvious disadvantages. The first method is not practical because it simply moves the contamination elsewhere. In addition, it creates problems and risks associated with the excavation, handling, and transport of the hazardous materials. Obviously, it becomes increasingly difficult and expensive to locate new landfill sites for final disposal of the

contaminated substance, which is an additional disadvantage. The cap-and-contain method solves the problem temporarily, because the contamination remains on site. This material then requires constant monitoring and maintenance of the isolation barriers long into the future (Vidali, 2001). Other current treatments include methods such as air-stripping, soil-vapour extraction or adsorption with activated carbon, which focuses on transfer from one phase to another. These processes however, often require further disposal decisions (da Cunha and Leite, 2000).

1.5.2 Biological remediation techniques

Biological processes offer an excellent alternative to many conventional techniques. These processes are simple and cost-effective and therefore, are very suitable for clean-up of contaminated environments (Vidali, 2001). A great deal of research has concluded that natural communities of microorganisms have remarkable physiological versatility and flexibility. It is therefore not surprising that, the controlled practical use of microorganisms for the destruction of chemical pollutants has been occurring much more frequently. Microorganisms have confirmed numerous times they are able to metabolize and often mineralize an enormous number of organic molecules. Probably every natural product, regardless of its complexity can be degraded by a suitable microbial species in a particular environment. Similarly, communities of bacteria and fungi metabolize a multitude of synthetic chemicals. The number of such molecules that can be degraded has yet to be counted, but literally thousands are known to be destroyed as a result of microbial activity in a given environment (Alexander, 1994). The list of compounds that may be subject to biological destruction by one/another bioremediation system is long.

Oil and oil products, gasoline and it's constituents, polycyclic aromatic hydrocarbons, chlorinated aliphatics such as TCE and PCE as well as some chlorinated aromatic hydrocarbons are widespread and represent significant health and ecological hazards. Due to the fact that these compounds are susceptible to microbial detoxification, extensive research has been directed to their remediation (Alexander, 1994). Microbes have the potential to transform these toxic chemicals into benign or harmless products thereby affording bioremediation a major advantage in comparison to chemical and physical techniques, where pollutants are often transferred into another phase. (Aulenta *et al*., 2005). The use of microorganisms is further advantageous because they have the ability to lead to complete mineralization of the contaminants to innocuous end products. Investigations have also concluded that most microbially mediated enzymatic reactions are generally faster than those of the same reaction in the absence of microorganisms (Bouwer, 1992).

1.5.3 Principles of bioremediation

By definition, bioremediation employs the use of living organisms, primarily microorganisms. These microorganisms that may be naturally occurring bacteria or fungi are able to degrade environmental contaminants that are hazardous to human health and the environment, to less toxic forms. Such microorganisms may be indigenous to the contaminated area, or they may be brought to the contaminated site (Vidali, 2001). Contaminant compounds are transformed by living organisms through reactions that take place as part of their metabolic processes. These organisms transform the environmentally available nutrients to forms that are functional for inclusion into cells

and the subsequent synthesis of cell polymers (Bouwer, 1992).

1.5.4 Factors governing bioremediation

Certain criteria should be met for bioremediation to be seriously considered as a practical means for treatment:

a) Microorganisms must have the relevant catabolic potential,

b) Such microorganisms must be capable of converting contaminants at satisfactory and practical rates, and reduce concentrations to levels that meet, or are below regulatory standards,

c) By-products that can be generated during the remediation process should not be toxic,

d) It is important that the site does not contain certain concentrations or combinations of chemicals that can affect the growth and ability of the biodegrading species,

e) The target compound must be readily bioavailable to the microorganisms.

f) Conditions that favour microbial growth or activity must exist (Alexander, 1994).

Degradation of contaminants is achieved by the interaction of various other environmental factors. These may include soil moisture, soil pH, oxygen content, nutrient content, temperature and type of soil (Vidali, 2001).

1.5.5 Bioremediation strategies

The degree of saturation and aeration of an area dictate which bioremediation technique may be employed. *In situ* techniques are defined as those that are applied to soil and ground water at the site with minimal disturbance. These techniques include *in situ* bioremediation, biosparging, bioventing and bioaugmentation (Table 6).

Table 1.6: Summary of different bioremediation strategies (Vidali, 2001).

TECHNOLOGY	EXAMPLES	BENEFITS	LIMITATIONS
In situ	*In situ* bioremediation Biosparging Bioventing Bioaugmentation Biostimulation	Most cost efficient Noninvasive Relatively passive Natural attenuation processes Treats soil & water	Environmental constraints Extended treatment time Monitoring difficulties
Ex situ	Landfarming Composting Biopiles	Cost efficient Low cost Can be done on site	Space requirements Extended treatment time Need to control abiotic loss
Bioreactors	Slurry reactors Aqueous reactors	Rapid degradation Optimised environmental parameters Enhances mass transfer Effective use of inoculants and surfactants	Soil requires excavation Relatively high cost capital Relatively high operating cost

Ex situ techniques are those that are applied to soil and ground water which has been removed from the site via excavation (soil) or pumping (water). This technology employs landfarming, composting and biopiles. Bioreactors are also used for bioremediation purposes, and these can be either slurry reactors or aqueous reactors. The individual advantages and disadvantages of each treatment differ with the types of technique used. Despite many factors that can hamper the remediation process, many of these techniques are currently being employed at a number of sites worldwide (Vidali, 2001).

Bioremediation of contaminated sites is a vast field of endeavour, and many new or altered technologies are appearing. Nevertheless, the utilization of microbial processes to destroy chemicals is neither a novel idea nor a new technology (Alexander, 1994). Table

6 summarises the various bioremediation strategies, also indicating their advantages and disadvantages.

1.6 SCOPE OF THIS STUDY

Bioremediation is gaining popularity in Europe, as it is frequently being used at many contaminated sites. There are various bioremediation technologies that may be implemented at contaminated sites. Although these techniques are not problematic, substantial expertise and skill are required to ensure the correct implementation and success of a bioremediation scheme (Vidali, 2001). Extensive chemical, geographical and biological research must be undertaken to determine the factors that will enable efficient detoxification of xenobiotic chemicals. In spite of the comprehensive research that provides information on the degradation of individual chlorinated aliphatic hydrocarbons, research that focuses on the degradation of mixtures of compounds is scarce. It has been estimated that more than half of all contaminated sites worldwide contain multiple pollutants, therefore this void in research seriously needs to be addressed (Adamson and Parkin, 1999). Mixtures of chemicals can present various problems that may affect remediation. Partial degradation of chemicals that can be present in a mixture can result in the formation of certain chemical species that may be more toxic than the parent compounds (Sims *et al*., 1991). The accumulation of such toxic metabolites in the environment has additional ramifications for human health and wildlife. In addition, when many compounds are present, factors such as toxicity and inhibition can affect the transformations of the chemicals in a mixture (Adamson and Parkin, 1999). Hughes and Parkin (1992) observed that some interactions can occur when compounds are present in

mixtures. It was noted that the presence of some compounds in a combination can have negative impacts on other compounds within that specific combination. Furthermore, while in a mixture, the occurrence of some compounds in a mixture may even have beneficial effects on the transformation of others.

While most chlorine compounds are recalcitrant, many have the potential to be transformed and degraded through microbially mediated processes under favourable conditions. A limited understanding of the factors that control biodegradation pathways and the reaction rates of many contaminants renders bioremediation an important scientific and engineering endeavour that demands further research. Thus it has become imperative to determine the factors and conditions that are able to permit the simultaneous biodegradation of individual compounds in mixtures.

Chapter two describes the selection and identification of microbial strains that were used during the bioaugmentation procedures; chapter three and four investigated the effects of the biostimulation and bioaugmentation processes respectively on CAHs' degradation in soil artificially contaminated with a mixture of CCl_4, DCM and 1, 2-DCA. A comparison of two different microcosm types was undertaken to determine the effects of each microcosm type on the bioremediation of CAH-contaminated soil. Chapter five is a general discussion and conclusion, including current and future directions for bioremediation research.

1.7 HYPOTHESIS

It is hypothesized that bioremediation of soil contaminated with a mixture of chlorinated aliphatic hydrocarbons will be improved by biostimulation and/or bioaugmentation processes. It is further hypothesized that circulating microcosms may be effective for the bioremediation of certain chlorinated aliphatic hydrocarbons.

1.8 OBJECTIVES

1.8.1 To evaluate the effects of biostimulation and/or bioaugmentation on soil contaminated with a mixture of CCl_4, DCM and 1, 2-DCA.

1.8.2 To assess the differences in bioremediation between a stationary microcosm and a circulating microcosm on certain CAHs.

1.9 AIMS

1.9.1 To construct laboratory-scale microcosms with soil artificially contaminated with a mixture of CCl_4, DCM and 1, 2-DCA.

1.9.2 To assess the ability of bacterial consortia displaying appreciable biodegradation of CAHs to be used for bioaugmentation.

1.9.3 To supplement contaminated soil with nutrients and/or known CAH degrading bacterial consortia and compare biodegradation in two different microcosm types.

1.9.4 To determine effects of biostimulation and/or bioaugmentation on the soil microbial biomass in the different types of microcosm.

1.9.5 To determine the final products of degradation of CAHs.

CHAPTER TWO

SELECTION AND IDENTIFICATION OF CAH-DEGRADING ORGANISMS FOR BIOAUGMENTATION STUDIES

2.1 INTRODUCTION

Bioaugmentation can be defined as a technique to improve the quality of a contaminated matrix. This is achieved by introducing specific competent strains or consortia of microorganisms, in order to eliminate or decrease pollutant concentrations. While bioaugmentation is already being practiced in some agriculture and wastewater treatment programmes, this technique still remains an experimental methodology, especially for the *in situ* bioremediation of contaminated sites (El Fantroussi and Agathos, 2005).

Augmenting contaminated soil with catabolically relevant microorganisms to accelerate remediation is the simple rationale that underpins bioaugmentation. Studies have revealed that most successful bioaugmentation treatments involved frequent application of competent pollutant degrading microorganisms. Selection of the necessary organisms is therefore an arduous task, dependant on various factors (Thompson *et al*., 2005). The isolation and identification of appropriate microbial strains is crucial to ensure a successful bioaugmentation regimen, and thus far many strategies have been developed for designing new and improved catalysts for bioremediation. Selective enrichment techniques have allowed the sourcing of microbial strains to be used for bioaugmentation, for at least the last 100 years. The strains from the polluted biotope can be enriched and grown in culture by using the contaminant as a sole source of carbon and nitrogen. Thus, the selection of strains that express the desired degradation ability can be achieved, but in

the specific conditions of the enrichment culture. Such enriched populations may not typically represent indigenous communities that may be present in the contaminated area, and it is this factor that probably leads to the failure of bioaugmentation. It has been suggested that the enrichment procedure is unlikely to influence other traits, also required for strains to be successful and competitive when introduced into their target environment (Thompson *et al*., 2005).

The addition of activated soils, which are soils that contain indigenous degrading populations that are exposed to the contaminant, have also demonstrated varying levels of success in bioaugmentation (El Fantroussi and Agathos, 2005). For obvious reasons, strain selection is restricted to those species that are not linked to human pathogens. It is often however, that a superbug is closely related to a human pathogen preventing its field implementation (Singer *et al*., 2005).

The biodegradation performance of a consortium to be used for bioaugmentation purposes can be easily improved by the addition of a specialist organism. Usually, in this strategy, a two or three member consortium is attained. One member initiates the catabolic reactions, and the other members may complete the sequence. Such consortia have been identified and used for the mineralization of bicyclic aromatic compounds such as chlorinated dibenzofurans and chlorinated biphenyls (Timmis and Pieper, 1999).

This chapter focused on determining the ability of organisms isolated in a previous study to grow with and degrade a mixture of CAHs i.e., carbon tetrachloride (CCl_4),

dichloromethane (DCM) and 1, 2-dichloroethane (1, 2-DCA). The isolates that demonstrated the best growth patterns were chosen to formulate several consortia. A preliminary biodegradation study was then undertaken to determine which consortia would be used for future bioaugmentation studies. In addition, the identities of these microorganisms were determined using 16 S rDNA sequence analysis.

2.2 MATERIALS AND METHODS

2.2.1 Growth, maintenance and inoculum preparation of bacterial isolates

Ten bacteria isolated during a previous biodegradation study (Olaniran, 2005), from various sources were used in this study. The microorganisms were maintained on nutrient agar plates at 4ºC. Long term storage was accomplished by preparing glycerol stocks of the organisms. This was achieved by adding 500 µl of an overnight nutrient broth culture of the respective bacteria and 500 µl of a 30% glycerol solution in a sterile 1.5 ml microfuge tube. This was then stored at -20ºC, until required. The inoculum used for growth measurement studies was prepared by centrifuging a 100 ml overnight nutrient broth culture at 8500 × *g* for 15 min at 4ºC, using a Beckman Model J2-21 centrifuge. The supernatant was discarded and the cell pellet was then suspended in 30 ml of 0.85% (w/v) saline solution, and again centrifuged. This washing step was repeated, and the cells were finally re-suspended in 5 ml saline solution. The cultures were then standardized to $OD_{(600nm)} = 2$ using an UltroSpec KB II spectrophotometer.

2.2.2 Growth measurement studies

In order to determine if the microorganisms were capable of using a mixture of CAHs as a carbon source for growth, their respective growth patterns were monitored individually. One hundred milliliters of minimal salts medium (Appendix I), 100 µl trace element solution (Appendix I), 100 µl vitamin solution (Appendix I), 10 µl of CCl_4, DCM and 1, 2-DCA together with a 1% inoculum (Section 2.2.1) were added to 250 ml glass screw cap bottles, with a rubber septa, mixed well and immediately sealed. The bottles were then incubated on a rotary shaker at 140 rpm, at 28ºC. Control bottles did not contain any microorganisms. Optical density measurements at 600 nm were taken every 12 hrs, for 36hrs. Samples for optical density readings were removed using a 1ml sterile syringe, through a rubber septum in the bottle cap, to prevent abiotic loss of the volatile CAHs'. The bacterial isolates that showed the highest optical density values were chosen as candidates for seven consortia. Consortia designated A-G were created by adding 200 µl of different standardized bacterial suspensions together in a 1.5 ml microfuge tube. This was gently mixed by vortexing for 10 sec. Experiments identical to those conducted for individual isolates, were then conducted, to establish which consortium showed best growth potential. Similarly, control bottles did not contain any consortium of microorganisms. The consortia that demonstrated appreciable growth potential after 4 days (96hrs) were then selected for preliminary biodegradation studies.

2.2.3 Preliminary biodegradation study

A preliminary biodegradation study was conducted to determine which consortia demonstrated the best biodegradation ability. Experiments identical to those described in

Section 2.2.2 above, were conducted, except that 250 ml Wheaton serum bottles equipped with teflon-lined rubber septa, were used. Reaction mixtures were then shaken at 150 rpm on a rotary shaker for 2 hrs, at 28ºC, to allow equilibration of the volatile CAHs in the headspace. Changes in CAH concentrations were then monitored by headspace analysis in a gas chromatograph as described in the following section (2.2.4).

2.2.4 Gas chromatography

CAH concentrations were monitored using a Varian model 3700 gas chromatograph equipped with a flame ionization detector (FID). Nitrogen was used as the carrier gas. The FID output was connected to a Millipore recording integrator. Four hundred microlitres of the headspace samples were injected into the chromatograph, using a gas tight syringe (Hamilton). The injector and detector temperatures were set at 200°C and the column temperature at 100°C. All CAH concentrations were calculated from peak area measurements, by creating a standard curve. This was accomplished by adding a known amount of each CAH to a serum bottle with the same headspace to liquid ratio as the experimental microcosms. The degradation values were calculated after incorporating peak areas into the equation derived from the standard curve, and according to the following formula:

$$\text{Degradation} = \frac{(A) - (B)}{(A)} \times 100$$

where A= the concentration of CAH at day zero (0),

B= the concentration of CAH at sampling time (Olaniran, 2005).

2.2.5 Identification of the bacterial isolates

2.2.5.1 Extraction of genomic DNA

Genomic DNA was isolated from nutrient broth cultures of the six bacterial isolates grown overnight (chosen during growth measurement studies, in Section 2.2.2) using the QiaAmp Mini DNA Isolation Kit (QIAGEN), according to the manufacturer's instructions. Extracted DNA was electrophoresed on a 1% agarose gel at 100 V for 2.5 hrs, using a 1 × Tris- acetate-EDTA (TAE) buffer. This was followed by staining; using a 0.5 µg/ml ethidium bromide solution and DNA was visualized using the ChemiGenius BioImaging System equipped with SynGene GeneSnap Software.

2.2.5.2 PCR-amplification of the bacterial 16S rRNA genes

Using the extracted DNA as a template, PCR was then conducted using the forward primer 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and reverse primer 1387r (5′-GGG CGG WGT GTA CAA GGC-3′), corresponding to identical positions on the 16S rRNA gene of *E. coli* (Marchesi *et al*., 1998). A 50µl reaction contained 25.5 µl double distilled water, 5 µl buffer, 2 µl (1mM) $MgCl_2$, 10 µl (200µM) dNTPs, 2.5 µl (0.5mM) each of the forward primer and reverse primer and 0.5 µl (2.5 U) *Taq* polymerase, with 2 µl template DNA. The PE-9600 PCR thermal cycler (Perkin- Elmer) was programmed to complete 30 cycles that consisted of 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min followed by a final extension step of 5 min at 72°C (Marchesi *et al*., 1998). PCR products were subsequently visualized by electrophoresis, as described in Section 2.2.5.1. These PCR products were then purified using a Qiaquick PCR purification kit (QIAGEN), according to the manufacturer's instructions. The purified PCR products

were again visualised by agarose gel electrophoresis (Section 2.2.5.1). Purified PCR products were then used as the insert DNA for a cloning reaction to aid identification of the bacterial isolates.

2.2.5.3 Ligation

Ligation of PCR purification products to the pGEM T Easy Vector Kit (Promega) vector was conducted according to the manufacturer's instructions.

2.2.5.4 Preparation of electrocompetent cells

Three milliliters of an overnight *E. coli* DH5α F' nutrient broth culture was added to 100 ml YT medium. This was incubated at 37ºC, at 150 rpm until the cell density reached an $OD_{(600nm)}$= 2. The culture was then immediately placed on ice to prevent further growth. Cells were then pelleted by centrifugation for 10 min at 10 000 × *g* at 4ºC. The resultant cell pellet was suspended in 100 ml cold 10% glycerol and centrifuged. Subsequently, the pellet was then resuspended in 50 ml cold glycerol, centrifuged and resuspended in 5 ml 10% cold glycerol. The suspension was centrifuged and then resuspended in a final volume of 2.5 ml 10% cold glycerol. Fifty microlitre aliquots were dispensed into Eppendorf tubes. Seventy percent ethanol (stored at -70ºC) was then used to snap-freeze the cells, which were subsequently stored at -70ºC until required.

2.2.5.5 Transformation

Electrocompetent cells were transformed by electroporation. Two microlitres of the ligation mixture obtained as described above, Section 2.2.5.4 was electroporated into

electrocompetent *E.coli* cells. The Gene Pulser apparatus (BioRad) was set at 25 F and 2.5 kV for 0.2 cm cuvettes (BioRad) and the Pulse controller was set to 200 Ohms for *E. coli* cells. One millilitre of SOC medium (Appendix I) was immediately added to the cuvette to enhance the recovery of transformants. This mixture was then placed at 37ºC at 150 rpm in 1.5ml microfuge tubes, for 1 hr.

2.2.5.6 Selection of transformants

Following a 1 hr incubation period, the mixtures (Section 2.2.5.5) were centrifuged for 30 sec to obtain a cell pellet. Subsequently, 50 µl X-gal (100 mg/ml stock solution), 20 µl IPTG and 100 µl of the cell suspension were plated onto Luria-Bertani (LB) agar plates containing 50 µg/ml. These plates were then incubated for 16 hr at 37ºC and transformants were identified by blue-white selection.

2.2.5.7 Sequencing and sequence analysis

Positive transformants (white in colour) were purified by four-way streaking and sequenced (Inqaba Biotech). Sequence chromatograms were visualized and edited using Chromas software (version 1. 45) and aligned with the DNAMAN software (Lynnon Biosoft, version 5.0). 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).

2.2.6 Statistical analysis

Statistical values were calculated using the T- Test for independent samples with SPSS 15 for Windows® software. All samples were compared to the controls, unless stated otherwise. A 95% confidence interval was set, therefore *P* values < 0.05 were regarded as statistically significant, while values > 0.05 were regarded as insignificant.

2.3 RESULTS

2.3.1 Screening of individual isolates

Ten bacteria isolated during a previous biodegradation were studied individually, to examine the effects of a mixture of CAHs on their respective growth patterns. Optical density values are reflected in Appendix I.

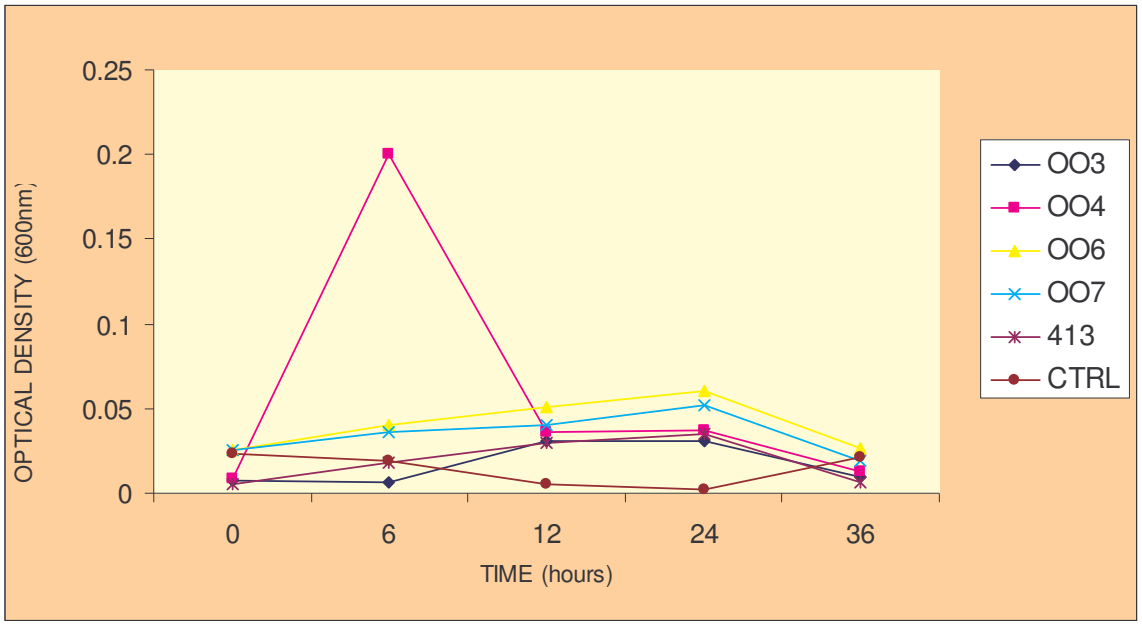

Fig. 2.1: The effect of a mixture of CAHs on the growth of bacterial isolates 003; 004; 006; 007; and 413.

A clear trend was evident when examining the growth patterns of ten isolates in media

supplemented with a mixture of CCl_4, DCM and 1, 2-DCA. With the exception of the control that did not contain any microorganisms, all isolates showed an increase in growth until 24 hr, followed by a decrease at 36 hr (Fig.2.1). After 24 hr, isolate 006 had reached the highest optical density value of 0.0605. Of the 10 isolates tested, this was the highest value observed at this sampling time. This was closely followed by isolate designated 007, which achieved its maximum growth value of 0.052 at 24 hr as well. Isolate 004 displayed a 2.5 fold increase in turbidity after just 6 hr, however, this value quickly decreased after 12 hr. At 24 hr, isolate 004 had the third highest growth, with a value of 0.037. Isolates 003 and 413 exhibited similar growth patterns showing values of 0.031 and 0.034, respectively. Isolate A01 had displayed a value of 0.037 at 12 hr (Fig. 2.2), which was approximately a 4-fold increase from the start of sampling (0 hr). Comparitively, isolate A01 had the 4th highest growth measurements among the 10 isolates.

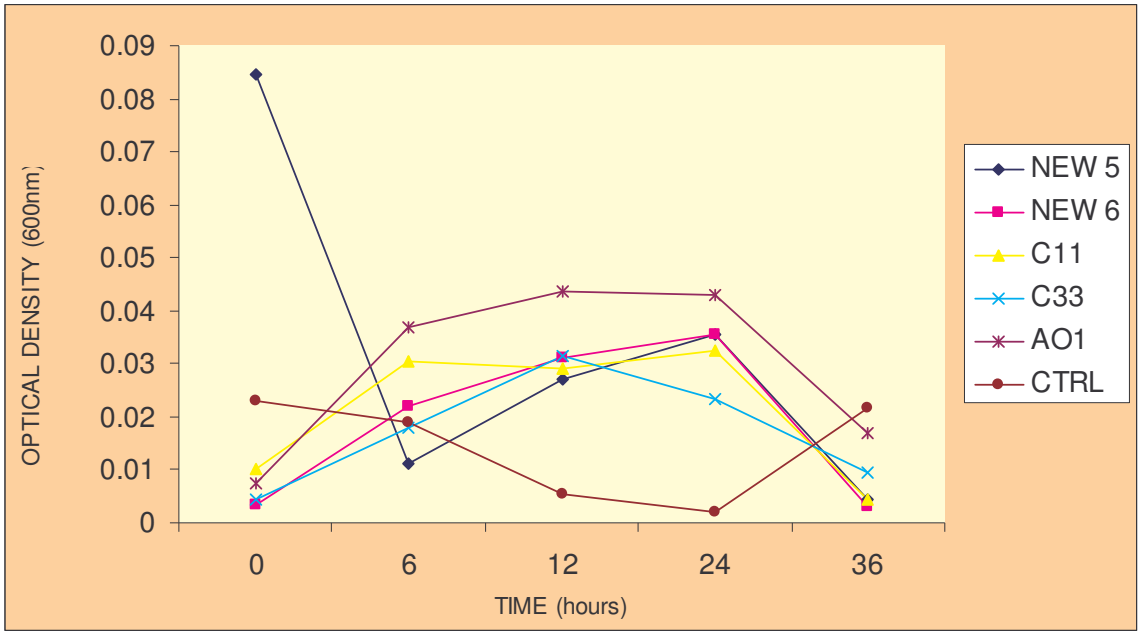

Fig. 2.2: The effect of a mixture of CAHs on the growth of bacterial isolates NEW 5; NEW 6; C 11; C 33 and AO1.

The New 6 isolate also reached appreciable growth levels, a 10% increase after 1 day (0 to 24 hr) was observed. Isolates C11 and C33 did not demonstrate growth patterns that were significant, compared to the other isolates. In fact, the lowest growth values, at most sampling times were recorded for isolate C33 (Fig. 2.2). With regard to isolate C11, it was the only isolate that demonstrated constant growth from 6 to 24 hr, after which a decrease in optical density was noted. The growth of isolate New 5 was seriously inhibited by the presence of a mixture of CAHs and this is evidenced by a drastic decrease after 6 hr. Subsequently, its maximum growth potential was observed to occur after 24 hr (Fig. 2.2) to a value of 0.035.

2.3.2 Screening of various consortia

The isolates that displayed the most favourable growth patterns and were statistically different from the controls at 24 hr were 006, AO1, 007, 413, 004 and New 6. These isolates were chosen to formulate several combinations of different consortia, and the growth patterns of those consortia were then monitored (Fig. 2.3). It was quite evident that many of the consortia behaved quite similarly and produced similar growth patterns. With the exception of consortium E, the other consortia attained peak growth measurement values at 36 hr. All values recorded at this time were above 0.1, and are displayed in Appendix I. The various consortia demonstrated much more sustained growth patterns than their respective monocultures (Fig. 2.3). The compositions of the different consortia are: A (004, 006, 007, 413, AO1); B (006, 007, 413, AO1, New 6); C (004, 007, 413, AO1, New 6); D (004, 006, 413, AO1, New 6); E (004, 006, 007, AO1, New 6); F (004, 006, 007, 413, New 6); G (004, 006, 007, 413, AO1, New 6).

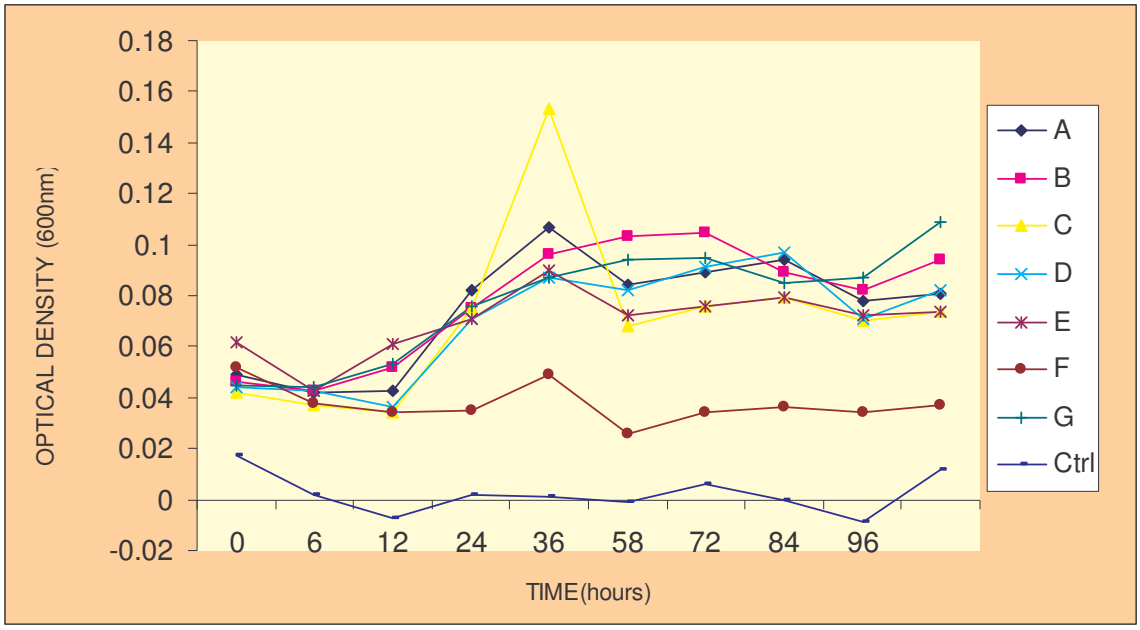

Fig. 2.3: The effect of a mixture of CAHs on the growth pattern of various bacterial consortia.

When isolates were evaluated individually, their growth became inhibited after 24 hr. When the isolates were present in various combinations, each consortium's growth peaks at 36 hr and subsequently had constant growth until 96 hr. Consortia designated B, C, D, E and G displayed the best growth potential and were statistically significant compared to the control at 36 hr.

2.3.3 Preliminary biodegradation study

A preliminary biodegradation study was conducted to determine these consortia's ability to degrade a mixture of CAHs.

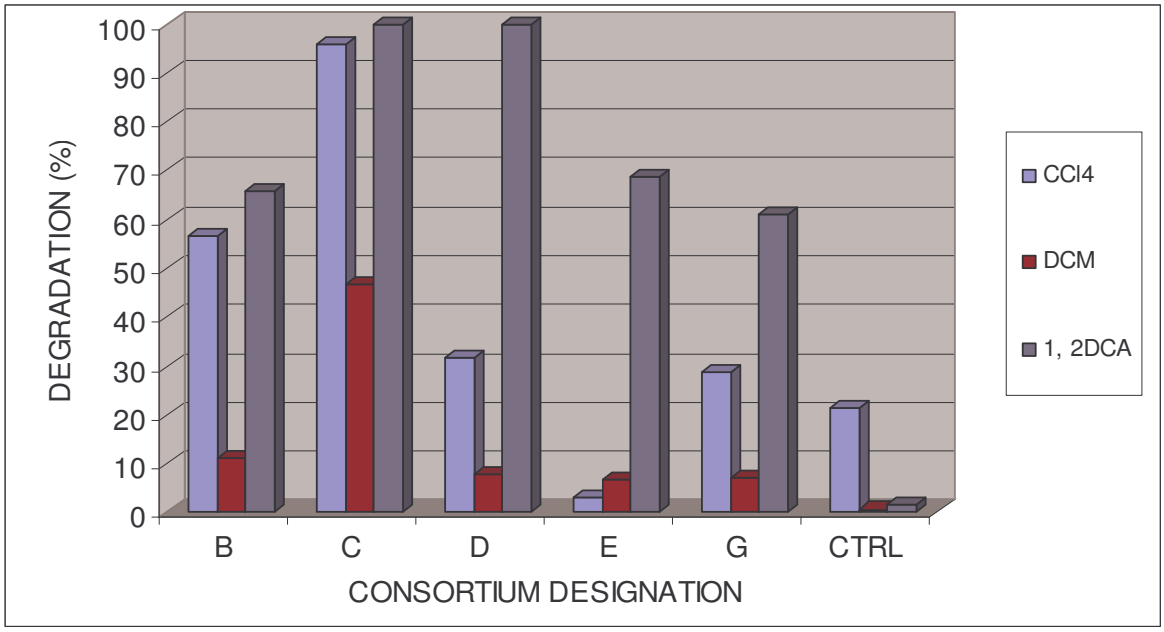

Fig. 2.4: Biodegradation profiles of the different CAHs present in the mixture by the various bacterial consortia after 7 days.

While CCl_4 DCM, 1, 2-DCA were added as a mixture for experimental purposes, their biodegradation is represented individually in Fig. 2.4 (Data is reflected in Appendix I).

The results of the growth measurement studies were analogous to those obtained during the preliminary biodegradation study. It was noted by day 7, that the consortia that was designated C had led to the greatest reduction in CCl_4 concentration (Fig. 2.4), a highly significant 95.7% (P = 0.017) degradation value was obtained compared to a 21.25% reduction in CCl_4 observed in the control. This was followed by consortia B and D. The greatest decrease in DCM concentration was observed in bottles that contained consortium C (Fig. 2.4). By day 7, 46.6% of DCM was degraded (P = 0.017). This was followed by consortium B and consortium D that resulted in an 11.82% and 6.63% degradation of DCM. The various consortia demonstrated much greater degradation potential for 1, 2-DCA. Figure 2.4 shows that consortia C and D led to 100% degradation

of this CAH. This was followed by consortia E and consortia B that displayed similar ability for 1, 2-DCA degradation. Consortia labeled B, C and D had displayed the greatest potential for degrading a mixture of CAHs and were therefore chosen for inclusion in the bioaugmentation experiments.

2.3.4 Identification of CAH-degrading organisms

PCR amplification of the genomic DNA of isolates 004, 006, 007, 413, AO1 and New 6, using the 63f and 1387r primer set yielded a product approximately 1350 kb (Fig. 2.5). The isolates' consensus sequences (Appendix I) were compared to those available in the GenBank Database, and Table 2.1 shows the accession numbers and names of species closely related to the bacteria isolates used in this study.

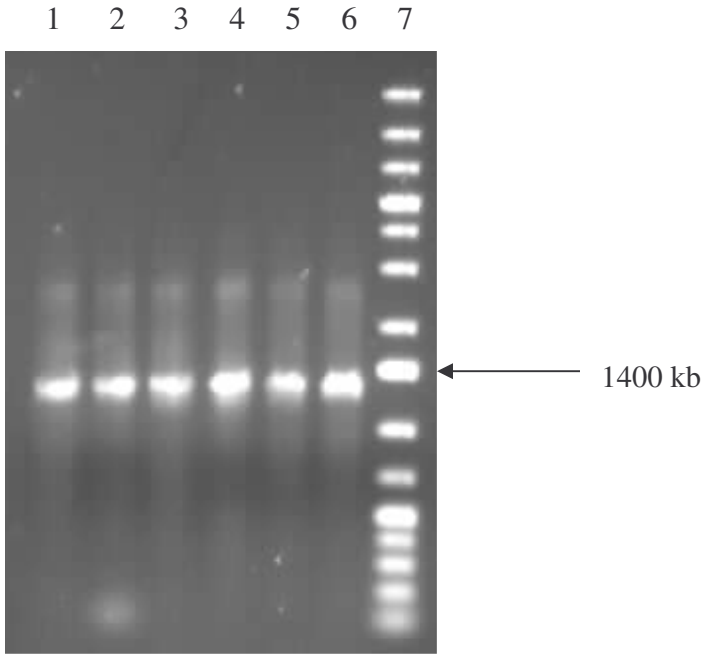

Fig. 2.5: PCR amplicons of the 16S rRNA genes of the six bacterial isolates (Lane 1-6: 004; 006; 007; 413; AO1; New 6; and lane 7: Fermentas DNA Ladder Plus).

Table 2.1: Identity of bacterial isolates selected for bioaugmentation.

ISOLATE DESIGNATION	IDENTITY	ACCESSION NUMERS OF RELATED ORGANISMS
004	*Bacillus subtilis* KCM RG5	AJ830709
006	*Bacillus sp*. LAMI 002	EU082292
007	*Bacillus sp*. G1DM-80	EU037268
413	*Bacillus sp*. Ni36	DQ643186
AO1	*Bacillus sp*. MB23	AB166892
New 6	*Alcaligenaceae* bacterium Lm-2-1	EF025348

2.4 DISCUSSION

The ability of an inoculant to proliferate and colonise soil is dependant on various physiological features. These features can differ and are often not well understood or known. It therefore becomes imperative, when searching for inoculants to conduct a systematic and thorough selection procedure (van Veen *et al*., 1997). Experiments with minimal media demonstrated that the ability of the 10 isolates to use a mixture of CAHs as a carbon source, varied. The fate and activity of microorganisms selected for introduction into another environment can be improved if prior knowledge of the ubiquity, population dynamics, and spatial and temporal distribution of microbial communities within sampled habitats is known (Thompson *et al*., 2005). The microorganisms tested in this study were previously isolated from pulp and paper mill effluent (004, AO1 and New 6), brewery effluent (006 and 007) and soil (413). These constitute diverse and harsh environments that may sometimes prove difficult for microbial metabolism. This is especially true for the pulp and paper mill effluent, wherein cellulose bleaching often results in the production of chlorinated compounds that are very toxic.

Xenobiotics and naturally occurring toxic compounds that have become prevalent in many environments, have affected microbial evolution tremendously. Such environmental disturbances have exerted selective pressure on microbial communities (Tondo *et al*., 1998). The consequence is that these organisms have evolved several mechanisms to survive their surroundings. Previous exposure of these cultures to complex environments can possibly explain their enhanced ability to degrade a mixture of CAHs. Klecka (1992) reported that aerobic conditions led to rapid degradation of DCM by acclimated cultures. The disappearance of 1 mg of C^{14} labelled DCM was observed after 3 hrs. After 50 hrs, 66% of the parent compound was recovered as $^{14}CO_2$. This observation clearly demonstrated the drastic effect of acclimated cultures on the biodegradation of certain organic chemicals. All the isolates appear to have decreased activity after 24 hrs, and this is most likely the result of the accumulation of some toxic metabolites, as a result of CAH metabolism. Alternatively, decreases in substrates and energy sources could have accounted for this result (Olaniran *et al*., 2001).

The various consortia grew more successfully in the presence of a mixture of CAHs than their respective monocultures. The growth and survival of bacteria can be vastly improved when they are in close contact with various other bacteria. This has been speculated to be as a result of interspecies interactions (Kuo and Genther, 1996; De Souza *et al*., 1998). In addition, a system can become more tolerant to environmental stress, as a result of the stability afforded by the presence of secondary co- degraders in a consortium. It can be assumed that no single strain has all the characteristics that may enable degradation of a particular chemical. However, many researchers propose that

these characteristics might be found within a microbial consortium (Radianingtyas *et al.*, 2003). The consortia had a slightly longer lag phase than the individual isolates. Dejonghe *et al*. (2003) proposed several reasons for the observation of extended lag phases, by xenobiotic degrading bacteria. These include: induction/derepression of catabolic genes, genetic exchange, mutations, and an increase in the initially small degrader population, an inclination towards utilization of other organic compounds before the chemical of interest, adaptation to toxins or inhibitors.

Picardal *et al*. (1995) stated that the mechanisms under which microorganisms can transform halogenated compounds are diverse. The degradation of organic contaminants by bacterial consortia should prove more effective mainly due to symbiotic metabolic activities. This trend probably occurs frequently in the environment, naturally. Two mechanisms have been postulated to explain this phenomenon: i) if degrading bacteria are fastidious, they often rely on the secondary strains in a consortium to complement their metabolic deficiencies; secondary strains probably provide essential growth factors and nutrients, and ii) association metabolism, in which strains with biosynthetic deficiencies will obtain amino acids or other vitamins from other bacteria , via metabolic cross- feeding (De Souza *et al*., 1998; Dejonghe *et al*., 2003). This is supported by the fact that interspecies variations in dehalogenation ability are also diverse.

Biodegradation is often considered to be a multifaceted process that can be affected by various factors. The contaminant itself and other inhibitory substances can profoundly alter its progression. Furthermore, bioavailability of nutrients or contaminants, physical

conditions (e.g. temperature, salinity, and pH) can also limit biodegradation (Langwaldt and Puhakka, 2000). Dehalogenation of CCl_4 by pure cultures can be attributed to the presence of vitamin B_{12} and other corrinoids in cells. However, it is also speculated that other unknown dechlorinating mechanisms may also be active in halogenated compound dechlorination (van Eekhert *et al*., 1998). This is also applicable with regards to DCM and 1, 2-DCA degradation. Often if various substrates are available for use by microorganisms, the microbes will opt to utilize the most readily available substrate first (Dejonghe *et al*., 2003). This is probably true for 1, 2-DCA. The high solubility coefficient of 1, 2-DCA enabled increased contact with the degrading bacteria, therefore higher biodegradation rates were noted for 1, 2-DCA, compared to the other 2 CAHs.

Isolates 004, 006, 007, 413 and AO1 were identified as members of the *Bacillus sp*. Three of the 4 isolates were isolated from a pulp and paper mill effluent and this is consistent with other investigators who have reported the isolation of members of this species and their ability to degrade chlorinated compounds (Tondo *et al*., 1998; Andretta *et al*., 2004; Matafonova *et al*., 2006; Nair *et al*., 2007). Various bacteria have been implicated in the biodegradation of many chemicals, and *Bacillus sp*. are regularly recognized as being competent degraders of many chemicals ranging from chlorophenols to oil. *Bacillus sp*. are gram positive, spore forming bacteria that are also frequently found in different soil types, at various depths. They often occur as spores, but will germinate upon the availability of readily decomposable organic matter (Vilain *et al*., 2006). These bacteria facilitate many biodegradation tasks, mainly because they have the natural ability to effectively adapt to diverse environments. This is due in part to their

extensive enzymatic capabilities. These bacteria are notorious for their ability to survive even in the presence of contaminants that are not commonly encountered (Tondo *et al*., 1998). *Bacillus sp*. are further characterized by superb metabolic versatility that enables them to break down complex structures of xenobiotics and thus are central to many bioremediation processes.

