Bacterial Community Structure in a Nitro-Organic Explosive Contaminated Soil Sample

David Andrew Coffey, B.Sc.

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ABSTRACT

Soil contamination from nitro-organic explosives such as 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and HMX poses a significant risk to human health and that of the surrounding eco-system. Removal of contaminants can be accomplished through soil washing, incineration, and chemical techniques and/or bioremediation. The current study focused on the characterization of microbes present in soils, obtained from an artillery firing range, that contained significant levels of nitroorganic explosives. Direct examination of metagenomic bacterial DNA extracted from soil samples was achieved using the 16S rRNA gene as a target for bacterial identification. The 16S rRNA gene was used to identify bacteria present in a bioreactor treated soil sample contaminated with nitro-organic explosives. The community structure was evaluated over a 90 day period with and without the addition of supplementary bacterial nutrients. The results were compared to previously reported results of bacteria capable of degrading these contaminants. Differences in the microbial community structure were noted between different bioreactor treatments, nucleic acid extractions, and bioreactor vessels. Soil was initially contaminated to the greatest extent with HMX, and possible TNT metabolites were also identified in the initial soil sample. Soil samples from control bioreactor runs showed no genera present in proportions greater than 10%, except at day 45 samples from the second bioreactor, which was largely inhabited by members of the genus *Methylotenera.* Fingerprints obtained from nutrient addition soil samples showed a significant presence of the genus *Nitrosospira.* The phylum *Acidobacteria* increased in abundance throughout the bioreactor treatments, and its abundance was previously reported to be inversely proportional to TNT concentration in the soil. None of the previously reported genera capable of degrading nitro-explosives were found in the soil microbial fingerprints. These genera may be present in lower abundance and would be detected with additional sampling. Phenomena such as horizontal gene transfer may allow for nitro-organic explosive degradation by bacteria that were not previously reported.

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"I and my colleagues here have been engaged in the pursuit of knowledge".

-Frederick Sanger

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

3'	3 prime end of a segment of nucleic acids
5'	5 prime end of a segment of nucleic acids
a	Amino
A260	Absorbance of 260nm light
A280	Absorbance of 280nm light
α	Alpha
ACE	Abundance based coverage estimator
AMO	Ammonia monooxygenase
AOB	Ammonia-oxidizing bacteria
β	Beta
C	Cytosine
°C	Degrees Celsius
CFB	Canadian Forces Base
cm	Centimetre
ddNTP	Dideoxyribonucleoside Triphosphate
DGGE	Denaturing Gradient Gel Electrophoresis
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNB	Dinitrobenzene
DNT	Dinitrotoluene
dNTP	Deoxyribonucleoside Triphosphate
e	Electron
E. coli	*Escherichia coli*

EPA	US Environmental Protection Agency
F	Forward PCR primer
FASTA	FAST-ALL
g	Gram or the force of gravity
G	Guanine
γ	Gamma
GEM	Genetically engineered microorganisms
HA	Hydroxyamino
HGT	Horizontal gene transfer
HMX	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine or High Melting Explosive
HPLC	High performance liquid chromatography
HPS	3-hexulosephosphate synthase
hr	Hour
IARC	Internation Agency for Research on Cancer
kb	Kilobase
kg	Kilogram
L	Litre
LB	Luria-Bertani or Liquid Broth
lacZ	Lactose catabolism operon
LAW	Light Anti-tank Weapon
M	Concentration in moles/litre
MEDINA	$O_2NNHCH_2NHNO_2$
µg	Microgram
µm	Micrometre
µM	Concentration in µmol/L
mg	Miligram

min	Minute
mL	Mililitre
mol	Mole
MspI	Type-2 restriction enzyme from *Moraxella sp.*
mV	Milivolt
NC	Nitrocellulose
NDAB	4-nitro-2,4-diazabutanal
NG	Nitroglycerin
NR	Nitroreductase
NT	Nitrotoluene
OTU	Operational taxonomic unit
PAH	Polyaromatic hydrocarbon
PCR	Polymerase chain reaction
PET	Petawawa
pH	Negative Logarithm of the molar concentration of dissolved hydrogen ions
PHI	Phospho-3-hexuloisomerase
PICT	Pollution-induced community tolerance
PPi	Pyrophosphate
ppm	Parts per million
PVC	Polyvinyl chloride
R	Reverse PCR primer
RDX	Hexahydro-1,3,5-trinitro-1,3,5-triazine or Royal Demolition Explosive or Research Department Explosive
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

rpm	Rotations per minute
RuMP	Ribulose monophosphate
SMRT	Single-molecule real-time DNA sequencing
S.O.C.	Super optimal broth with Catabolite repression
sp.	Species
SSCP	Single strand conformation polymorphism
TAT	2,4,6-Triaminotoluene
Tetryl	2,4,6-Trinitrophenylmethylnitramine
TNB	1,3,5-Trinitrobenzene
TNT	2,4,6-Trinitrotoluene
TOPO	Topoisomerase
t-RFLP	Terminal-restriction fragment length polymorphism
UXO	Unexploded ordinance
V	Volt
VHb	Bacterial haemoglobin
w/v	Weight per unit volume
X	Times
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
XpAI	Cytochrome P450 like-enzyme that metabolizes RDX

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

1.1 SOIL CONTAIMINATION

Soil contamination resulting from human activity that causes the release of xenobiotic compounds into the environment is a serious concern to human health and has detrimental effects on the surrounding ecosystems. It is estimated that in Canada alone there are 17,800 federally-owned contaminated sites (OAG, 2008). Activities that may result in soil contamination include the application of pesticides, rupture of underground storage tanks, demolitions, leaching from landfill sites, and a variety of activities arising from the oil industry, as well as the manufacturing, storage and discharge of munitions. Prevention and treatment of polluted soil environments is a concern of the Canadian government as outlined in the Environmental Protection Act of 1999 (CEPA, 1999). The Canadian Government has also developed guidelines for acceptable levels of soil contamination that would not pose a significant risk factor for human health. For example, a detailed list of acceptable levels of many prominent contaminants is provided by the Canadian Environmental Quality Guidelines (CEQGs). These CEQGs are used by federal, provincial, and territorial governments to maintain high levels of environmental quality across their jurisdictions and prohibit activities that would cause contaminant levels to exceed the guideline amount.

1.1.1 NITRO-ORGANIC EXPLOSIVES SOIL CONTAMINANTS

An environmental contaminant of concern is a group of nitro-substituted explosives that are dispersed as a result of mining, demolitions, weapons manufacturing, deployment and dispersal. Examples of such explosives include TNT,

RDX, HMX, TNB whose chemical structures are shown in Figure 1.1. TNT, RDX and HMX are the most widely used nitro-organic explosives as they are often detected in soil and groundwater contamination (Hawari *et al.,* 2000). In military installations, concentrations of nitroaromatic explosives in soil have been reported to be as high as 87,000 mg/kg of soil (Talmage et *al.,* 1999). Mixtures of two or more of these explosives may be used as is the case in Comp B which is a mixture of TNT and RDX of 39/60 and 1% wax (Taylor *et al.,* 2006). There are general hazards associated with soil contaminated with these explosives. The most dramatic concern is the potential for large pieces of crystallized residual explosives to explode. Soil containing 12% or less explosive concentration has been shown to pose no detonation or explosive threat when heated under confinement (Kristoff *et al.,* 1987). Human health concerns may arise through the exposure of residual explosives via dust inhalation, soil ingestion and dermal absorption (IARC, 2002). TNT and RDX are classified as possible carcinogens while TNT-metabolites 2,4-DNT and 2,6-DNT are classified as probable human carcinogens (IARC, 2002). Other health effects reported as a result of exposure to residual explosives include skin haemorrhages, liver abnormalities, anemia and convulsions.

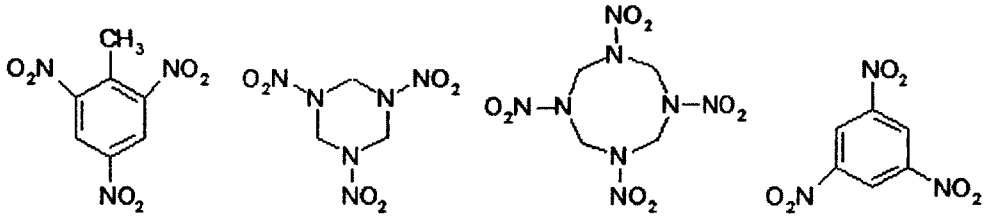

Figure 1.1: Chemical structures of four commonly used nitro-organic explosives: 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and 1,3,5-trinitrobenzene (TNB).

1.1.2 EXPLOSIVES/SOIL SORPTION

Contamination of soil and ground waters by nitro-organic explosives are reported in military firing ranges such as at CFB Petawawa (McAllister, 2011). The primary cause of such contamination is the incomplete detonation of military ordinance (Morley *et al.,* 2006). The environmental fate of these residual explosives is largely dependent on their chemical properties which are summarized in Table 1.1, as well as the composition of the soil. Nitramine explosives such as RDX and HMX are reported to have lower affinities for sorption to inorganic soil components than the nitroaromatic explosive TNT (Lynch *et al.,* 2002). Clay soils also allow for high sorption of TNT (Eriksson *et al.,* 2004). Soil composition can be a factor in the transformation of residual explosives into their metabolites as reported for vermiculite soils promoting the transformation of TNT (Jaramillo *et al.,* 2011). Such soil would be ideal for use in firing ranges to encourage degradation of TNT. High sorption will allow for the persistence of contamination in the soil and increase the potential for detrimental exposure when it is desorbed.

Table 1.1: Chemistry summary for three nitro-organic explosives TNT, RDX and HMX (Walsh *et al.*, 1995).

Compound	*Molecular Formula*	*Appearance*	*Molecular Weight (g/mol)*	*Melting Point (°C)*	*Boiling Point (°C)*	*Density (g/cm^3)*	*Water Solubility (mg/L)*
TNT	$C_6H_2(CH_3)(NO_2)_3$	Yellow Crystals[1]	227.13	80.1-81.6	240 (explodes)	1.65[1]	130
RDX	$C_3H_6N_6O_6$	White Granular[2]	222.26	205-206	(decomposes)	1.82[2]	40-60
HMX	$C_4H_8N_8O_8$	White Crystals[3]	296.16	276-286	(decomposes)	1.91[3]	6.63

[1]: Safety Data for 2,4,6-Trinitrotoluene (2003).[2]: RDX MSDS (2011).[3]: HMX MSDS (2011).

1.2 SOIL REMEDIATION

Explosives in the environment are recalcitrant to degradation and sites have been reported to exhibit high levels of contamination in soils and ground water 20-50 years after the discontinuation of activities where explosives were used (Craig *et al.,* 1995). As a result, a variety of approaches have been developed in order to decontaminate these sites, including physical remediation, chemical remediation and thermal remediation. However, these methods can be costly, destructive to the environment and impractical on large scales. Alternatively, bacteria capable of degrading xenobiotic contaminants may be exploited for soil decontamination in a process known as bioremediation.

1.2.1 PHYSICAL REMEDIATION

Physical remediation methods involve the collection of soil for treatments either *in-situ* or off site. The simplest form of physical remediation is excavation, which has the goal of transferring the contamination elsewhere. Soil may also be washed off site which is most effective for contamination by non-volatile hydrophilic and hydrophobic organic compounds as well as heavy metals (Tadesse *et al.,* 1994). A major drawback of this technique is that it is relatively ineffective on large scales and the fluid run-through requires treatment itself as it dissolves the original contaminants.