The isolate New 6 was identified as an affiliate of the *Alcaligenes sp*. They are gram-negative, non-motile bacteria, are usually non-spore-forming and have peritrichous flagella. These bacteria have been implicated in the degradation of a variety of compounds including 2, 4 dichloro-phenoxyacetate (2, 4-D); 3 dichlorobenzoate; monochlorophenols; 4- fluorobenzoate; dichlorobenzoates; chlorobiphenyls and DDT (Valuenzuela *et al*., 1997; Krooneman *et al*., 1999; Gallega *et al*., 2001; Rehfuss and Urban, 2005; Chirnside *et al*., 2007). Some *Alcaligenes sp*. have also been shown to degrade halogenated aliphatic compounds under aerobic conditions. The range of compounds that are subject to degradation by many *Alcaligenes sp.* is likely due to the organism's ability to generate oxygenase enzymes with broad substrate specificity (Chirnside *et al*., 2007). In addition, many *Alcaligenes sp*. harbour plasmids, which often contain the genes responsible for many catabolic tasks. Plasmids are highly transmissible, and it is quite likely that such plasmids may be spread to other members of a microbial consortium. The fact that many degradative plasmids show homology is evidence to support this fact (Rothmel and Chakrabarty, 1990).

The importance of concise strain selection was aptly demonstrated by Duhamel *et al*.

(2002), who suggested that the main reasons for stalled or incomplete dechlorination in the field may not necessarily be the lack of appropriate conditions, but rather the lack of appropriate organisms to carry the dechlorination reaction to completion. Such aspects must be thoroughly investigated before application of degrader organisms. Considering that inoculum survival in most bioaugmentation regimens is currently regarded as the proverbial 'Achilles heel' (Singer *et al*., 2005), it is necessary to also ensure that the selected organisms also display the appropriate ability to survive in harsh environments such as soil and groundwater.

CHAPTER THREE

THE EFFECTS OF BIOSTIMULATION ON THE DEGRADATION OF A MIXTURE OF CAHs IN CONTAMINATED SOIL

3.1 INTRODUCTION

Biostimulation can be defined as a method to improve the activity of indigenous populations that may be involved in the bioremediation process. This is accomplished by the addition of nutrients and/or a terminal electron acceptor/ donor and relies on the degradation capacity of indigenous microbes (Vezzulli *et al*., 2004).

According to Margesin and Schinner (2001), contaminated sites frequently have a population of active degrading organisms. Often, the microbes that are inhabitants of such sites may have developed the ability to degrade toxic chemicals and other contaminants. However, large inputs of carbon sources in the form of contamination, results in a rapid loss of nutrients, decreasing the microbes' inherent catabolic ability, thus rendering biodegradation ineffective. Another premise for biostimulation, may be rate of biodegradation. If a contaminated site is left undisturbed, it will definitely undergo biodegradation. This bioremediation treatment is termed natural attenuation. However, it is often the case that this natural rate of *in situ* biodegradation is too slow. In addition, natural attenuation does not always ensure the complete destruction of all pollutants. It may not always be feasible to implement a natural attenuation approach, because, with increased time, contaminants can sorb with soil organics, and this can lead to further pollution of groundwater. In this case, the addition of amendments to contaminated areas

can improve the rate of natural biodegradation (Margesin and Schinner *et al*., 2001).

One of the most commonly added nutrients at most bioremediation sites is nitrogen. Primarily, nitrogen can be used as an alternate electron acceptor. It can also be utilised as a source for cellular growth in the form of nitrate (NO_3^-) and ammonium (NH_4^+). Both forms maybe incorporated as urea, ammonium chloride, ammonium nitrate or as any ammonium salt. In these forms, nitrogen is easily absorbed by bacteria and integrated into their metabolism (Liebeg and Cutright, 1999).

Phosphorus, often added as a source for cellular growth, is the second regularly added nutrient. Phosphorus is supplied in various forms including potassium phosphate, sodium phosphate or orthophosphoric and polyphosphate salts. If phosphorus salts or orthophosphate are to be employed, several points must be considered. These substances are known to form precipitants, and can therefore lead to clogging of aquifers during a bioremediation project. The bioavailability of phosphorus is also important to consider. It demonstrates a high affinity for most soils, and this decreases its ability to be transported. In this way it becomes less available for biological activity (Liebeg and Cutright, 1999).

Although bioremediation is deemed to be more financially feasible, considerable expense may be incurred if full-scale nutrient supplementation to contaminated sites is necessary. Investigations to establish which nutrients promote the greatest biological activity are required (Liebeg and Cutright, 1999). Therefore, the main objective of this chapter was to establish if degradation of a mixture of chlorinated aliphatic hydrocarbons (CAHs) by

indigenous soil populations was improved by the addition of nutrients to contaminated soil. This was accomplished by comparing biostimulation of artificially contaminated soil using two different soil microcosm types. In addition, soil microbial biomass was measured by analyzing the phospholipid phosphate content and end products of biodegradation were determined using gas chromatography/mass spectrometry.

3.2 MATERIALS AND METHODS

3.2.1 Soil collection

Loam soil was collected from areas within the grounds of the University of KwaZulu-Natal (Westville campus). The soil was sieved with a metal sieve (pore size = 6mm) in order to remove particulate matter and debris. The soil was then stored at 4ºC until further use.

3.2.2 Stationary/ Static microcosm (Type S)

For all treatments, stationary soil microcosms were constructed using 250 ml serum bottles (Wheaton). Each bottle contained 100 g of soil and 75 ml of synthetic groundwater. Synthetic groundwater contained 1.5 mM $MgCl_2$, 0.12 mM KCl, 0.03 mM NH_4NO_3, 1.0 mM $CaCl_2$, 1.5 mM $Ca(OH)_2$, and 8.5 mM $NaHCO_3$ in deionised water at pH 7.8 (Klier *et al*., 1999). To examine the effects of biostimulation, 1.5 g of glucose, fructose or fertilizer (N: P: K ratio = 3:1:6, Kompel Chemicult) was added individually to each bottle. All reaction mixtures were then purged with oxygen gas at 400 kPA, for several min using a 0.2 micron filter to simulate an aerobic environment (Olaniran *et al*., 2006). Ten microlitres each of CCl_4, DCM and 1, 2-DCA were added to artificially

contaminate the soil. Microcosms were then immediately sealed using teflon faced butyl rubber caps, due to the volatility of the test compounds. Adequate reaction mixtures were prepared in order to permit sacrifice of microcosms during weekly soil sampling. Microcosms were then incubated at 200 rpm on a rotary shaker for 2 hrs at 28°C to allow for equilibration of gases before gas chromatographic analysis. Following the initial gas chromatographic sampling, microcosms were incubated at 28°C, without shaking, in the dark to prevent photodegradation of the chlorinated compounds. Two controls, an autoclaved and unautoclaved soil control were used for the duration of the investigation. For the autoclaved soil control, soil was divided into 2 portions, and then autoclaved thrice for 15 minutes at 121ºC. The unautoclaved soil control did not contain any nutrients and was employed to determine the effect of indigenous soil microbial populations.

3.2.3 Circulating microcosm (Type C)

Circulating microcosms were constructed by the Academic Instrumentation Unit of the University of KwaZulu-Natal (Westville Campus). These microcosms were cylindrical perspex tubes (Fig. 3.1) that were 24 cm in length with a radius of 8 cm, and had screw-on lids at either ends of the tube. Lids at both ends were equipped with a Viton® gasket. These gaskets were essential to decrease evaporation of the volatile test compounds. It was necessary to lubricate Viton® seals with silicon grease, prior to microcosm closure. This was to ensure that the seals maintained their flexibility and did not become rigid or dry, thus eliminating volatilization by improper sealing. The upper lid contained an outlet (height, 1 cm; radius, 0.02 cm) and a rubber septum. The microcosm was divided into 2

reservoirs, and separated by a circular platform (radius, 7.5 cm; thickness, 0.75 cm), also composed of perspex.

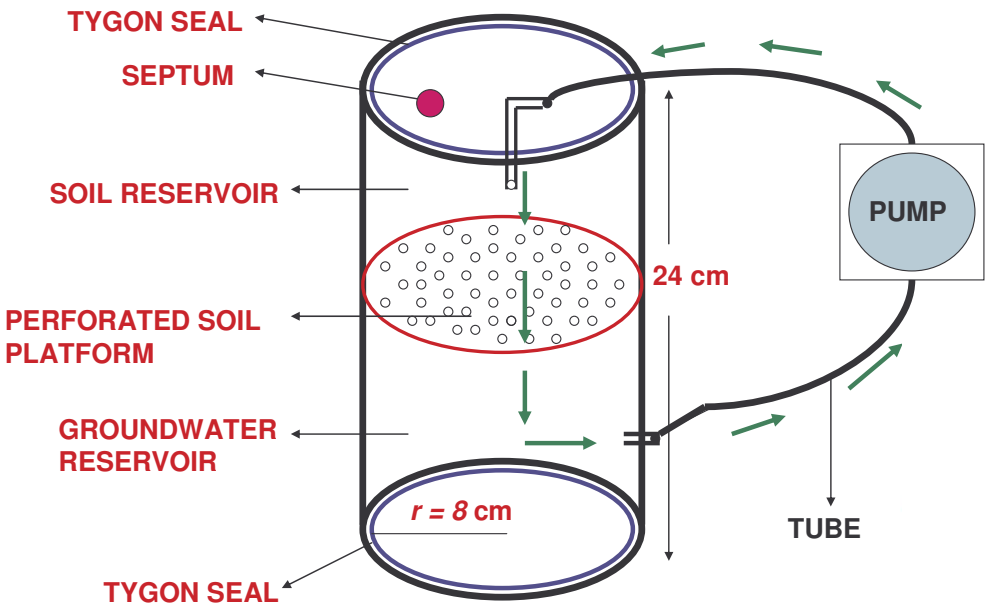

Fig. 3.1: Cross-sectional view of the circulating microcosm (green arrows indicate the flow of groundwater).

This platform contained a plastic sieve (radius, 5 cm) with numerous pores (size, 0.02 cm). Two hollow perspex rods (inner diameter, 0.02 cm; length 15 cm) were inserted into the platform. This rod served to equilibrate the gas and pressure build-up between the two compartments in the vessel. The lower reservoir of the microcosm contained 1.2 litres of synthetic groundwater. The upper reservoir housed the soil. Whatman filter paper grade 1 (radius, 9 cm) was placed on the plastic sieve, and then moistened with approximately 5 ml synthetic groundwater. This was followed by the addition of 600 g of soil. Nine grams of glucose, fructose or fertilizer, dissolved in 150 ml synthetic groundwater (Section

3.2.2) were then added to the soil, individually and mixed well. Polypropylene tubing (dimensions: inner diameter, 3 mm; outer diameter, 5 mm) was connected to an outlet (identical dimensions as outlet on upper reservoir lid) that emerged from the lower liquid reservoir, to the outlet on the lid of the upper soil reservoir. The connection between the outlets and tubing were very firm, therefore the outlets had to be lubricated with silicon grease prior to connection. Polypropylene tubing was important as it was very durable, and was important for minimizing evaporation of the volatile CAHs.

The microcosms were then purged with oxygen gas at 400 kPa, for several minutes using a sterile 0.2 micron filter. Sixty microlitres of CCl_4, DCM and 1, 2-DCA were added and the microcosms were immediately sealed, using a specially designed tool. They were then placed on a rotary shaker for a minimum of 2 hr at 28ºC, to allow for equilibration of gases. Following gas chromatography analysis, the tubing was then connected to a Gilson Minipuls 2/3 Peristaltic Pump that maintained a flow rate of 5.5 ml/ min. The peristaltic action allowed the groundwater from the liquid reservoir to enter the upper soil reservoir. This groundwater then mixed with, and spread through the contaminated soil. The addition of the perforated platform then enabled the groundwater to re-enter the liquid reservoir, thus circulating the groundwater, continuously. The cycle was maintained for the duration of the experiment. Type C microcosms were incubated at 28ºC, for a period of 15 days. As with Type S microcosms, adequate reactions were prepared to permit sacrifice, for sampling. Since perspex was unautoclavable, an alternative disinfection procedure was used. Microcosms were first washed with laboratory soap, rinsed once with tap water, twice with distilled water and air-dried. Disinfection with 70 % alcohol

was not feasible, as it was incompatible with perspex. Therefore, sterilization by exposure to UV light for a period of 16-24 hours was conducted to ensure asepsis. Polypropylene tubing was sterilized by rinsing once with sterile distilled water and twice with 70% alcohol. Tubes were rinsed twice again with sterile water to remove any residual alcohol.

3.2.4 Gas chromatography (GC)

Gas chromatography for both microcosm types was conducted on the day of microcosm set-up (Day 0). Subsequently, headspace GC analysis was conducted on Type S microcosms every 7 days for a period of 28 days. Type C microcosms were sampled every 3 days for a period of 9 days, and then on day 15. GC analysis and degradation rates for both microcosm types were calculated as described in Section 2.2.4. For Type C microcosms, 600 µl of gas was injected directly into the chromatograph.

3.2.5 Phospholipid phosphate analysis

Microbial biomass was calculated by determining the amount of lipid-bound phosphates. Phospholipids were measured according to the method of Findlay *et al* (1989). Briefly, 28.5 ml of an extraction mixture containing chloroform, methanol and phosphate buffer (50 mM, pH 7.4), in the ratio 1: 2: 0.8 was added to 2 ml of soil slurry in 50 ml plastic centrifugation tubes. The tubes were shaken and allowed to stand for 2 hr. The solvents were then partitioned by the addition of 7.5 ml of water and 7.5 ml of chloroform. The tubes were again shaken and centrifuged for 10 min at 2500 rpm. This mixture was then allowed to stand overnight. Following overnight incubation, the upper (aqueous) phase

(15 ml) was aspirated, filtered and collected in screw cap glass test tubes, in order to facilitate recovery of chloroform layer. A stream of nitrogen was then applied to the filtrate. Phosphates were liberated from samples by potassium persulfate digestion. Two millilitres of chloroform was subsequently added to the filtrate to dilute the lipid samples. Two replicate 100 µl portions were dispensed into 2.5 ml glass vials, and the solvent was removed, again, under a stream of nitrogen. Four hundred and fifty microlitres of a saturated solution of potassium persulfate (5 g added to 100 ml of 0.36 N H_2SO_4), was added to the vials, which were sealed with rubber stoppers and crimped with aluminium caps. These vials were then incubated at 95ºC, overnight. In order to measure liberated phosphates, 100 µl of ammonium molybdate (2.5% $(NH_4)_6$ Mo_7O_{24}. $4H_2O$ in 5.72 N H_2SO_4) was added to the vials. This was allowed to stand for 10 minutes. Thereafter, 450 µl of a solution of malachite green and polyvinyl alcohol in water (0.111% polyvinyl alcohol dissolved in water at 95ºC, was cooled, and 0.011% malachite green added), and allowed to stand for a further 30 minutes. The absorbance values at 610 nm were then read. Deionized water was used as the blank. Finally, concentrations of phosphate were calculated by incorporating absorbance values into an equation derived from a standard curve, prepared by digesting various concentrations of glycerol phosphate with ammonium molybdate and malachite green and polyvinyl alcohol as described above. Unautoclaved soil was used as the control for these experiments.

3.2.6 Gas chromatography/Mass spectrometry (GC/MS)

Headspace GC/MS analysis was conducted to determine the final degradation products. This procedure was only performed on Type S and Type C microcosms that had

appreciable degradation values with respect to the autoclaved controls, after treatments at days 28 and day 15, respectively.

3.2.7 Statistical analysis

Statistical analysis was performed as described in Section 2.2.6. With regards to degradation studies, all experimental samples were compared to the autoclaved controls, unless stated otherwise. Phospholipid phosphates were compared at various sampling times, except when in comparison to another additive.

3.3 RESULTS

3.3.1 Biostimulation of CAH-contaminated soil

While CCl_4, DCM and 1, 2-DCA were added together as a mixture to contaminate soil, their degradation profiles (for Type S and Type C microcosms) are represented individually. Degradation was calculated from values reflected in Appendix II.

The nutrients glucose, fructose or fertiliser were used to biostimulate contaminated soil in order to determine whether these nutrients were important at reducing the contaminant concentrations. At day 28, it was observed that all nutrients led to complete removal of CCl_4 (Fig. 3.2). Microcosms that were amended with fructose showed a drastic 81.1% decrease in CCl_4 concentration, within only 7 days. In comparison to the other nutrients and the autoclaved soil, fructose led to 100% biodegradation of CCl_4, which had occurred at day 14 (P = 0.011). Glucose was the 2nd most effective nutrient, and led to a 71.5% reduction within 14 days (P = 0.017), and 100% degradation by day 21.

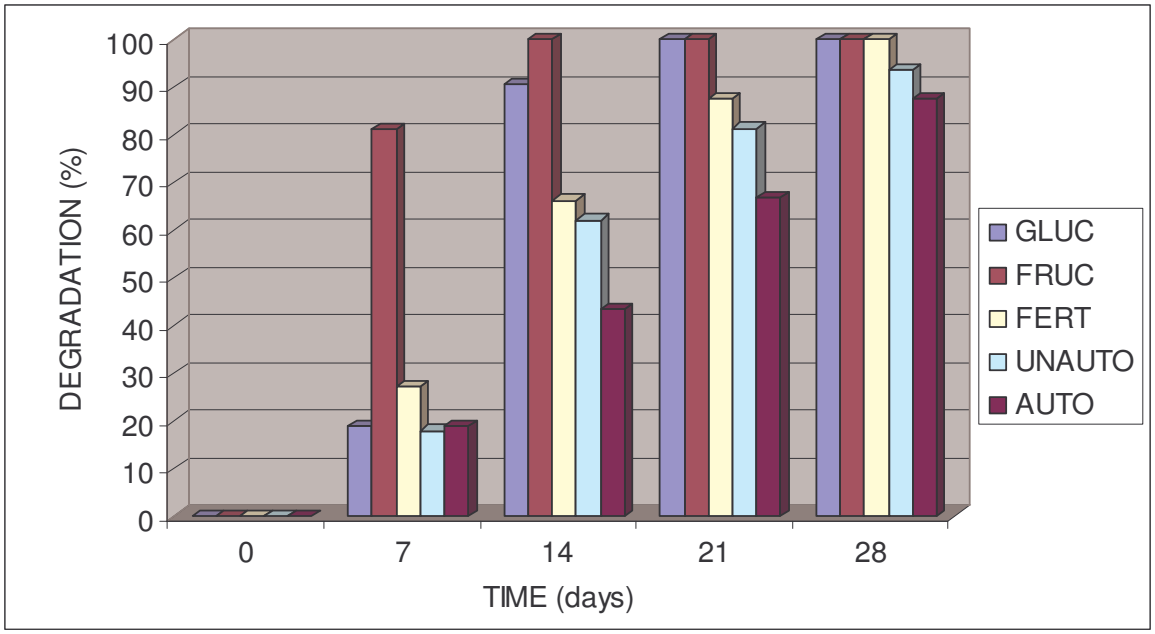

Fig. 3.2: The effect of biostimulation on the biodegradation of CCl_4 in Type S microcosms.

The fertiliser also resulted in 100% elimination of CCl_4, but was not significant in comparison to the unautoclaved soil, at the same sampling time ($P = 0.122$). With regards to microcosms that received fertilizer, an initial decrease of 27.14%, at day 7 was observed This was followed by increases in degradation that amounted to approximately half those of preceding weeks, i.e. degradation at day 14 increased by 39.12% and subsequently at day 21 degradation increased by 21.17%. The degradation potential of autochthonous microorganisms is clearly evidenced by a 93.52% degradation value in the unautoclaved control at day 28. A 87.52% reduction of CCl_4 was also noticed in the autoclaved control.

The addition of fructose to contaminated soil led to the greatest decrease of DCM in Type S microcosms (Fig. 3.3). Similar to the biodegradation of CCl_4, most microcosms

exhibited an initial considerable decrease in concentration of DCM, and this was then followed by small less substantial decreases in concentration. Microcosms that received fertiliser had a similar degradation pattern to those that were obtained for CCl_4 biodegradation. Biodegradation of DCM due to the presence of fertiliser and fructose at day 28 was 64.52% and 64.82% respectively (P = 0.985). DCM concentration was observed to decrease to 68.43%, at day 21, in microcosms that contained glucose. By day 28, however, the concentration of DCM increased to 63.37%. The autoclaved and unautoclaved controls exhibited degradation values of 29.6% and 36.14% respectively, at day 28 (P = 0.589).

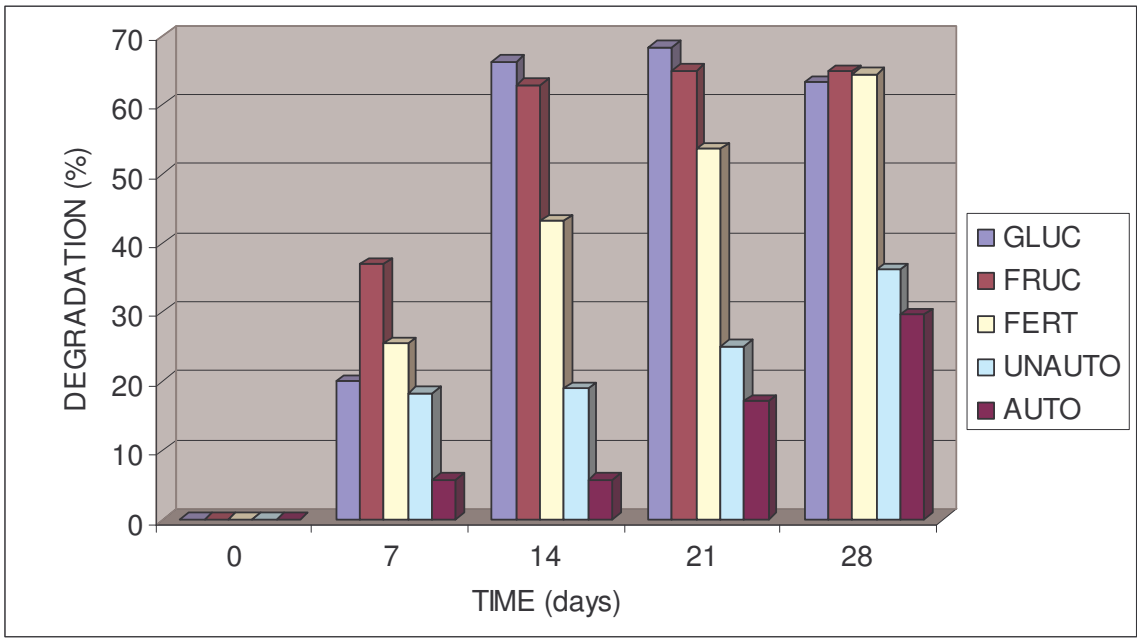

Fig. 3.3: The effect of biostimulation on the biodegradation of DCM in Type S microcosms.

In this regard, DCM proved to be less amenable to abiotic loss or volatilization than CCl_4. 1, 2-DCA was most effectively biodegraded in soil that was biostimulated by glucose (Fig. 3.4). At day 7, 34.41% of 1, 2-DCA biodegradation had occurred. By day

28 this value had increased to 76.61%. In comparison, fructose was the next appreciable agent (P = 0.028). The degradation observed at day 7 of 26.08% had, however, only just doubled by day 28. Indigenous microorganisms without nutrient supplementation led to a decrease of 33.2% at day 28, while autoclaved soil showed a 54.65% loss of 1, 2-DCA (P = 0.881).

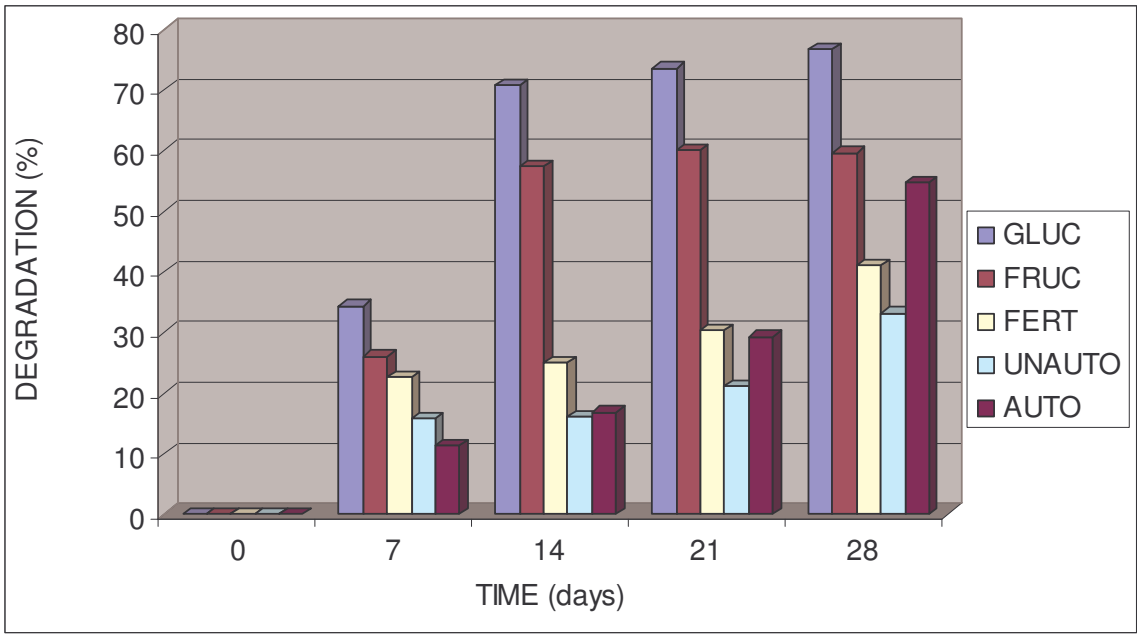

Fig. 3.4: The effect of biostimulation on the biodegradation of 1, 2-DCA in Type S microcosms.

Type C microcosms aimed to establish if biodegradation was different with a constant circulation of groundwater through artificially contaminated soil. Degradation values are depicted in Appendix II.

As with Type S microcosms, the addition of glucose and fructose led to the greatest decrease in CCl_4 concentration in Type C microcosms (Fig. 3.5). Fructose increased the degradation of CCl_4 to 100% at day 15, while the difference caused by glucose addition

was 95.38% (P = 0.110). The differences in degradation as observed for microcosms amended with fertiliser, in the first nine days were very minor, but by day 15, an improvement in degradation of up to 54.9% was noted, compared to the values obtained at day 9. CCl_4 depletion in the autoclaved control amounted to 98.49%, at day 15. At day 15, the nutrients that led to the most notable degradation of DCM were glucose and fertilizer (Fig. 3.6), resulting in a significant 90.6% (P = 0.013) and 83.64% (P = 0.028) increase, in degradation respectively. In comparison, biostimulation by fructose lead to a degradation value of 73.72% at day 15.

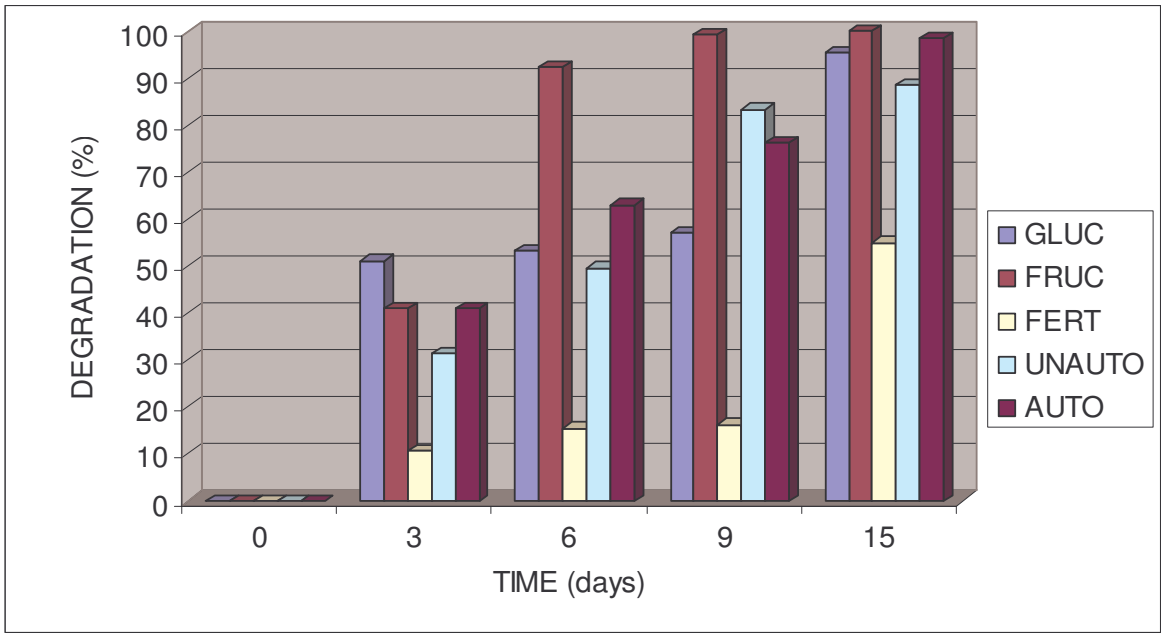

Fig. 3.5: The effect of biostimulation on the biodegradation of CCl_4 in Type C microcosms.

The removal of DCM by all nutrients tested in Type C microcosms was higher than that observed for the Type S microcosms. However, high degradation amounts of 66.5 % for unautoclaved and 62.8% for autoclaved soil was also observed.

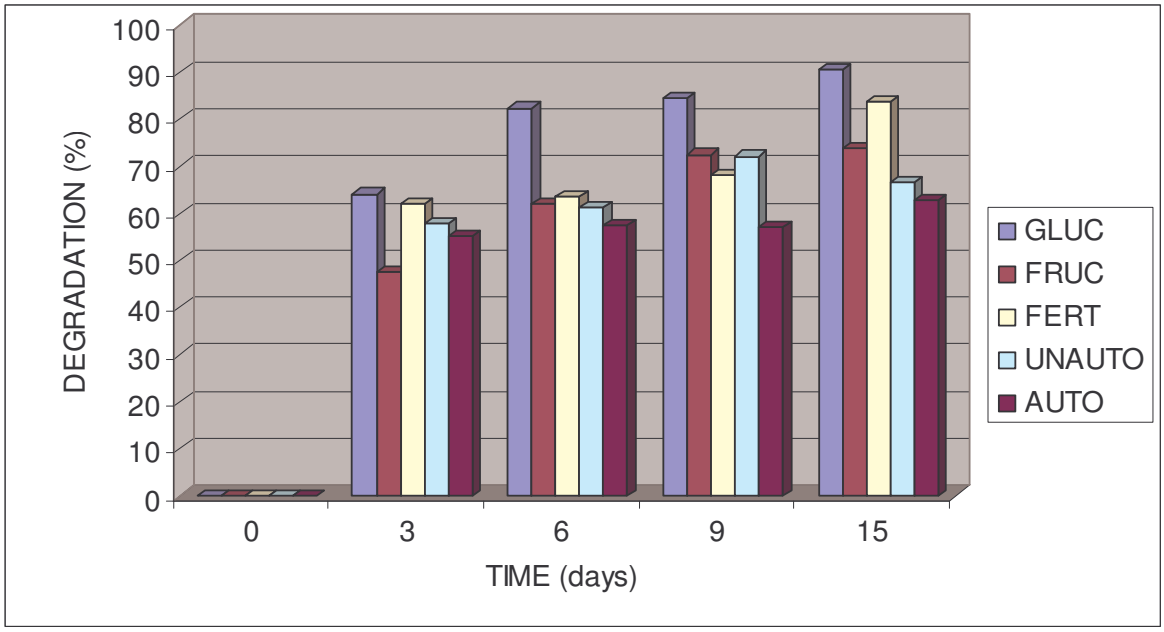

Fig. 3.6: The effect of biostimulation on the biodegradation of DCM in Type C microcosms.

In Type C microcosms, glucose also proved to be the most effective at improving 1, 2-DCA degradation (Fig. 3.7). A 77.48% change in 1, 2-DCA concentration was noted within 3 days. In contrast to Type S microcosms, fertilizer produced a larger decrease in 1, 2-DCA concentration, in Type C microcosms, that amounting to 82.21% at day 15 (P = 0.028). Unautoclaved soil displayed slightly greater degradation after 15 days than soil that was adjusted with fructose. Indigenous organisms in soil without any nutrients resulted in a 78.73% (P = 0.014) decrease in 1, 2-DCA concentration.

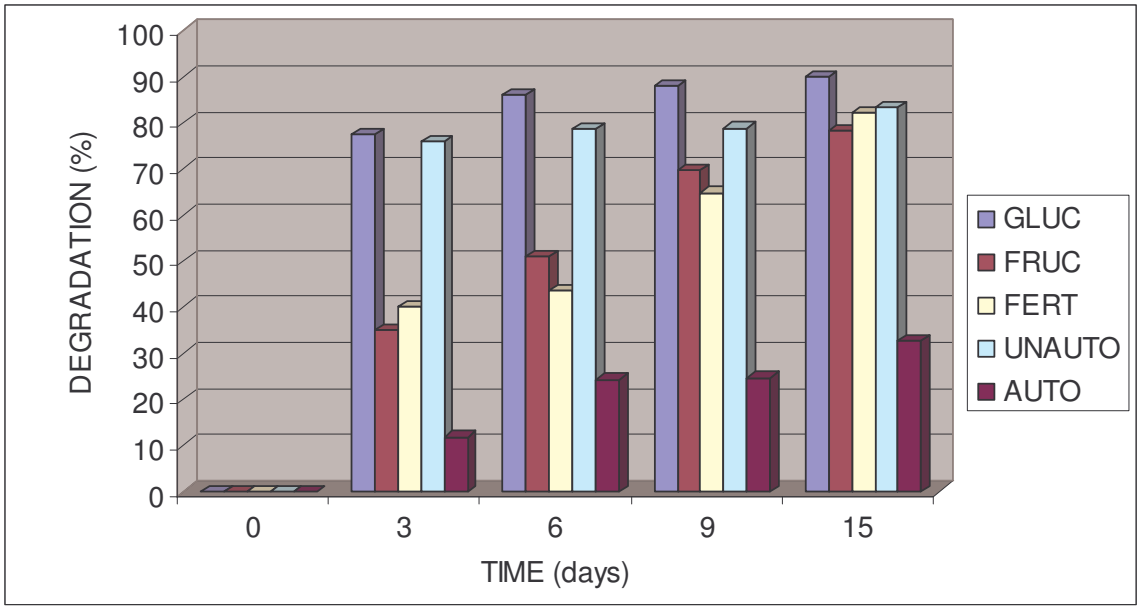

Fig. 3.7: The effect of biostimulation on the biodegradation of 1, 2 DCA in Type C microcosms.

To summarise, for both microcosm types, glucose proved to be the most efficient at remediating soil contaminated with a mixture of CCl_4, DCM and 1, 2-DCA. Comparatively, however, fructose led to larger percentage removal of all compounds in Type S, while, fertiliser demonstrated improved degradation in Type C microcosms.

3.3.2 Microbial biomass yield in the different microcosms under various biostimulation conditions

Data sets used for calculating phospholipid phosphates are reflected in Appendix II. A clear trend was evident in microbial biomass pattern for Type S biostimulated microcosms (Fig. 3.8). Regardless of which nutrient was added, a decrease of the phospholipids was observed within the first week. Microcosms amended with glucose displayed the most noticeable decrease ($P = 0.038$) of 8.8 grams per nanomole phosphate (g. nm^{-1}). At day 7, phosphate measured in microcosms with fertiliser was 12.637 g.

nm^{-1}. Fructose led to the smallest decrease of phosphate within the first week, and this was consistent with its ability to stimulate biodegradation.

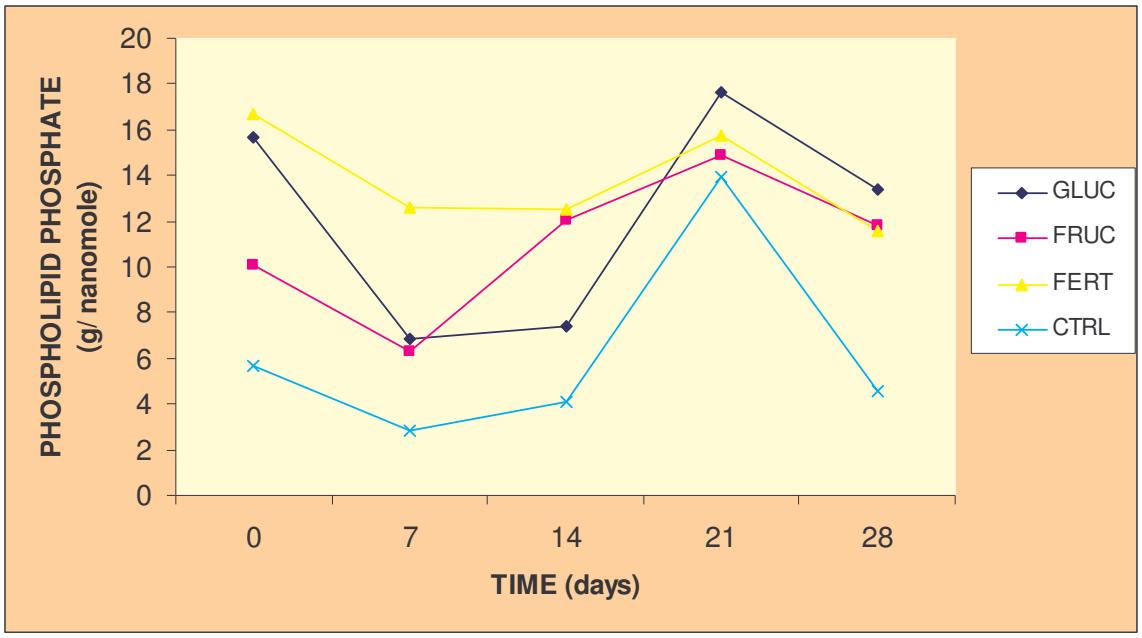

Fig. 3.8: Effect of nutrient addition on microbial biomass in Type S microcosms.

Both glucose and fertiliser containing microcosms did not lead to any noticeable changes from days 7 to 14. However, at day 21, the addition of glucose resulted in a significant (P = 0.019) 2.4-fold increase in biomass yield. ˉA similar trend was evident in fertiliser containing microcosms, but a smaller insignificant increase of 3.2 g. nm^{-1} was observed (P = 0.482). At day 28, all these values decreased again. Control soil, i. e. unautoclaved soil, followed a similar pattern to that of glucose. The phosphates measured at all sampling times are much lower than those obtained for nutrient amended bottles, indicating that indigenous soil microorganisms without any nutrients were able to thrive with the mixture of CAHs, but did not grow as abundantly in soil that has been biostimulated by glucose. Type C biostimulated microcosms were sampled on days 0, 5,

10 and 15. A pattern also emerged in Type C biostimulated microcosms (Fig. 3.9). Control and fertiliser amended soil produced similar patterns, while glucose and fructose-amended soils displayed parallel results.

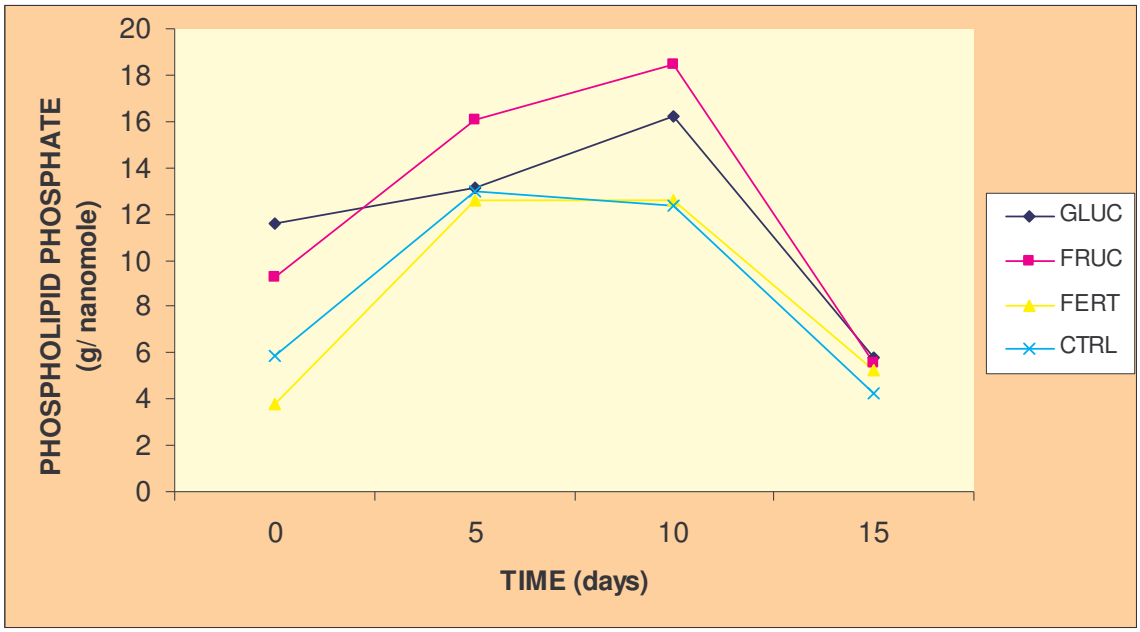

Fig. 3.9: Effect of nutrient addition on microbial biomass in Type C microcosms.

In contrast to Type S microcosms, all Type C biostimulated microcosms showed an initial increase in biomass till day 5. Phospholipids in fructose- containing microcosms appeared to have doubled from day 0 to day 10, from 9.25 to 18.45 g phospholipid phosphate per nanomole ($P = 0.02$) after which a large decline in biomass was noted ($P = 0.027$). A similar pattern arose for glucose, however, a slightly less significant increase of 4.69 g. nm^{-1} phospholipid was observed ($P = 0.764$). Fertiliser and control Type C microcosms showed the similar period of little or no change in biomass concentrations, from day 5 to day 10 ($p = 0.977$). The addition of fertiliser did not lead to the highest biomass value; however this soil had the greatest increase in terms of g. nm^{-1}

phospholipid. A significant (P = 0.009) 3.3-fold increase in phospholipid content was observed in Type C microcosms that received fertiliser, at day 5 compared to the initial value.

3.3.3 Identification of end-products of degradation

DCM (methylene chloride) and 1, 2-DCA were detected in both microcosm types at the end of the respective time periods for determining biodegradation (Table 3.1). Carbon dioxide (CO_2) formation was observed in Type S microcosms amended with fructose and Type C microcosms amended with fertiliser. Other common intermediates of chlorinated hydrocarbon degradation that included trichloroacetyl chloride (TCA) and chloroform ($CHCl_3$) were also identified. Nitrous oxide (N_2O) was only present in Type C microcosms that contained glucose.