1.2.2 CHEMICAL REMEDIATION

Chemical remediation techniques remove contamination by eliminating the xenobiotics through chemical reactions. Commonly used reactions include neutralization of acid and alkali contaminants, oxidation, photolysis in shallow soils where light can penetrate, precipitation, and reduction. Under alkaline conditions (ph 14), complete transformation of TNT and partial mineralization has been reported through hydrolysis (Saupe *et al.,* 1998). The addition of lime to soil contaminated with TNT, RDX, and HMX due to the establishment of a basic pH for alkaline hydrolysis to take place (Davis *et al.,* 2006). Common drawbacks involved with these methods are the risk of explosive reactions and the possible production of more toxic and hazardous substances such as the production of toluene through the chemical remediation of TNT (Tadesse et *al.,* 1994).

1.2.3 THERMAL REMEDIATION

Thermal remediation uses heat to destroy and/or detoxify contaminants. Methods used include a fluidized bed for cyanides, a rotary kiln for combustible contaminants and pyrolysis for soils containing heavy metal residues. These techniques all require the use of expensive machinery and can only be used for relatively small amounts of soils. The heating apparatus requires a large amount of energy to operate and the combustion of contaminants will release gases that can detrimentally affect the environment such as CO_2, NO_x, and SO_2 (Cappuyns *et al.,* 2011).

1.2.4 BIOLOGICAL REMEDIATION

Biological remediation or bioremediation utilizes microorganisms that can break down contaminants into simpler compounds or detoxifiy hazardous xenobiotics. On average, in one gram of soil there are an estimated 10^7 - 10^{10} prokaryotic cells (Gans *et al.,* 2005). It is therefore possible that some of these cells may adapt to metabolise any particular contaminant. We can exploit this natural ability of certain microbes to detoxify a polluted site in a process known as bioremediation. Both aerobic and anaerobic microbes may be used for bioremediation. Furthermore, genes allowing for the bioremediation of contaminants can be isolated and expressed in plants to clean up contaminated sites. Currently, bioremediation is not widely used and much research is still required in order to use it to its full potential.

One method used to study the bioremediation potential of bacteria uses bioslurry treatments. In this method, soil samples are taken from contaminated sites and placed in a bioreactor that provides controlled conditions such as pH, dissolved oxygen and temperature while also mixing and aerating the soil. This controlled environment allows for the growth of bacteria able to metabolise the soil contaminants. A nutrient supplement may also be added to the bioreactor to enhance microbial xenobiotic metabolism. The fermented soil may then be re-introduced into the contaminated site. The bacteria that thrived in the bioreactor would then be present in high enough quantities to facilitate the remediation of the contaminated soil. In one example, soil contaminated with high concentrations of 2,4,6-trinitrophenylmethylnitramine (tetryl)

was treated with a bioslurry that resulted in a 99.9% reduction of tetryl concentrations within 200 days (Fuller, *et al.,* 2003). Soil slurries are also employed to identify bioremediators in laboratory studies. Soil slurry studies have been conducted in samples contaminated with TNT (Newcombe & Crawford, 2007), RDX, and HMX (Guiot *et al.,* 1999). These bioslurry studies are used to determine which bacteria can thrive in contaminated soil and to what extent the contaminant concentration is decreased.

1.3 TNT

2,4,6-trinitrotoluene (TNT) is an extensively used aromatic explosive and concentrations up to 40g/kg can be found in heavily used sites such as mines and munitions disposal sites (IARC, 2002). TNT can have a negative effect on human health due to its mutagenicity and suspected carcinogenicity (IARC, 2002). TNT may also cause rashes, skin haemorrhages, mucous and blood disorders in humans (Makris *et al.,* 2010). Prolonged exposure to TNT has been shown to cause anemia and abnormal liver functions as well as having possible effects on male fertility (Public Health Service, 1995). The suspected mechanism of TNT toxicity is a single-electron reduction of the nitro groups that results in the production of reactive oxygen species (Georges *et al.,* 2008). The cytotoxicity of TNT has also been explored. At concentrations of 100 ppm, TNT killed approximately 85 percent of a mammalian test cell line NG108 neuroblastoma (Banerjee *et al.,* 1999). Its primary metabolite 2-amino-4,6-

dinitrotoluene showed increased cytotoxicity compared to TNT resulting in 90 percent of the test cells dying. When concentrations of TNT are high (e.g. 80g/L) many bacteria become unculturable due to the toxic effects of TNT (Fuller and Manning, 1997). A further hindrance to TNT bioremediation is its biological unavailability due to its close association with humic soil components (Thorn & Kennedy, 2002).

1.3.1 TNT BIOREMEDIATORS

TNT contamination in soil has been shown to effect microbial diversity. Comparison of a site contaminated with high concentrations of TNT compared to a control soil sample showed a dramatic decrease in microbial biodiversity in the TNT site as revealed through DGGE fingerprinting (Georges *et al.,* 2008). The prevalent genus in the TNT soil was *Pseudomonas*, which was hypothesized to be a result of its ability to metabolize TNT-derivatives that accumulate in the soil. Other less prominent genera identified in this same study include *Brevundimonas, Acidovorax* and *Rhodanobacter.* Further studies on TNT-contaminated soils via 16S rRNA gene sequence alignments confirm the role of *Pseudomonas* on the species level as *Pseudomonas aeruginosa* (Kalafut *et al.,* 1998). This species was able to survive in media spiked with up to 50 mg/L of TNT with no adverse effects on its growth rate. Other species of *Pseudomonas* that are able to grow in the presence of TNT include *P. savastanoi, P. fluorescens, P. chlororaphis, P. putida,* and *P. marginalis* (Martin *et al.,* 1997).