Table 3.1: Products of CAH degradation detected in biostimulated microcosms.

	CO_2	DCM	1, 2-DCA	$CHCl_3$[a]	TCA[b]	N_2O[c]
[1]GLUC		√	√	√		
[1]FRUCT	√	√	√			
[2]GLUC		√	√			√
[2]FERT	√	√	√		√	

[a] Chloroform; [b] Trichloroaetyl chloride; [c] Nitrous oxide; [1] Type S microcosms; [2] Type C microcosms.

3.4 DISCUSSION

The methodical and proper execution of the necessary and relevant treatability studies will determine if an *in situ* approach will be successful. The degradation patterns for both microcosm types and for the 3 contaminants proved very similar. This pattern is analogous to other investigations pertaining to biodegradation, especially regarding the behaviour of organic chemicals in soil. All treatments resulted in an initial large decrease

in the concentration of the test compound followed by small or transitory changes. These initial large decreases were noted by day 14, for Type S, and day 9 for Type C microcosms. Such degradation profiles are described as the 'hockey- stick phenomenon'. Alexander (1994) explained that this probably resulted due to depletion of nutrients and the descent of microbial populations. Microorganisms would have also exploited the test compounds as a source of carbon for growth, thus rendering them less bioavailable. In addition, the higher recalcitrance of the residual contaminants could also provide a rationale for this phenomenon.

A significant loss of the test compounds was observed in autoclaved soil controls. In some cases, especially with CCl_4, it became difficult to speculate on the effect of the nutrient amendment without ambiguity, based on this fact. Decrease of the test compounds in these autoclaved controls could have been as a result of abiotic loss or evaporation. All the test compounds are highly volatile, so this is not unusual. However, Carter *et al*. (2007) reported that while autoclaved soil produced a significant loss of biomass and microbially mediated metabolism, some soil enzymes may still retain their activity. It is not yet known if these soil enzymes were instrumental in the decrease of CAH concentrations. Decreases in CCl_4 in autoclaved controls are not uncommon and have been reported by several authors (Scherer and Sahm, 1981; Egli *et al*., 1988; Egli *et al*., 1990; Holliger *et al*., 1992; van Eekhart *et al*.,1998). van Eekhart *et al*. (1998) observed degradation of CCl_4 by autoclaved sludge. While unadapted living sludge led to degradation rates that were 2 to 3 fold lower than those for autoclaved sludges, it was suggested that abiotic processes could have been instrumental in the degradation

observed for autoclaved sludge. It is hypothesized that cofactors like vitamin B_{12} and other corrinoids and factor F_{430} could have mediated these abiotic processes. The presence of live cells is not necessary, because dead cells are also able to release these stable transition metal complexes (Ferguson and Pietari, 2000). These biochemicals are then available to participate in the transformation process (Sims *et al*., 1991). Glucose was ideal as a biostimulation agent, mainly due to the fact that it represents the dominant carbon compound that enters soil. Therefore it would be fundamental to soil carbon cycling (Paul and Clark, 1996). Glucose and fructose are simple carbohydrates that are easily assimilable and commonly used as energy sources. These characteristics enabled them to be effective at biostimulation. Glucose, in particular, has displayed much success at increasing the biodegradation of various chlorinated compounds (Luo *et al*., 2008; Hirschorn *et al*., 2007; Gao and Skeen, 1999; Kao and Prosser, 1999).

Under anaerobic conditions, CCl_4 can be metabolized within 14 to 16 days (Fellenberg, 2000). Theoretically, a progression from aerobic to anaerobic (methanogenic) conditions occurs, in any environment that sustains microbial activity. A definite sequence of electron acceptors are utilized in this progression through distinctly different redox states (Azadpour-Keeley *et al*., 1999). Even though microcosms were aerated by purging their headspace with oxygen during set-up, it can be easily assumed that conditions became anaerobic. It is also widely accepted that increases in soil moisture can favour the development of anaerobic conditions, supporting the CAH's rapid degradation values in Type C microcosms. Rapid biodegradation of CCl_4 under anaerobic conditions is not uncommon and concurs with Ferguson and Pietari (2000), who used serum bottles to

investigate the effects of a mixture of organic substrates on the degradation of CCl_4. The disappearance of this compound was observed after only 1 day, although under strict anaerobic conditions.

DCM concentration when present in a mixture can be considered a function of CCl_4 degradation. This is due to the fact that CCl_4 transformation is characterized by the formation of DCM (Ferguson and Pietari, 2000). Type S microcosms that were amended with glucose and fructose led to a greater decrease in CCl_4 concentration, by day 21, resulting in slightly elevated concentrations of DCM at the same sampling time. Increases in concentration of 5.06% and 0.22% were observed for Type S glucose and fructose containing microcosms. This discrepancy can be substantiated by CCl_4 depletion. However, glucose was able to initiate DCM degradation again, if only slightly, by day 28. Glucose amended soils would have resulted in the development of methanogenic conditions faster. It is possible that the initiation of DCM degradation was due to the activity of methanogens in the soil community. Unautoclaved soil and soil that was biostimulated by fertiliser did not produce any drastic change in CCl_4 concentrations and therefore the increases in DCM concentration were not observed. The concomitant increase in DCM concentration as a result of CCl_4 degradation was not evident in Type C microcosms, compared to Type S micrcosms. The reason for this is still unclear. If anaerobic conditions were favoured in this type of microcosm, the degradation by the methanogenic population can also be responsible for this result.

Carbon sources (in the form of contamination) when added in large amounts to various

environments, have the ability to cause an exhaustion of nutrients. Aulenta *et al*. (2005) used a microcosm study to evaluate the potential for *in situ* natural or enhanced bioremediation in chloroethane- and chloroethene- contaminated groundwater and found that the addition of growth factors had beneficial effects on the degradation process in soil. This was likely due to the fact that activity of soil microorganisms was limited by the lack of micronutrients. The nutrients, nitrogen and phosphorus are commonly reduced or sometimes absent within contaminated sites. Therefore almost any fertiliser, even if applied in small quantities, has the ability to greatly affect soil biota (Paul and Clark, 1996), and positive effects following treatment with N-P-K or oleophilic fertilizers have been confirmed (Margesin and Schinner, 2001). The introduction of fertiliser as a method to biostimulate microbial populations in contaminated soil proved most effective in microcosm Type C, with highest degradation values observed for DCM and 1, 2-DCA. Fertiliser contains the macronutrients, nitrogen and phosphorus that are essential to the growth of microorganisms. These nutrients are important for the synthesis of DNA, RNA and other necessary cellular constituents (Azadpour-Keeley *et al*., 1999). The continuous circulation of groundwater probably rendered it more accessible to soil microorganisms, thereby allowing easier utilization by soil microbial communities. Thus, the rate of removal of the contaminants was increased, explaining its difference in behaviour in microcosm Type C. During preparation, the fertiliser demonstrated greater water solubility than glucose and fructose. This difference can therefore also account for enhanced degradation rates in microcosms that had a continuous circulation of groundwater.

1, 2-DCA is commonly linked to groundwater contamination, mainly due to its high aqueous solubility (8000 mg/L) and a low sorption coefficient (De Wildeman *et al*., 2004). Water is known to lead to a dilution of toxicants and an increased mixing of nutrients and bacteria. The aqueous groundwater fraction that probably retained 1, 2-DCA in Type S microcosms most likely settled on the soil surface. Thus it can be assumed that localized biodegradation of 1, 2-DCA perhaps occurred, with the overall consequence that greater degradation of 1, 2-DCA resulted in Type C than in Type S microcosms. Type C microcosms revealed a net difference of 41.29% and 3.36% for microcosms amended with fertiliser and glucose, respectively, when comparing 1, 2-DCA degradation between the two microcosm types. In Type C microcosms, the movement of the groundwater resulted in better distribution of 1, 2-DCA rendering it more available to soil populations for utilization. Sanin *et al*. (2000) established that moisture infiltration was important to mineralisation of 1, 2-DCA. The authors reported that 1, 2-DCA mineralisation became inhibited in the absence of additional moisture in simulated laboratory-scale landfills. This study had demonstrated the difference in biodegradation that is evident when environmental conditions, such as water availability are altered. The bioavailable fractions of contaminants that need to be degraded is a fundamental criterion when proposing a bioremediation scheme; and the increased degradation of 1, 2-DCA in Type C microcosms clearly confirms this aspect. Therefore, it is vital to ensure that the chemical characteristics (e.g., water solubility and sorption coefficients) of the contaminants that need to be remediated are the subject of intense scrutiny.

Measurements of soil microbial biomass can be relatively easy to estimate within a laboratory using single species cultures, because it corresponds to the turbidity of a culture. However, in densely populated environments such as soil, biomass measurements can prove to be quite tricky. This is largely due to the abundance and diversity of the species present and the resultant difficulties involved in separating out the microorganisms (Ritz *et al*., 1995).

Constantly changing environmental conditions affect soil communities immensely, mainly because they are so susceptible to ecosystem perturbations. This fact is clearly evidenced by the rapid decrease in the amount of phospholipids measured in Type S microcosms within the first 7 days, irrespective of the nutrient added, and in the unautoclaved control. These results are consistent with the degradation profiles, considering that small changes in degradation were noted at this time. When present in environments that are not conducive to their growth and sustainability, microbes will rapidly adapt (Hazen and Stahl, 2006). The relatively stationary biomass curves produced between days 7 to 14 corresponds to this phenomenon. Substrate concentration and availability of alternative carbon sources have also been implicated in the acclimation and adaptation of a microbial population (Klecka, 1992). CAH degradation that occurred during the first week could have resulted in concentrations that were slightly less toxic to soil microorganisms, with the consequence that no further decrease in biomass was observed. Decreased contaminant toxicity and increased biodegradation can also explain the huge biomass increase observed at day 21. The presence of the nutrients cannot be held solely responsible for this increase, based on the fact that increases in biomass also

occurred in the unautoclaved control. Elevated biomass values produced in fructose containing Type S microcosms, however, are also consistent with the fact that fructose led to the highest degradation rates at the respective sampling times. The decline in phospholipids, by day 28, could have resulted due to the depletion of nutrients or even the amount of available CAHs, which may be linked to decreased CAH biodegradation as well. In addition the formation of some toxic by- products as a result of CAH degradation could have led to this occurrence.

The increase in phospholipids in Type C microcosms can be attributed to the aqueous solubility of the CAHs. The lower solubility values for CCl_4 and DCM may well have determined their adsorption to soil colloids. Labud *et al*. (2007) explained that the toxic effect of some hydrocarbons is a function of the adsorption of the hydrocarbon onto the soil particles. The subsequent desorption of hydrocarbons and mineralization of organic matter can lead to the increase of hydrocarbon concentration in aqueous and gaseous phases. As a result, the toxicity on microbes will become more evident. This observation could provide an explanation for the increase in biomass in Type C microcosms from day 0 to day 5. In this instance, the degradation profiles do not correspond with the biomass values, suggesting that some other mechanism and not the microorganisms themselves were responsible for degradation. The theory that fertiliser probably provided a nutrient supply to existing microorganisms, while not necessarily increasing the microbial population, is supported by the data that reveal that unautoclaved controls also did not lead to significant increases in phospholipids. However, the possibility of biofilm formation at interfaces within the microcosm that was constantly in contact with the

flowing liquid, becomes imminent when considering the fact that fertiliser led to the greatest decrease of CAH concentrations in Type C microcosms. Bacteria have a greater chance of survival in environments that may be detrimental to their growth by the formation of biofilms. It has also been observed that these microorganisms are better suited to contaminant degradation, due to their close proximity and mutually beneficial interactions (Singh *et al*., 2006).

Gas chromatography coupled to mass spectrometry (GC/MS) allows researchers to gain analytical information that is concise and invaluable for the analysis of environmental samples. GC/MS can provide particularly valuable results, especially when the organic compounds in the samples to be analysed are amenable to GC analysis (Santos and Galceran, 2002). A decrease in concentration of DCM (or methylene chloride) and 1, 2-DCA in both microcosm types occurred but these compounds were not completely biodegraded by biostimulation. Therefore, this verifies their presence in GC/MS spectra in both microcosm types. Under suitable environmental conditions, oxygen is the preferred electron acceptor, for many chemical reactions. In oxygen limiting situations, many organic chemicals, including the halogenated compounds will serve as electron acceptors. The halogenated compound itself; becomes reduced. This process is known as reductive dechlorination and involves the replacement of a chloride molecule by a hydrogen atom (Sims *et al*., 1991). CCl_4 degradation has been suggested to occur as a result of biological catalysts and some chemical mechanisms (in the absence of a catalyst). Enzyme co-factors are instrumental in the abiotic degradation processes (van Eekhert *et al*., 1998). In this case, CCl_4 has been reductively dechlorinated to produce

chloroform (Fig 1.1).

The transformation of CCl_4 can occur under a variety of different environmental conditions (Semprini *et al*., 1992). If reductive dechlorination is the central degradation mechanism, then nitrogen either from the soil or groundwater, could have acted as the electron donor, if the chlorinated compound was the electron acceptor. $CHCl_3$ can be further reductively dehalogenated to form DCM (Fig 1.1). While complete DCM degradation did not occur, it can be assumed that a fraction of the DCM that remained could be due to reductive dechlorination of CCl_4. Trichloromethyl chloride is believed to initiate the biodegradation of CCl_4. This intermediate binds temporarily to a metal cofactor. A single electron is transferred from a transition metal to CCl_4 which is accompanied by the formation of a chloride anion (http://biocyc.org/META/new-image?type=PATHWAY&object=PWY-5372). Fe (II) porphyrins (e.g. cytochromes), Co complexes (e.g. vitamin B_{12}) and Ni complexes (e. g. F_{430}) are probably responsible for reductive dehalogenation in natural environments and have key roles as metal co- factors (Ferguson and Pietari, 2000; Hashsham and Freedman, 1999; Sims *et al*., 1991). These corrinoids and F_{430} have also been implicated in the reductive dechlorination of 1, 2 DCA (Holliger *et al*., 1992).

The formation of N_2O was also observed. Ninety percent of global N_2O can be ascribed to the presence of soil (Mosier *et al*., 1993). Microbial nitrification and denitrification have been recognized as processes that can affect the production and utilization of N_2O (Russow *et al*., 2008). Oxygen availability, which is a function of soil water content and

soil respiration, determines the nitrification and denitrification processes. Denitrification of nitrate (NO_3^-) becomes the major source of N_2O, when anaerobic conditions (> 60 % waterfilled pores) develop (Russow *et al*., 2008). Type C microcosms contained approximately 185% more groundwater than Type S. Denitrification of ammonium nitrate (component of synthetic groundwater, Section 3.2.4) can explain the formation of N_2O. In addition, Renault and Stengel (1994) suggested that formation of N_2O can also occur within anaerobic microsites that may occur in aerobic soils.

The formation of CO_2 could be the result of various reactions. Firstly, the correlation of the depletion of the contaminant with the production of CO_2 is as a consequence of pollutant mineralization. A fraction of the CO_2 formed could be attributed to CAH mineralisation. Secondly, microbial respiration can account for a substantial amount of CO_2 that occurs in soils. This is generally as a result of microbial decomposition of soil organic matter (Rodeghiero and Cescatti, 2008). With regards to CCl_4, van Eekhert *et al*. (1998) proposed that CCl_4 degradation can be accomplished by bacteria via 2 major pathways, a reductive or oxidative pathway. Corrinoids and cofactor F_{430} initiate the reductive route by the formation of lower chlorinated methanes. In the oxidative or substitutive pathway, CCl_4 is transformed to CO_2 by reductive dechlorination of formate or CO, formed by a 2 electron transfer. Brunner and Leisinger (1980) stated that a halidohydrolase enzyme is responsible for the oxidation of DCM by a *Pseudomonas sp*. In this reaction mechanism, a hydroxyl group is substituted for a chlorine substituent of DCM, yielding monochloromethanol. Formaldehyde is then formed after the spontaneous decomposition of the monochloromethanol intermediate. This decomposition results in

the release of the second chlorine substituent. Formaldehyde is then consumed by microbial growth or further oxidized to CO_2. Formation of CO_2 can also occur due to transformation of 1, 2-DCA. During carbon 14 measurements, Bouwer and McCarty (1983) detected 38% of 1, 2-DCA as CO_2. It was suggested that the reduction of another organic compound, such as acetate was coupled to the electrons generated in the oxidation of CO_2, under methanogenic conditions.

Ultimately, any bioremediation strategy should demonstrate that products formed during biodegradation are less toxic, or more environmentally acceptable than the parent compound. In addition, such studies should also demonstrate that biodegradation is occurring at a desirable rate. This study illustrated that the addition of glucose stimulated the indigenous microbial population immensely, with the net result biodegradation rates were vastly improved in both microcosm types. However, the formation of undesirable toxic by-products of CAH degradation in both microcosm types was also noted. Indeed, the toxic products are intermediates in degradative pathways, and their formation can be possibly prevented or decreased by longer incubation times, probably meriting subsequent use of glucose. It must be noted, however, that contaminants in a mixture pose different problems compared to their individual counterparts. This can easily be justified by the increases in DCM concentration, following transformation of CCl_4. Such occurrences are obviously undesirable at field scale, as it would serve to possibly increase the concentrations of some contaminants. 1, 2-DCA proved to be more efficiently degraded while groundwater was being re-circulated. It can be assumed that because DCM has a greater aqueous solubility than 1, 2-DCA, degradation of DCM in Type C

microcosms, if added singly, would probably have proven more effective. However, the degradation of DCM, in both microcosm types could not be accurately predicted, mainly due to the reactions of CCl_4. This information clearly demonstrates the importance of understanding the reaction mechanisms that occur in the presence of a mixture of contaminants.

CHAPTER FOUR

THE EFFECTS OF BIOAUGMENTATION AND A COMBINATION OF BIOAUGMENTATION AND BIOSTIMULATION ON THE DEGRADATION OF A MIXTURE OF CAHs IN CONTAMINATED SOIL

4.1 INTRODUCTION

Bioaugmentation finds its roots in the ancient art of food preparation. Cheese, yoghurt and beer production processes all employ small quantities of the previous batch of fermented product to commence the process yet again. This had increased the possibility to duplicate the identical product with each consecutive batch. In spite of the fact that bioaugmentation has been used for many years without knowledge, it is only recently that many scientists have used newer technologies to improve its application (Singer *et al*., 2005).

The bioaugmentation process is based on the premise that toxic pollutants are mineralised by the addition of excess, active degrading microorganisms, and that remediation should occur at a more desirable rate than would ordinarily occur by the means of indigenous microorganisms. The efficiency of bioaugmentation is supported by studies showing the incompetence of indigenous microorganisms in some cases and the apparent enhanced bioremediation rates soon after the addition of competent organisms (El Fantroussi and Agathos, 2005; Singer *et al*., 2005). Natural microbial flora that are capable of degrading pollutants are already present in contaminated soils and aquifers. However, the local environmental conditions are not always conducive to optimal microbial growth.

Therefore, such indigenous microbial communities may not usually display the appropriate metabolic potential to degrade/mineralise the target pollutants into innocuous end-products, eg., methane and water. In most cases the target pollutant is a complex molecule or a mixture of compounds that can only be broken down by a specific consortium of microorganisms that follow defined pathways. The contamination of environments with recalcitrant xenobiotics can therefore be decreased by inoculating the contaminated biotope with specific populations of microorganisms (El Fantroussi and Agathos, 2005).

Microorganisms that are often implemented in soil bioaugmentation regimens include *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus*, *Flavobacterium, Nocardia* and *Pseudomonas*. Fungi that may be used include members of the species *Trichoderma, Rhodotorula, Mortierella, Aspergillus* and *Penicillium* (Paul and Clark, 1996). The perceptions about bioaugmentation are varied; it still seems that this technique is not generally accepted as an efficient method for soil bioremediation. Many researchers still continue to demonstrate possible advantages and research aimed at examining the effectiveness of bioaugmentation for soil remediation continues (Vogel, 1996; El Fantroussi and Agathos, 2005).

Bioremediation strategies that involve the implementation of bioaugmentation in conjunction with biostimulation offers the possibility of further accelerating bioremediation. It is speculated that the introduction of nutrients will be beneficial to both exogenous and indigenous microorganisms (El Fantroussi and Agathos, 2005). Silva *et*

al.(2004) reported that a *Pseudomonas* sp. when inoculated into atrazine contaminated sites, only lead to limited mineralization of the contaminant. When the organism was added in combination with citrate or succinate biostimulation, atrazine mineralization and cell survival was markedly improved. Therefore, this chapter investigated the ability of exogenous bacterial consortia to enhance bioremediation of soil contaminated with a mixture of chlorinated aliphatic hydrocarbons (CAHs) viz., CCl_4, DCM and 1, 2-DCA. Furthermore, the effects of combined biostimulation and bioaugmentation approaches on the remediation process were also evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Soil collection

Soil collection was conducted as described in Section 3.2.1

4.2.2 Stationary/ Static microcosm (Type S)

Stationary microcosms were constructed as described in Section 3.2.2. To investigate the effects of bioaugmentation, 2 ml of different consortia, prepared as described in Section 2.2.2, was added to the bottles.

4.2.3 Circulating microcosm (Type C)

Circulating liquid microcosms were constructed as described in Section 3.2.3. The exception, however, was the addition of 12 ml of different consortia prepared as described in Section 2.2.2.

4.2.4 Gas chromatography

Gas chromatography was performed as described in Sections 2.2.4 and 3.2.4.

4.2.5 Phospholipid phosphate analysis

Microbial biomass was measured according to the procedure outlined in Section 3.2.5.

4.2.6 Gas chromatography/ Mass spectrometry (GC/MS)

GC/MS was performed as stated in Section 3.2.6.

4.2.7 Biostimulation and bioaugmentation

The consortium and the nutrient that demonstrated the best degradation potential were chosen to determine the effects of a combined biostimulation and bioaugmentation approach on the remediation of soil contaminated with CAHs. Preliminary screening of 7 consortia revealed that consortia designated B, C and D displayed the greatest ability to degrade a mixture of CAHs. These consortia comprised of aerobic *Bacillus* and *Alcaligenes sp.* (Chapter 2). These microcosms were constructed identically to previously described biostimulated or bioaugmented microcosms. For stationary microcosms, 1.5 g of nutrient and 2 ml of consortium (prepared as described in Section 2.2.2) were added together to the bottles. For the circulating microcosms, 9 g of the nutrient plus 12 ml of consortium were added. Controls were also prepared as described in Sections 3.2.2 and 3.2.3. Gas chromatography, phospholipid phosphate analysis and GC/MS were conducted as described in Sections 3.2 4; 3.2.5 and 3.2.6, respectively.

4.2.8 Statistical analysis

Statistical values were calculated as described in Section 3.2.7.

4.3 RESULTS

4.3.1Bioaugmentation of CAH-contaminated soil

A mixture of CCl_4, DCM and 1, 2-DCA was used to artificially contaminate soil. The degradation profiles for both microcosm types are represented individually. Degradation values were calculated from data as per Appendix III.

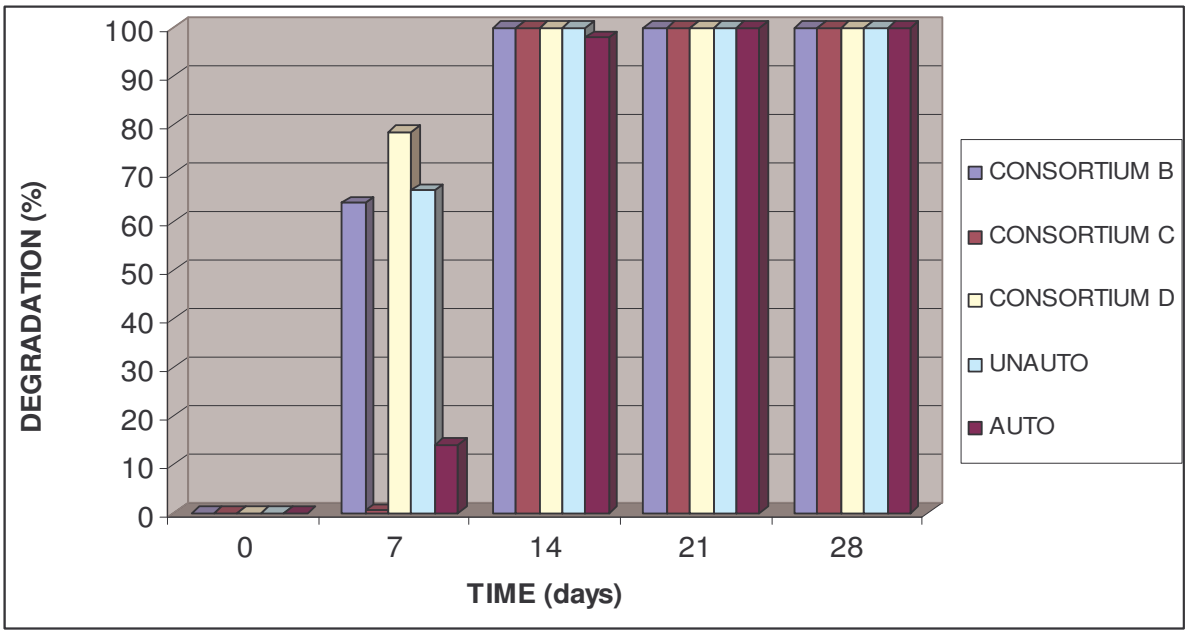

Fig. 4.1: The effect of bioaugmentation on the biodegradation of CCl_4 in Type S microcosms.

CCl_4 was rapidly degraded during bioaugmentation experiments, in Type S microcosms (Fig. 4.1). All consortia led to a total removal of CCl_4 after 14 days. At day 7, consortium D led to the highest biodegradation value of 78.51%, which was highly significant compared to the autoclaved control (P = 0.001). At this sampling time, unautoclaved soil

showed a 66.62% degradation value. By day 14, there was a total disappearance of the compound in all bioaugmented microcosms and unautoclaved soil. After 3 weeks of incubation, a total disappearance of the compound was also observed in autoclaved soil.

The decreases in DCM concentration that were observed in the autoclaved control appeared higher at day 7 than those observed for the bioaugmented soil. (Fig. 4.2). This trend continued until day 14. At day 21, however, consortium D proved to have the greatest effect on DCM concentration. By day 28, this consortium had led to a 91.56% decrease in DCM concentration, in comparison to the autoclaved control (P = 0.031). This was followed by consortium B that had a degradation value of 80.36% at the same sampling time.

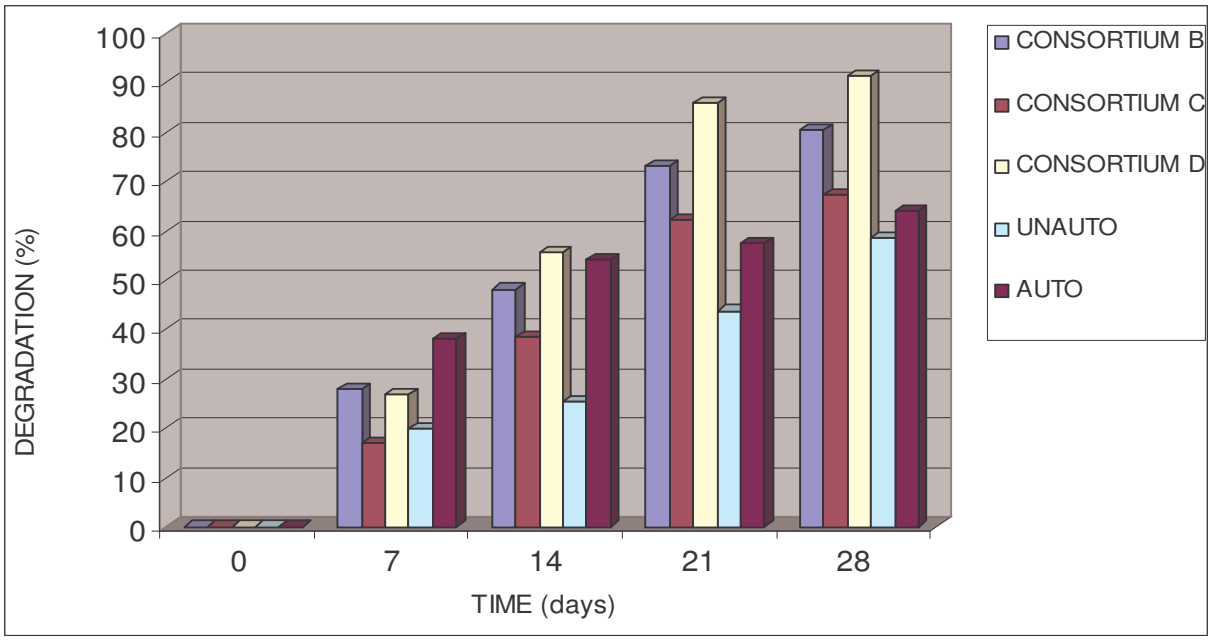

Fig. 4.2: The effect of bioaugmentation of the biodegradation of DCM in Type S microcosms.

At day 7, biodegradation of 1, 2-DCA (Fig. 4.3) proved quite rapid in Type S microcosms that contained consortium B, but at day 14, consortium D clearly proved more competitive and led to the greatest biodegradation. Consortium D was instrumental in attaining a final degradation value of 98.33% by day 28, which was significantly different compared to the autoclaved control ($P = 0.002$). Unautoclaved soil displayed a lower degradation value ($P = 0.005$), compared to the autoclaved control and other bioaugmented microcosms, in the first week, and relatively stationary degradation values were noted for preceding weeks. Degradation by consortium C was almost constant for 3 weeks. As with the other experiments, there was considerable decrease of the test compound from autoclaved controls.

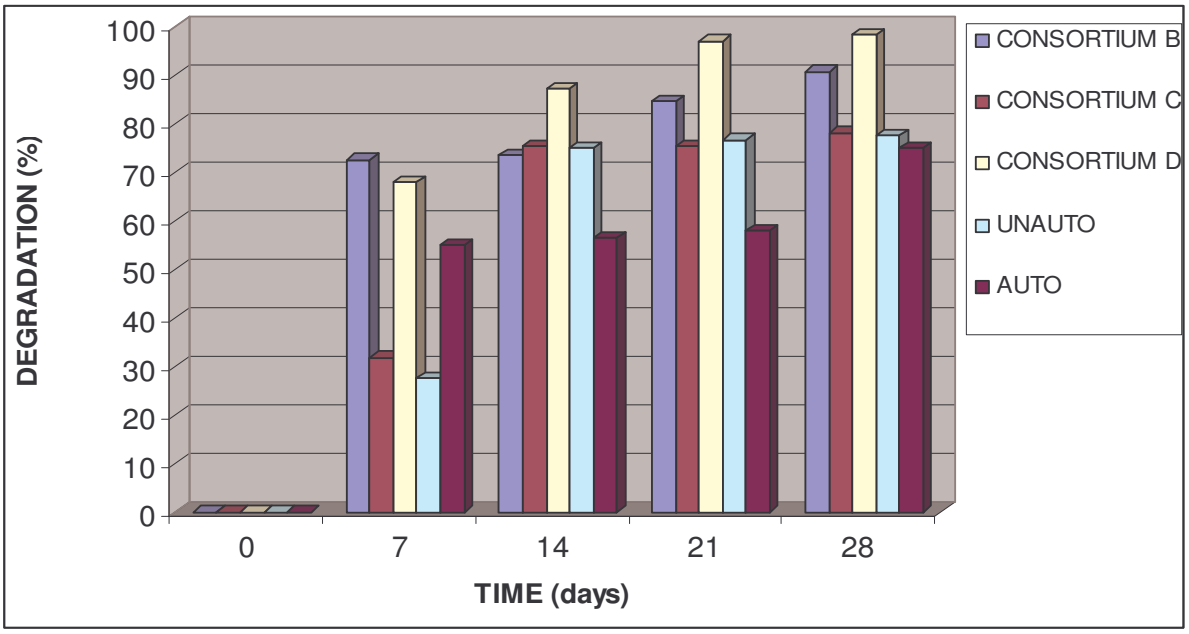

Fig. 4.3: The effect of bioaugmentation on the biodegradation of 1, 2-DCA in Type S microcosms.

Similar to Type S microcosms, CCl_4 concentration was observed to decline at a faster rate in autoclaved controls, than in the Type C microcosms amended with the various consortia, up

until day 15 (Fig. 4.4). At this sampling time, a total (100%) reduction was observed in microcosms that contained consortium B. Unautoclaved soil had the least degradative capacity in this case.

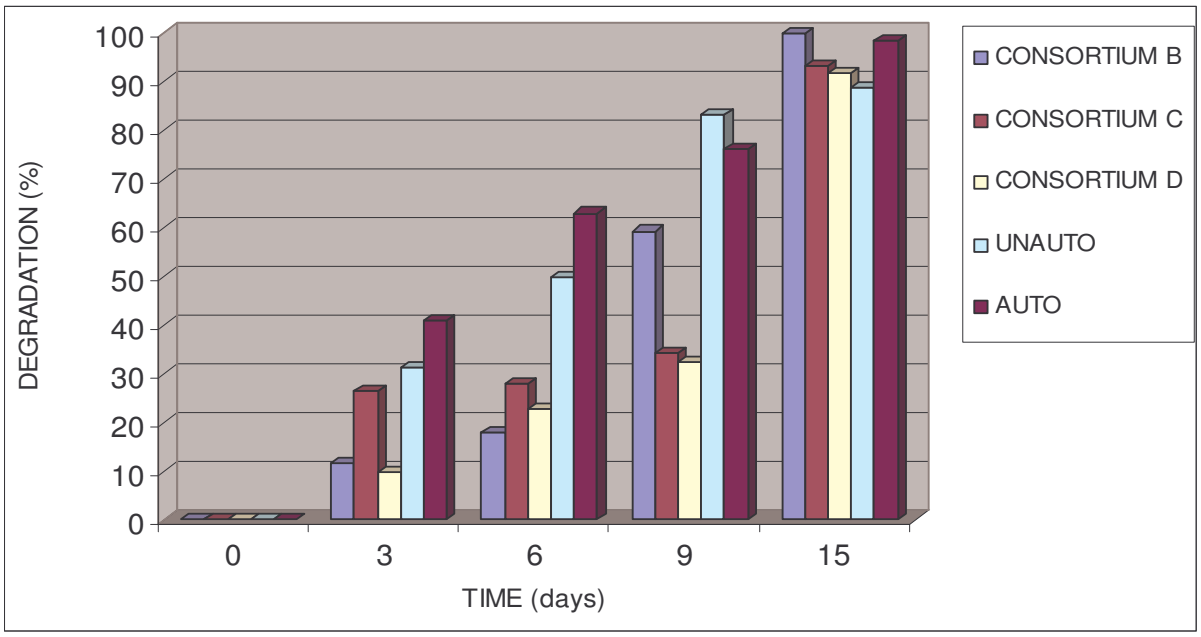

Fig. 4.4: The effect of bioaugmentation on the biodegradation of CCl_4 in Type C microcosms.

Addition of consortium B to the Type C microcosms resulted in the highest biodegradation (75.64%) of DCM (Fig. 4.5). Microcosms that contained consortium C did not display any considerable degradation capabilities, and maintained degradation rates below 35%, for the duration of the experiment. Similar to other microcosms, the decrease in concentration of DCM in autoclaved controls were higher than microcosms that contained consortia C and D. Unautoclaved soil appeared to have reached the highest value of 72.15% by day 9. This was characteristic of DCM in Type S as well.

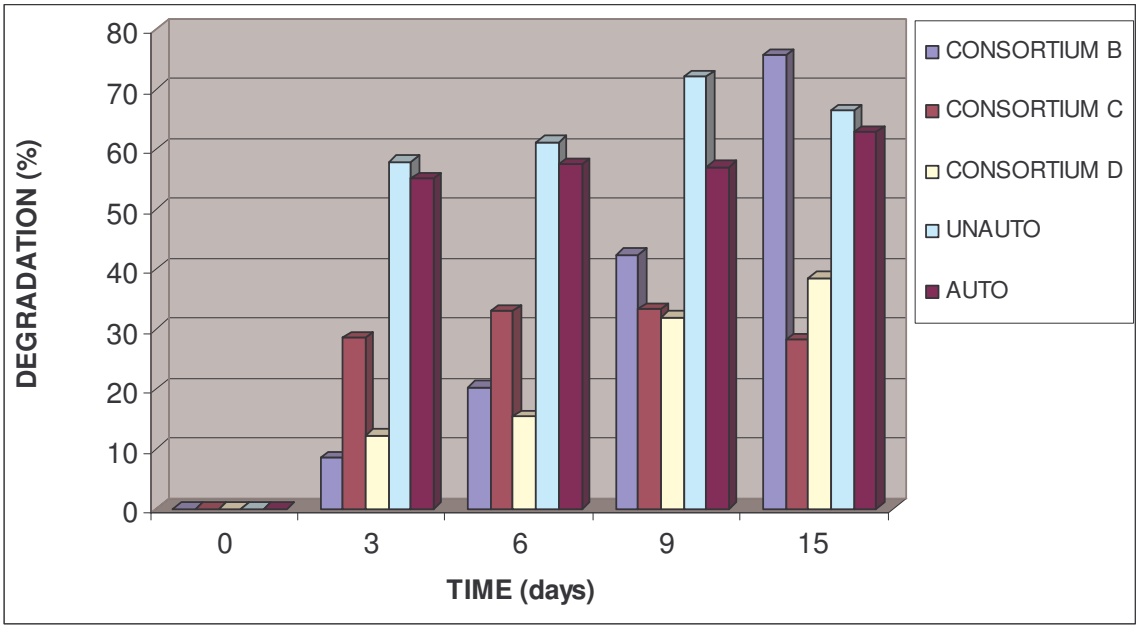

Fig. 4.5: The effects of bioaugmentation on the biodegradation of DCM in Type C microcosms.

Unautoclaved soil clearly proved to the best at maintaining high degradation values of 1, 2-DCA in Type C microcosms that were bioaugmented (Fig. 4.6). By day 15, 83.42% of 1, 2-DCA was degraded, which was significant (P = 0.04) compared to the autoclaved control. Addition of consortium B resulted in 52.77% biodegradation of 1, 2-DCA within 3 days, and attained a final degradation of 70.7% by day 15 (P = 0.016), which was highly significant compared to the autoclaved control.

Consortium D proved most ineffective at degrading 1, 2-DCA with values below 15% until day 15, when a degradation value of 40.93% was observed. Consortium C also did not produce any significant results, compared to the other consortia, and consistently maintained rates below 50% after 15 days.

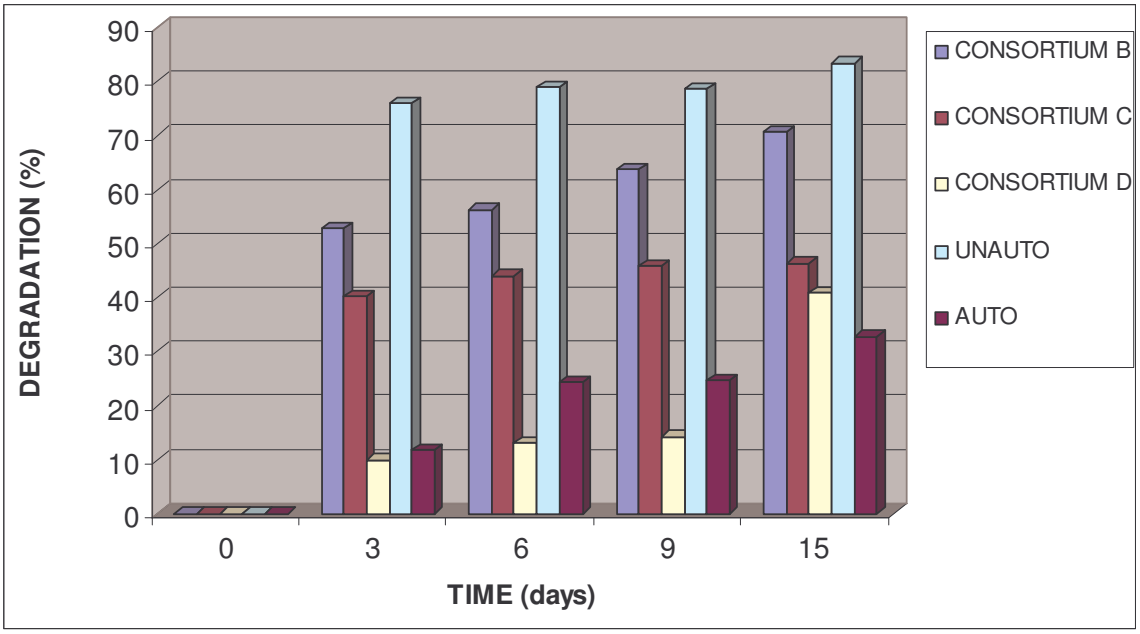

Fig. 4.6: The effect of bioaugmentation on the biodegradation of 1, 2-DCA in Type C microcosms.

4.3.2 The combination of biostimulation and bioaugmentation on CAH-contaminated soil

Figure 4.7 shows the effect of a combined biostimulation and bioaugmentation approach on the biodegradation of CCl_4 in Type S microcosms. Previous experiments had revealed that glucose and fructose had shown to be the most effective biostimulation agents, while consortium D had produced the greatest decrease in CAH concentrations during bioaugmentation experiments. Therefore, these 2 nutrients and consortium were chosen to determine the effects of a combination of biostimulation and bioaugmentation on the bioremediation of CAH-contaminated soil. In microcosms that were amended with glucose and consortium D, CCl_4 was totally removed after 7 days.

Within 2 weeks, microcosms that contained fructose and consortium D, also led to 100% degradation of the test compound, while total disappearance was observed by day 21 in unautoclaved soil. Decreases within the autoclaved controls also occurred exponentially from days 7 to 28. At 28 days, there was no significant difference between the microcosms that contained the nutrients and consortium and the autoclaved control.

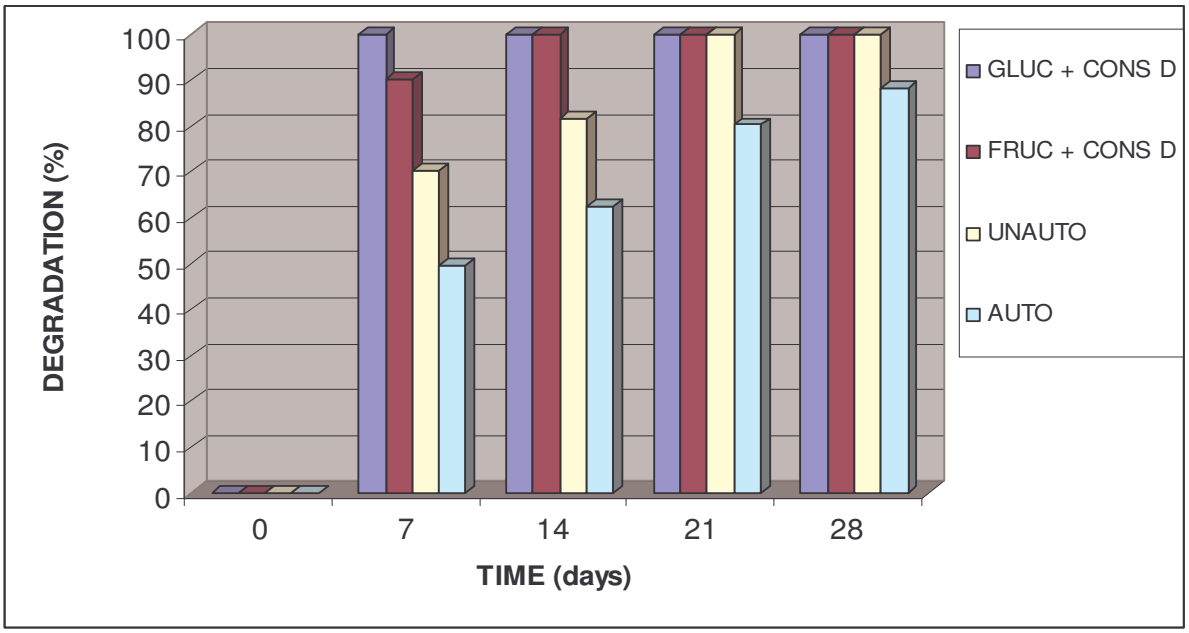

Fig. 4.7: The combined effect of bioaugmentation and biostimulation on the biodegradation of CCl_4 in Type S microcosms.

Glucose, together with consortium D showed a 62.5% reduction in DCM concentration in just 14 days (Fig. 4.8). Subsequently, microcosms that contained fructose and consortium D led to an 84.24% decrease in total DCM concentration by day 28 ($P = 0.001$), compared to the autoclaved control. During the combination of biostimulation and bioaugmentation, there was no increase in DCM concentration (Fig. 4.8).

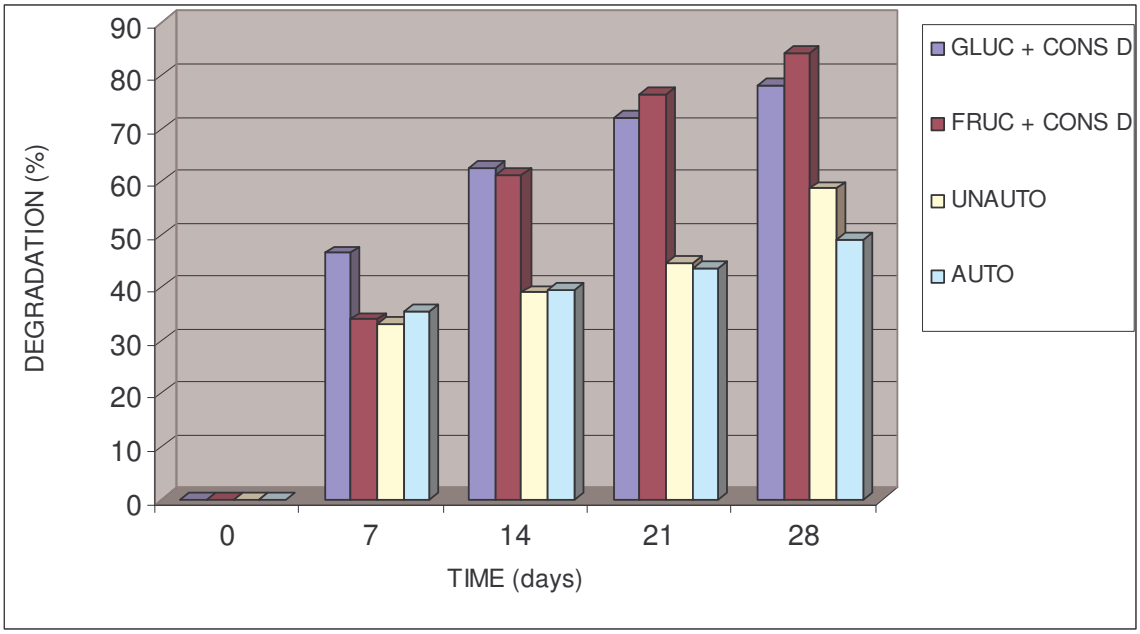

Fig. 4.8: The combined effect of biaugmentation and biostimulation on the biodegradation of DCM in Type S microcosms.

Figure 4.9 indicates the combined biostimulation and bioaugmentation effect on the biodegradation of 1, 2-DCA. The addition of consortium D with either glucose or fructose had resulted in similar biodegradation of 1, 2-DCA at day 7. At day 14, it was observed that glucose and consortium D, followed closely by fructose and the same consortium had attained the highest degradation percentages of 54.07% and 47.26%, respectively.

A similar trend of the greater decrease of 1, 2-DCA concentration was evident in autoclaved controls, from the start of the experiment, till day 21. By day 28, the potential of indigenous organisms is clearly evidenced by a 79.35% decrease in 1, 2-DCA concentration in unautoclaved soil.

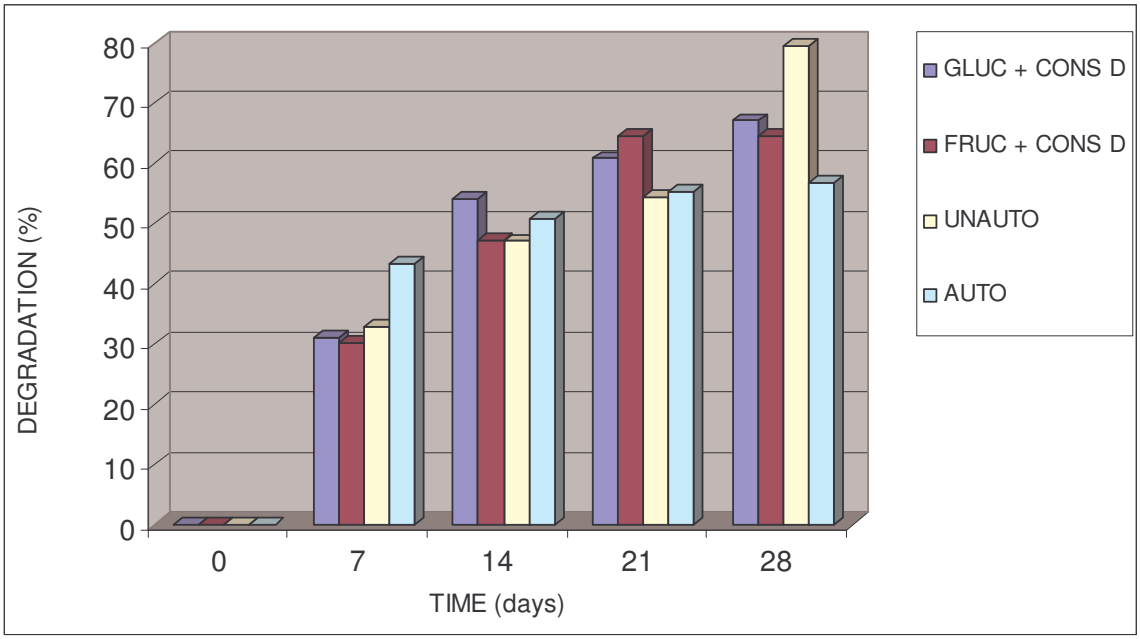

Fig. 4.9: The combined effect of bioaugmentation and biostimulation on the biodegradation of 1, 2-DCA in Type S microcosms.

The nutrients; glucose and fertiliser, and consortium B were chosen for inclusion in the combined biostimulation and bioaugmentation strategy, for Type C microcosms Type C biostimulated and bioaugmented microcosms showed that glucose and consortium B led to the highest degradation of CCl_4 (Fig. 4.10). A significant ($P = 0.009$) 90.2% decrease in CCl_4 concentration was noted in microcosms that contained glucose and consortium B at day 6, compared to the autoclaved control. Unautoclaved soil had led to a similar degradation rate as glucose and consortium B, but only by day 15.

As with other microcosms evaluating the biodegradation of CCl_4, the autoclaved controls, also showed a large percentage decrease of the test compound. The combination of fertiliser and consortium B did not produce the anticipated degradation values. As at day 15, an 87.72% reduction in CCl_4 was detected.

Glucose and consortium B proved to be an appreciable combination for increasing biodegradation of DCM in Type C microcosms (Fig. 4.11). This was followed by the unautoclaved soil, with 63.58%. The combination of fertiliser and consortium B displayed a 42.96% increase in degradation, until day 9. This was followed by a decrease of 29.35% that was characteristic in other microcosms investigating DCM degradation.

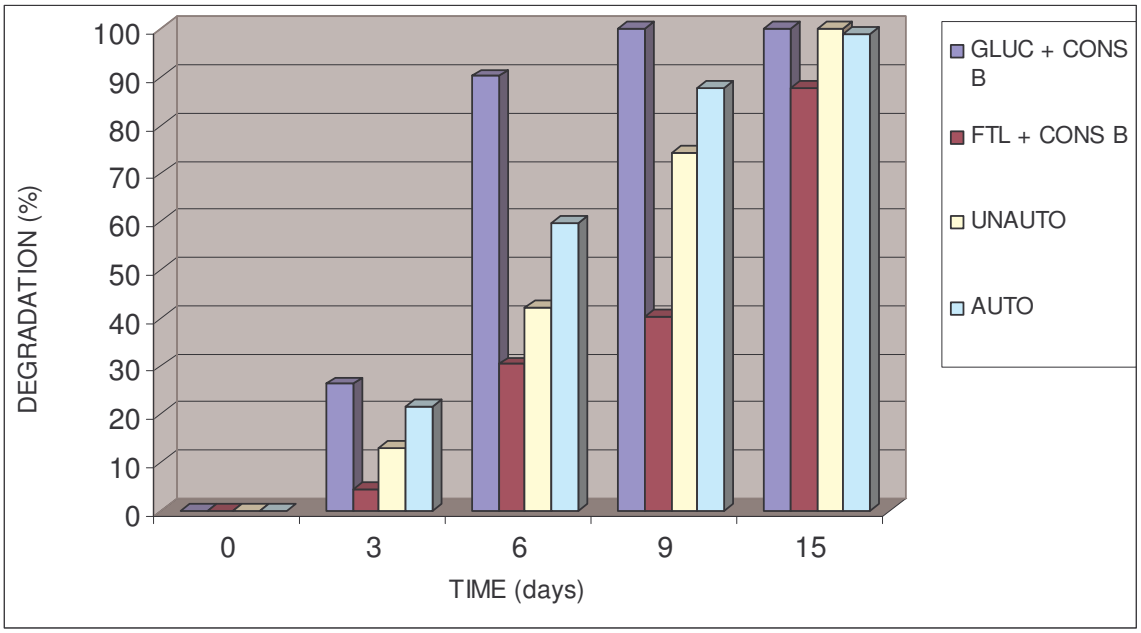

Fig. 4.10: The combined effect of bioaugmentation and biostimulation on the biodegradation of CCl_4 in Type C microcosms.

In contrast to bioaugmented microcosms, there was a large reduction of 1, 2-DCA, when a biostimulation and bioaugmentation approach was investigated in Type C microcosms (Fig. 4.12). In addition, unautoclaved soil was slightly less effective at decreasing contaminant concentrations. This microcosm type showed that fertiliser and consortium B had the highest degradation value ($P = 0.025$).

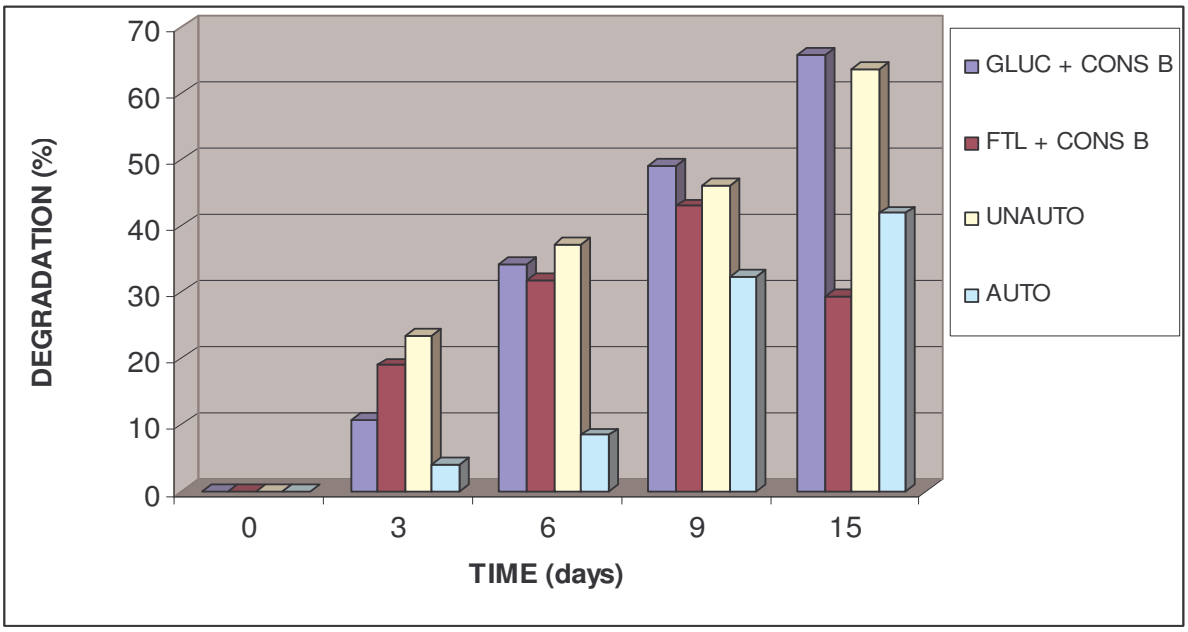

Fig. 4.11: The combined effect of bioaugmentation and biostimulation on the biodegradation of DCM in Type C microcosms.

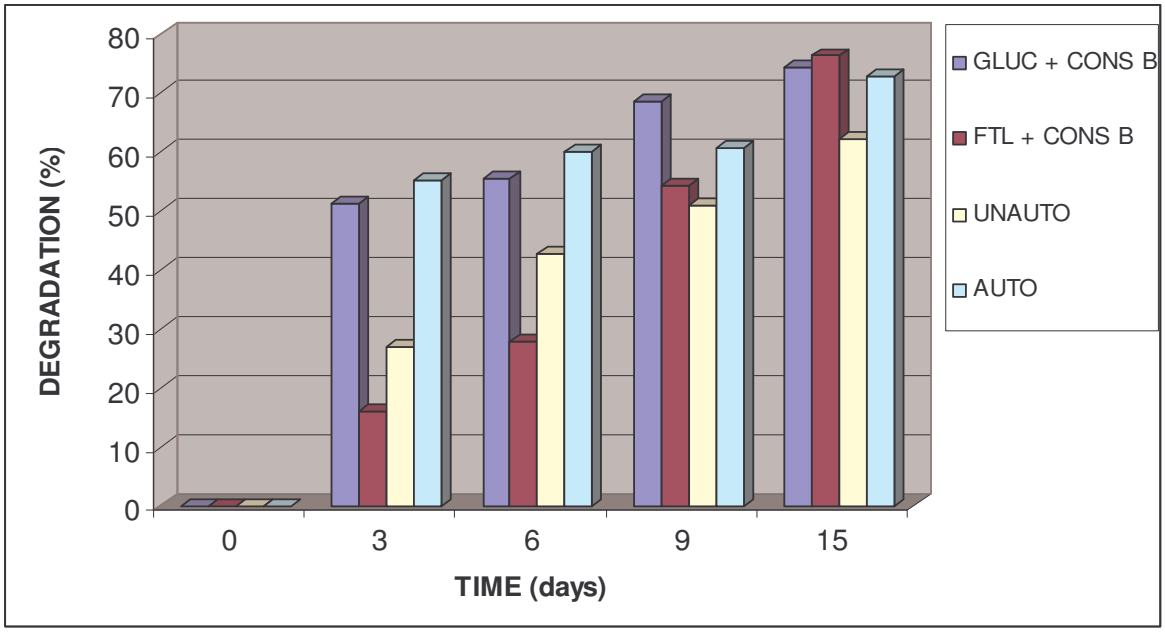

Fig. 4.12: The combined effect of bioaugmentation and biostimulation on the biodegradation of 1, 2-DCA in Type C microcosms.

4.3.3 Microbial biomass yield in the different microcosms under various bioremediation conditions

Data used to calculate phospholipid phosphates are reflected in Appendix III. Phospholipids became drastically reduced within 14 days, in microcosms that contained consortium B and C (Fig. 4.13). At day 14, an increase of up to 11.35 grams per nanomole phosphate (g. nm^{-1} phosphate) followed by a 2.8-fold decrease occurred in microcosms that were bioaugmented by consortium B, by day 28.

The biomass pattern produced by consortium D also showed a slight decrease within the first week (P = 0.059). This was then followed by a sharp increase to 18.086 g. nm^{-1} phosphate, after which a significant (P = 0.01) decrease again occurred. Finally, between days 21 to 28, biomass appeared to escalate again.

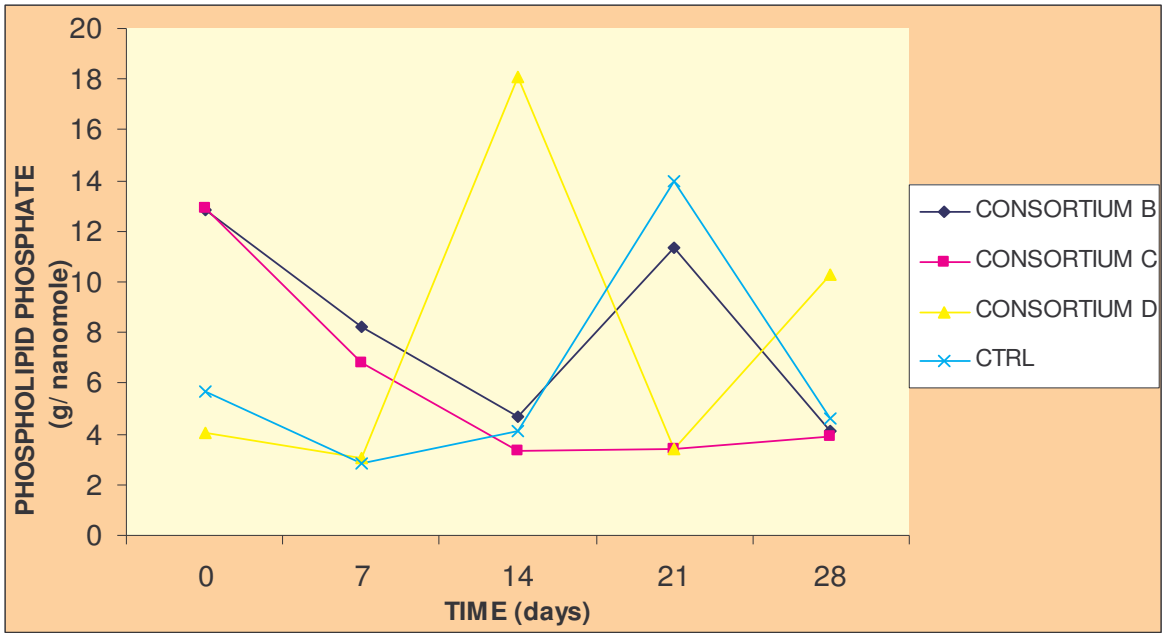

Fig. 4.13: Effect of consortium addition on microbial biomass in Type S microcosms.

There was no specific trend that could be established with regards to biomass in Type S microcosms that were biostimulated and bioaugmented (Fig. 4.14). While glucose and consortium D had attained the highest value of 17.52 g. nm^{-1} phosphate at day 14, it was the fructose and consortium D combination that had led to the greatest % increase in biomass. A significant (P = 0.017) increase of 13.0798 g. nm^{-1} phosphate was measured in fructose and consortium D containing microcosms from days 7 to day 21

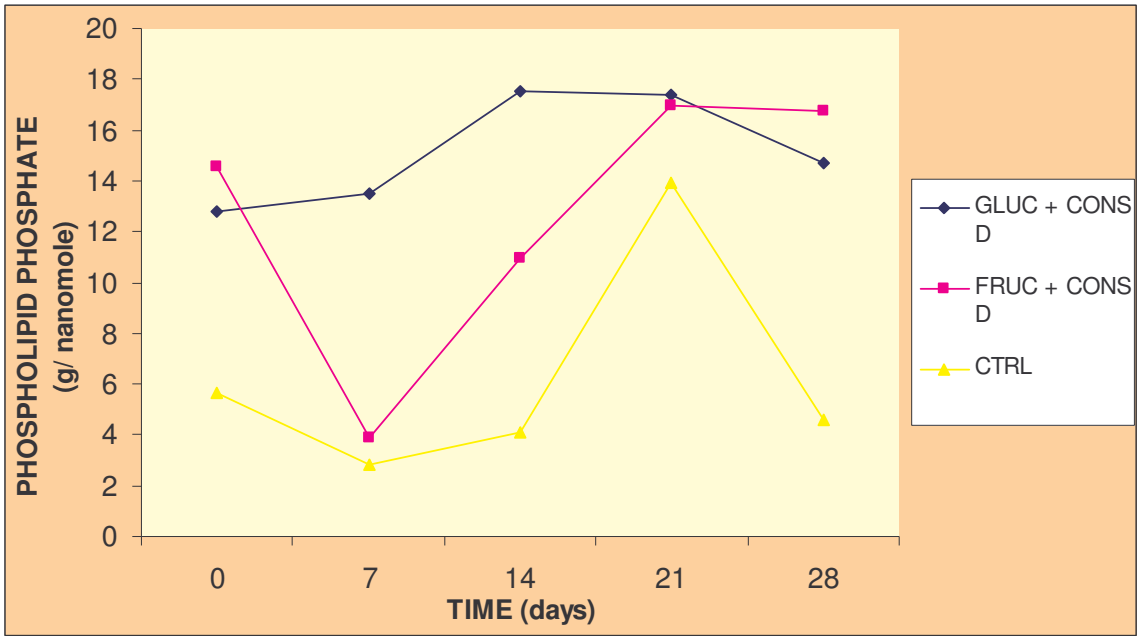

Fig. 4.14: Effect of nutrient and consortium addition on microbial biomass in Type S microcosms.

Similar to combination experiments in Type S microcosms, biomass values varied considerably (Fig. 4.15) in Type C microcosms. The greatest phospholipid increases were evident in controls that were unautoclaved soil, in the first 5 days (P = 0.013). In the same time period, a major phosphate decrease was observed in microcosms that received consortium C. Consortiums B and D produced similar values of 7.2 and 7.5 g. nm^{-1}

phosphate by day 10, respectively.

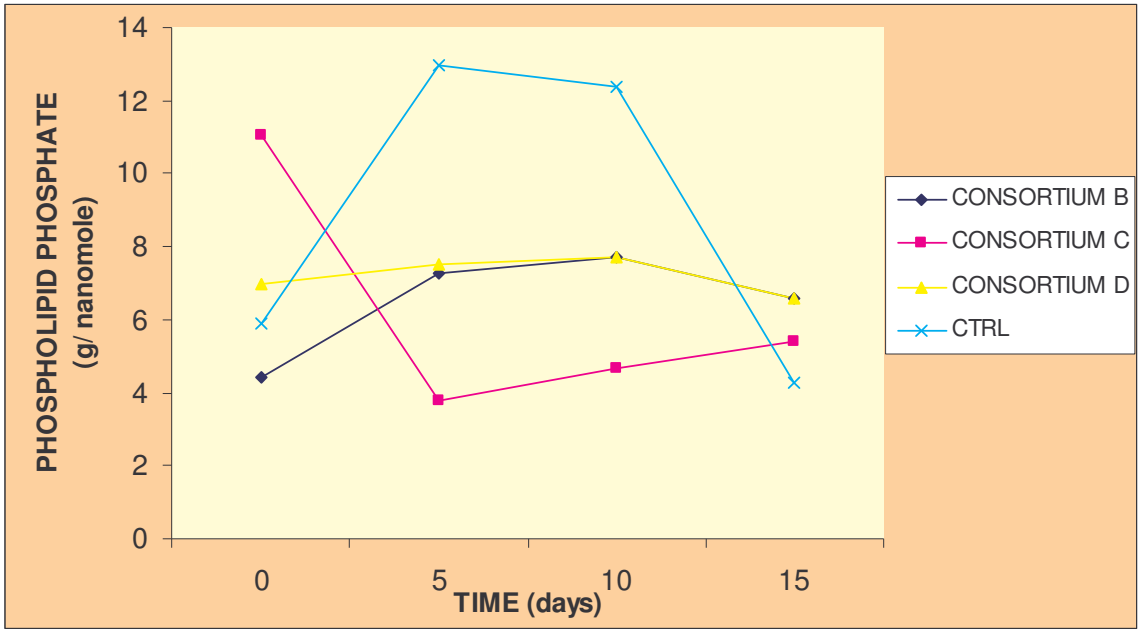

Fig. 4.15: Effect of consortium addition on microbial biomass in Type C microcosms.

Both experimental microcosms that investigated the effects of biostimulation and bioaugmentation in Type C microcosms (Fig. 4.16) were characterized by increases of 5 and 7 g. nm^{-1} phosphate, for glucose and consortium B, and fertiliser and consortium B, respectively, from day 0 to day 5. Thereafter, phospholipids in microcosms with glucose and consortium B continued to decline until day 15. Biomass measured in microcosms with fertiliser and consortium B remained constant from day 10 to day 15. At day 10, control microcosms had significantly higher phospholipid values than both experimental microcosms.

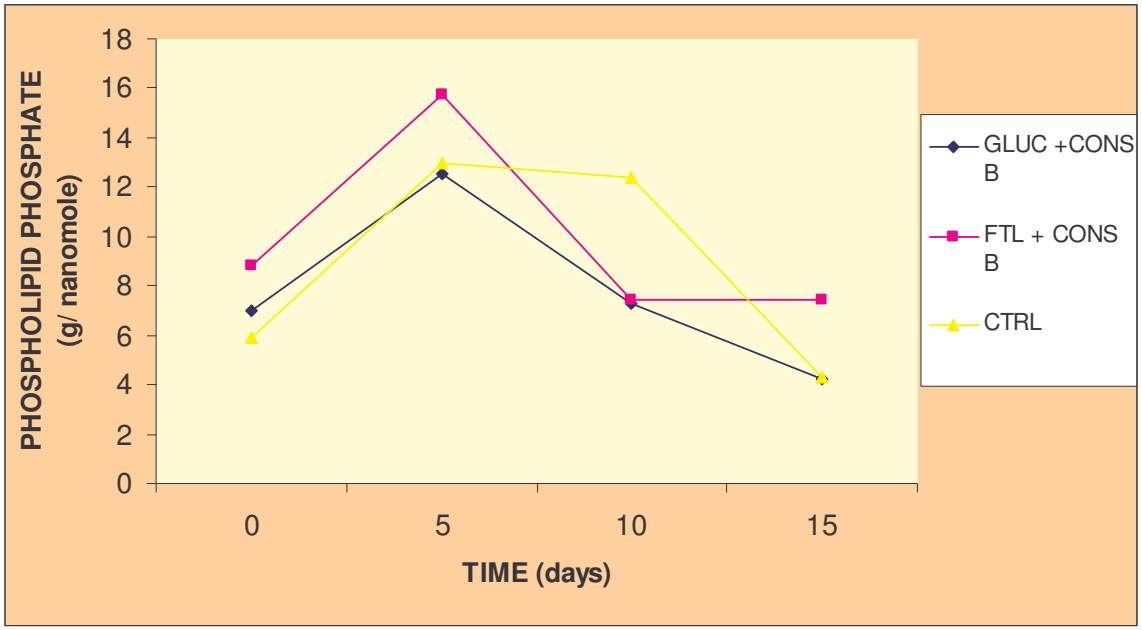

Fig. 4.16: Effect of nutrient and consortium addition on microbial biomass in Type C microcosms.

4.3.4 Identification of end-products of degradation

DCM and 1, 2-DCA were not completely degraded after the respective degradation periods, therefore their appearance in the GC/MS spectra (Appendix III) was expected. Carbon dioxide (CO_2) was also detected in both microcosm types, and for all treatments (Table 4.1). Ammonia (NH_3) was present in microcosms that received fertilizer and consortium B, and microcosms that were bioaugmented with consortium D. Trichloroacetyl chloride (TCA) and triethylene glycol (TEG) which are by-products of CCl_4 and 1, 2-DCA transformation, respectively, were also identified.

Table 4.1: Products of CAH degradation detected in bioaugmented or combination microcosms.

	NH_3	CO_2	DCM	1, 2-DCA	TEG[a]	TCA[b]
[1]CONS D	√	√				
[1]GLUC + D		√	√	√		
[1]FRUC + D		√	√	√	√	
[2]CONS B		√	√	√		
[2]GLUC + B		√	√	√		
[2]FERT + B	√	√	√	√		√

[a] Triethylene glycol; [b] Trichloroacetyl chloride; [1] Type S microcosms; [2] Type C microcosms.

4.4 DISCUSSION

Several chemicals including chlorinated solvents, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, oil, pentachlorophenol and pesticides such as atrazine and carbofuran have been successfully degraded following bioaugmentation with specific organisms (Gentry *et al*., 2004).

The CAHs used in this study were subject to abiotic decomposition to varying degrees. Decreases observed for autoclaved controls can also be ascribed to use of biocidal treatments such as autoclaving, thus resulting in varying concentrations of CAHs in autoclaved controls. This is consistent with the findings of Carter *et al*. (2007). An experimental approach widely used to differentiate between biodegradation and non-biological dissipation mechanisms involves the comparison of sterile and non-sterile treatments. Some sterilisation methods such as autoclaving have been reported to induce changes in the soils physico-chemical properties. The sterilization of soil may result in alterations to solvent extractability and perhaps even the abiotic decomposition of the test compound. Thus, this could lead to incorrect estimates of biodegradation (Shaw *et al*., 1999). It must be noted, however, that decreases in autoclaved controls with

regards to CCl_4 biodegradation is not uncommon and have been reported by several authors (Scherer and Sahm, 1981; Egli *et al*., 1988; Egli *et al*., 1990; Holliger *et* al., 1992; van Eekhert *et al*., 1998). Factors such as vitamin B_{12} and factor F_{430} play a role in decreases of the compound from autoclaved controls. It is speculated that these biochemicals can still be released from dead cells (Ferguson and Pietari, 2000; Hashsham and Freedman, 1999; Sims *et al*., 1991).

Degradation of CCl_4 under aerobic conditions is rare. Many anaerobic bacteria capable of degrading this CAH have been isolated and identified. *Shewanella putrefaciens* 200 was only able to begin biodegradation of CCl_4 after all available oxygen was depleted in an initially oxic headspace (Picardal *et al*., 1995). At oxygen concentrations of less than 1%, a change from aerobic to anaerobic metabolism occurs. It has been suggested that aerobic bacteria play a preparatory role in producing an environment suitable for the growth of the anaerobes. These aerobic bacteria grow within the soil microsites, consume stored oxygen and thereby enhancing the development of the anaerobes (Paul and Clark, 1996; Azadpour- Keeley *et al*., 1999). De Wildeman *et al*. (2004) reported that an anaerobic bacterium, *Desulfitobacterium dichloroeliminans* strain DCA1 was not capable of dechlorinating 1, 2-DCA, when inoculated into serum bottles with an oxic headspace (20% oxygen in air). When the bacterium was again inoculated into an unsterile groundwater medium, oxygen was probably consumed within 24 hrs by most aerobic and anaerobic facultative organisms, and degradation was again initiated by strain DCA1. The presence of facultative aerobic and anaerobic organisms within soil can therefore also account for the continued degradation, even after the inoculants may have lost

viability. This is supported by increases in the unautoclaved controls.

At day 28, biodegradation of CCl_4 could not be attributed to the addition of any of the consortia, based on the observation that this CAH was also totally absent from Type S microcosms and the autoclaved controls. Furthermore, the insignificant net degradation value obtained by the addition of consortium B only in Type C microcosms is probably also as a result of other non-specific chemical reactions, and not necessarily inoculant addition. This was also true with respect to the degradation of CCl_4 when a combination of biostimulation and bioaugmentation in Type C microcosms were investigated. A combined biostimulation and bioaugmentation approach was used to examine if greater biodegradation rates or shorter transformation times would result. Picardal *et al*. (1995) reported that the rates of stationary phase CCl_4 transformation by a nonfermentative bacterium, *Shewanella putrefaciens* 200, to dehalogenate CCl_4 was similar, irrespective of whether glucose, lactate, formate, pyruvate or glycerol were added. This is consistent with the differences observed for the biodegradation of CCl_4 in Type S combination experiments that showed that the glucose and consortium D combination did not produce drastically different degradation profiles compared to the fructose and consortium D combination. In these combination experiments, however, with Type S microcosms, a low final degradation rate of 11.76% was observed for all microcosms, including unautoclaved soil, by day 28. It can therefore be deduced that the observed degradation, was not as a result of the addition of the nutrient and consortium, but rather the activities of the soil indigenous communities.

DCM was considered non-biodegradable for many years (Klecka, 1992). However, substantial evidence has now emerged that indicates that some bacteria are capable of using DCM as a growth source. In Type C microcosms, the simultaneous addition of glucose and consortium B produced a degradation value (23.96%) that was approximately double the degradation value attained by consortium B only. Considering that glucose alone led to an effective degradation of 27.8%, it is logical to assume that the increases in degradation observed in the combination experimental microcosms were not due to the addition of the organisms, but rather the ability of glucose to stimulate the naturally occurring microbes.

The fact that degradation of DCM in Type C microcosms in unautoclaved soil (soil indigenous microorganisms only) in both bioaugmentation and combination experiments was also quite substantial strengthens the argument that the indigenous microbial community degraded majority of the DCM, while the degradation due to the introduction of the exogenous population was probably very transient and negligible. This explanation is also applicable to Type S bioaugmented and combination microcosms that examined the biodegradation of 1, 2-DCA, as a similar trend was evident.

There can be several explanations for this occurrence. Abiotic stresses such as temperature, pH, nutrients, water content and high toxicity of contaminated environments are important factors that can affect the efficacy of bioaugmentation. Introduced organisms also face the challenge of frequent competition from indigenous organisms for limited nutrients. This is because indigenous organisms have developed under

competitive stresses and have thus evolved strategies to maintain themselves. Aspects such as antagonism associated with antibiotic production by competing organisms, as well as predation by protozoa and bacteriophages, can also have a negative impact on the survival of introduced inocula (Paul and Clark, 1996; Gentry *et al*., 2004).

There has been increasing evidence to suggest that the best way to overcome the ecological barriers such as predation and competition with autochthonous microorganisms, is to search for microorganisms from the same ecological niche as the polluted area. Watanabe *et al*. (2002) augmented activated sludge with foreign *phc* genes, which code for phenol hydroxylase and its transcriptional regulators. These genes that originated from *Comamonas testosteroni* R5 were introduced into an indigenous bacterium *Comamonas sp.* rN7, which was the dominant catabolic population of the activated sludge community. Degradation was improved and probably due to the insertion of the foreign catabolic genes that were maintained by an already 'fit' member of the natural ecosysytem (i.e. a major population).

There can be several factors that were instrumental in the lack of appreciable degradation with respect to bioaugmentation in Type C microcosms, but this was most accurately explained by Gannon *et al*. (1991) who determined the relationship between cell surface properties and the associated impacts on bacterial movement through soil. Nineteen strains of bacteria, including two *Bacillus sp*. were investigated. The percentage of cells transported through soil varied considerably, even among the 2-chlorobenzene degrading *Bacillus sp*. used in the study. The authors concluded that various traits such as

hydrophobicity, net surface electrostatic charge, cell size and presence of flagella can determine whether bacterial cells can be transported through soil with moving water. However, this is in contrast with a recent report by Vilain *et al*. (2006) that found that several *Bacillus sp*. are able to translocate easily through soil. It was established that *B. subtilis* translocates through soil by certain cell elongation forces, that result in the bacterial locomotion phenomenon known as sliding. Moreover, it was hypothesized that other *Bacillus sp*. were able to travel far from the original point of inoculation, mainly due to their ability to switch to a multicellular form of growth.

Biodegradation of DCM in bioaugmented Type S microcosms differed from that of Type C. The fructose and consortium D combination produced a 7.81% difference in degradation of DCM in comparison to consortium D alone. A difference of 32.94% in degradation between consortium D and unautoclaved soil control (day 28) eliminates the possibility that the degradation was due to the growth of facultative aerobes/ anaerobes. Similar degradation rates would have been observed in the unautoclaved soil, if the anaerobe population became dominant. This marked variation demonstrated that the introduced population indeed became competitive enough to increase biodegradation rates. The experimental design of Type S microcosms could explain this result. In all likelihood, the introduced microorganisms would have been in closer and much more frequent contact with the contaminant. It is well recognized that contact between organisms and contaminants are essential for effective biodegradation.

In the case of Type C microcosms, both bioaugmentation and the combination

experiments did not allow appreciable degradation of 1, 2-DCA, with the unautoclaved controls serving as a better option for the biodegradation of this CAH. When two organisms have an active demand on a resource in excess of the immediate availability of that resource, competition between the organisms occurs (Paul and Clark, 1996). The competition encountered by the introduced organisms with indigenous soil populations for the nutrient, would present another explanation for the less than desirable degradation profile. In this instance, it appears as if neither the introduced population nor the indigenous population gained the competitive advantage, with the final consequence that unautoclaved soil showed a 58.7% difference in degradation of 1, 2-DCA at day 15, in Type C microcosms. This is in contrast to a report by Olaniran *et al*. (2006) who reported an overall 14% and 18% increase in biodegradation of *cis*-dichloroethene and *trans*-dichloroethene, respectively within 2 weeks. These authors showed that a combination of biostimulation and bioaugmentation will prove to be a successful solution for the remediation of soil and groundwater contaminated with chloroethenes.

In addition, Luo *et al*. (2008) explained that in the presence of an easily utilizable substance, such as glucose, organisms would preferentially use it as a carbon source, rejecting the contaminant. Consequently, genes that may be required for degradation are not expressed. Furthermore, Petersen and Astaf'eva (1962) stated that the secretion of stable extracellular enzymes by several microbial generations can accumulate in soil. These accumulated enzymes have been postulated to be involved in nutrient cycling, especially during initial stages of decomposition and conditions that are inhibitory to microbial proliferation. The possibility exists that these extracellular enzymes can also

account for increased degradation of the CAHs in unautoclaved soil.

Differences in degradation in unautoclaved soils can also be due to the nature of the soil sample. Attempts were made to use composite soil samples, collected during the same season. In some cases, however, further soil collection was necessary. Different soil sampling times could explain certain experimental variations. It is well recognised that soil microbial communities are subject to physical factors such as temperature fluctuations. These temperature changes can also lead to shifts within the microbial community. This fact can therefore provide a rationale for some inherent contradictions during sampling for different treatments.

Many different types of soil, including loam soil have the capacity to retain organic contaminants. The active surfaces of humic substances and soil minerals play a vital role in imparting adsorption ability to soil that is believed to be important in contaminant distribution within soil particles. Bioavailability of contaminants to degrader organisms can be significantly reduced, hence it is important to monitor the binding of contaminants to organic matter (Dercova *et al*., 2006). Bioavailability of contaminants is crucial to the biodegradation process, and could also account for variations observed for unautoclaved soil controls during the course of the experiments. No direct correlation between the biomass patterns and degradation profiles for Type C and Type S, bioaugmented or bioaugmented and biostimulated microcosms could be established. There are several explanations for these observations. As previously mentioned, for this set of experiments further soil sample collection was necessary. It is possible that biomass variations can be

as a result of the differences in the microbial communities from the samples collected during this exercise. Secondly, any increases in biomass in microcosms that contained consortia of degraders, after the development of anaerobic conditions will definitely be variable. Most members of the consortium were the aerobic or facultatively anaerobic spore forming *Bacillus sp*. *Bacilli*, in addition to many other microbes occur in soil as spores, and will germinate and be active upon the availability of readily decomposable organic matter and if soil moisture is high (Vilain *et al*., 2006). The germination of spores can perhaps explain the fluctuation of biomass levels between different sampling times. Microbial biomass patterns for biostimulation and combination experiments proved similar, further iterating the assumption that biodegradation in combination experiments was due to nutrient supplementation and not consortium introduction.

Lorenz *et al*. (2006) studied the effect of soil storage on soil microbial biomass, total DNA yields, enzyme activities and fatty acid microbial biomarkers. These authors concluded that DNA extraction and ester-linked fatty-acid-methyl-esters (EL-FAME) analyses should be performed on fresh soil and preferable storage method for soil is – 20 ºC. It was also concluded that microbial biomass, microbial respiration and aryl-sulfatase activity and most EL-FAME were typically altered by storage and observed differences that do occur, can be avoided if fresh soils are used. The monthly and fort-nightly incubation times for Type S and Type C microcosms, respectively, did not allow the sampling of fresh soils. In addition, soil samples that were used were stored at 4ºC. Konopka *et al*. (1999) suggest that results regarding microbial biomass and activity may not always correlate the effects of hydrocarbon pollution. Differences in the chemical

properties of the hydrocarbons used can account for this observation.

In bioaugmented Type S microcosms, DCM and 1, 2-DCA was not present in the GC/MS spectra. This data conflicts with the fact that GC still detected appreciable concentrations of these CAHs, after day 28. A possible sampling error or library mismatch with the GC/MS appears to be the only reasonable explanation. Ammonia can be commonly found in soil due to various sources, usually it volatilizes into the atmosphere. In this case, however, it is probably due to the ammonification of soil organic matter. Normally, ammonia can be nitrified to nitrate, which can leach into soil, or be subject to denitrification to form nitrous oxide and nitrogen (Paul and Clark, 1996).

Jeffers *et al*. (1989) demonstrated that vinyl chloride was detected upon abiotic transformation of 1, 2-DCA, via an alkaline hydrolysis reaction. Reactions at neutral pH support a hydrolytic substitution reaction, with the formation of ethylene glycol. The half-life for this reaction at neutral pH and 25ºC is 7 years. However, the abiotic reactivity can be improved by the existence of certain anions often present in natural environments (Klecka *et al*., 1998). Triethylene glycol identified in fructose and consortium D Type C microcosms is perhaps the co-product of the formation of ethylene glycol.