1.3.2 TNT BIOREMEDIATION

There are two major degradation pathways for the bioremediation of TNT. The first pathway is initiated through the reduction of nitro groups in the aromatic ring via nitroreductase enzymes as observed in TNT metabolism by *Pseudomonas aeruginosa* (Figure 1.2) (Kalafut *et al.,* 1998). This initial nitroreduction produces 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene. Complete reduction of all of the aromatic nitro groups results in the production of 2,4,6-triaminotoluene (TAT). Bacteria such as *Desulfovirio sp.* are able to further reduce TAT, which results in the production of toluene (Boopathy & Kulpa, 1992). The formation of TAT requires a redox potential below -200 mV, which can only be provided under strictly anoxic conditions (Esteve-Nunez *et al.,* 2001).

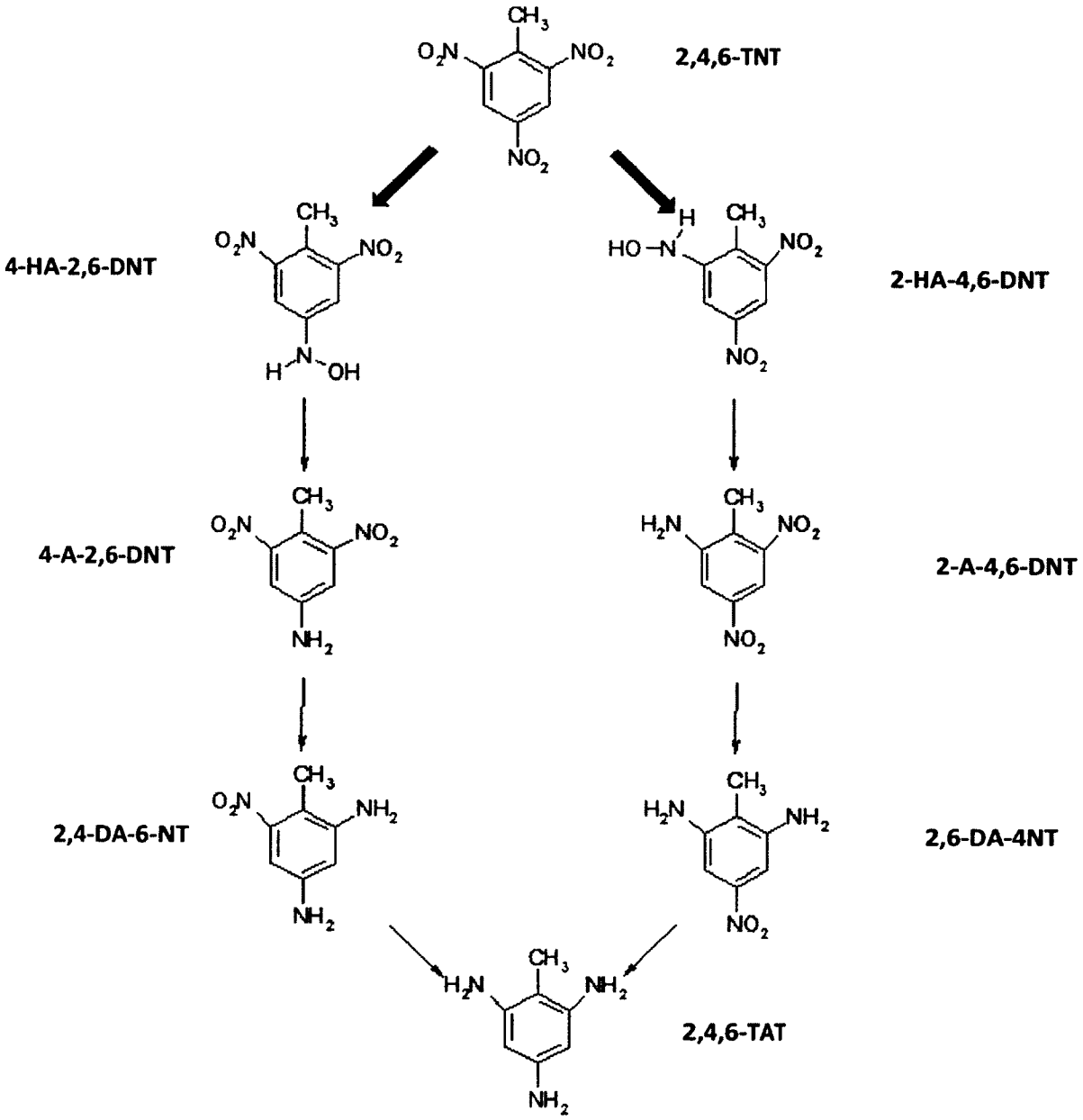

Figure 1.2: Reduction of the nitro groups during microbial metabolism of TNT. The two sides of the figure differ according to the nitro group that is reduced first. The last step in the reaction mechanism only occurs under anoxic conditions (Kalafut *et al.*, 1998).

Another major pathway for the biodegradation of TNT involves the denitrification of the aromatic ring as observed in *Pseudomonas savastanoi* (Figure 1.3) (Martin *et al.,* 1997). Denitrification is accomplished through the formation of what is known as a Meisenheimer complex (Figure 1.4), in which aromatic ring reduction occurs through hydride ion addition and aromaticity is restored through the elimination of a nitrite group via electron shuffling (Vorbeck *et al.,* 1994). Biodegration by denitrification results in the release of nitrite ions and the ultimate production of toluene.