It is compulsory to determine conditions that permit the simultaneous degradation of several halogenated aliphatic compounds, due to contamination by complex mixtures of organic chemicals (Chang and Alvarez-Cohen, 1996). While bioaugmentation is a

feasible option, inoculum survival is paramount to its success. The distribution of degrader organisms through contaminated matrices is vital to ensure successful bioremediation. However, aspects such as predation, lysis, starvation and parasitism have been implicated in loss of cell viability during movement. Other factors such as sorption and filtration can also have a profound effect on bacterial viability (Gannon *et al*., 1991). It is these factors that have led to many failures of bioaugmentation at the field-scale. The ultimate purpose of searching for novel 'super-bugs' lies with their ability to degrade contaminants more efficiently than their naturally occurring environmental counterparts. It is often the case, that many of these 'super-bugs' often display tremendous degradation ability in the laboratory, however, fail to become competitive when introduced into the environment. Therefore, many researchers are currently directing their efforts towards determining methods that can allow introduced microorganisms to remain viable for long periods of time and support effective degradation. Encapsulation technologies will definitely make bioaugmentation a more feasible option for the bioremediation of contaminated sites.

CHAPTER FIVE

CONCLUDING REMARKS

5.1 THE RESEARCH IN PERSPECTIVE

Bioremediation is often considered a multidisciplinary technology. Microbiology, ecology, chemistry, geology and engineering are important aspects that demand attention before execution of any bioremediation regimen (Evans *et al*., 2004). Nevertheless, *in situ* bioremediation has shown enormous potential as a technique for the effective treatment of sites contaminated with chlorinated aliphatic hydrocarbons (CAHs). Bioremediation is frequently hampered because it is quite difficult to prove its success at a given site (Heraty *et al*., 1999). Therefore, it has become mandatory to perform the necessary and relevant laboratory feasibility tests to determine the effectiveness of bioremediation at a particular site, before its implementation on a field-scale. Such treatability studies can vary, and are highly dependant on various factors. These factors include the characteristics of the site, as well as the type and nature of the contaminants present. Treatability studies should provide the rationale for the occurrence of biodegradation, after the assessment of the site characteristics (Rittmann and McCarty, 2001).

The United States Air Force and United States Environmental Protection Agency have recommended microcosm testing procedures, to obtain valuable preliminary information prior to instituting a bioremediation project (Findlay and Fogel, 2000). Full-scale field studies in biogeochemistry are often costly and complex, therefore most microbiological

investigations are dependant on laboratory microcosms. Laboratory microcosms may be as simple as possible ranging from simple glass jars, batch reactors or columns, to sophisticated artificial aquifer constructs that comprise soil/aquifer material and reproduced water (Mandelbaum *et al*., 1997). Due to the emphasis on preliminary feasibility studies, the main objective for this study was to investigate the bioremediation of soil contaminated with a mixture of CAHs using two simple laboratory microcosm types by either biostimulation, bioaugmentation or a combination of both processes.

In order to justify any practical application, bioremediation feasibility tests should reveal that removal of contaminants is the primary effect of biodegradation and that the degradation rate is greater than the natural rate of decontamination (Bento *et al*., 2005). In this respect, biostimulation in both Type S and Type C microcosms proved to be more efficient for the bioremediation of soils contaminated with dichloromethane (DCM) and 1,2-dichloroethane (1,2-DCA). Currently, there are no guidelines to detail which nutrient sources will produce the desired results in a particular contaminated site. Specific carbon-nitrogen-phosphorus ratios can only be established after the rate and extent of degradation of chemicals present, nutrient bioavailability and soil types, are thoroughly assessed. Nutrients are also inherent to a contaminated site, thus additional nutrients can interact with the existing soil populations. It is presumed that these interactions that occur can, to a large extent, affect the final result of a bioremediation venture (Liebeg and Cutright, 1999).

A variety of natural habitats are characterized by the close association of microbes with

surfaces and interfaces. This type of association gives rise to biofilms, which are assemblages of single/multiple microbial populations that become attached to surfaces. This attachment is known to occur as a result of the secretion of an extrapolymeric substance. The main biofilm reactors used for degradation studies are usually distinguished according to the method used. This can include an upflow sludge blanket (USB), biofilm fluidized bed (BFB), expanded granular sludge blanket (EGSB); biofilm airlift suspension (BAS) and internal circulation (IC). In the USB, BFB and EGSB reactors, an upward liquid flow keeps the particles fluidized. In BAS reactors, air is pumped into the system, allowing a suspension to be acquired. In IC reactors, the production of gas in system enables circulation and mixing of solids and liquids (Singh *et al*., 2006). The principle of microcosm type C was very similar to that of an IC reactor, except that circulation of liquid was driven by a peristaltic pump.

Since, such bioreactors promote biofilm formation, the increased degradation rates of 1,2-DCA in Type C microcosms can also be ascribed to this microbial phenomenon. Bacteria have a tendency to grow in various interfaces and in flowing environments, this interfacial growth is very beneficial to the organisms. It is believed that there is a certain degree of protection afforded by the matrix within which the cells are contained. These cells have a greater chance of survival and adaptation than planktonic microorganisms. Due to the fact that microorganisms are in assemblages and close to each other, mutually beneficial physiological and physical interactions can occur. Contact between the cells is known to promote the utilization of xenobiotics and therefore, degradation can be accelerated (Langwaldt and Puhakka, 2000; Singh *et al*., 2006).

Bioaugmentation, since its initiation as a bioremediation option, still remains a highly controversial issue. It has even been described as a costly and unpromising equivalent to voodoo medicine. On the other hand, its commercialization has proven to be extremely beneficial to those willing to exploit its virtues (Thompson *et al*., 2005). Most research in the laboratory has been devoted to the discovery of organisms with significant catabolic potential. It has proven to be extremely difficult, though, to achieve as good or better, results in the field as in the laboratory. Exogenous/introduced microorganisms have been repeatedly unable to elicit a significant result when in combination with the autochthonous community. This could very well be due to their inability to compete with the natural populations already present in contaminated sites (Vezzuilli *et al*., 2004). The inability of some introduced inocula to permeate the subsurface environment and their possible sorption to minerals have also been identified as key factors in limiting its success (Mandelbaum *et al*., 1997).

The microorganisms tested from an existing culture collection displayed satisfactory ability to degrade a mixture of CAHs in minimal media. Most cultures utilized in biodegradation and bioremediation studies often harbor various degradative enzymes or plasmids that enable them to effectively degrade many xenobiotics. Further research to determine the factors and mechanisms by which these consortia can degrade CAHs is warranted. While these consortia were able to attain appreciable degradation rates in liquid media, as with many other bioaugmentation experiments, they did not produce similar results when inoculated into soil. Usually, microbial inoculants are introduced into soil as live microorganisms in liquid culture form. Attachment of cells onto a carrier

material is often the preferred method, since soil is such a harsh environment. The exploitation of carrier materials has proven to be ideal, because it can provide temporary nutrition and a protective environment for the introduced organisms (Gentry *et al*., 2004). It is possible that this application could perhaps have enabled the introduced consortium to attain their degradative potential, and thus facilitate efficient biodegradation.

It was difficult to establish whether the nutrients or even the consortia had led to biodegradation of CCl_4, based on the losses observed in autoclaved controls. The fate of most CAHs in the environment is determined by many factors, most importantly, their chemical properties. Chemical and biological transformations that can occur under various environmental conditions have the potential to be affected by the number and position of the chlorinated substituents on the chlorinated hydrocarbon. A common observation with regards to CAH degradation is that a greater number of halogen substituents is equivalent to a greater rate of reduction increases. Likewise, the less chlorinated the compound; the higher the rate of oxidation (Chaudhry and Chapalamadugu, 1991).

The reduction of CCl_4 has been observed to occur mostly under anaerobic conditions, and reports of CCl_4 degradation under aerobic conditions are rare, if not totally absent. It was impossible to maintain an aerobic environment, by a continuous flow of oxygen in microcosms, mainly due to the high vapour pressure of 1, 2-DCA and DCM. Thus, the development of anaerobic conditions in both microcosm types was unavoidable. The degradation of CCl_4 under anaerobic conditions is usually accomplished by strictly

anaerobic bacteria. However, many investigators suggest that facultative anaerobic or iron-reducing bacteria can also be instrumental in the dehalogenation of CCl_4 (Picardal *et al*., 1995).

Thus, anaerobic treatment options would appear more feasible if recalcitrant chemicals such as CCl_4, which cannot be easily treated under aerobic conditions, are present. Anaerobic treatment processes can be advantageous, in that, little biomass is produced, oxygen is unnecessary, and less energy may be required for the degradation process (de Best *et al*., 2000). However, contaminated sediments generally comprise of intermittent aerobic/anaerobic zones and conditions. When investigating a bioremediation approach, it is therefore essential to scrutinize the chemicals to be remediated, and make sound decisions about the prevailing and future environmental conditions. A great deal of effort is currently being directed towards sequenced bioremediation strategies that involve intermittent aerobic and anaerobic conditions (Devlin *et al*., 2004). This area of research will probably prove very valuable for sites containing various contaminants that may require different conditions.

Bioremediation by biostimulation or bioaugmentation is, and has proven to have an excellent track record at remediating polluted environments. As with most techniques, it has its disadvantages. With biostimulation, there are many concerns regarding the introduction of nutrients into a contaminated site. Nutrients, especially fertilisers can have negative ramifications on the existing flora and fauna of the surrounding environment. Substantial concern has also been expressed about the introduction of

laboratory grown cultures into the environment. This is especially true for genetically engineered microorganisms. Their interactions with other microorganisms may not always be feasible. Therefore, the introduction of such microorganisms is strongly regulated by many environmental laws and agencies (Boopathy, 2000). Another common point of contention is the removal of bioaugmented organisms after the remediation process is complete. With this in mind, another unit of bioremediation, phytoremediation can prove very useful. Phytoremediation involves the use of green plants to remove, accumulate or render harmless environmental contaminants. Plants are known to stimulate the growth and metabolism of soil microorganisms (Kas *et al*., 1997). It is well known that the rhizosphere, which is the zone of soil associated with the plant roots contain a large community of microorganisms. Plants can provide beneficial primary substrates to microbes by releasing nutrients (simple sugars, amino acids, vitamins etc.) and transporting oxygen to their roots. As a result, the growth of specific microbial populations is encouraged. This complex mutualistic relationship has now been suggested to be responsible for the improved degradation of many xenobiotics in the presence of plants (Maćek *et al*., 2000). Given the current global warming debacle, phytoremediation can be beneficial not only to decontaminating polluted sites, but can also perhaps curb some carbon dioxide emissions, with plants acting as potential carbon sinks.

Traditionally, the efficiency of *in situ* biodegradation may be determined by monitoring CAH concentrations in groundwaters or soil, using gas chromatography. This was one of the first chromatographic separation techniques developed, and is still used for the identification and estimation of many halogenated compounds. Today these techniques

are used routinely to monitor the concentration and fate of specific pollutants in different environments (Santos and Galceran, 2002). However, many physical processes, including evaporation, adsorption, dilution, even biodegradation, can lead to variations in CAH concentrations (Huang *et al*., 1999). In addition, the complexity of environmental samples can further complicate the effective monitoring of a bioremediation strategy. Thus, the use of stable isotopes can minimize the limitations associated with regularly used analytical techniques like gas chromatography. Stable isotopes, commonly of carbon or hydrogen may be used in fractionation experiments or as tracers, and have shown much potential as valuable indictors for biodegradation/bioremediation processes. Compound specific isotope analysis (CSIA) has already been used *in situ* and in microcosm studies, and is now emerging as a popular method in biodegradation studies. Biodegradation *in situ* can be directly measured using CSIA, whereby changes in the isotopic fractionation of the residual pool of contaminants, or even the resultant metabolites may be measured (Coleman *et al*., 2003; Scow and Hicks, 2005). This method would have easily eliminated the discrepancies associated with losses in autoclaved controls. It would have been easier to justify degradation after determining the exact amounts of chloride released and easier identification of concise reaction mechanisms would have also been possible.

The dynamic nature of microbial biomass is continuously under investigation mainly because it accounts for a large proportion of sedimentary organic matter. As such, precise biomass measurements can enable accurate predictions regarding the trophic interactions of bacteria (Findlay *et al*., 1989). Phospholipids are located in the membranes of all

living cells, although not in storage products. These lipids are generally regarded as excellent signature molecules mainly due to the fact that they are rapidly turned over on cell death (Paul and Clark, 1996). In most cases the accumulation of microbial biomass correlates with the degradation profiles. This was not consistent in many microcosms that had higher net degradation, but did not reflect increased biomass values. Overall, Wirth (1999) state that evaluation of soil microbial biomass is vital to indicate soil quality. These measurements may assist in establishing changes and possible future trends with regards to soil organic matter levels and equilibria. This is important to determine the fate and behaviour of microbial communities in contaminated soils. These values may also aid in determining if further or increased amounts of nutrients are necessary, with respect to the duration of a bioremediation scheme. Furthermore, information about soil quality and subsequent predictions concerning the state of the ecosystem after bioremediation is completed can be attained.

The identification of the products of biodegradation is fundamental to understanding the chemical reactions that take place. The final aim of bioremediation of chemicals is the transformation of the toxic molecules and the subsequent production of non-toxic molecules (Lloyd, 2003). With regards to xenobiotic degradation, especially chlorinated compound degradation, it is often the case that the intermediate products of degradation are equally/more toxic than the parent compound. Effective bioremediation is characterized by the formation of innocuous end-products like carbon dioxide, which was observed in some microcosms. Chloroform and nitrous oxide were also formed in some microcosms, which is undesirable according to bioremediation guidelines. Chloroform is

extremely toxic and is even familiar as a narcotic, and in some instances is more persistent than CCl_4 in some environments. Furthermore, it has been designated a suspected carcinogen by many regulatory agencies (Tatara *et al*., 1993). The formation of nitrous oxide can also be detrimental, because it is infamous as an atmospheric pollutant and assumed to have 300 times the global warming potential of carbon dioxide. This chemical is known to contribute directly towards the greenhouse effect and thought to be instrumental in the demise of the stratospheric ozone layer (Ma *et al*., 2007; Russow *et al*., 2008).

Many factors must be considered before bioremediation is chosen as the option to decontaminate environments. Examples of such non-technical factors include the site to be remediated, the cost compared to other remediation methods, as well as the contaminants that remain after the bioremediation scheme is complete. Due to the fact that bioremediation is a relatively new technology, public opinion can also impact its potential (Boopathy, 2000). Ultimately, it must be recognized that bioremediation is a site-specific process. It cannot be assumed that a certain combination of nutrients and/or microorganisms that has shown to be satisfactory at one site will produce similar results at a different location. In addition, degradation rates may differ for other chemicals. It is important that a thorough knowledge of the effects of the nutrient type and quantity are gained. Consequently, this can easily assist with comparisons with many different sites, and may even decrease the time required for feasibility studies. However, the success of such generalizations will be dependant on the evaluation of various site specific characteristics (Liebeg and Cutright, 1999).

It is difficult to speculate if bioremediation in the present study was totally effective. The reason for this conclusion is two-fold. Firstly, bioremediation is a function of biodegradation, and some microcosms displayed the appropriate results. However, the amount of degradation that occurred in autoclaved controls seriously retarded a well defined conclusion. Secondly, the formation of some toxic by-products is undesirable. In this regard, it can be assumed that longer incubation periods, would have alleviated this issue. Likewise, these products can appear as intermediates in the biodegradation of CAHs regardless of the time-frame.

On a positive note, the formation of carbon dioxide occurred, which is deemed as acceptable in accordance with bioremediation standards. Ultimately, this study showed that biostimulation is an effective method for the bioremediation of soil contaminated with a mixture of chlorinated aliphatic hydrocarbons. Nevertheless, continued research regarding bioremediation will definitely be advantageous and is best described in the words of Nobel laureate Prof. Lord George Porter: 'There is no way that humans can foresee all the consequences of their actions…The only sure foundation in this technological world is to have a science base which is continually asking whatever questions seem interesting and is always there to advise and to act when the need emerges' (Fischli, 1996).

5.2 POTENTIAL FOR FUTURE DEVELOPMENT OF THE STUDY

Occasionally, microcosm testing procedures may be more definitive than those of field monitoring, mainly due to the high degree of control that microcosms can offer (Findlay and Fogel, 2000; Rittmann and McCarty, 2001). With this in mind, microcosms based on bioreactor type constructs, will prove to assist greatly in decontamination strategies. Bioreactors are currently exploited in many treatment processes. However, it is essential to avoid complications with volatilization or adsorption, and based on the results of this study, chemicals that are highly water soluble will probably be effectively remediated using this approach. Therefore further studies including such compounds will prove highly beneficial. In nature, xenobiotic organics are readily integrated into soil organic matter. Detoxification of hazardous chemicals can be facilitated by exploiting this process, known as immobilization. Immobilization has shown great promise as a decontamination method mainly because there is no negative impact of bound xenobiotics on the environment. Some reports have documented that immobilisation processes in soil can lead to significant decreases in bioavailability and toxicity of xenobiotic compounds (Dercova *et al*., 2006). Immobilisation studies encompassing bioreactors will probably have a fortuitous impact on bioremediation strategies.

Molecular biology techniques are rapidly invading every area of scientific research. New nucleic acid based methods have become powerful tools for evaluation of biodegradation and linking these processes to specific microbial populations. Such methods have become influential because they offer information about bioremediation by supporting data of biodegradation. Often, this leads to the detection of specific organisms or populations in

biotransformation events. In addition, environmentally relevant organisms are able to be quantified. Most pollutant degrading organisms isolated and studied in the laboratory are now believed to only facilitate minor changes in bioremediation (Watanabe, 2001; Scow and Hicks, 2005). Considering that a large percentage of soil microorganisms are not amenable to routinely used cultural techniques employed in most laboratories, it is now essential to determine the impact of the unculturable soil communities. Techniques such as denaturing gradient gel electrophoresis (DGGE) and single stranded conformational polymorphisms (SSCP) along with many others have now brought to the fore an abundance of natural populations previously undescribed. With the increasing shift towards understanding microbial ecology and diversity, molecular approaches in bioremediation projects, such as within the present study are definitely advised. Such investigations will certainly equip the relevant stakeholders with a better understanding of the remarkable network of microbial interactions and thus enable sound decision making when implementing bioremediation schemes.

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APPENDIX 1:

Figures or tables in parenthesis are indicated within the text.

Trace metal solution (milligrams per litre): (as per Section 2.2.2)

530 mg $CaCl_2$

200 mg $FeSO_4$. $7H_2O$

10 mg $ZnSo_4$. $7H_2O$

10 mg H_3BO_3

10 mg $CoCl_2$. $6H_2O$

4 mg $MnSO_4$. $5H_2O$

3 mg Na_2Mo_4. $2H_2O$

2 mg $NiCl_2$. $6H_2O$

Vitamin solution (grams per litre): (as per Section 2.2.2)

0.012 g biotin

1 g choline chloride

1 g calcium (d) pantothenate

2 g *i*- inositol, 1 g nicotinic acid

1 g pyridoxine chloride

1 g thiamine chloride

0.2 g *p*- aminobenzoic acid

0.01 g cyanocobalamin

50 X TAE Buffer (grams per litre) (as per Section 2.2.5)

242 g tris base

57.1 ml acetic acid

100 ml 0.5 M EDTA, pH 8.5

SOC medium (grams per litre) (as per Section 2.2.9)

20 g bacto- tryptone,

5 g bacto- yeast extract

20 mM glucose

0.5 g NaCl

2.5 mM KCl

1 mM $MgCl_2$

Table 5: Duplicate optical density values for different isolates during growth measurement studies (Fig. 1.)

TIME (hrs)	003	004	006	007	413	NEW 5	NEW 6	C 11	C 33	AO1	Ctrl
0	0.006	0.007	0.04	0.01	0.004	0.152	0.001	0.012	0.004	0.004	0.02
	0.008	0.009	0.011	0.04	0.006	0.017	0.006	0.008	0.005	0.011	0.026
6	0.002	0.016	0.05	0.028	0.016	0.004	0.025	0.048	0.022	0.034	0.02
	0.01	0.024	0.031	0.045	0.021	0.018	0.019	0.013	0.014	0.04	0.018
12	0.037	0.035	0.054	0.033	0.031	0.028	0.029	0.036	0.03	0.051	0.008
	0.025	0.038	0.048	0.047	0.029	0.026	0.033	0.022	0.033	0.036	0.003
24	0.03	0.043	0.063	0.05	0.044	0.033	0.04	0.032	0.02	0.049	0.002
	0.032	0.031	0.058	0.054	0.025	0.038	0.031	0.033	0.027	0.037	0.002
36	0.009	0.012	0.02	0.014	0.006	0.006	0.001	0.002	0.01	0.019	0.019
	0.01	0.014	0.032	0.024	0.007	0.003	0.005	0.007	0.009	0.015	0.0024

Table 6: Duplicate optical density values for different consortia during growth measurement studies (Fig. 3).

TIME (hrs)	A	B	C	D	E	F	G	Ctrl
0	0.045	0.044	0.048	0.036	0.040	0.050	0.038	0.011
	0.053	0.042	0.036	0.052	0.084	0.054	0.052	0.023
6	0.040	0.042	0.046	0.049	0.041	0.057	0.040	0.001
	0.042	0.044	0.028	0.037	0.045	0.047	0.048	0.001
12	0.036	0.054	0.035	0.032	0.063	0.039	0.062	-0.009
	0.050	0.050	0.033	0.034	0.059	0.029	0.044	-0.023
24	0.071	0.058	0.040	0.062	0.081	0.045	0.078	0.000
	0.093	0.092	0.035	0.080	0.099	0.025	0.074	0.002
36	0.110	0.090	0.158	0.089	0.085	0.050	0.084	0.000
	0.104	0.102	0.148	0.085	0.095	0.048	0.090	0.001
58	0.089	0.105	0.069	0.043	0.089	0.022	0.099	-0.003
	0.079	0.101	0.067	0.045	0.055	0.030	0.089	0.002
72	0.101	0.099	0.071	0.099	0.071	0.030	0.098	0.004
	0.077	0.111	0.075	0.081	0.075	0.038	0.092	0.002
84	0.096	0.117	0.082	0.098	0.080	0.047	0.088	0.000
	0.092	0.061	0.076	0.096	0.080	0.025	0.082	0.000
96	0.080	0.081	0.043	0.090	0.090	0.031	0.090	-0.005
	0.076	0.081	0.027	0.052	0.054	0.037	0.084	-0.004
102	0.082	0.093	0.078	0.031	0.071	0.034	0.110	0.011
	0.080	0.095	0.070	0.051	0.077	0.004	0.108	0.013

Table 7: Duplicate gas chromatography peak area values for CCl_4 biodegradation in liquid media (Fig. 4).

DAY	B	C	D	E	G	CTRL
0	2142	2152	1942	1578	1824	1890
	2060	1856	2126	1480	1980	1716
7	1180	427	1497	1486	1533	1553
	964	335	1618	1564	1340	1905

Table 8: Duplicate gas chromatography peak area values for DCM biodegradation in liquid media (Fig. 5)

DAY	B	C	D	E	G	CTRL
0	799	863	998	964	976	1350
	827	804	1003	975	1006	1168
7	770	440	913	891	943	1482
	647	413	928	912	915	1823

Table 9: Duplicate gas chromatography peak area values for 1, 2-DCA biodegradation in liquid media (Fig. 6).

DAY	B	C	D	E	G	CTRL
0	1102	1819	797	1270	1344	1502
	850	1831	784	1470	1382	1472
7	680	0	0	388	585	1482
	346	0	0	488	495	1823

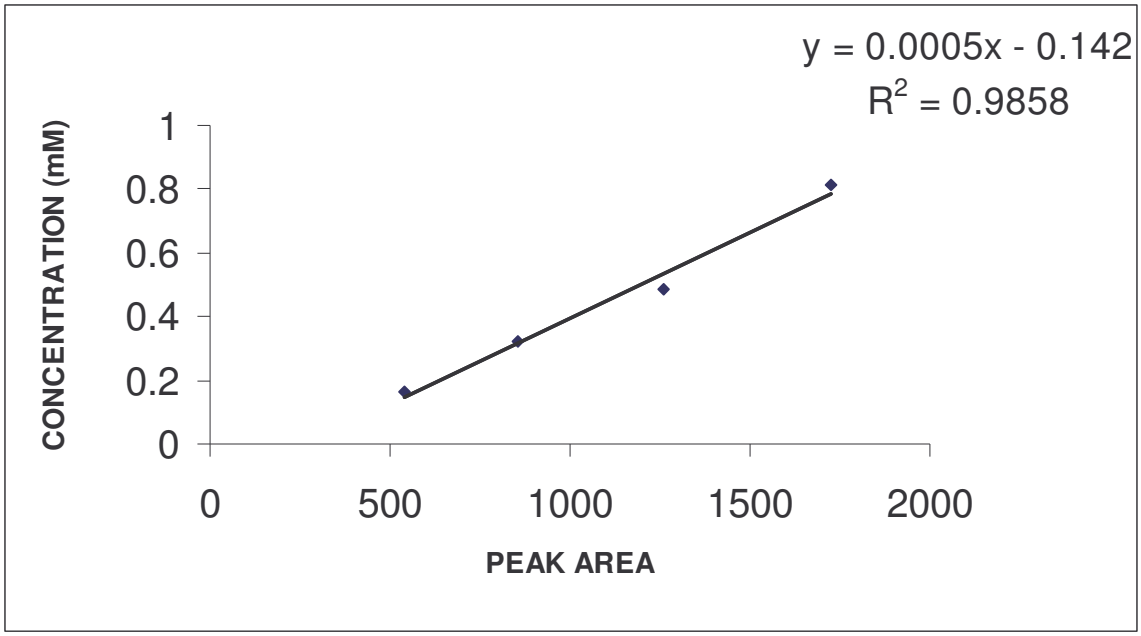


Fig. 5: Standard curve for determination of CCl_4 concentration in liquid media (Section 2.2.4)

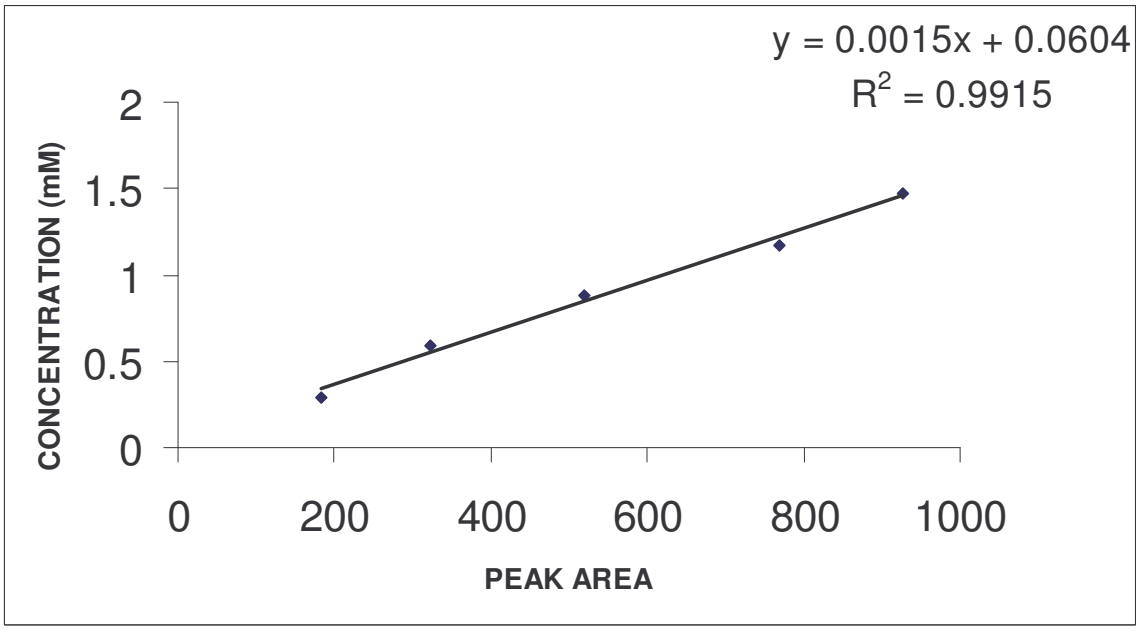


Fig. 6: Standard curve for determination of DCM concentration in liquid media (Section 2.2.4).

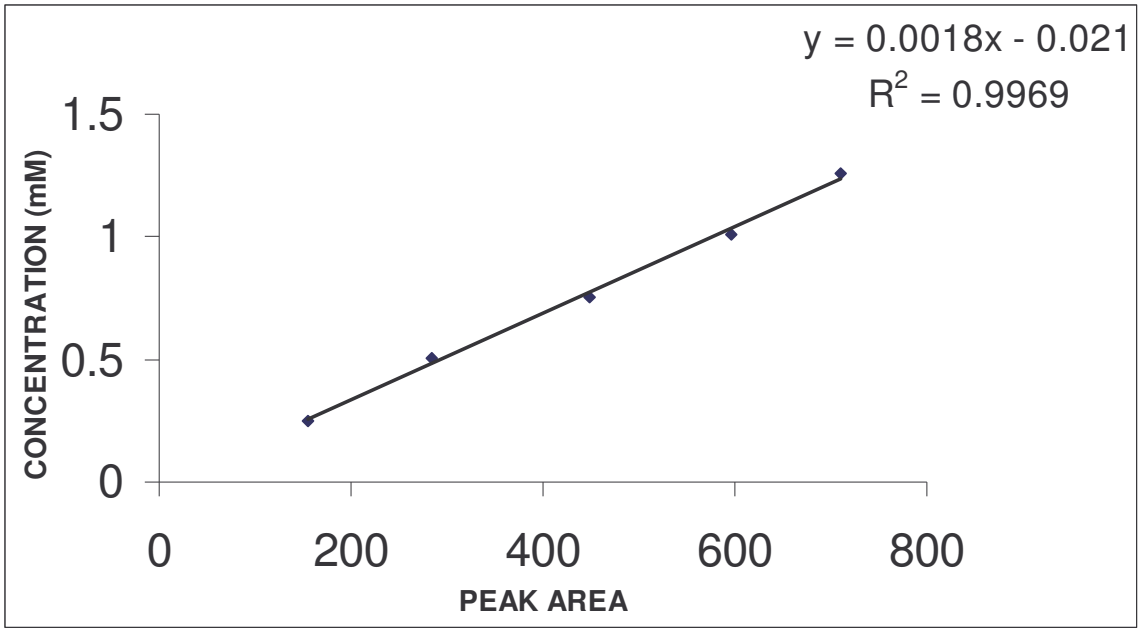


Fig. 7: Standard curve for determination of 1, 2-DCA concentration in liquid media (Section 2.2.4).

Table 10: Gas chromatography peak area values for construction of standard curve in liquid media as per Figs. 33, 34 and 35.

	CCl_4		DCM		1, 2 DCA	
VOLUME (µl)	**PEAK AREA**	**CONC. (mM)**	**PEAK AREA**	**CONC. (mM)**	**PEAK AREA**	**CONC. (mM)**
2.5	539	0.162	184	0.294	156	0.252
5	857	0.324	323	0.588	284.5	0.505
7.5	1260	0.487	519	0.882	449	0.757
12.5	1723	0.811	770	1.176	710.5	1.262

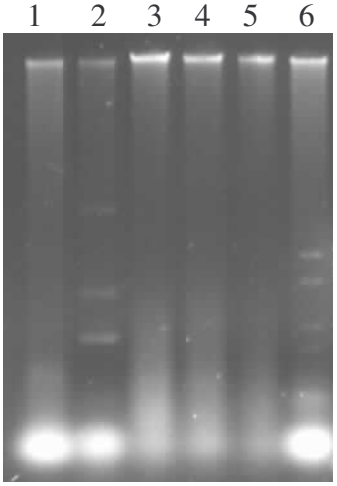


Fig. 8: Agarose gel electrophoresis of the DNA of the six isolates comprising the various consortia (Lane 1- 6: 004, 006, 007, 007, 413, AO1 and New 6- Section 2.2.5).

16 S rRNA gene sequences of the bacterial isolates.

GCTCCGGCCG CCATGGCGGC CGCGGGAATT CGATTCAGGC CTAACACATG CAAGTCGAGC
GGACAGATGG GAGCTTGCTC CCTGATGTTA GCGGCGGACG GGTGAGTAAC ACGTGGGTAA
CCTGCCTGTA AGACTGGGAT AACTCCGGGA AACCGGGGCT AATACCGGAT GCTTGTTTGA
ACCGCATGGT TCAAACATAA AAGGTGGCTT CGGCTACCAC TTACAGATGG ACCCGCGGCG
CATTAGCTAG TTGGTGAGGT AATGGCTCAC CAAGGCAACG ATGCGTAGCC GACCTGAGAG
GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGG CAGCAGTAGG
GAATCTTCCG CAATGGACGA AAGTCTGACG GAGCAACGCC GCGTGAGTGA TGAAGGTTTT
CGGATCGTAA AGCTCTGTTG TTAGGGAAGA ACAAGTACCG TTCGAATAGG GCGGTACCTT
GACGGTACCT AACCAGAAAG CCACGGCTAA CTACGTGCCA GCAGCCGCGG TAATACGTAG
GTGGCAAGCG TTGTCCGGAA TTATTGGGCG TAAAGGGCTC GCAGGCGGTT TCTTAAGTCT
GATGTGAAAG CCCCCGGCTC AACCGGGGAG GGTCATTGGA AACTGGGGAA CTTGAGTGCA
GAAGAGGAGA GTGGAATTCC ACGTGTAGCG GTGAAATGCG TAGAGATGTG GAGGAACACC
AGTGGCGAAG GCGACTCTCT GGTCTGTAAC TGACGCTGAG GAGCGAAAGC GTGGGGAGCG
AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT GAGTGCTAAG TGTTAGGGGG
TTTCCGCCCC TTAGTGCTGC AGCTAACGCA TTAAGCACTC CGCCTGGGGA GTACGGTCGC
AAGACTGAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTCGAAGCAA CGCGAAGAAC CTTACCAGGT CTTGACATCC TCTGACAATC CTAGAGATAG
GACGTCCCCT TCGGGGGCAG AGTGACAGGT GGTGCATGGT TGTCGTCAGC TCGTGTCGTG
AGATGTTGGG TTAAGTCCCG CAACGAGCGC AACCCTTGAT CTTAGTTGCC AGCATTCAGT
TGGGCACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA GGTGGGGATG ACGTCAAATC
ATCATGCCCC TTATGACCTG GGCTACACAC GTGCTACAAT GGACAGAACA AAGGGCAGCG
AAACCGCGAG GTTAAGCCAA TCCCACAAAT CTGTTCTCAG TTCGGATCGC AGTCTGCAAC
TCGACTGCGT GAAGCTGGAA TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT
TCCCGGGCCT TGTACACACC GCCCAATCAC TAGTGAATTC GCGGCCGCCT GCAGGTCGAC
CATATGGGAG AGCTCCCAAC GCATSTATCT

Consensus sequence for isolate 004 (Table: 2.1).

TTGGGAGCTC TCCCATATGG TCGACCTGCA GGCGGCCGCG AATTCACTAG TGATTCAGGC
CTAACACATG CAAGTCGAGC GGACAGATGG GAGCTTGCTC CCTGATGTTA GCGGCGGACG
GGTGAGTAAC ACGTGGGTAA CCTGCCTGTA AGACTGGGAT AACTCCGGGA AACCGGGGCT
AATACCGGAT GCTTGTTTGA ACCGCATGGT TCAAACATAA ARGGKGGCCT CGGCTACCAC
TTACAGATGG ACCCGCGGCG CATTAGCTAG TTGGTGAGGT AATGGCTCAC CAAGGCAACG
ATGCGTAGCC GACCTGAGAG GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC
CTCCGGGAGG CAGCAGTAGG GTATCTTCCG CAATGGACGA AAGTCTGACG GAGCAACGCC
GCGTGAGTGA TGAAGGTTTT CGGATCGTAA AGCTCTGTTG TTAGGGAAAA AACAAGTACC
GTTCGAATAG GGSGGTACCT TGGCTGGTAC CTAACCAAGA AAGCCACGGC TATCTACGTG
CCAGCAGCCG GCGGTTAATA CGTAGGTGGC AGGCGTTGTC CGAAATTATA GGGCGTAAAG
GGCTCGCAGG CGGTTTCTTA AGTCTGATGT GAAGCCCCCC GGCTCAACTG GGGAGGGTCA
TTAGCAAACT GGGAAACTTG AGTGCAAAAG AGGAGAGTGC AATTCCACCG TGTAGCGGTG
AAATGCGTAA AGATGTGGAG GATCCCCAAG TGGCGGAAGG CGACTCTCTG KRACGCTGAG
GARCGAARGC KTGGGGRRGC GAMCAGGATT AGATWCCTTG CGTWAAACAA TGAGTGCTAA
GKGTTAGGGG KKTTYCCGCC CCCTTAGKGS TGRCASWTAA GGGGAGRTAC YTGAATCTCA
AAAGGAATGA ACGGGGGGCC CGCCCAAGCG GTGGGAGCAT GTGGTTTAAT TCGAAGCAAC
GCGAAGAACC TTACCAGGTC TTGACATCCT CTGACAATCC TAGAGATAGG ACGTCCCCTT
CGGGGGCAGA GTGACAGGTG GKGCATGGTT GTCGTCAGCT CGTGTCGTGA GATGTTGGGT
TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA GCATTCAGTT GGGCACTCTA
AGGTGACTGC CGGTGACAAA CCGGAGGAAG GTGGGGATGA CGTCAAATCA TCATGCCCCT
TATGACCTGG GCTACACACG TGCTACAATG GACAGAACAA AGGGCAGCGA AACCGCGAGG
TTAAGCCAAT CCCACAAATC TGTTCTCAGT TCGGATCGCA GTCTGCAACT CGACTGCGTG
AAGCTGGAAT CGCTAGTAAT CGCGGATCAG CATGCCGCGG TGAATACGTT CCCGGGCCTT
GTACACTCCG CCCAATCGAA TTCCCGCGGC CGCCATGGCG GC

Consensus sequence for isolate 006 (Table: 2.1).

CCGGCCGCCA TGGCGGCCGC GGGAATTCGA TTCAGGCCTA ACACATGCAA GTCGAGCGGA
CAGATGGGAG CTTGCTCCCT GATGTTAGCG GCGGACGGGT GAGTAACACG TGGGTAACCT
GCCTGTAAGA CTGGGATAAC TCCGGGAAAC CGGGGCTAAT ACCGGATGGT TGTTTGAACC
GCATGGTTCA AACATAAAAG GTGGCTTCGG CTACCACTTA CAGATGGACC CGCGGCGCAT
TAGCTAGTTG GTGAGGTAAT CGGCTCACCA AGGCAACGAT GCGTAGCCGA CCTGAGAGGG
TGATCGGCCA CACTGGGACT TGAGACACGG CCCAGACTCC TACGGGAGGC AGCAGTAGGG
AATCTTCCGC AATGGACGAA AGTCTGACGG AGCAACGCCG CGTGAGTGAT GAAGGTTTTC
GGATCGTAAA GCTCTGTTGT TAGGGAAGAA CAAGTACCGT TCGAATAGGG CGGTACCTTG
ACGGTACCTA ACCAGAAAGC CACGGCTAAC TACGTGCCAG CAGCCGCGGT AATACGTAGG
TGGCAAGCGT TGTCCGGAAT TATTGGGCGT AAAGGGCTCG CAGGCGGTTT CTTAAGTCTG
ATGTGAAAGC CCCCGGCTCA ACCGGGGAGG GTCATTGGAA ACTGGGGAAC TTGAGTGCAG
AAGAGGAGAG TGGAATTCCA CGTGTAGCGG TGAAATGCGA AGAGATGTGG AGGAACACCA
GTGGCGAAGG CGACTCTCTG GTCTGTAACT GACGCTGAGG AGCGAAAGCG TGGGGAGCGA
ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG AGTGCTAAGT GTTAGGGGGT
TTCCGCCCCT TAGTGCTGCA GCTAACGCAT TAAGCACTCC GCCTGGGGAG TACGGTCGCA
AGACTGAAAC TCAAAGGAAT TGACGGGGGC CCGCACAAGC GGTGGAGCAT GTGGTTTAAT
TCGAAGCAAC GCGAAGAACC TTACCAGGTC TTGACATCCT CTGACAATCC TAGAGATAGG
ACGTCCCCTT CGGGGGCAGA GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA
GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA GCATTCAGTT
GGGCACTCTA AGGTGACTGC CGGTGACAAA CCGGAGGAAG GTGGGGATGA CGTCAAATCA
TCATGCCCCT TATGACCTGG GCTACACACG TGCTACAATG GACAGAACAA AGGGCAGCGA
AACCGCGAGG TTAAGCCAAT CCCACAAATC TGTTCTCAGT TCGGATCGCA GTCTGCAACT
CGACTGCGTG AAGCTGGAAT CGCTAGTAAT CGCGGATCAG CATGCCGCGG TGAATACGTT
CCCGGGCCTT GTACACTCCG CCCAATCACT AGTGAATTC

:Consensus sequence for isolate 007 (Table: 2.1)

GGCCGCGGGA TTCGATTGGG CGGTGTGTAC AAGGCCCGGG AACGTATTCA CCGCGGCATG
CTGATCCGCG ATTACTAGCG ATTCCAGCTT CACGCAGTCG AGTTGCAGAC TGCGATCCGA
ACTGAGAACA GATTTGTGGG ATTGGCTTAA CCTCGCGGTT TCGCTGCCCT TTGTTCTGTC
CATTGTAGCA CGTGTGTAGC CCAGGTCATA AGGGGCATGA TGATTTGACG TCATCCCCAC
CTTCCTCCGG TTTGTCACCG GCAGTCACCT TAGAGTGCCC AACTGAATGC TGGCAACTAA
GATCAAGGGT TGCGCTCGTT GCGGGACTTA ACCCAACATC TCACGACACG AGCTGACGAC
AACCATGCAC CACCTGTCAC TCTGCCCCCG AAGGGGACGT CCTATCTCTA GGATTGTCAG
AGGATGTCAA GACCTGGTAA GGTTCTTCGC GTTGCTTCGA ATTAAACCAC ATGCTCCACC
GCTTGTGCGG GCCCCCGTCA ATTCCTTTGA GTTTCAGTCT TGCGACCGTA CTCCCCAGGC
GGAGTGCTTA ATGCGTTAGC TGCAGCACTA AGGGGCGGAA ACCCCCTAAC ACTTAGCACT
CATCGTTTAC GGCGTGGACT ACCAGGGTAT CTAATCCTGT TCGCTCCCCA CGCTTTCGCT
CCTCAGCGTC AGTTACAGAC CAGAGAGTCG CCTTCGCCAC TGGTGTTCCT CCACATCTCT
ACGCATTTCA CCGCTACACG TGGAATTCCA CTCTCCTCTT CTGCACTCAA GTTCCCCAGT
TTCCAATGAC CCTCCCCGGT TGAGCCGGGG GCTTTCACAT CAGACTTAAG AAACCGCCTG
CGAGCCCTTT ACGCCCAATA ATTCCGGACA ACGCTTGCCA CCTACGTATT ACCGCGGCTG
CTGGCACGTA GTTAGCCGTG GCTTTCTGGT TAGGTACCGT CAAGGTACCG CCCTATTCGA
ACGGTACTTG TTCTTCCCTA ACAACAGAGC TTTACGATCC GAAAACCTTC ATCACTCACG
CGGCGTTGCT CCGTCAGACT TTCGTCCATT GCGGAAGATT CCCTACTGCT GCCTCCCGTA
GGAGTCTGGG CCGTGTCTCA GTCCCAGTGT GGCCGATCAC CCTCTCAGGT CGGCTACGCA
TCGTTGCCTT GGTGAGCCAT TACCTCACCA ACTAGCTAAT GCGCCGCGGG TCCATCTGTA
AGTGGTAGCC GAAGCCACCT TTTATGTTTG AACCATGCGG TTCAAACAAG CATCCGGTAT
TAGCCCCGGT TTCCCGGAGT TATCCCAGTC TTACAGGCAG GTTACCCACG TGTTACTCAC
CCGTCCGCCG CTAACATCAG GGAGCAAGCT CCCATCTGTC CGCTCGACTT GCATGTGTTA
GGCCTGAATC ACTAGTGAAT TC

: Consensus sequence for isolate 413 (Table: 2.1)

TCGATTGGGC GGAGTGTACA AGGCCCGGGA ACGTATTCAC CGCGGCATGC TGATCCGCGA
TTACTAGCGA TTCCAGCTTC ACGCAGTCGA GTTGCAGACT GCGATCCGAA CTGAGAACAG
ATTTGTGGGA TTGGCTTAAC CTCGCGGTTT CGCTGCCCTT TGTTCTGTCC ATTGTAGCAC
GTGTGTAGCC CAGGTCATAA GGGGCATGAT GATTTGACGT CATCCCCATC TTCCTCCGGT
TTGTCACCGG CAGTCACCTT AGAGTGCCCA ACTGAATGCT GGCAACTAAG ATCAAGGGTT
GCGCTCGTTG CGGGACTTAA CCCAACATCT CACGACACGA GCTGACGACA ACCATGCACC
ACCTGTCACT CTGCCCCCGA AGGGGACGTC CTATCTCTAG GATTGTCAGA GGATGTCAAG
ACCTGGTAAG GTTCTTCGCG TTGCTTCGAA TTAAACCACA TGCTCCACCG CTTGTGCGGG
CCCCCGTCAA TTCCTTTGAG TTTCAGTCTT GCGACCGTAC TCCCCAGGCG GAGTGCTTAA
TGCGTTAGCT GCAGCACTAA GGGGCGGAAA CCCCCTAACA CTTAGCACTC ATCGTTTACG
GCGTGGACTA CCAGGGTATC TAATCCTGTT CGCTCCCCAC GCTTTCGCTC CTCAGCGTCA
GTTACAGACC AGAGAGTCGC CTTCGCCACT GGTGTTCCTC CACATCTCTA CGCATTTCAC
CGCTACACGT GGAATTCCAC TCTCCTCTTC TGCACTCAAG TTCCCCAGTT TCCAATGACC
CTCCCCGGTT GAGCCGGGGG CTTTCACATC AGACTTAAGA AACCGCCTGC GAGCCCTTTA
CGCCCAATAA TTCCGGACAA CGCTTGCCAC CTACGTATTA CCGCGGCTGC TGGCACGTAG
TTAGCCGTGG CTTTCTGGTT AGGTACCGTC AAGGTACCGC CCTATTCGAA CGGTACTTGT
TCTTCCCTAA CAACAGAGCT TTACGATCCG AAAACCTTCA TCACTCACGC GGCGTTGCTC
CGTCAGACTT TCGTCCATTG CGGAAGATTC CCTACTGCTG CCTCCCGTAG GAGTCTGGGC
CGTGTCTCAG TCCCAGTGTG GCCGATCACC CTCTCAGGTC GGCTACGCAT CGTTGCCTTG
GTGAGCCGTT ACCTCACCAA CTAGCTAATG CGCCGCGGGT CCATCTGTAA GTGGTAGCCA
AAGCCACCTT TTATGTTTGA ACCATGCGGT TCAAACAACC ATCCGGTATT AGCCCCGGTT
TCCCGGAGTT ATCCCAGTCT TACAGGCAGG TTACCCACGT GTTACTCACC CGTCCGCCGC
TAACATCAGG GAGCAAGCTC CCATCTGTCC GCTCGACTTG CATGTGTTAG GCCTGAATCA
CTAGTGAATT C

Consensus sequence for isolate AO1 (Table: 2.1).

TCCGGCCGCC ATGGCGGCCG CGGGAATTCG ATTGGGCGGT GTGTACAAGG CCCGGGAACK
TATTCACCGC GACATGCTGA TCCGCGATTA CTAGCGATTC CGACTTCACG CAGTCGAGTT
GCAGACTGCG ATCCGGACTA CGATCGGGTT TCTGGGATTG GCTCCCCCTC GCGGGTTGGC
GACCCTCTGT CCCGACCATT GTATGACGTG TGAAGCCCTA CCCATAAGGG CCATGAGGAC
TTGACGTCAT CCCCACCTTC CTCCGGTTTG TCACCGGCAG TCTCATTAGA GTGCCCTTTC
GTAGCAACCA ATGACAAGGG TTGCGCTCGT TGCGGGACTT AACCCAACAT CTCACGACAC
GAGCTGACGA CAGCCATGCA GCACCTGTGT TCCAGTTCTC TTGCGAGCAC TGCCAAATCT
CTTCGGCATT CCAGACATGT CAAGGGTAGG TAAGGTTTTT CGCGTTGCAT CGAATTAATC
CACATCATCC ACCGCTTGTG CGGGTCCCCG TCAATTCCTT TGAGTTTTAA TCTTGCGACC
GTACTCCCCA GGCGGTCAAC TTCACGCGTT AGCTGCGCTA CCAAGGTCCG AAGACCCCAA
CAGCTAGTTG ACATCGTTTA GGGCGTGGAC TACCAGGGTA TCTAATCCTG TTTGCTCCCC
ACGCTTTCGT GCATGAGCGT CAGTGTTATC CCAGGAGGCT GCCTTCGCCA TCGGTGTTCC
TCCGCATATC TACGCATTTC ACTGCTACAC GCGGAATTCC ACCTCCCTCT GACACACTCT
AGCCCGGTAG TTAAAAATGC AGTTCCAAAG TTAAGCTCTG GGATTTCACA TCTTTCTTTC
CGAACCGCCT GCGCACGCTT TACGCCCAGT AATTCCGATT AACGCTTGCA CCCTACGTAT
TACCGCGGCT GCTGGCACGT AGTTAGCCGG TGCTTATTCT GCAGGTACCG TCAGTTTCGC
GGGGTATTAA CCCACGACGT TTCTTTCCTG CCAAAAGTGC TTTACAACCC GAAGGCCTTC
ATCGCACACG CGGGATGGCT GGATCAGGGT TTCCCCCATT GTCCAAAATT CCCCACTGCT
GCCTCCCGTA GGAGTCTGGG CCGTGTCTCA GTCCCAGTGT GGCTGGTCGT CCTCTCAAAC
CAGCTACGGA TCGTCGCCTT GGTGAGCCGT TACCCCACCA ACTAGCTAAT CCGATATCGG
CCGCTCCAAT AGTGCAAGGT CTTGCGATCC CCTGCTTTCC CCCGTAGGGC GTATGCGGTA
TTAGCTACGC TTTCGCGTAG TTATCCCCCG CTACTGGGCA CGTTCCGATA CATTACTCAC
CCGTTCGCCA CTCGCCACCA GACCGAAGTC CGTGCTGCCG TTCGACTTGC ATGTGTTAGG
CSTGAATCAC TAGTGAATTC

Consensus sequence for isolate New 6 (Table: 2.1).

Data present in Tables 10- 30 appear in Section 2.3

Table 10: Independent T- Test comparing isolate 003 and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	118813348365 07430.000	.000	-29.000	2	.001	-.02900	.00100	-.03330	-.02470	
	Equal variances not assumed			-29.000	1.000	.022	-.02900	.00100	-.04171	-.01629	

Table 11: Independent T- Test comparing isolate 004 and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	.	.	-5.833	2	.028	-.03500	.00600	-.06082	-.00918	
	Equal variances not assumed			-5.833	1.000	.108	-.03500	.00600	-.11124	.04124	

Table 12: Independent T- Test comparing isolate 006 and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	.	.	-23.400	2	.002	-.05850	.00250	-.06926	-.04774	
	Equal variances not assumed			-23.400	1.000	.027	-.05850	.00250	-.09027	-.02673	

Table 13: Independent T- Test comparing isolate 007 and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	.	.	-25.000	2	.002	-.05000	.00200	-.05861	-.04139	
	Equal variances not assumed			-25.000	1.000	.025	-.05000	.00200	-.07541	-.02459	

Table 14: Independent T- Test comparing isolate 413 and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	.	.	-20.000	2	.002	-.04000	.00200	-.04861	-.03139	
	Equal variances not assumed			-20.000	1.000	.032	-.04000	.00200	-.06541	-.01459	

Table 15: Independent T- Test comparing isolate new 5 and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	.	.	-7.444	2	.018	-.03350	.00450	-.05286	-.01414	
	Equal variances not assumed			-7.444	1.000	.085	-.03350	.00450	-.09068	.02368	

Table 16: Independent T- Test comparing isolate new 6 and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	11881334836507430.000	.000	-61.000	2	.000	-.03050	.00050	-.03265	-.02835	
	Equal variances not assumed			-61.000	1.000	.010	-.03050	.00050	-.03685	-.02415	

Table 17: Independent T- Test comparing isolate c 11 and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	28101216869600000.000	.000	-6.143	2	.025	-.02150	.00350	-.03656	-.00644	
	Equal variances not assumed			-6.143	1.000	.103	-.02150	.00350	-.06597	.02297	

Table 18: Independent T- Test comparing isolate c 33 and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	.	.	-6.833	2	.021	-.04100	.00600	-.06682	-.01518	
	Equal variances not assumed			-6.833	1.000	.093	-.04100	.00600	-.11724	.03524	

Table 19: Independent T- Test comparing isolate AO1 and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	118813348365 07430.000	.000	-22.500	2	.002	-.04500	.00200	-.05361	-.03639	
	Equal variances not assumed			-22.500	1.000	.028	-.04500	.00200	-.07041	-.01959	

Table 20: Independent T- Test comparing consortium A and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	.	.	-35.017	2	.001	-.10650	.00304	-.11959	-.09341	
	Equal variances not assumed			-35.017	1.056	.015	-.10650	.00304	-.14066	-.07234	

Table 21: Independent T- Test comparing consortium B and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	.	.	-63.246	2	.000	-.10000	.00158	-.10680	-.09320	
	Equal variances not assumed			-63.246	1.220	.004	-.10000	.00158	-.11327	-.08673	

Table 22: Independent T- Test comparing consortium C and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	.	.	-98.663	2	.000	-.15600	.00158	-.16280	-.14920	
	Equal variances not assumed			-98.663	1.220	.002	-.15600	.00158	-.16927	-.14273	

Table 23: Independent T- Test comparing consortium D and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	160476878709 90540.000	.000	-41.959	2	.001	-.08650	.00206	-.09537	-.07763	
	Equal variances not assumed			-41.959	1.125	.010	-.08650	.00206	-.10674	-.06626	

Table 24: Independent T- Test comparing consortium E and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
rate	Equal variances assumed	191513001127 07040.000	.000	-36.085	2	.001	-.09200	.00255	-.10297	-.08103	
	Equal variances not assumed			-36.085	1.080	.013	-.09200	.00255	-.11925	-.06475	

Table 25: Independent T- Test comparing consortium F and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	297033370912 6863.000	.000	-43.380	2	.001	-.04850	.00112	-.05331	-.04369	
	Equal variances not assumed			-43.380	1.471	.003	-.04850	.00112	-.05542	-.04158	

Table 26: Independent T- Test comparing consortium G and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	134924756653 41790.000	.000	-28.441	2	.001	-.08650	.00304	-.09959	-.07341	
	Equal variances not assumed			-28.441	1.056	.019	-.08650	.00304	-.12066	-.05234	

Table 27: Independent T- Test for CCl_4 biodegradation comparing consortium B and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	399289605581 82130.000	.000	-7.644	2	.017	-35.70800	4.67153	-55.80799	-15.60801	
	Equal variances not assumed			-7.644	1.006	.082	-35.70800	4.67153	-94.28908	22.87308	

Table 28: Independent T- Test for DCM biodegradation comparing consortium C and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
rate	Equal variances assumed	.	.	8.223	2	.014	25.07000	3.04867	11.95262	38.18738
	Equal variances not assumed			8.223	1.892	.017	25.07000	3.04867	11.20482	38.93518

Table 29: Independent T- Test for DCM biodegradation comparing consortium B and consortium D.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
rate	Equal variances assumed	4651155847036630.000	.000	.509	2	.661	4.40000	8.63919	-32.77145	41.57145
	Equal variances not assumed			.509	1.007	.700	4.40000	8.63919	-103.68821	112.48821

Table 30: Independent T- Test for 1, 2-DCA biodegradation comparing consortium B and control.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
rate	Equal variances assumed	9985233214759740.000	.000	15.140	2	.004	61.18920	4.04146	43.80018	78.57822
	Equal variances not assumed			15.140	1.011	.041	61.18920	4.04146	11.18477	111.19363

APPENDIX II:

Figures or tables in parenthesis are indicated within the text.

Table 31: Duplicate gas chromatography peak area values for biodegradation of CCl_4 in biostimulated Type S microcosms (Fig. 3.2).

DAY	GLUC	FRUC	FERT	UNAUTO	AUTO
0	6632	9968	1997	6899	5101
	4976	8296	2443	7869	5699
7	4589	1728	1894	6155	4286
	4883	1952	1418	6013	4522
14	669	0	785	2810	3258
	701	0	901	2976	2968
21	0	0	410	1448	1846
	0	0	396	1544	1944
28	0	0	0	644	896
	0	0	0	578	700

Table 32: Duplicate gas chromatography peak area values for biodegradation of DCM in biostimulated Type S microcosms (Fig. 3.3).

DAY	GLUC	FRUC	FERT	UNAUTO	AUTO
0	7411	7056	3570	5511	5808
	7005	8000	4710	4321	6000
7	6069	4850	2089	4593	4927
	5411	4526	4007	4711	4665
14	2551	2400	2590	4599	4860
	2111	3002	1990	4601	4660
21	2338	2616	1883	4112	4543
	2012	2490	1799	4256	4227
28	2442	2625	1345	3334	3747
	2652	2447	1403	3500	3687

Table 33: Duplicate gas chromatography peak area values for biodegradation of 1, 2-DCA in biostimulated Type S microcosms (Fig. 3.4).

DAY	GLUC	FRUC	FERT	UNAUTO	AUTO
0	5556	6652	4353	5562	3300
	5500	5848	5887	5990	3220
7	3585	4781	4221	4397	2472
	3847	4995	4483	4667	3366
14	1707	2864	4462	3800	2513
	1901	3000	4210	5000	2999
21	1752	2423	3962	3503	2276
	1558	3111	4222	4711	2488
28	1478	2800	3789	3363	1659
	1510	2782	3225	3661	2111

Table 34: Duplicate gas chromatography peak area values for biodegradation of CCl_4 in biostimulated Type C microcosms (Fig. 3.5).

DAY	GLUC	FRUC	FERT	UNAUTO	AUTO
0	3259	5240	3009	5222	6623
	3683	3782	5789	6420	4735
3	2570	2928	4652	3969	3200
	970	2596	3246	4115	3632
6	1652	422	3520	2466	2503
	1748	536	2972	3536	1879
9	1521	132	3522	1111	1674
	1621	82	3876	1059	1236
15	152	0	1639	718	140
	294	0	2475	800	128

Table 35: Duplicate gas chromatography peak area values for biodegradation of DCM in biostimulated Type C microcosms (Fig. 3.6).

DAY	GLUC	FRUC	FERT	UNAUTO	AUTO
0	5002	7200	7226	6199	5922
	5263	5932	6472	5233	9520
3	1548	3741	3049	2142	3580
	1986	3477	2555	2992	3722
6	1240	2600	2442	1738	3600
	1114	2818	2904	3102	3362
9	1058	1599	2300	1762	3411
	1100	2501	2530	1900	3603
15	867	1865	1694	1692	2898
	699	2075	1102	2576	3262

Table 36: Duplicate gas chromatography peak area values for biodegradation of 1, 2 DCA in biostimulated Type C microcosms (Fig. 3.7).

DAY	GLUC	FRUC	FERT	UNAUTO	AUTO
0	5479	4891	4031	5336	3211
	8669	4359	4003	7734	3101
3	2507	3175	1237	1680	2312
	1645	3273	4085	2400	3402
6	1492	2410	2594	1833	2496
	1296	2762	2490	1917	2588
9	1368	3208	1455	1550	2109
	1258	456	2181	1658	2955
15	1269	1247	1135	1669	2222
	1261	1735	1279	2093	2430

Table 37: Duplicate absorbance values for phospholipid phosphates in biostimulated Type S microcosms (Fig.3.8).

DAY	GLUC	FRUC	FERT	CTRL
0	2.560	2.575	2.593	1.476
	2.00	0.600	2.24	0.294
7	1.819	1.81	1.558	0.594
	0.546	0.422	2.256	0.772
14	1.945	1.631	1.402	1.1175
	0.521	2.035	2.634	0.567
21	2.356	1.719	2.298	2.729
	2.702	2.655	2.037	1.412
28	1.288	2.614	2.642	0.889
	2.717	0.998	0.910	0.909

Table 38: Duplicate absorbance values for phospholipid phosphates in biostimulated Type C microcosms (Fig.3.9).

DAY	GLUC	FRUC	FERT	CTRL
0	2.322	2.469	0.923	1.181
	1.228	0.501	0.681	0.945
5	1.931	2.770	1.184	1.551
	1.996	2.156	2.611	2.342
10	2.108	2.655	1.061	1.225
	2.602	2.620	0.905	2.526
15	0.971	1.511	1.355	0.565
	1.131	0.542	2.452	1.158

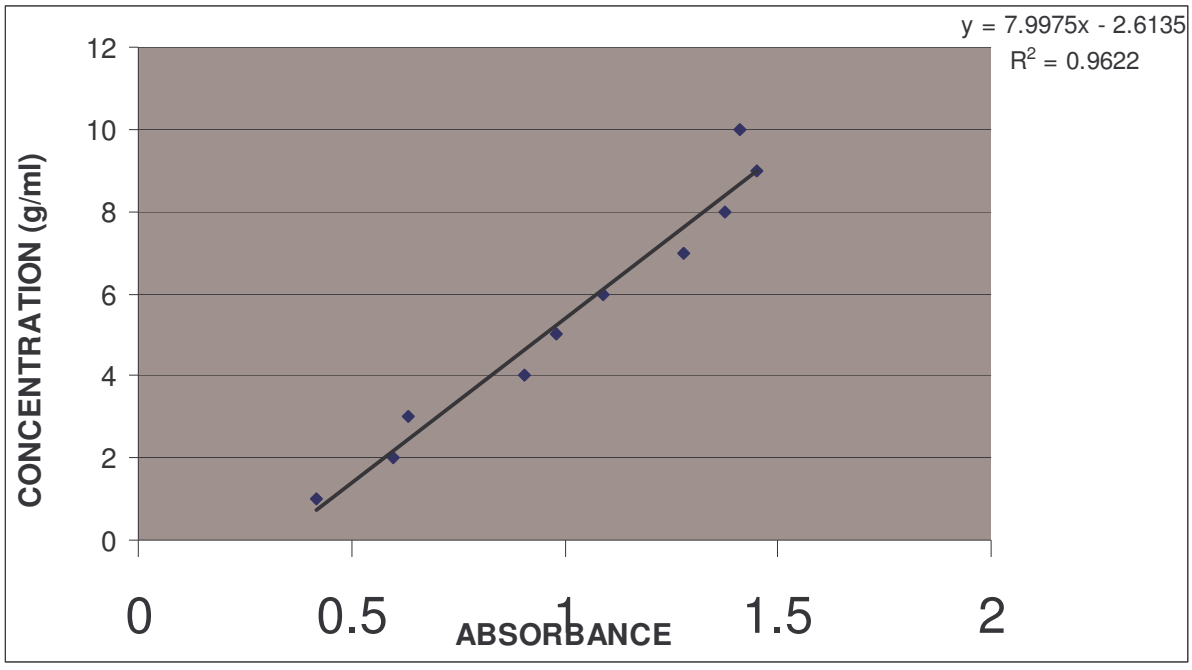


Fig. 9: Standard curve for determination of phospholipid phosphates in soil (Section 3.2.5)

Table 39: Absorbance values for standard curve for estimation of phospholipid phosphates as per Fig. 10.

ABSORBANCE	CONC. (g/ml)	ABSORBANCE	CONC. (g/ml)
0.419	1	1.091	6
0.599	2	1.28	7
0.634	3	1.376	8
0.904	4	1.451	9
0.979	5	1.412	10

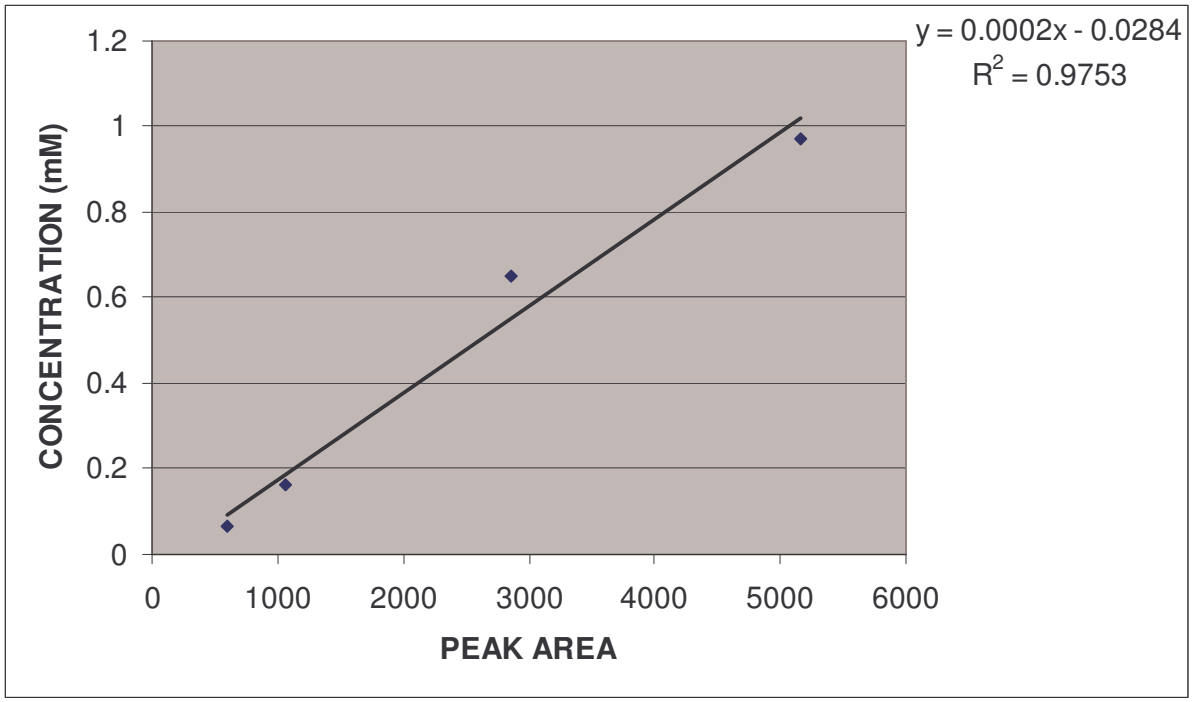


Fig. 10: Standard curve for determination of CCl_4 in Type S microcosms (Section 3.2.2).

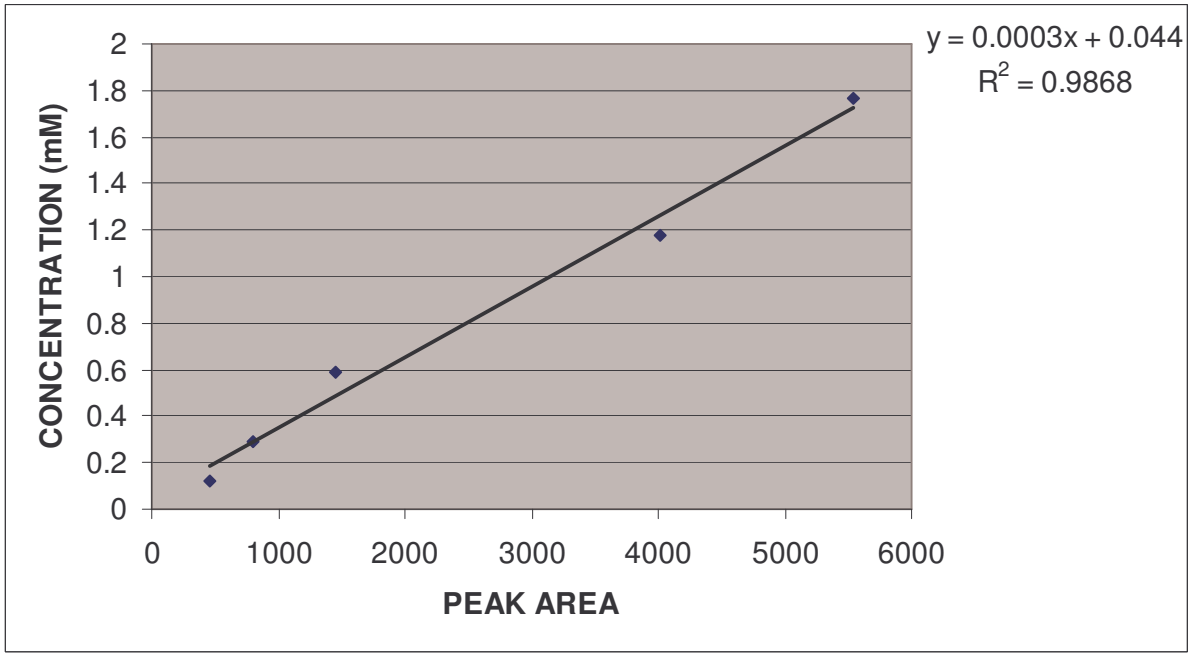


Fig. 11: Standard curve for determination of DCM concentration in Type S microcosms (Section 3.2.2).

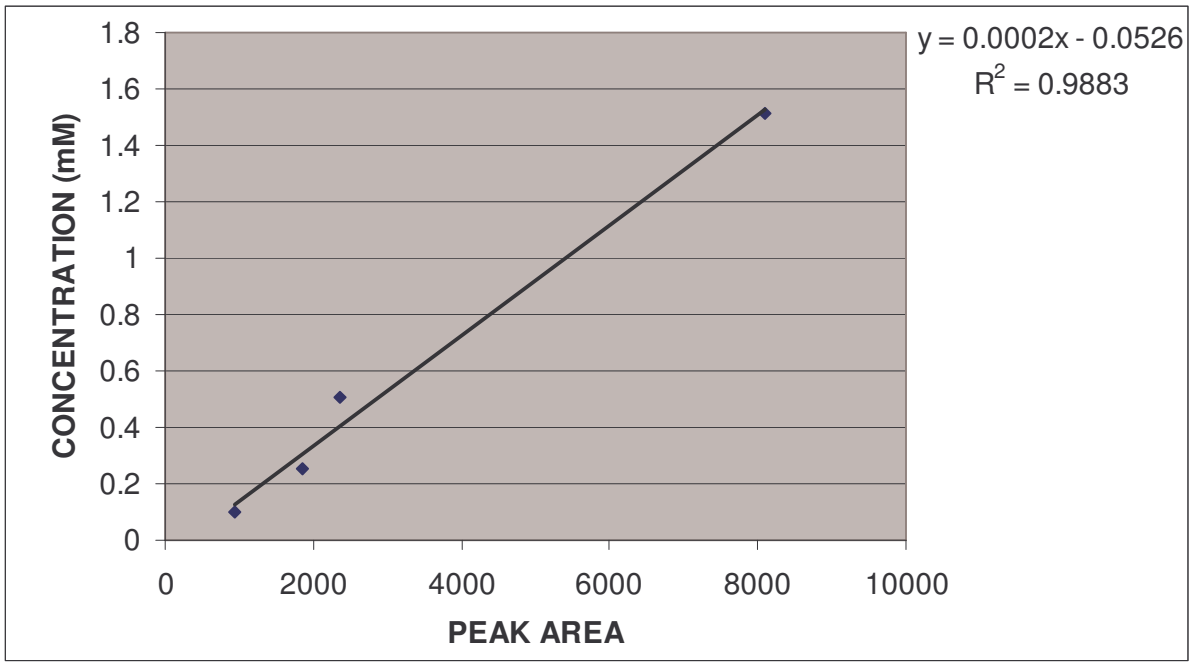


Fig. 12: Standard curve for determination of 1, 2- DCA concentration in Type S microcosms (Section 3.2.2)

Table 40: Gas chromatography peak area values for construction of standard curve in Type S microcosms as per Figs. 3.2, 3.3 and 3.4.

CCl_4			DCM			1, 2-DCA		
VOL (µl)	**PEAK AREA**	**CONC. (mM)**	**VOL (µl)**	**PEAK AREA**	**CONC. (mM)**	**VOL (µl)**	**PEAK AREA**	**CONC. (mM)**
1	600	0.066	1	462.5	0.1176	1	936	0.1008
2.5	1066	0.162	2.5	796.75	0.294	2.5	1862	0.252
10	2853	0.649	5	1447	0.588	5	2355	0.505
15	5164	0.972	15	5542	1.764	15	8097	1.512

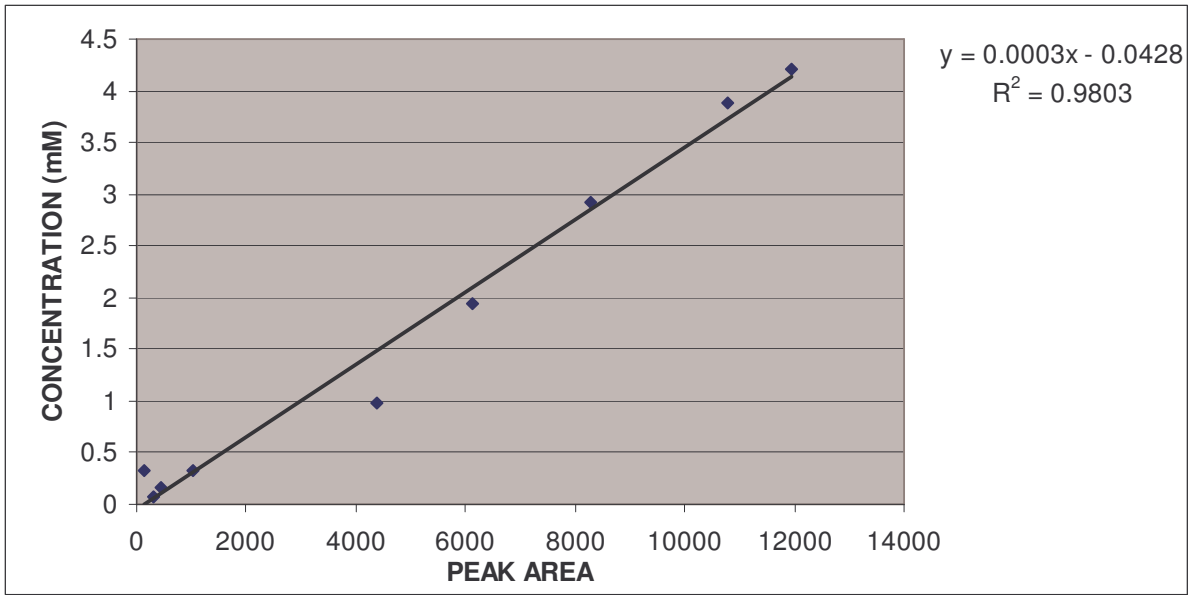


Fig. 13: Standard curve for determination of CCl_4 concentration in Type C microcosms (Section 3.2.3).

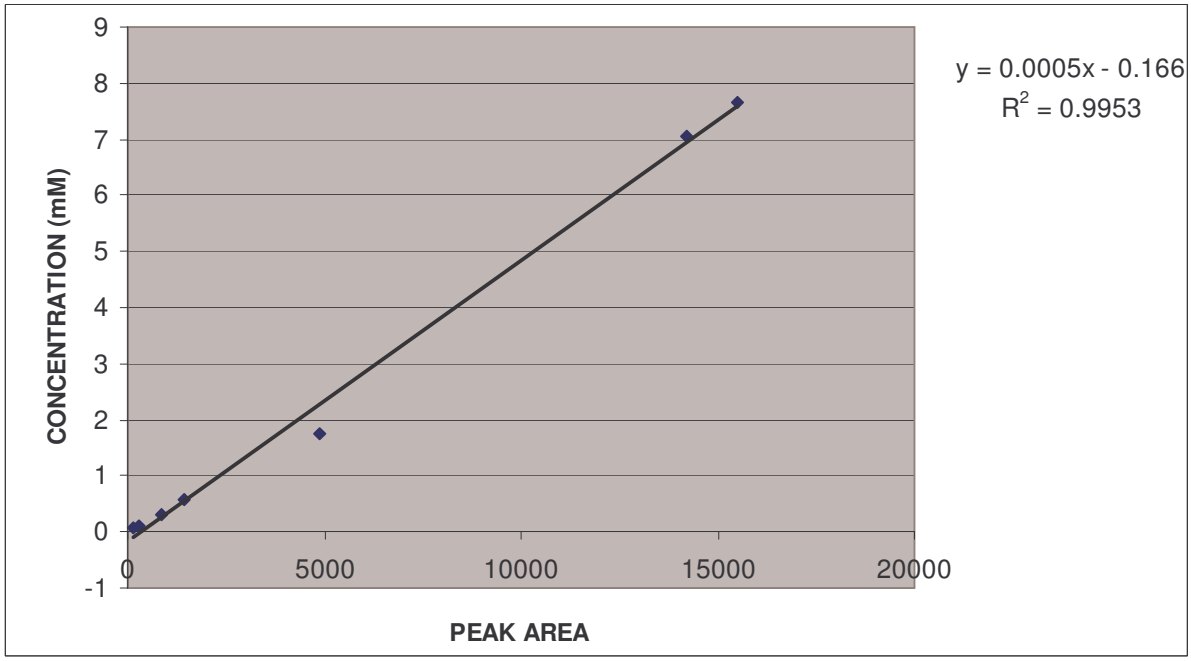


Fig. 14: Standard curve for of determination DCM concentration in Type C microcosms (Section 3.2.3).

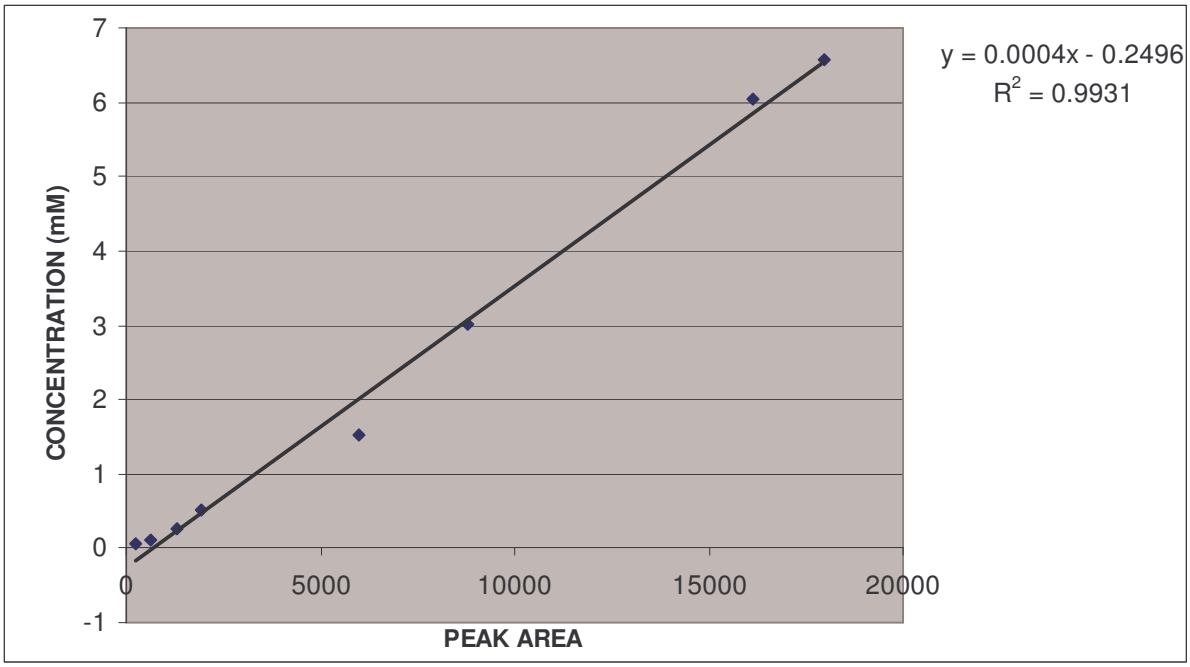


Fig. 15: Standard curve for determination of 1, 2-DCA concentration in Type C microcosms (Section 3.2.3).

Table 41: Gas chromatography peak area values for CCl_4 standard curve in Type C microcosms as per Fig.3.5.

PEAK AREA	CONC. (mM)	VOLUME (µl)	PEAK AREA	CONC. (mM)	VOLUME (µl)
130	0.33	0.5	6131	1.944	30
314	0.066	1	8289	2.916	45
462	0.162	2.5	10794	3.888	60
1039	0.324	5	11951	4.212	65
4375	0.972	15			

Table 42: Gas chromatography peak area values for DCM standard curve in Type C microcosms as per Fig. 3.6.

PEAK AREA	CONC. (mM)	VOLUME (µl)	PEAK AREA	CONC. (mM)	VOLUME (µl)
128	0.0588	0.5	4878	1.764	15
311	0.1176	1	14203	7.056	60
855	0.294	2.5	15520	7.644	65
1453	0.588	5			

Table 43: Gas chromatography peak area values for 1, 2 DCA standard curve in Type C microcosms as per Fig.3.7.

PEAK AREA	CONC. (mM)	VOLUME (µl)	PEAK AREA	CONC. (mM)	VOLUME (µl)
244	0.0504	0.5	5981	1.512	15
616	0.1008	1	8800	3.024	30
1282	0.252	2.5	16121	6.048	60
1952	0.505	5	17953	6.565	65

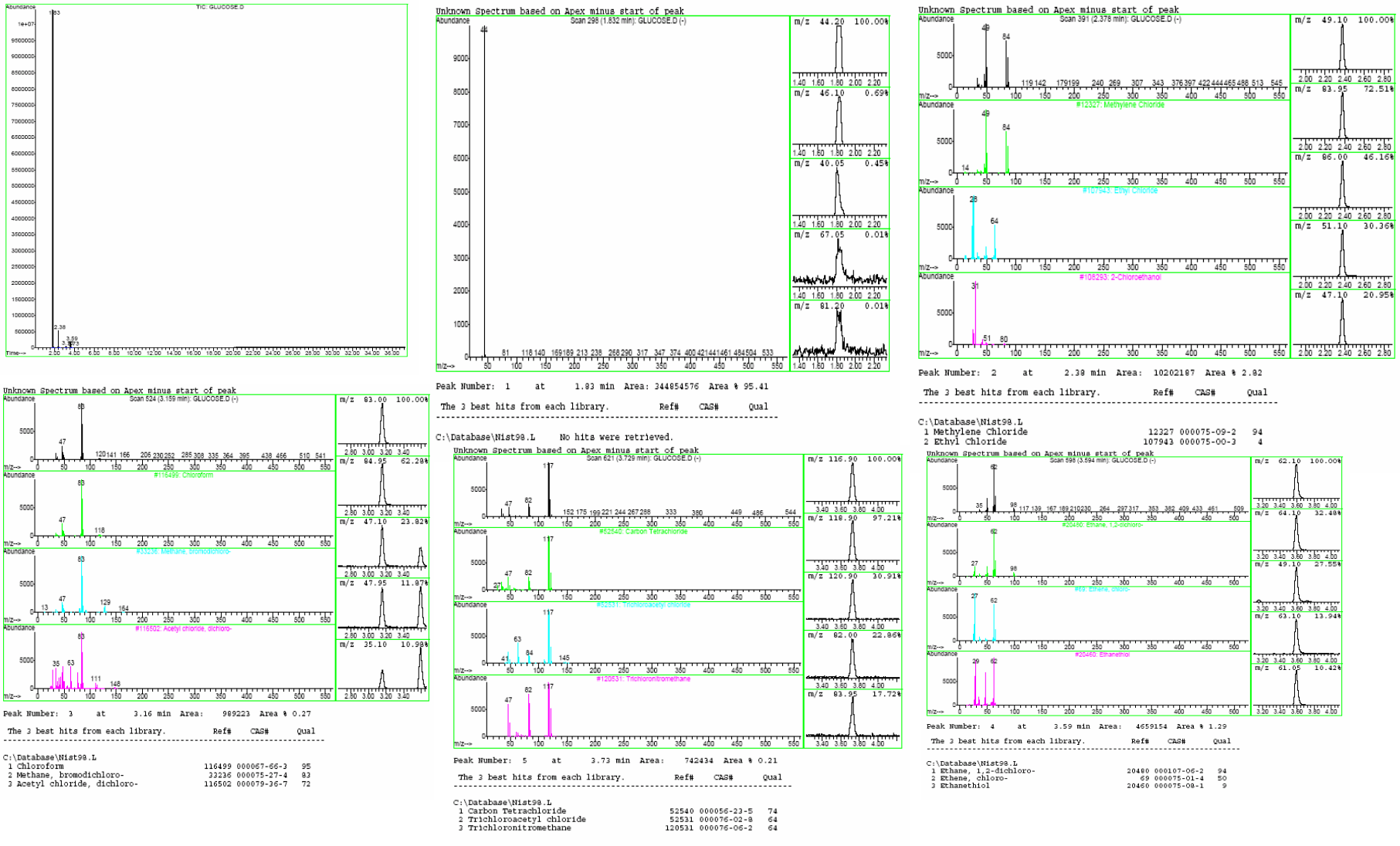


Fig 16: GC-MS spectra for Type S microcosms with fructose (Table: 3.1).