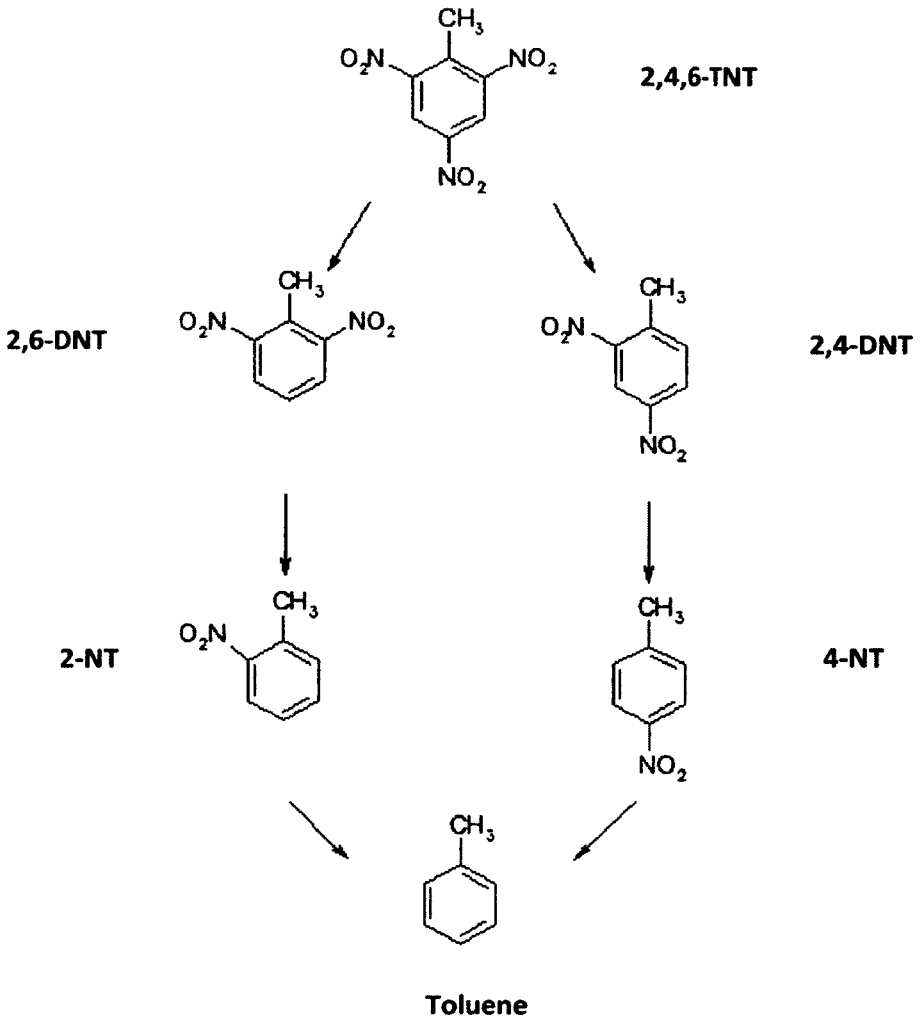

Figure 1.3: Catabolism of TNT via denitrification. The two sides of the pathway differ by what nitro-group is first eliminated (Martin *et al.*, 1997).

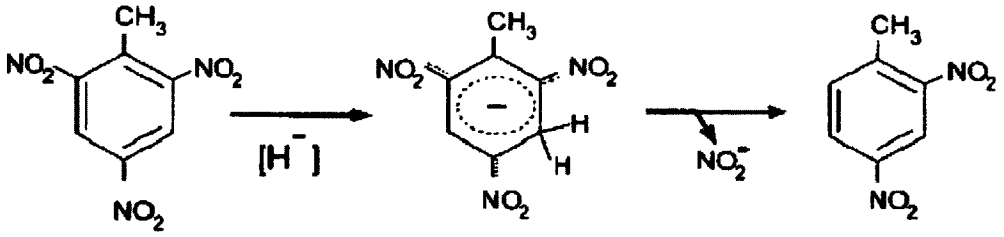

Figure 1.4: Formation of a Meisenheimer complex in the denitration of TNT (Esteve-Nunez *et al.*, 2001).

Mineralization (conversion of an organic substrate to inorganic products) of TNT is not commonly observed due to the inability to cleave the aromatic ring. However, bacteria capable of mineralizing the TNT metabolite 2,4-DNT and 2,6-DNT have been found. Enrichment culturing with 2,4-DNT revealed the presence of a species of *Pseudomonas* capable of using the substrate as its sole source of carbon and energy (Spanggord *et al.,* 1991). Mineralization is accomplished through the dioxygenation of 2.4-DNT to 4-methyl-5-nitrocathecol which itself is oxygenated to 2-hydroxy-5-methylquinone and then reduced to 2,4,5-trihydroxytoluene. Another oxidation reaction produces 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid, which subsequently undergoes ring cleavage that results in mineralization (Haigler *et al.,* 1994). This reaction mechanism is illustrated in Figure 1.5. Bacteria such as *Hydrogenophaga palleronii* JS863 are able to mineralize 2,6-DNT in a similar way and *Burkholderia cepacia* JS922 is able to degrade both isomers (Nishino *et al.,* 1999).