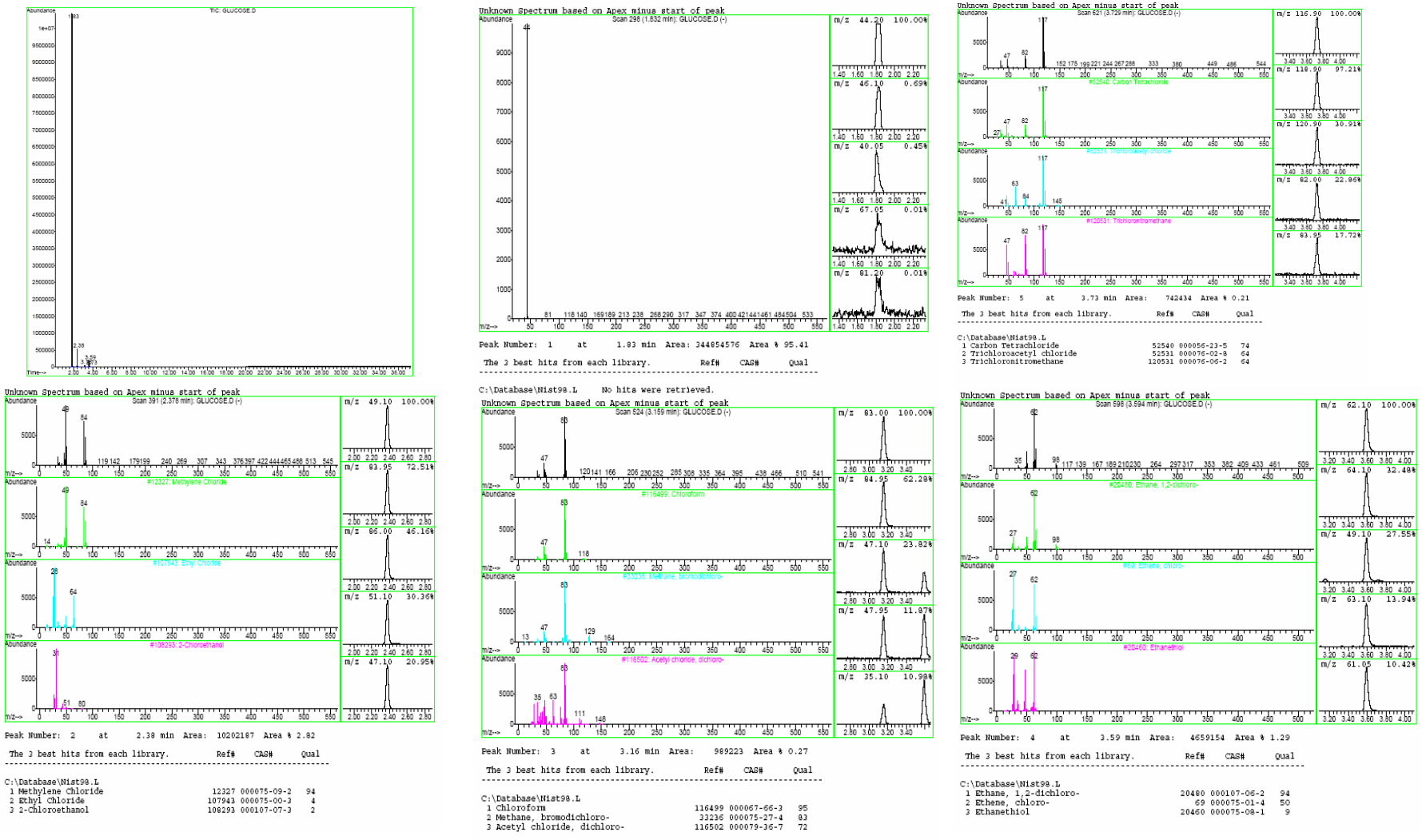


Fig. 17: GC-MS spectra for Type S microcosms with glucose (Table: 3.1).

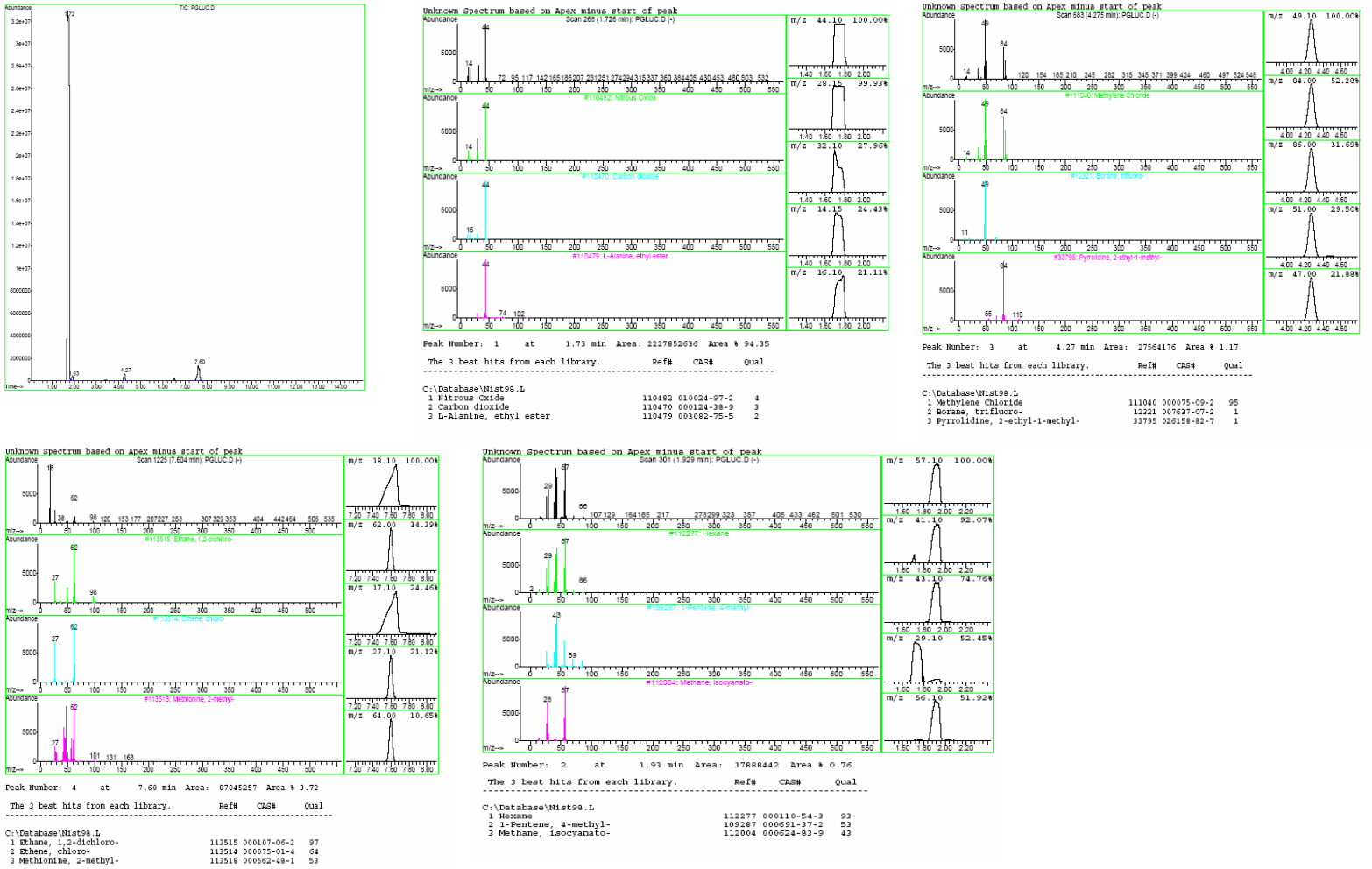


Fig. 18: GC-MS spectra for Type C microcosms with glucose (Table: 3.1).

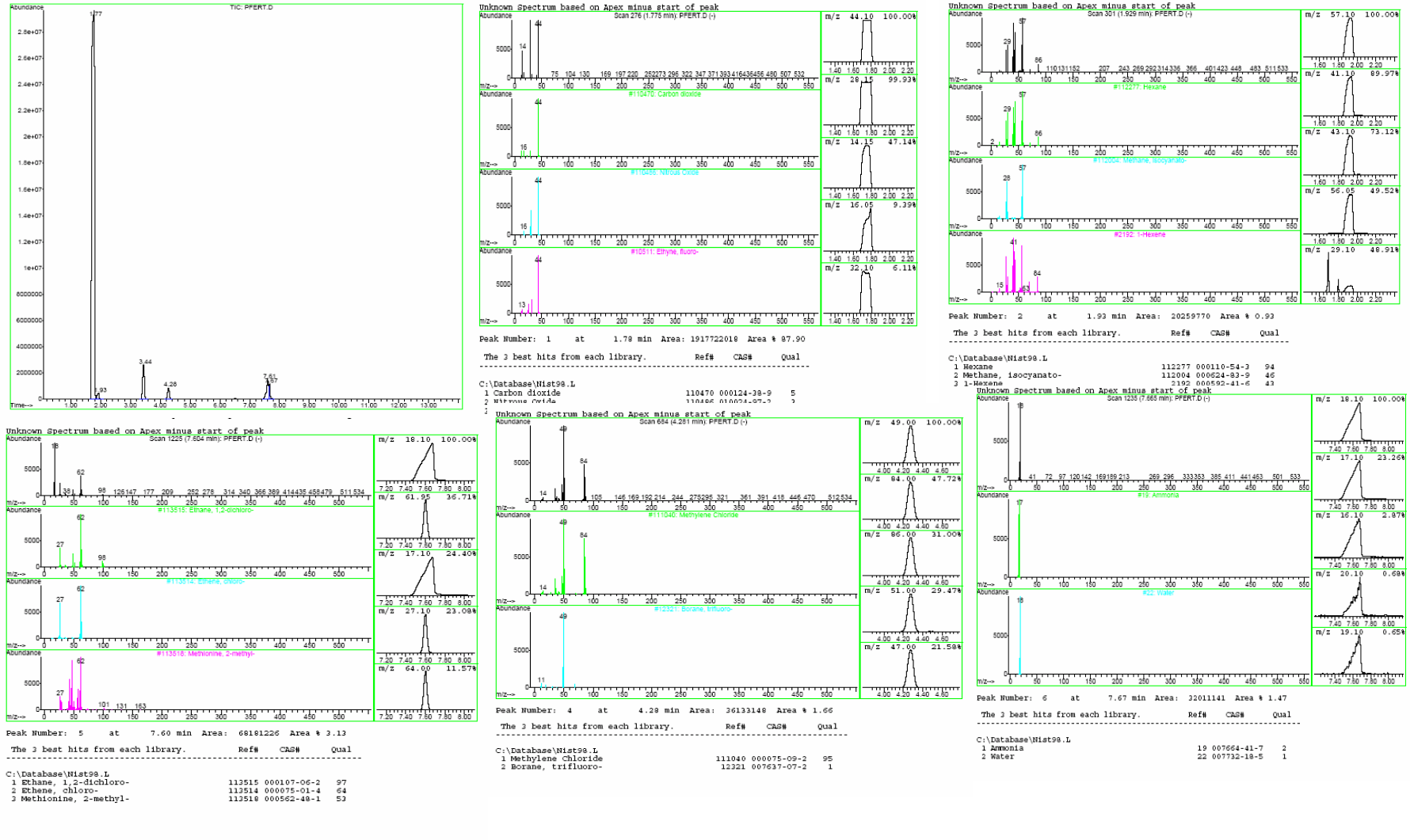


Fig. 19: GC-MS spectra for Type C microcosms with fertiliser (Table: 3.1).

Data present in Tables 43-86 appear in Sections 3.3.1 and 3.3.2

Table 43: Independent T- Test data for CCl_4 biodegradation comparing glucose with autoclaved soil in Type S microcosms at day 14.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	534208449843 6140.000	.000	7.542	2	.017	47.000	6.232	20.186	73.814
	Equal variances not assumed			7.542	1.164	.063	47.000	6.232	-10.150	104.150

Table 44: Independent T- Test data for CCl_4 biodegradation comparing glucose with fertilizer in Type S microcosms at day 14.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	752246372337 0470.000	.000	12.528	2	.006	23.980	1.914	15.744	32.216
	Equal variances not assumed			12.528	1.451	.019	23.980	1.914	11.907	36.053

Table 45: Independent T- Test data for CCl_4 biodegradation comparing glucose with unautoclaved soil in Type S microcosms at day 14.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	907025857638 158.000	.000	12.713	2	.006	28.235	2.221	18.679	37.791
	Equal variances not assumed			12.713	1.923	.007	28.235	2.221	18.305	38.165

Table 46: Independent T- Test data for CCl_4 biodegradation comparing fertiliser with unautoclaved soil in Type S microcosms at day 14.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	309766537450 2832.000	.000	2.599	2	.122	4.255	1.637	-2.788	11.298	
	Equal variances not assumed			2.599	1.634	.149	4.255	1.637	-4.539	13.049	

Table 47: Independent T- Test data for CCl_4 biodegradation comparing fertiliser with autoclaved soil in Type S microcosms at day 14.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	106836102472 73800.000	.000	9.491	2	.011	56.850	5.990	31.077	82.623	
	Equal variances not assumed			9.491	1.000	.067	56.850	5.990	-19.260	132.960	

Table 48: Independent T- Test data for CCl_4 biodegradation comparing glucose with autoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	135349324572 57500.000	.000	.897	2	.465	2.780	3.101	-10.562	16.122	
	Equal variances not assumed			.897	1.742	.476	2.780	3.101	-12.643	18.203	

Table 49: Independent T- Test data for DCM biodegradation comparing glucose with autoclaved soil in Type S microcosms at day 14 and day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	.	.	.247	2	.828	1.205	4.872	-19.760	22.170	
	Equal variances not assumed			.247	1.459	.835	1.205	4.872	-29.287	31.697	

Table 50: Independent T- Test data for DCM biodegradation comparing glucose with autoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	455027104418 92200.000	.000	6.300	2	.024	29.130	4.624	9.237	49.023	
	Equal variances not assumed			6.300	1.235	.069	29.130	4.624	-8.775	67.035	

Table 51: Independent T- Test data for DCM biodegradation comparing glucose with unautoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	132998093839 86020.000	.000	3.387	2	.077	36.515	10.780	-9.869	82.899	
	Equal variances not assumed			3.387	1.379	.126	36.515	10.780	-36.981	110.011	

Table 52: Independent T- Test data for DCM biodegradation comparing glucose with fertiliser in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	410562545657 50.080	.000	.212	2	.851	1.275	6.003	-24.552	27.102
	Equal variances not assumed			.212	1.993	.852	1.275	6.003	-24.641	27.191

Table 53: Independent T- Test data for DCM biodegradation comparing glucose with fructose in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	.161	2	.887	.930	5.785	-23.959	25.819
	Equal variances not assumed			.161	1.961	.887	.930	5.785	-24.442	26.302

Table 54: Independent T- Test data for 1, 2 DCA biodegradation comparing glucose with fructose in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	249608637070 89370.000	.000	5.899	2	.028	16.765	2.842	4.537	28.993
	Equal variances not assumed			5.899	1.046	.099	16.765	2.842	-15.820	49.350

Table 55: Independent T- Test data for 1, 2 DCA biodegradation comparing glucose with fertiliser in Type S microcosms at day 28

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	262537179968 87440.000	.000	2.697	2	.114	41.910	15.541	-24.957	108.777
	Equal variances not assumed			2.697	1.001	.226	41.910	15.541	-154.859	238.679

Table 56: Independent T- Test data for 1, 2 DCA biodegradation comparing fertiliser with fructose in Type S microcosms at day 28

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	181806042956 03440.000	.000	1.593	2	.252	25.145	15.787	-42.781	93.071
	Equal variances not assumed			1.593	1.065	.346	25.145	15.787	-148.580	198.870

Table 57: Independent T- Test data for 1, 2 DCA biodegradation comparing glucose with autoclaved soil in Type S microcosms at day 28

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	501175787737 9280000.000	.000	4.322	2	.050	30.720	7.108	.138	61.302
	Equal variances not assumed			4.322	1.007	.143	30.720	7.108	-58.086	119.526

Table 58: Independent T- Test data for 1, 2 DCA biodegradation comparing glucose with unautoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	191848954663385400.000	.000	16.489	2	.004	29.375	1.781	21.710	37.040
	Equal variances not assumed			16.489	1.120	.028	29.375	1.781	11.746	47.004

Table 59: Independent T- Test data for 1, 2 DCA biodegradation comparing fertiliser with unautoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	532581244741 9520.000	.000	3.821	2	.062	12.610	3.300	-1.588	26.808
	Equal variances not assumed			3.821	1.663	.082	12.610	3.300	-4.737	29.957

Table 60: Independent T- Test data for 1, 2 DCA biodegradation comparing fructose with autoclaved soil in Type S microcosms at day 28

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	838378263501 97300.000	.000	1.829	2	.209	13.955	7.631	-18.879	46.789
	Equal variances not assumed			1.829	1.306	.271	13.955	7.631	-42.828	70.738

Table: 61 Independent T- Test data for CCl_4 biodegradation comparing glucose with fructose in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	299239064261 04740.000	.000	-5.687	2	.030	-3.640	.640	-6.394	-.886	
	Equal variances not assumed			-5.687	1.000	.111	-3.640	.640	-11.772	4.492	

Table 62: Independent T- Test data for CCl_4 biodegradation comparing glucose with fertiliser in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	190547560640 202800.000	.000	8.795	2	.013	20.615	2.344	10.529	30.701	
	Equal variances not assumed			8.795	1.160	.053	20.615	2.344	-1.016	42.246	

Table 63: Independent T- Test data for CCl_4 biodegradation comparing glucose with unautoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	164833529965 57390.000	.000	11.386	2	.008	7.525	.661	4.681	10.369	
	Equal variances not assumed			11.386	1.132	.042	7.525	.661	1.126	13.924	

Table 64: Independent T- Test data for CCl_4 biodegradation comparing glucose with autoclaved soil in Type C microcosms at day 15

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	299239064261 04740.000	.000	-5.687	2	.030	-3.640	.640	-6.394	-.886	
	Equal variances not assumed			-5.687	1.000	.111	-3.640	.640	-11.772	4.492	

Table 65: Independent T- Test data for CCl_4 biodegradation comparing fructose with unautoclaved soil in Type C microcosms at day 15

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	.	.	67.667	2	.000	11.165	.165	10.455	11.875	
	Equal variances not assumed			67.667	1.000	.009	11.165	.165	9.068	13.262	

Table 66: Independent T- Test data for DCM biodegradation comparing glucose with fructose in Type C microcosms at day 15

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	.	.	10.220	2	.009	22.610	2.212	13.091	32.129	
	Equal variances not assumed			10.220	1.415	.027	22.610	2.212	8.124	37.096	

Table 67: Independent T- Test data for DCM biodegradation comparing fructose with fertiliser in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	.	.	-4.272	2	.051	-15.910	3.724	-31.934	.114	
	Equal variances not assumed			-4.272	1.134	.124	-15.910	3.724	-51.868	20.048	

Table 68: Independent T- Test data for DCM biodegradation comparing glucose with unautoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	.	.	16.141	2	.004	35.440	2.196	25.993	44.887	
	Equal variances not assumed			16.141	1.383	.015	35.440	2.196	20.550	50.330	

Table 69: Independent T- Test data for DCM biodegradation comparing glucose with autoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	.	.	9.916	2	.010	33.230	3.351	18.812	47.648	
	Equal variances not assumed			9.916	1.851	.013	33.230	3.351	17.638	48.822	

Table 70: Independent T- Test data for DCM biodegradation comparing fertiliser with unautoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	7.737	2	.016	28.740	3.714	12.758	44.722
	Equal variances not assumed			7.737	1.123	.065	28.740	3.714	-7.847	65.327

Table 71: Independent T- Test data for DCM biodegradation comparing fertiliser with autoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	5.902	2	.028	26.530	4.495	7.189	45.871
	Equal variances not assumed			5.902	1.848	.033	26.530	4.495	5.587	47.473

Table 72: Independent T- Test data for 1,2-DCA biodegradation comparing fertiliser with autoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	162863145875994700.000	.000	9.390	2	.011	50.800	5.410	27.523	74.077
	Equal variances not assumed			9.390	1.366	.033	50.800	5.410	13.385	88.215

Table 73: Independent T- Test data for 1,2-DCA biodegradation comparing fertiliser with unautoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	262162231606 22160.000	.000	1.864	2	.203	4.265	2.288	-5.580	14.110
	Equal variances not assumed			1.864	1.241	.274	4.265	2.288	-14.349	22.879

Table 74: Independent T- Test data for 1,2-DCA biodegradation comparing glucose with autoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	828093076443 2710.000	.000	9.561	2	.011	56.775	5.938	31.225	82.325
	Equal variances not assumed			9.561	1.730	.017	56.775	5.938	26.989	86.561

Table 75: Independent T- Test data for 1,2-DCA biodegradation comparing glucose with unautoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	168405060627 12270.000	.000	3.056	2	.092	10.240	3.351	-4.179	24.659
	Equal variances not assumed			3.056	1.107	.182	10.240	3.351	-23.769	44.249

Table 76: Independent T- Test data for 1,2-DCA biodegradation comparing fructose with fertiliser in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	942466589955 10200.000	.000	-5.944	2	.027	-28.550	4.803	-49.216	-7.884
	Equal variances not assumed			-5.944	1.476	.053	-28.550	4.803	-58.104	1.004

Table 77: Independent T- Test data for 1,2-DCA biodegradation comparing fructose with glucose in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	302822038944 3913.000	.000	6.404	2	.024	34.525	5.391	11.329	57.721
	Equal variances not assumed			6.404	1.867	.028	34.525	5.391	9.677	59.373

Table 78: Independent T- Test data for phospholipid phosphate analysis comparing addition of glucose at day 14 and 21 in Type S microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
rate	Equal variances assumed	689769087201 4380000.000	.000	-7.113	2	.019	-1.24900	.17558	-2.00447	-.49353
	Equal variances not assumed			-7.113	1.060	.080	-1.24900	.17558	-3.20226	.70426

Table 79: Independent T- Test data for phospholipid phosphate analysis comparing addition of fertiliser at day 14 and 21 in Type S microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
rate	Equal variances assumed	.	.	-.856	2	.482	-.14950	.17460	-.90076	.60176
	Equal variances not assumed			-.856	1.973	.483	-.14950	.17460	-.91074	.61174

Table 80: Independent T- Test data for phospholipid phosphate analysis comparing addition of glucose at day 7 and 14 in Type S microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
rate	Equal variances assumed	338866059052 3347.000	.000	4.994	2	.038	1.07200	.21467	.14834	1.99566
	Equal variances not assumed			4.994	1.753	.049	1.07200	.21467	.01195	2.13205

Table 81: Independent T- Test data for phospholipid phosphate analysis comparing addition of glucose at day 0 and 15 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
rate	Equal variances assumed	167779255813 00630.000	.000	1.310	2	.321	.72400	.55282	-1.65459	3.10259
	Equal variances not assumed			1.310	1.043	.408	.72400	.55282	-5.65483	7.10283

Table 82: Independent T- Test data for phospholipid phosphate analysis comparing addition of fructose at day 0 and 10 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
rate	Equal variances assumed	724728467435 4820.000	.000	-24.679	2	.002	-1.07750	.04366	-1.26536	-.88964
	Equal variances not assumed			-24.679	1.369	.009	-1.07750	.04366	-1.37835	-.77665

Table 83: Independent T- Test data for phospholipid phosphate analysis comparing addition of fructose at day 15 and 10 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
rate	Equal variances assumed	121291570406 69170.000	.000	5.970	2	.027	1.31750	.22069	.36793	2.26707
	Equal variances not assumed			5.970	1.013	.104	1.31750	.22069	-1.40523	4.04023

Table 84: Independent T- Test data for phospholipid phosphate analysis comparing addition of glucose at day 0 and 5 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
rate	Equal variances assumed	210475958424 03770.000	.000	-.344	2	.764	-.18850	.54796	-2.54620	2.16920
	Equal variances not assumed			-.344	1.007	.789	-.18850	.54796	-7.03675	6.65975

Table 85: Independent T- Test data for phospholipid phosphate analysis comparing addition of fertilizer with unautoclaved control at day 10 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	377052898512 521.900	.000	.033	2	.977	.02800	.85088	-3.63306	3.68906
	Equal variances not assumed			.033	1.945	.977	.02800	.85088	-3.73501	3.79101

Table 86: Independent T- Test data for phospholipid phosphate analysis comparing addition of fertilizer day 0 and day 5 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	163814466072 17010.000	.000	-10.467	2	.009	-1.14450	.10934	-1.61495	-.67405
	Equal variances not assumed			-10.467	1.168	.043	-1.14450	.10934	-2.14090	-.14810

APPENDIX III:

Figures or tables in parenthesis are indicated within the text.

Table 86: Duplicate gas chromatography peak area values for biodegradation of CCl_4 in bioaugmented Type S microcosms (Fig. 4.1).

DAY	B	C	D	UNAUTO	AUTO
0	3520	1732	2687	6996	2329
	3170	1814	2001	6988	3001
7	1268	1688	652	2303	2067
	1322	1844	576	2553	2555
14	0	0	0	0	192
	0	0	0	0	198
21	0	0	0	0	0
	0	0	0	0	0
28	0	0	0	0	0
	0	0	0	0	0

Table 87: Duplicate gas chromatography peak area values for biodegradation of DCM in bioaugmented Type S microcosms (Fig. 4.2).

DAY	B	C	D	UNAUTO	AUTO
0	4296	6676	3682	5030	8902
	4882	7110	3600	5122	9002
7	2984	6294	3254	6901	7098
	3570	5110	2000	5889	9002
14	2392	4369	1856	4385	6275
	2226	3991	1300	3677	4665
21	1233	2460	453	2970	3877
	1003	2574	299	2602	3535
28	714	2285	196	2032	2124
	852	1999	150	1996	4112

Table 88: Duplicate gas chromatography peak area values for biodegradation of 1, 2-DCA in bioaugmented Type S microcosms (Fig. 4.3).

DAY	B	C	D	UNAUTO	AUTO
0	5488	7367	6070	6074	7203
	7010	8001	8120	7910	6497
7	1820	5645	3017	3866	6114
	1988	4999	2333	6402	6078
14	1925	2050	1255	2146	2512
	1777	2134	999	1746	3102
21	1138	2158	421	1732	2717
	1234	2000	501	1930	3047
28	730	2254	392	1588	2454
	900	1520	362	1940	2980

Table 89: Duplicate gas chromatography peak area values for biodegradation of CCl_4 in biostimulated and bioaugmented Type S microcosms (Fig. 4.1).

DAY	GLUC + D	FRUC + D	UNAUTO	AUTO
0	5999	7660	6989	10446
	5785	7910	8103	10314
7	0	1237	2373	4375
	0	778	2301	6107
14	0	0	1196	3333
	0	0	1820	4791
21	0	0	0	1624
	0	0	0	2648
28	0	0	0	1161
	0	0	0	1503

Table 90: Duplicate gas chromatography peak area values for biodegradation of DCM in biostimulated and bioaugmented Type S microcosms (Fig. 4.2).

DAY	GLUC + D	FRUC + D	UNAUTO	AUTO
0	8081	8806	7124	8580
	7103	9100	8402	9074
7	4012	6009	4721	5163
	3970	5699	5555	6103
14	2052	3142	4386	4162
	3000	3620	4962	6410
21	1067	2523	3770	4790
	2941	1437	4690	5034
28	1611	1322	3145	4755
	1479	2579	3089	4107

Table 91: Duplicate gas chromatography peak area values for biodegradation of 1,2-DCA in biostimulated and bioaugmented Type S microcosms (Fig. 4.3).

DAY	GLUC + D	FRUC + D	UNAUTO	AUTO
0	6916	7649	8250	7542
	7800	8201	8902	9014
7	4302	1237	2373	4375
	5998	778	2301	6107
14	0	0	1196	3333
	0	0	1820	4791
21	0	0	0	1624
	0	0	0	2648
28	0	0	0	1161
	0	0	0	1503

Table 92: Duplicate gas chromatography peak area values for biodegradation of CCl_4 in bioaugmented Type C microcosms (Fig. 4.4).

DAY	B	C	D	UNAUTO	AUTO
0	5222	725	7499	5222	6623
	6106	5589	5873	6420	4735
3	6421	4263	5866	3969	3200
	3613	4877	6200	4115	3632
6	4840	3956	5182	2466	2503
	4522	5000	5210	3536	1879
9	2344	4785	4521	1111	1674
	2434	3425	4609	1059	1236
15	0	667	500	718	140
	0	411	862	800	128

Table 93: Duplicate gas chromatography peak area values for biodegradation of DCM in bioaugmented Type C microcosms (Fig. 4.5).

DAY	B	C	D	UNAUTO	AUTO
0	5916	5133	4533	6199	5922
	6000	4717	5017	5233	9520
3	5522	1286	3903	2142	3580
	5432	5946	4561	2992	3722
6	4799	3522	3394	1738	3600
	4837	3214	3320	3102	3362
9	2996	3551	4511	1762	3411
	4148	3248	3673	1900	3603
15	350	3555	3265	1692	2898
	588	2605	2861	2576	3262

Table 94: Duplicate gas chromatography peak area values for biodegradation of 1, 2-DCA in bioaugmented Type C microcosms (Fig. 4.6).

DAY	B	C	D	UNAUTO	AUTO
0	3500	3514	8284	5336	3211
	3652	3118	6550	7734	3101
3	2786	2401	3119	1680	2312
	2000	3699	4545	2400	3402
6	2584	3030	3633	1833	2496
	1998	2900	3553	1917	2588
9	2335	3400	2889	1550	2109
	2113	2466	3303	1658	2955
15	1852	2253	2447	1669	2222
	2560	2175	3449	2093	2430

Table 95: Duplicate gas chromatography peak area values for biodegradation of CCl_4 in biostimulated and bioaugmented Type C microcosms (Fig. 4.7).

DAY	GLUC + B	FTL + B	UNAUTO	AUTO
0	3328	3334	5215	4615
	3300	5888	6335	6387
3	2409	4477	3963	1009
	2555	4345	6111	4055
6	362	3110	3296	1994
	456	3394	3516	2610
9	0	1968	1764	775
	0	3652	1428	811
15	0	566	0	0
	0	816	0	0

Table 96: Duplicate gas chromatography peak area values for biodegradation of DCM in biostimulated and bioaugmented Type C microcosms (Fig. 4.8).

DAY	GLUC + B	FTL + B	UNAUTO	AUTO
0	3660	3500	5868	4606
	5360	4026	8202	5872
3	4118	2551	5518	4207
	3994	3675	5420	5877
6	2935	2544	2143	4301
	3221	2896	6955	5349
9	2500	1960	5236	3408
	2330	2618	2652	3900
15	1899	2009	1990	3170
	1685	3503	3556	3200

Table 97: Duplicate gas chromatography peak area values for biodegradation of 1, 2-DCA in biostimulated and bioaugmented Type C microcosms (Fig. 4.9).

DAY	GLUC + B	FTL + B	UNAUTO	AUTO
0	5662	3332	2535	6998
	5224	2114	6503	5920
3	2956	2151	2999	3331
	3000	2625	3937	3149
6	3222	1503	3528	2912
	2316	2777	2448	3000
9	1842	1707	2372	2819
	2448	1461	2696	3007
15	1651	122	1899	1966
	2079	120	2257	2462

Table 98: Duplicate absorbance values for phospholipid phosphates in bioaugmented Type S microcosms (Fig. 4.13).

DAY	B	C	D	CTRL
0	2.378	1.327	0.675	1.476
	1.481	2.558	0.992	0.294
7	0.508	1.558	0.589	0.594
	2.207	0.799	0.821	0.772
14	1.024	0.82	2.534	1.1175
	0.798	0.673	2.650	0.567
21	1.577	0.507	0.540	2.729
	1.923	1.002	0.967	1.412
28	1.237	0.811	1.142	0.889
	0.443	0.814	2.091	0.909

Table 99: Duplicate absorbance values for phospholipid phosphates in bioaugmented Type C microcosms (Fig. 4.15).

DAY	B	C	D	CTRL
0	1.138	0.771	1.354	1.181
	0.621	2.645	1.040	0.945
5	1.402	0.712	1.214	1.551
	1.632	0.859	1.148	2.342
10	1.923	0.423	1.033	1.225
	0.664	1.396	2.473	2.526
15	1.057	0.907	0.809	0.565
	1.243	1.098	2.581	1.158

Table 100: Duplicate absorbance values for phospholipid phosphates in biostimulated and bioaugmented Type S microcosms (Fig. 4.14).

DAY	GLUC + D	FRUC + D	CTRL
0	1.357	1.731	1.476
	2.503	2.563	0.294
7	1.471	0.803	0.594
	2.553	0.825	0.772
14	2.507	0.850	1.1175
	2.528	2.544	0.567
21	2.482	2.537	2.729
	2.516	2.362	1.412
28	1.785	2.342	0.889
	2.539	2.495	0.909

Table 101: Duplicate absorbance values for phospholipid phosphates in biostimulated and bioaugmented Type C microcosms (Fig. 4.15).

DAY	GLUC + B	FTL + B	CTRL
0	1.230	1.155	1.181
	1.178	1.695	0.945
5	1.278	2.156	1.551
	2.585	2.430	2.342
10	1.653	1.635	1.225
	0.818	0.874	2.526
15	0.918	1.182	0.565
	0.787	1.334	1.158

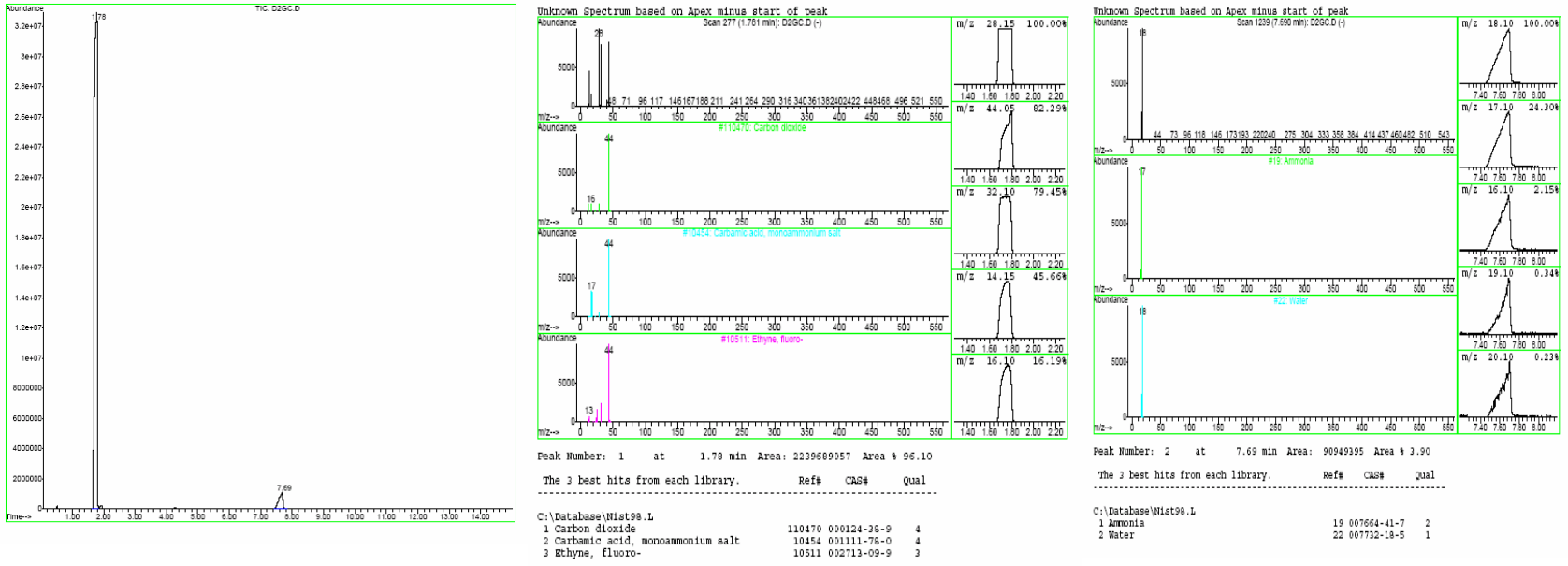


Fig. 20: GC- MS spectra for Type S microcosms with consortium D (Table: 4.1)

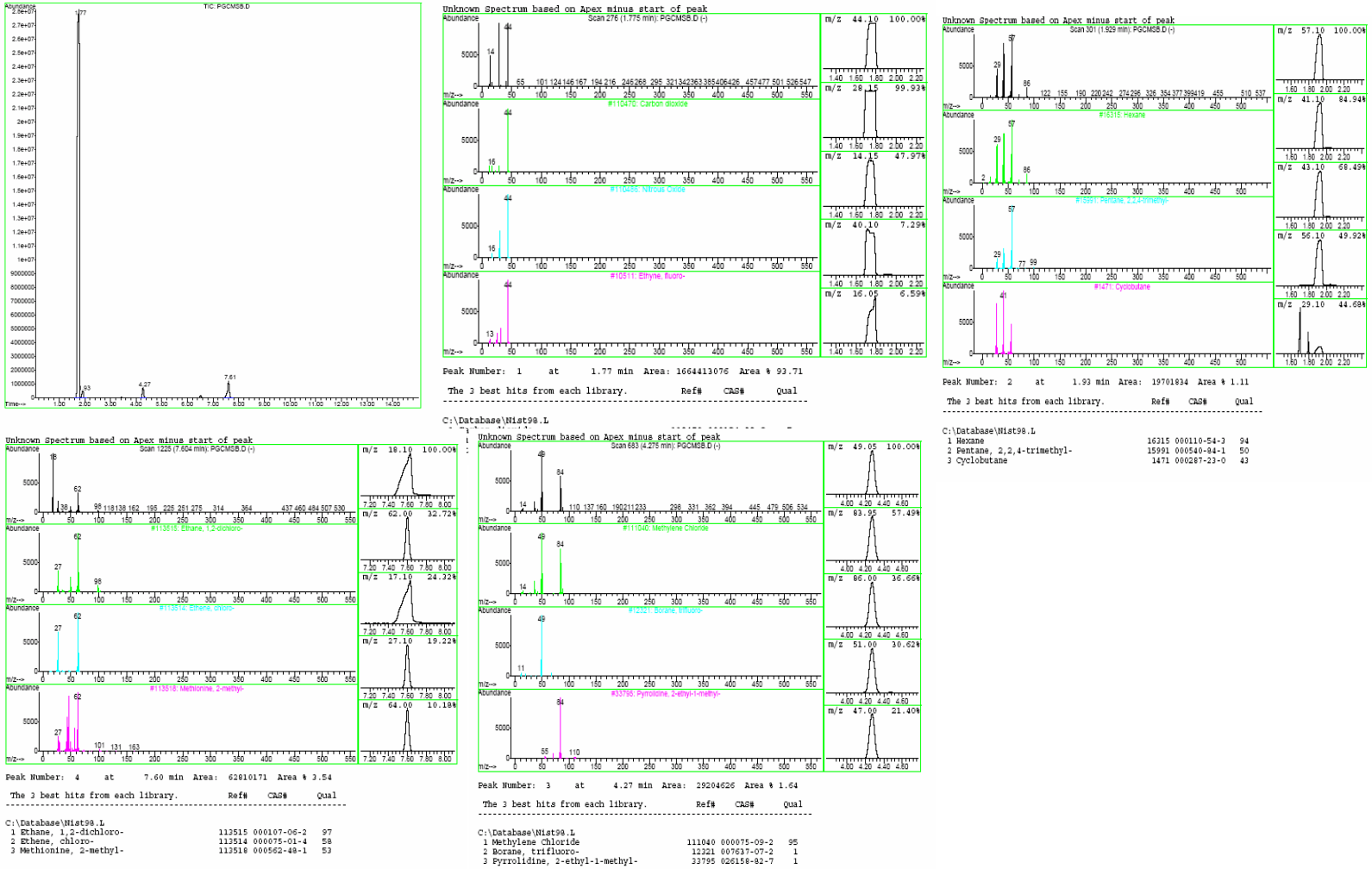


Fig. 21: GC-MS spectra for Type C microcosms with consortium B (Table: 4.1)

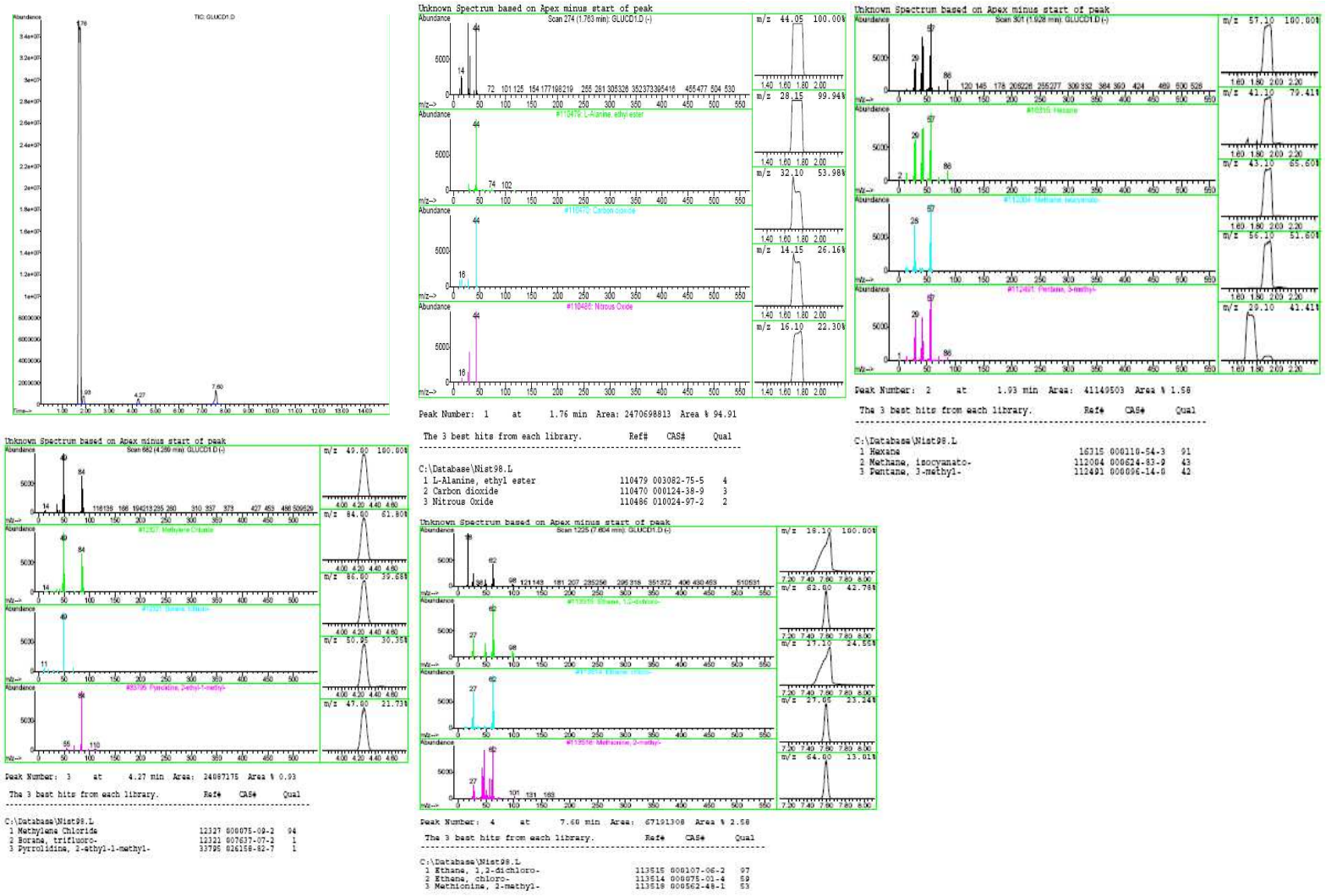


Fig. 22: GC-MS spectra for Type S microcosms with consortium D and glucose (Table: 4.1)