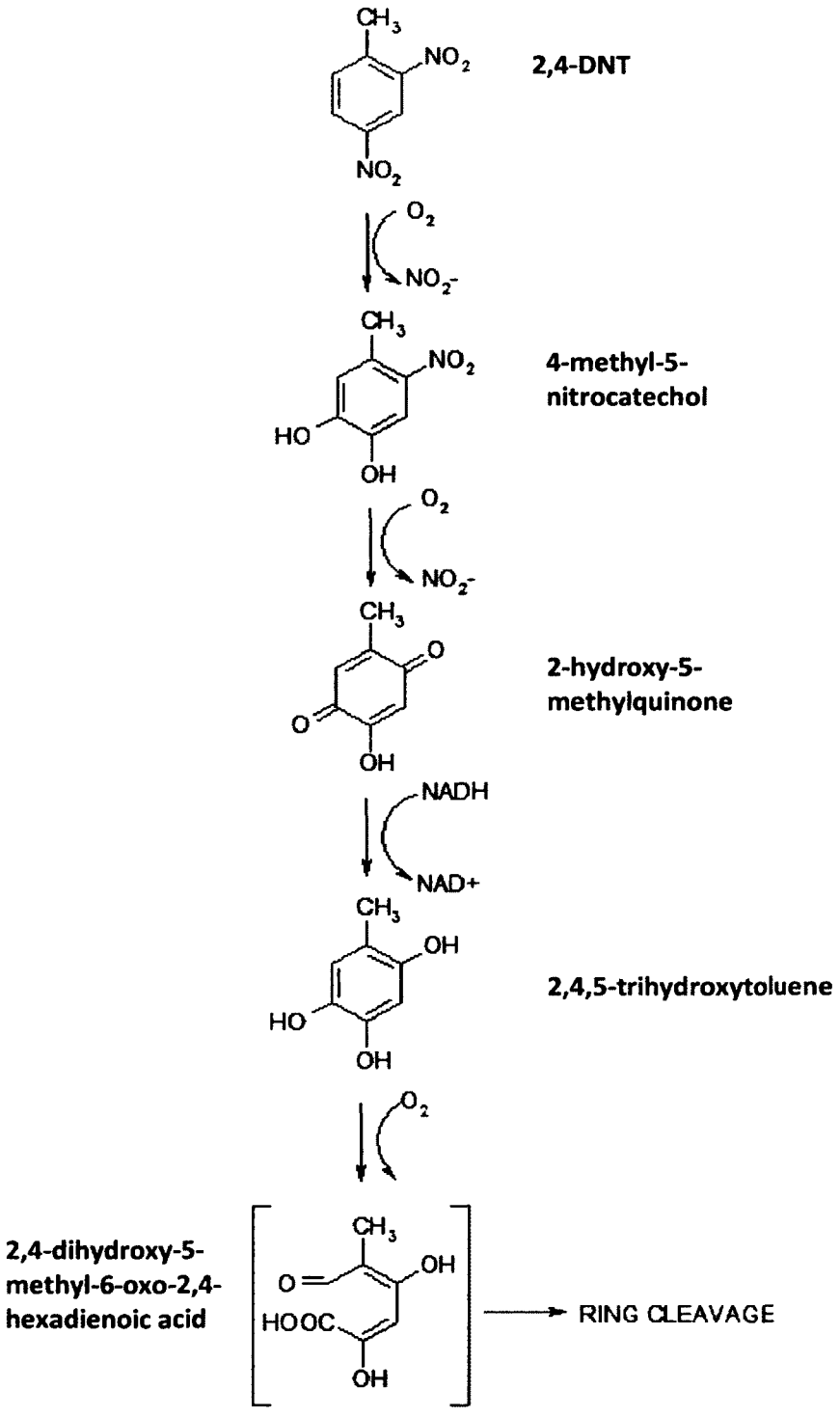

Figure 1.5: Mineralization of 2,4-DNT by *Pseudomonas sp.* (Haigler *et al.*, 1994).

1.4 TNB BIOREMEDIATION

1,3,5-Trinitrobenzene is a compound of similar structure and chemistry to TNT that is often produced as a minor byproduct during the manufacturing of TNT and can thereby be detonated in military firing ranges and industrial mining activities. Bioremediation of TNB is not as well known, however the bacterium *Pseudomonas vesicularis* was shown to use TNB as its sole source of nitrogen and remove the nitro-aromatic from solution (Davis *et al.,* 1997). Metabolism of TNB by *Pseudomonas vesicularis* is carried out through a series of reductions resulting in the removal of nitro groups from the aromatic ring (Figure 1.6). One of the nitro groups is first reduced to produce dinitroaniline and this product is then deaminated by the loss of ammonia to produce dinitrobenzene (DNB). Subsequently, DNB is reduced to produce nitroaniline, which is deaminated to nitrobenzene (NB) as a final product. No further transformation of NB is observed.

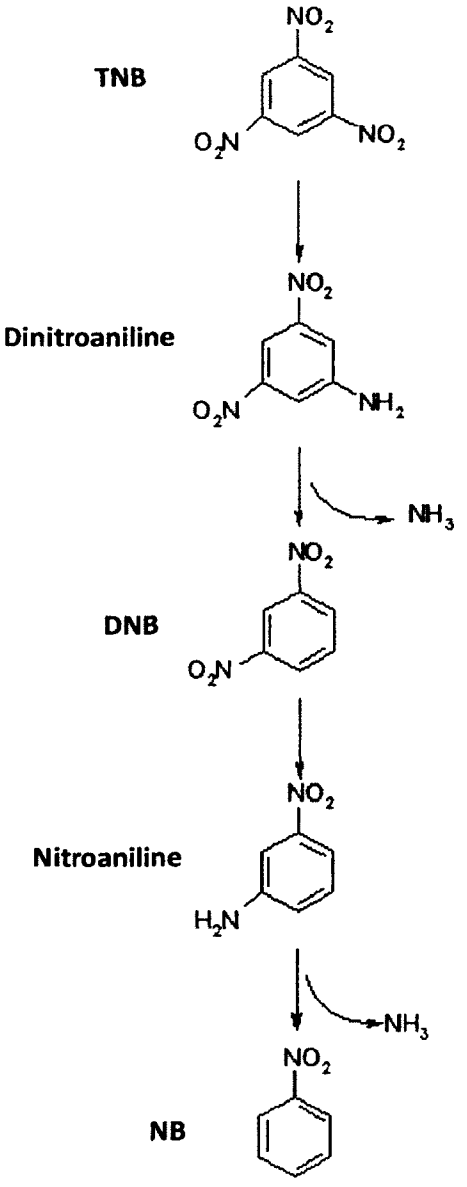

Figure 1.6: Transformation of TNB by *Pseudomonas vesicularis* (Davis et *al.*, 1997).

1.5 RDX

Another commonly used explosive used in mining and munitions is nitroamine hexahydro-1,3,5-trinitro-1,3,5-triazine, also known as RDX (Royal Demolition Explosive/Research Department Explosive). It has been a commonly used explosive in military campaigns dating back to World War II, and its civilian applications include fireworks, demolition blocks, heating fuel for food rations and even as a rodenticide (Global Security, 2011). It can cause convulsions when inhaled or ingested and is classified as a possible human carcinogen (EPA, 1988).

1.5.1 RDX BIOREMEDIATORS

Culturing methods were used to identify RDX degraders from an explosive contaminated site as *Morganella* morganii, *Providencia rettgeri* and *Citrobacter freundii* (Kitts *et al.,* 1994). There have also been numerous metagenomic studies to identify RDX metabolizing bacteria for possible bioremediation purposes. Bacteria in a groundwater sample contaminated with RDX were identified at the genus level through DGGE. Predominant genera were *Pseudomonas sp., Rhodococcus sp., Enterobacter sp., Shewanella sp., Clostridium sp.* (Fuller et *al.,* 2010). In addition, there were several 16S sequence alignments with no known genera, which emphasizes that many key players in RDX metabolism have yet to be identified. *Rhodococcus rhodochrous* has previously been shown to aerobically metabolize RDX. A gene coding for a cytochrome P450 like-enzyme XplA was isolated from this species as a probable RDX-degrading enzyme (Seth-Smith et *al.,* 2008). This hypothesis was supported by the observation that no RDX

degradation was observed in the presence of the cytochrome P450 inhibitor metyrapone compared to near complete depletion of RDX in the absence of metyrapone.

1.5.2 RDX BIOREMEDIATION

Aerobic metabolism of RDX by cytochrome P450 XplA is believed to result in denitration of the triazine ring, with the concomitant release of nitric oxide. Subsequently, an unstable ring formation spontaneously breaks down into formaldehyde, nitrous oxide and carbon dioxide (Seth-Smith *et al.*, 2002). Ring breakage is possible in RDX metabolism but not for TNT due to the lack of aromaticity in the former compound (Van Aken & Agathos, 2001). Aerobic bacteria *Williamsia sp. KTR4* and *Gordonia sp. KTR9* have been shown to be capable of using RDX as a sole source of carbon and nitrogen (Thompson *et al.*, 2005); this was the first reported case of RDX being degraded aerobically with no additional carbon or nitrogen sources required.

Methanogens are able to break down RDX using an alternative pathway (Hawari *et al.*, 2000). As illustrated in Figure 1.7, this degradative pathway begins with the direct cleavage of the triazine ring resulting in the production of methylenedinitramine and bis(hydroxymethyl)nitramine. When dissolved in water, methylenedinitramine will decompose into nitrous oxide and formaldehyde (Halasz *et al.*, 2002). An additional pathway, observed in an aerobic mixed culture, includes the sequential reduction of RDX to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) and finally to hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)

prior to ring cleavage that yields methanol, formaldehyde and hydrazine, and dimethylhydrazine (Figure 1.8) (McCormick *et al.,* 1981). Hydrazine and dimethylhydrazine are not reported as byproducts in a very similar reduction of RDX by a species of the genus *Clostridium* (Zhao *et al.,* 2003). While both aerobic and anaerobic degradation of RDX is possible, anaerobic degradation is significantly faster and is observed more extensively in the environment (Sagi-Ben Moshe *et al.,* 2012).