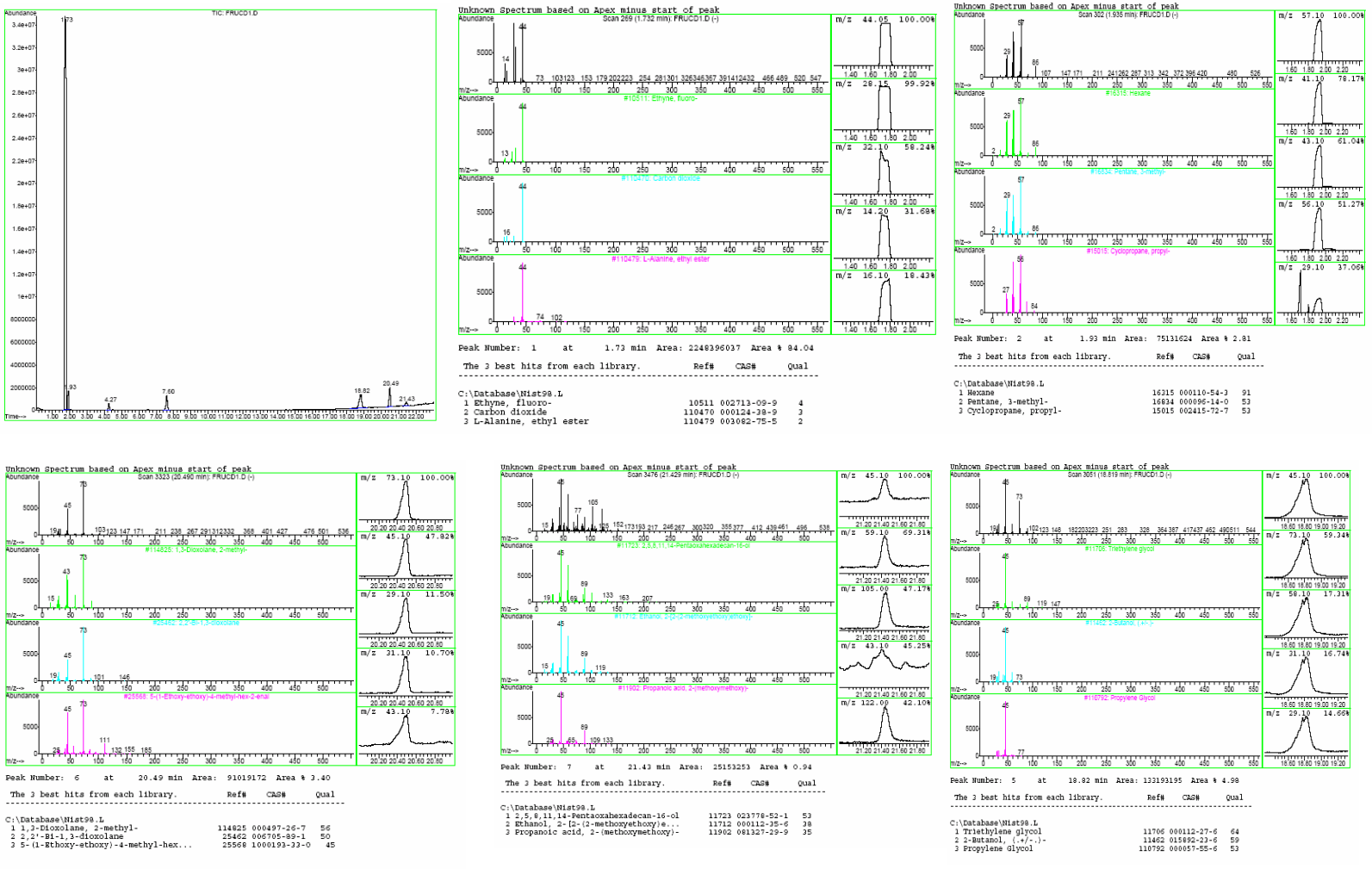
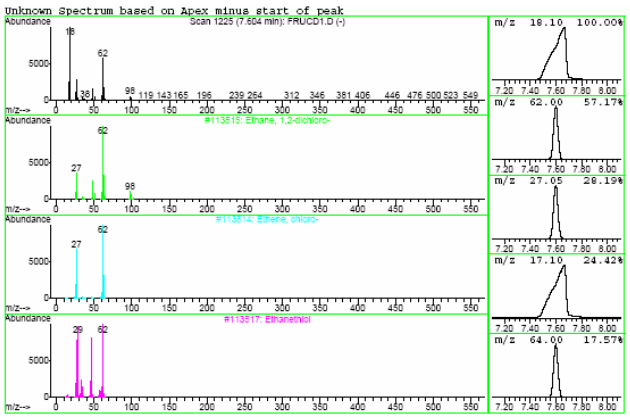


Fig. 23: GC-MS Spectra for Type S microcosms with consortium D and fructose (Table: 4.1)



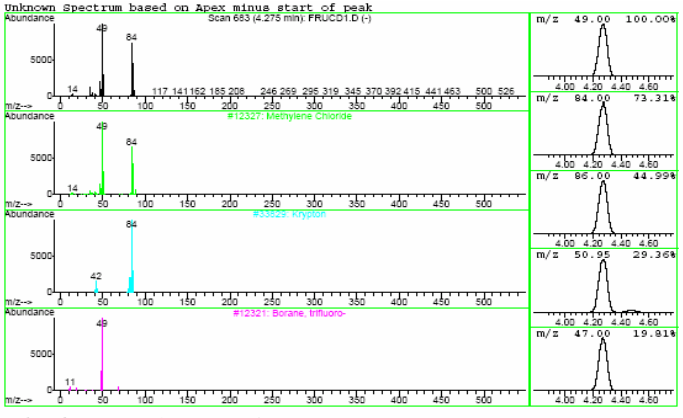


Fig 24: GC-MS Spectra for Type S microcosms with consortium D and fructose (contd)

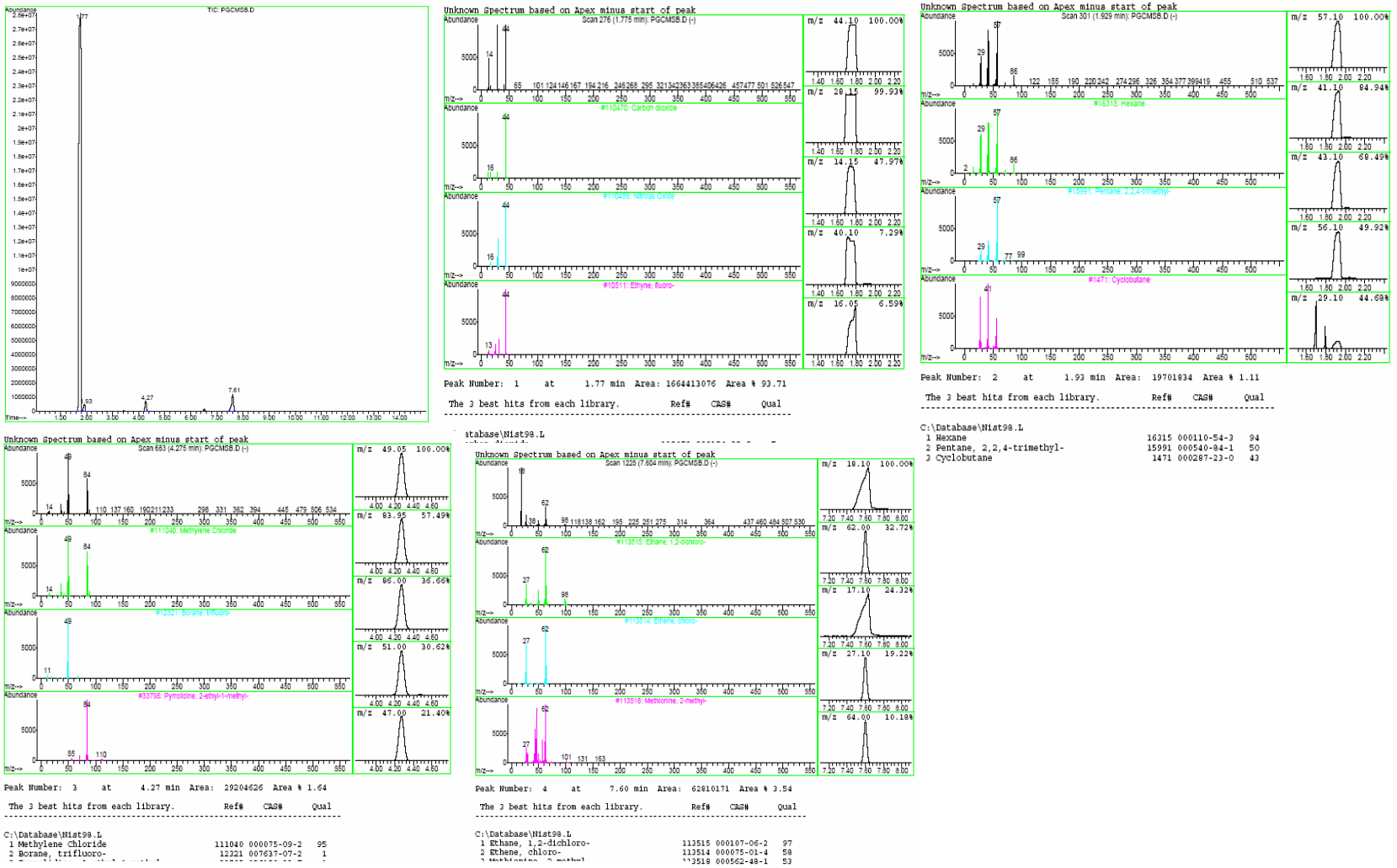


Fig. 25: GC- MS spectra for Type C microcosms with consortium B (Table: 4.1)

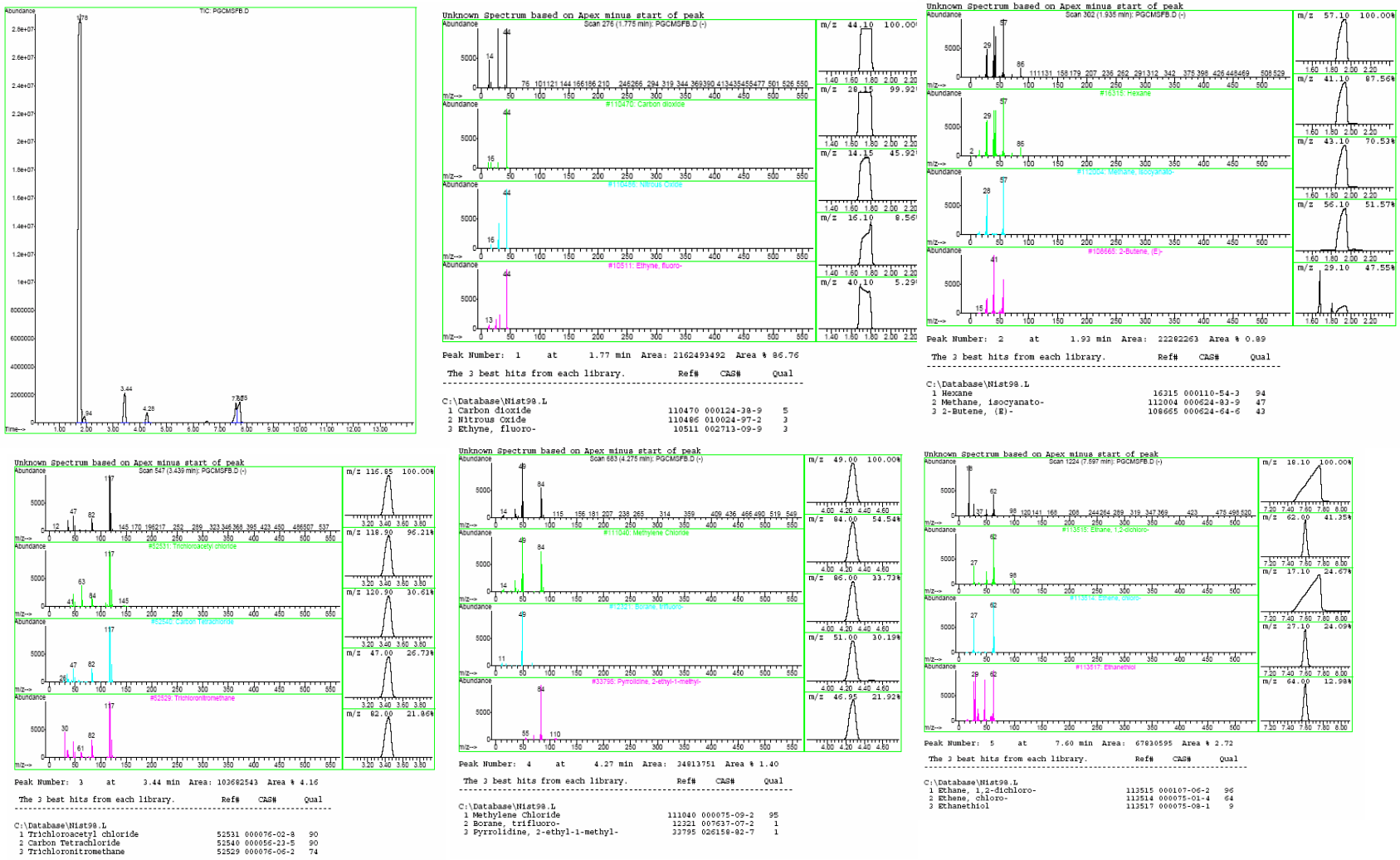


Fig. 26: GC-MS Spectra for Type C microcosms with consortium B and fertiliser (Table:4.1)

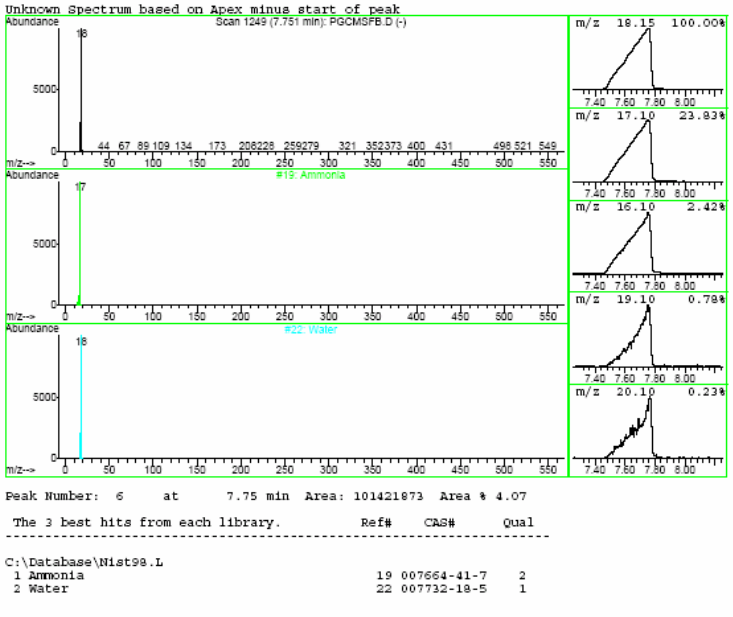


Fig. 27: GC-MS Spectra for Type C microcosms with consortium B and fertiliser (contd)

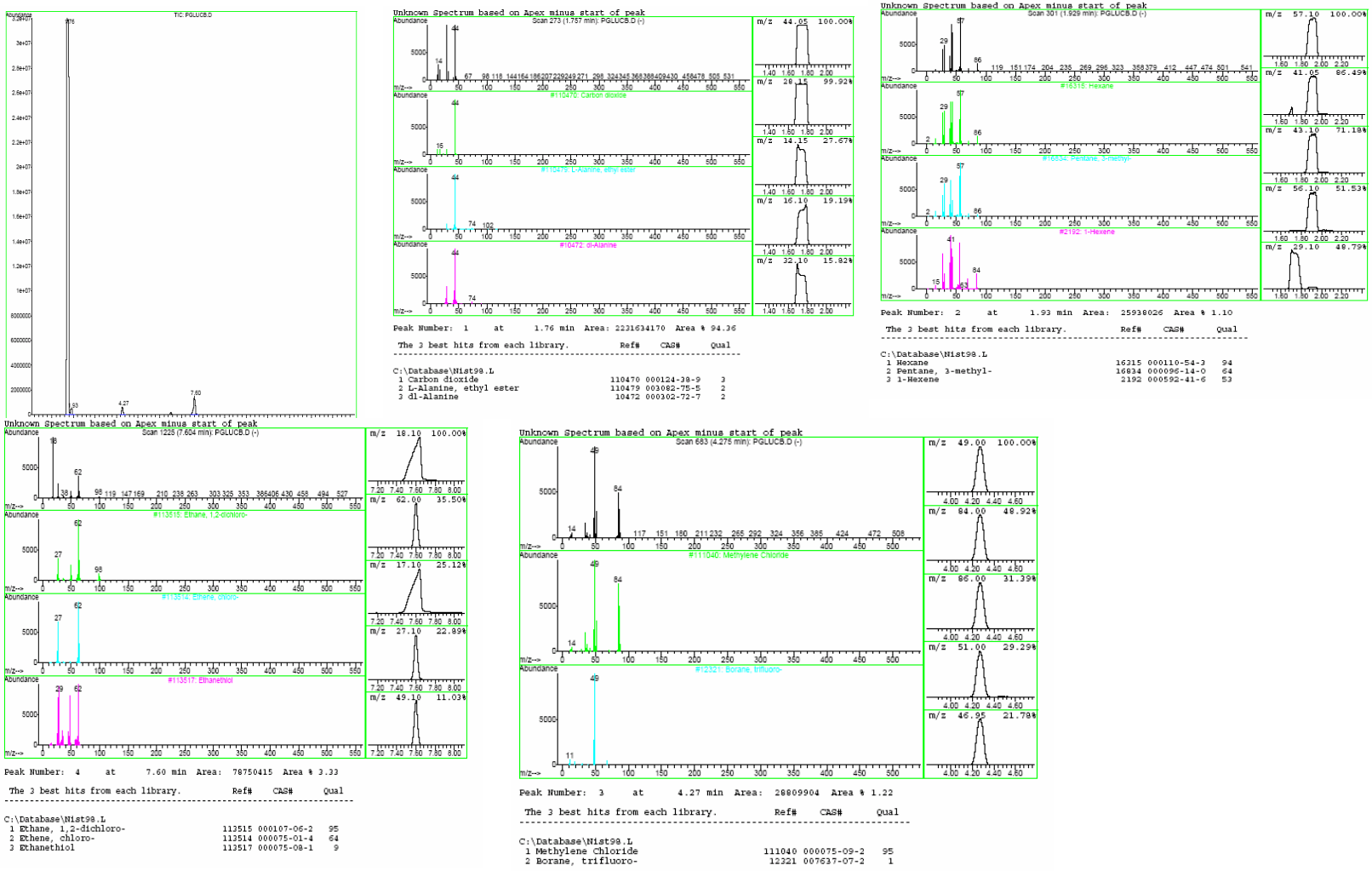


Fig. 28: GC-MS spectra for Type C microcosms with consortium B and glucose (Table: 4.1)

Data present in Tables 101- 174 appear in Sections 4.3.1, 4.3.2 and 4.3.3

Table 101: Independent T- Test data for CCl_4 biodegradation comparing addition of consortium B and C in Type S microcosms at day 7.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	244175361975 05730.000	.000	21.535	2	.002	61.581	2.859	49.277	73.884
	Equal variances not assumed			21.535	1.064	.025	61.581	2.859	30.004	93.157

Table 102: Independent T- Test data for CCl_4 biodegradation comparing addition of consortium B and D in Type S microcosms at day 7.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	525888534440 3330.000	.000	-4.428	2	.047	-14.460	3.265	-28.510	-.410
	Equal variances not assumed			-4.428	1.618	.069	-14.460	3.265	-32.227	3.307

Table 103: Independent T- Test data for CCl_4 biodegradation comparing addition of consortium D and unautoclaved soil in Type S microcosms at day 7.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	394506789728 050.200	.000	4.712	2	.042	11.680	2.479	1.016	22.344
	Equal variances not assumed			4.712	1.977	.043	11.680	2.479	.895	22.465

Table 104: Independent T- Test data for CCl_4 biodegradation comparing addition of consortium B and unautoclaved soil in Type S microcosms at day 7.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	343830061389 7242.000	.000	-.826	2	.496	-2.780	3.366	-17.262	11.702	
	Equal variances not assumed			-.826	1.725	.507	-2.780	3.366	-19.712	14.152	

Table 105: Independent T- Test data for CCl_4 biodegradation comparing addition of consortium D and autoclaved soil in Type S microcosms at day 7.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	182831281506 148.400	.000	26.309	2	.001	64.525	2.453	53.972	75.078	
	Equal variances not assumed			26.309	1.984	.002	64.525	2.453	53.891	75.159	

Table 106: Independent T- Test data for CCl_4 biodegradation comparing autoclaved and unautoclaved soil in Type S microcosms at day 7.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	821121420432 1.670	.000	20.446	2	.002	52.845	2.585	41.724	63.966	
	Equal variances not assumed			20.446	1.999	.002	52.845	2.585	41.720	63.970	

Table 107: Independent T- Test data for DCM biodegradation comparing addition of consortium B and D in Type S microcosms at day 14.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	7687573344206420000.000	.000	4.260	2	.051	12.990	3.049	-.131	26.111	
	Equal variances not assumed			4.260	1.012	.144	12.990	3.049	-24.648	50.628	

Table 108: Independent T- Test data for DCM biodegradation comparing addition of consortium C and D in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	1551578855174650000.000	.000	-7.837	2	.016	-24.170	3.084	-37.440	-10.900	
	Equal variances not assumed			-7.837	1.058	.072	-24.170	3.084	-58.595	10.255	

Table 109: Independent T- Test data for DCM biodegradation comparing addition of consortium B and D in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	5064087156357020.000	.000	37.429	2	.001	32.940	.880	29.153	36.727	
	Equal variances not assumed			37.429	1.833	.001	32.940	.880	28.803	37.077	

Table 110: Independent T- Test data for DCM biodegradation comparing addition of consortium C and unautoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	178831301610 2897000.000	.000	2.809	2	.107	8.770	3.122	-4.662	22.202
	Equal variances not assumed			2.809	1.109	.198	8.770	3.122	-22.786	40.326

Table 111: Independent T- Test data for DCM biodegradation comparing addition of consortium B and autoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	213904063864 79540.000	.000	1.512	2	.270	16.180	10.703	-29.870	62.230
	Equal variances not assumed			1.512	1.001	.372	16.180	10.703	-119.488	151.848

Table 112: Independent T- Test data for DCM biodegradation comparing addition of consortium D and autoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	211319058931 809600.000	.000	11.757	2	.007	18.110	1.540	11.482	24.738
	Equal variances not assumed			11.757	1.253	.031	18.110	1.540	5.797	30.423

Table 113: Independent T- Test data for 1,2-DCA biodegradation comparing addition of consortium B and D in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	244327736075 52.590	.000	17.357	2	.003	15.710	.905	11.816	19.604
	Equal variances not assumed			17.357	2.000	.003	15.710	.905	11.815	19.605

Table 114: Independent T- Test data for 1,2-DCA biodegradation comparing addition of consortium D and unautoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	12.612	2	.006	19.575	1.552	12.897	26.253
	Equal variances not assumed			12.612	1.868	.008	19.575	1.552	12.422	26.728

Table 115: Independent T- Test data for 1,2-DCA biodegradation comparing addition of consortium D and autoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	24.194	2	.002	38.295	1.583	31.485	45.105
	Equal variances not assumed			24.194	1.910	.002	38.295	1.583	31.166	45.424

Table 116: Independent T- Test data for 1,2-DCA biodegradation comparing addition of consortium B and unautoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	10.257	2	.009	11.635	1.134	6.754	16.516
	Equal variances not assumed			10.257	1.755	.014	11.635	1.134	6.041	17.229

Table 117: Independent T- Test data for 1,2-DCA biodegradation comparing autoclaved and unautoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	13.713	2	.005	18.720	1.365	12.846	24.594
	Equal variances not assumed			13.713	1.995	.005	18.720	1.365	12.831	24.609

Table 118: Independent T- Test data for 1,2-DCA biodegradation comparing addition of consortium B and autoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	25.809	2	.001	30.355	1.176	25.294	35.416
	Equal variances not assumed			25.809	1.704	.003	30.355	1.176	24.350	36.360

Table 119: Independent T- Test data for CCl_4 biodegradation comparing addition of consortium B and C in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	229670198871288700.000	.000	3.737	2	.065	6.24000	1.67000	-.94543	13.42543	
	Equal variances not assumed			3.737	1.000	.166	6.24000	1.67000	-14.97936	27.45936	

Table 120: Independent T- Test data for CCl_4 biodegradation comparing addition of consortium C and D in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	450939674207 85.200	.000	-.726	2	.543	-1.76800	2.43493	-12.24466	8.70866	
	Equal variances not assumed			-.726	1.993	.543	-1.76800	2.43493	-12.27994	8.74394	

Table 121: Independent T- Test data for CCl_4 biodegradation comparing addition of consortium B and unautoclaved control in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	.	.	-20.774	2	.002	-11.01000	.53000	-13.29041	-8.72959	
	Equal variances not assumed			-20.774	1.000	.031	-11.01000	.53000	-17.74429	-4.27571	

Table 122: Independent T- Test data for CCl_4 biodegradation comparing addition of consortium C and unautoclaved control in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	107024056241 932800.000	.000	-2.722	2	.113	-4.77000	1.75208	-12.30861	2.76861
	Equal variances not assumed			-2.722	1.199	.189	-4.77000	1.75208	-19.93725	10.39725

Table 123: Independent T- Test data for CCl_4 biodegradation comparing addition of consortium D and unautoclaved control in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	705736193584 2650.000	.000	-1.623	2	.246	-3.00200	1.84956	-10.96003	4.95603
	Equal variances not assumed			-1.623	1.177	.323	-3.00200	1.84956	-19.58325	13.57925

Table 124: Independent T- Test data for DCM biodegradation comparing addition of consortium B and C in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	897239425554 865000.000	.000	7.280	2	.018	55.730	7.655	22.794	88.666
	Equal variances not assumed			7.280	1.018	.084	55.730	7.655	-37.488	148.948

Table 125: Independent T- Test data for DCM biodegradation comparing addition of consortium B and D in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	975716051822335000.000	.000	7.311	2	.018	58.115	7.949	23.915	92.315	
	Equal variances not assumed			7.311	1.017	.084	58.115	7.949	-38.975	155.205	

Table 126: Independent T- Test data for DCM biodegradation comparing addition of consortium C and D in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	.	.	.217	2	.848	2.385	10.987	-44.888	49.658	
	Equal variances not assumed			.217	1.997	.848	2.385	10.987	-44.953	49.723	

Table 127: Independent T- Test data for DCM biodegradation comparing addition of consortium B and unautoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	423202304561289.000	.000	13.140	2	.006	20.805	1.583	13.992	27.618	
	Equal variances not assumed			13.140	1.503	.016	20.805	1.583	11.307	30.303	

Table 128: Independent T- Test data for DCM biodegradation comparing addition of consortium B and autoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	152786158657 2526.000	.000	33.862	2	.001	43.165	1.275	37.680	48.650
	Equal variances not assumed			33.862	1.788	.002	43.165	1.275	37.008	49.322

Table 129: Independent T- Test data for 1,2-DCA biodegradation comparing addition of consortium B and autoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	173724461917 0955000.000	.000	7.806	2	.016	21.035	2.695	9.441	32.629
	Equal variances not assumed			7.806	1.123	.065	21.035	2.695	-5.492	47.562

Table 130: Independent T- Test data for 1,2-DCA biodegradation comparing addition of consortium B and D in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	535029375292 7910.000	.000	-7.938	2	.016	-17.450	2.198	-26.908	-7.992
	Equal variances not assumed			-7.938	1.190	.056	-17.450	2.198	-36.769	1.869

Table 131: Independent T- Test data for 1,2-DCA biodegradation comparing addition of consortium C and D in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	608308644288339.000	.000	-9.302	2	.011	-33.380	3.589	-48.821	-17.939
	Equal variances not assumed			-9.302	1.819	.015	-33.380	3.589	-50.387	-16.373

Table 132: Independent T- Test data for 1,2-DCA biodegradation comparing addition of consortium D and unautoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	432878728731 9025.000	.000	-2.005	2	.183	-4.475	2.232	-14.077	5.127
	Equal variances not assumed			-2.005	1.254	.253	-4.475	2.232	-22.282	13.332

Table 133: Independent T- Test data for 1,2-DCA biodegradation comparing addition of unautoclaved and autoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	127381947699411700.000	.000	15.784	2	.004	42.960	2.722	31.249	54.671
	Equal variances not assumed			15.784	1.166	.027	42.960	2.722	18.074	67.846

Table 134: Independent T- Test data for CCL_4 biodegradation comparing addition of glucose and consortium D and fructose and consortium D in Type S microcosms at day 7.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	465565258850 38500.000	.000	3.567	2	.070	11.380	3.190	-2.345	25.105	
	Equal variances not assumed			3.567	1.000	.174	11.380	3.190	-29.153	51.913	

Table 135: Independent T- Test data for CCL_4 biodegradation comparing addition of glucose and consortium D with unautoclaved soil in Type S microcosms at day 7.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	179037450046 91120.000	.000	257.286	2	.000	27.015	.105	26.563	27.467	
	Equal variances not assumed			257.286	1.000	.002	27.015	.105	25.681	28.349	

Table 136: Independent T- Test data for CCL_4 biodegradation comparing addition of glucose and consortium D with autoclaved soil in Type S microcosms at day 7.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	867147571835 88800.000	.000	8.480	2	.014	46.515	5.485	22.915	70.115	
	Equal variances not assumed			8.480	1.000	.075	46.515	5.485	-23.179	116.209	

Table 137: Independent T- Test data for CCL_4 biodegradation comparing addition of fructose and consortium D with autoclaved soil in Type S microcosms at day 7.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	931359875429 7370.000	.000	5.537	2	.031	35.135	6.345	7.834	62.436
	Equal variances not assumed			5.537	1.607	.049	35.135	6.345	.324	69.946

Table 138: Independent T- Test data for DCM biodegradation comparing addition of glucose and consortium D with fructose and consortium D in Type S microcosms at day 21.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	359597206447 36550.000	.000	46.861	2	.000	42.505	.907	38.602	46.408
	Equal variances not assumed			46.861	1.077	.010	42.505	.907	32.760	52.250

Table 139: Independent T- Test data for DCM biodegradation comparing addition of glucose and consortium D with autoclaved soil in Type S microcosms at day 21.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	426137525356 6139.000	.000	27.563	2	.001	41.180	1.494	34.752	47.608
	Equal variances not assumed			27.563	1.845	.002	41.180	1.494	34.204	48.156

Table 140: Independent T- Test data for DCM biodegradation comparing addition of fructose and consortium D with autoclaved soil in Type S microcosms at day 21.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	110573007680 66160.000	.000	29.660	2	.001	27.485	.927	23.498	31.472	
	Equal variances not assumed			29.660	1.074	.017	27.485	.927	17.462	37.508	

Table 141: Independent T- Test data for DCM biodegradation comparing addition of fructose and consortium D with unautoclaved soil in Type S microcosms at day 21.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	147049400625 5014.000	.000	17.370	2	.003	26.160	1.506	19.680	32.640	
	Equal variances not assumed			17.370	1.864	.004	26.160	1.506	19.206	33.114	

Table 142: Independent T- Test data for DCM biodegradation comparing addition of glucose and consortium D with fructose and consortium D in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	236522636342 35870.000	.000	-3.021	2	.094	-4.210	1.394	-10.206	1.786	
	Equal variances not assumed			-3.021	1.309	.154	-4.210	1.394	-14.548	6.128	

Table 143: Independent T- Test data for DCM biodegradation comparing addition of glucose and consortium D with autoclaved soil in Type S microcosms at day 28.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	147610663436 7934000.000	.000	5.787	2	.029	29.145	5.036	7.475	50.815
	Equal variances not assumed			5.787	1.021	.105	29.145	5.036	-31.802	90.092

Table 144: Independent T- Test data for CCl_4 biodegradation comparing addition of glucose and consortium B with unautoclaved soil in Type C microcosms at day 6.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	155695872831 951400.000	.000	16.304	2	.004	51.115	3.135	37.626	64.604
	Equal variances not assumed			16.304	1.484	.012	51.115	3.135	31.965	70.265

Table 145: Independent T- Test data for CCl_4 biodegradation comparing addition of glucose and consortium B with fertiliser and consortium Bin Type C microcosms at day 6.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	16.123	2	.004	12.495	.775	9.160	15.830
	Equal variances not assumed			16.123	1.000	.039	12.495	.775	2.648	22.342

Table 146: Independent T- Test data for CCl_4 biodegradation comparing addition of glucose and consortium B with fertiliser and consortium B in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	21.683	2	.002	64.365	2.969	51.593	77.137
	Equal variances not assumed			21.683	1.252	.014	64.365	2.969	40.598	88.132

Table 147: Independent T- Test data for CCl_4 biodegradation comparing addition of glucose and consortium B with autoclaved soil in Type C microcosms at day 6.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	.	.	10.453	2	.009	30.825	2.949	18.137	43.513
	Equal variances not assumed			10.453	1.223	.038	30.825	2.949	6.215	55.435

Table 148: Independent T- Test data for DCM biodegradation comparing addition of glucose and consortium B with autoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	354450464939183000.000	.000	10.082	2	.010	26.930	2.671	15.437	38.423
	Equal variances not assumed			10.082	1.087	.052	26.930	2.671	-1.235	55.095

Table 149: Independent T- Test data for DCM biodegradation comparing autoclaved with unautoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	128903790859564000000.000	.000	9.334	2	.011	24.410	2.615	13.158	35.662
	Equal variances not assumed			9.334	1.000	.068	24.410	2.615	-8.804	57.624

Table 150: Independent T- Test data for DCM biodegradation comparing addition of glucose and B with unautoclaved soil in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	222718363436 53910.000	.000	4.619	2	.044	2.520	.546	.173	4.867
	Equal variances not assumed			4.619	1.004	.135	2.520	.546	-4.344	9.384

Table 151: Independent T- Test data for DCM biodegradation comparing addition of glucose and B with fertilizer and B in Type C microcosms at day 15.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
RATE	Equal variances assumed	754434608243 26800.000	.000	25.718	2	.002	41.045	1.596	34.178	47.912
	Equal variances not assumed			25.718	1.259	.011	41.045	1.596	28.403	53.687

Table 152: Independent T- Test data for DCM biodegradation comparing addition of glucose and B with autoclaved soil in Type C microcosms at day3.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
RATE	Equal variances assumed	.	.	20.238	2	.002	21.970	1.086	17.299	26.641	
	Equal variances not assumed			20.238	1.021	.030	21.970	1.086	8.821	35.119	

Table 153: Independent T- Test data for 1,2-DCA biodegradation comparing addition of glucose and B with fertiliser and B in Type C microcosms at day15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	.	.	-26.329	2	.001	-21.19500	.80500	-24.65864	-17.73136	
	Equal variances not assumed			-26.329	1.000	.024	-21.19500	.80500	-31.42349	-10.96651	

Table 154: Independent T- Test data for 1,2-DCA biodegradation comparing addition of fertiliser and B with unautoclaved soil in Type C microcosms at day15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	.	.	-24.000	2	.002	-48.00000	2.00000	-56.60531	-39.39469	
	Equal variances not assumed			-24.000	1.000	.027	-48.00000	2.00000	-73.41241	-22.58759	

Table 155: Independent T- Test data for 1,2-DCA biodegradation comparing addition of fertiliser and B with autoclaved soil in Type C microcosms at day15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	347015197622 25840.000	.000	-24.616	2	.002	-33.35500	1.35500	-39.18509	-27.52491	
	Equal variances not assumed			-24.616	1.000	.026	-33.35500	1.35500	-50.57191	-16.13809	

Table 156: Independent T- Test data for 1,2-DCA biodegradation comparing addition of glucose and B with autoclaved soil in Type C microcosms at day15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	571735664169 7830.000	.000	-7.715	2	.016	-12.16000	1.57609	-18.94135	-5.37865	
	Equal variances not assumed			-7.715	1.628	.028	-12.16000	1.57609	-20.66784	-3.65216	

Table 157: Independent T- Test data for 1,2-DCA biodegradation comparing addition of glucose and B with unautoclaved soil in Type C microcosms at day15.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	.	.	-12.433	2	.006	-26.80500	2.15593	-36.08121	-17.52879	
	Equal variances not assumed			-12.433	1.316	.025	-26.80500	2.15593	-42.65303	-10.95697	

Table 158: Independent T- Test data for phospholipid phosphate analysis comparing addition of consortium D at day 0 and 7 in Type S microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	.	.	-4.074	2	.055	-.22300	.05474	-.45853	.01253
	Equal variances not assumed			-4.074	1.919	.059	-.22300	.05474	-.46833	.02233

Table 159: Independent T- Test data for phospholipid phosphate analysis comparing addition of fructose and consortium D at day 7 and 21 in Type S microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	258006022407 27280.000	.000	-7.492	2	.017	-.79100	.10557	-1.24525	-.33675
	Equal variances not assumed			-7.492	1.022	.081	-.79100	.10557	-2.06625	.48425

Table 160: Independent T- Test data for phospholipid phosphate analysis comparing addition of consortium D at day 14 and 21 in Type S microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	.	.	8.310	2	.014	1.83850	.22124	.88659	2.79041
	Equal variances not assumed			8.310	1.147	.058	1.83850	.22124	-.24987	3.92687

Table 161: Independent T- Test data for phospholipid phosphate analysis comparing unautoclaved soil at day 0 and 5 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	653990343087381.000	.000	-8.777	2	.013	-1.38350	.15762	-2.06169	-.70531
	Equal variances not assumed			-8.777	1.971	.013	-1.38350	.15762	-2.07128	-.69572

Table 162: Independent T- Test data for phospholipid phosphate analysis comparing unautoclaved soil at day 5 and 15 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	351701473393 65780.000	.000	-4.743	2	.042	-1.08500	.22876	-2.06929	-.10071
	Equal variances not assumed			-4.743	1.493	.071	-1.08500	.22876	-2.47033	.30033

Table 163: Independent T- Test data for phospholipid phosphate analysis comparing addition of consortium C at day 0 and 5 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	577311300147 7690.000	.000	28.777	2	.001	1.90650	.06625	1.62144	2.19156
	Equal variances not assumed			28.777	1.209	.012	1.90650	.06625	1.34179	2.47121

Table 164:. Independent T- Test data for phospholipid phosphate analysis comparing addition of consortium C at day 5 and 15 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	708354972430447000.000	.000	-2.058	2	.176	-.20100	.09768	-.62126	.21926	
	Equal variances not assumed			-2.058	1.092	.271	-.20100	.09768	-1.22040	.81840	

Table 165: Independent T- Test data for phospholipid phosphate analysis comparing addition of consortium C and unautoclaved soil at day 5 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	24618409976452360.000	.000	15.447	2	.004	1.64500	.10649	1.18680	2.10320	
	Equal variances not assumed			15.447	1.077	.034	1.64500	.10649	.50006	2.78994	

Table 166: Independent T- Test data for phospholipid phosphate analysis comparing addition of consortium B and unautoclaved soil at day 5 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	120830322798485 5.000	.000	8.924	2	.012	1.20865	.13543	.62593	1.79137	
	Equal variances not assumed			8.924	1.930	.014	1.20865	.13543	.60514	1.81216	

Table 167: Independent T- Test data for phospholipid phosphate analysis comparing addition of consortium D and unautoclaved soil at day 10 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	172293376289 98610.000	.000	15.295	2	.004	1.11150	.07267	.79882	1.42418
	Equal variances not assumed			15.295	1.066	.035	1.11150	.07267	.31291	1.91009

Table 168: Independent T- Test data for phospholipid phosphate analysis comparing addition of consortium D at day 5 and 10 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	.	.	.105	2	.926	.01450	.13752	-.57719	.60619
	Equal variances not assumed			.105	1.122	.932	.01450	.13752	-1.34265	1.37165

Table 169: Independent T- Test data for phospholipid phosphate analysis comparing addition of consortium D at day 5 and 15 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	840675727691 919000000.000	.000	2.674	2	.116	.35700	.13350	-.21741	.93141
	Equal variances not assumed			2.674	1.000	.228	.35700	.13350	-1.33918	2.05318

Table 170: Independent T- Test data for phospholipid phosphate analysis comparing addition of glucose and consortium B at day 0 and 5 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	313658713532786900.000	.000	-7.884	2	.016	-1.22750	.15569	-1.89736	-.55764
	Equal variances not assumed			-7.884	1.057	.072	-1.22750	.15569	-2.96937	.51437

Table 171: Independent T- Test data for phospholipid phosphate analysis comparing addition of glucose and consortium B at day 5 and 5 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	.	.	9.911	2	.010	1.52900	.15428	.86518	2.19282
	Equal variances not assumed			9.911	1.020	.061	1.52900	.15428	-.34113	3.39913

Table 172: Independent T- Test data for phospholipid phosphate analysis comparing addition of fertiliser and consortium B at day 5 and 15 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Rate	Equal variances assumed	241306312589 3699.000	.000	6.606	2	.022	1.03500	.15667	.36091	1.70909
	Equal variances not assumed			6.606	1.562	.040	1.03500	.15667	.14321	1.92679

Table 173: Independent T- Test data for phospholipid phosphate analysis comparing addition of fertiliser and consortium B at day 0 and 5 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	794365245808 4710.000	.000	-8.341	2	.014	-1.16550	.13973	-1.76672	-.56428	
	Equal variances not assumed			-8.341	1.080	.065	-1.16550	.13973	-2.65710	.32610	

Table 174: Independent T- Test data for phospholipid phosphate analysis comparing addition of fertiliser and consortium B at day 0 and 15 in Type C microcosms.

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference		
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	
Rate	Equal variances assumed	721310815540 92000.000	.000	-1.615	2	.248	-.13050	.08082	-.47825	.21725	
	Equal variances not assumed			-1.615	1.257	.314	-.13050	.08082	-.77255	.51155	