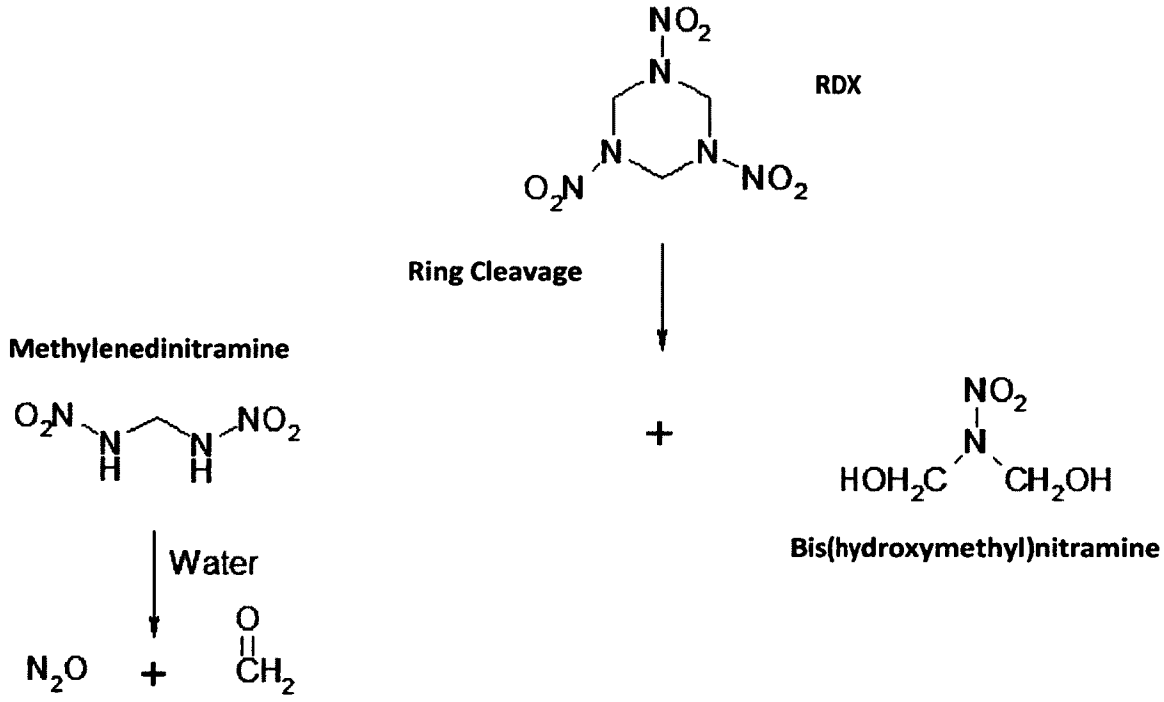

Figure 1.7: Catabolism of RDX through direct ring cleavage as observed in methanogens (Halasz *et al.*, 2002).

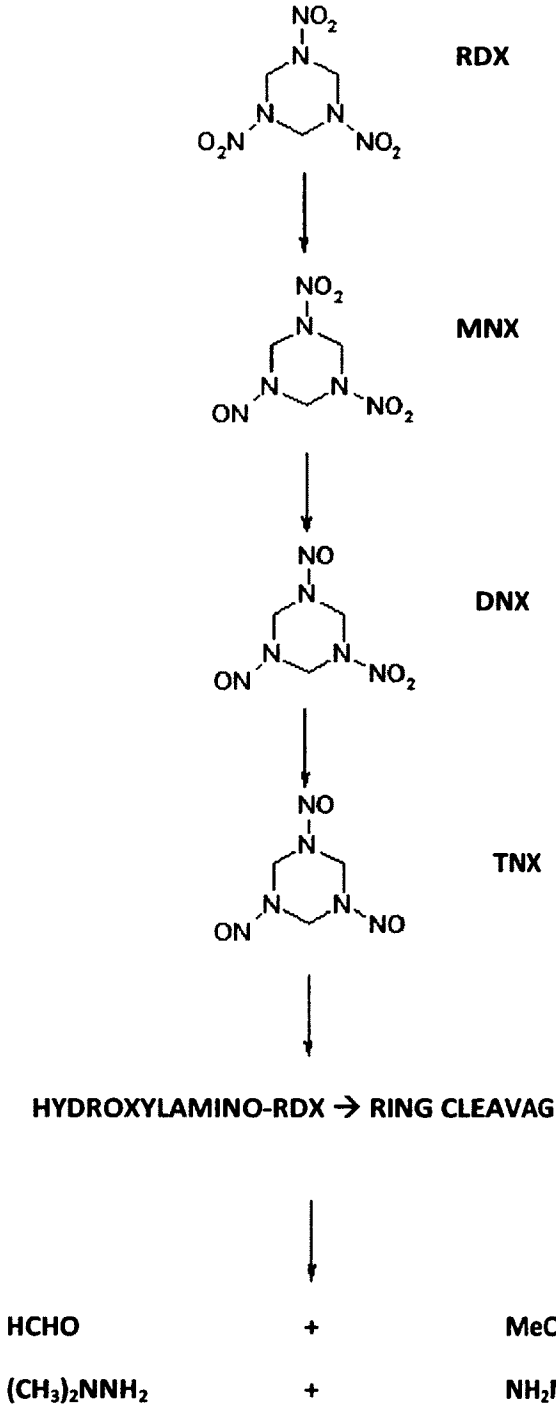

Figure 1.8: Reduction of the nitro groups in an alternate pathway for the catabolism of RDX (McCormick *et al.*, 1981).

1.6 HMX

Another nitro-aromatic explosive used extensively for military purposes is octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, also known as high melting explosive (HMX). HMX shows no significant toxicological effects on soil microbes in concentrations as high as 12.5 g/kg of soil (Gong *et al.,* 2002).

1.6.1 HMX BIOREMEDIATORS

Biodegradation of HMX occurs at a much slower rate than RDX due to the former's lower water solubility and higher chemical stability (Hawari *et al.,* 2001). Anaerobic bacteria capable of HMX degradation were isolated from a marine unexploded ordnance site. Sediments were first tested for their ability to remove spiked HMX and then predominant clusters of taxa were identified through 16S rRNA sequence alignment. HMX-degrading genera identified include *Clostridiales, Paenibacillus, Tepidibacter* and *Desulfovibrio* (Zhao et *al.,* 2007). In a study by Kitts et al. (1994) the bacteria identified were also shown to be capable of degrading HMX, though to a lesser extent than RDX. It was also found that HMX degradation by *Providencia rettgeri* and *Citrobacter freundii* was inhibited by the presence of RDX which may indicate a preference for RDX as a substrate over HMX (competitive biodegradation).

1.6.2 HMX BIOREMEDIATION

Anaerobic degradation of HMX occurs through two possible pathways. In the first pathway (Figure 1.9), nitro groups are sequentially reduced to nitroso groups followed by a spontaneous ring cleavage to produce nitrous oxide and formaldehyde (Zhao *et al.*, 2007). In the other pathway (Figure 1.10), denitration similar to the mechanism described in TNT degradation occurs to produce $O_2NNHCH_2NHNO_2$, which mineralizes to produce nitrous oxide and formaldehyde (Zhao et *al.*, 2007). HMX degradation through nitro reduction and denitration is observed in genera *Clostridiales*, *Paenibacillus*, *Tepidibacter* and *Desulfovibrio* (Zhao et *al.*, 2007).

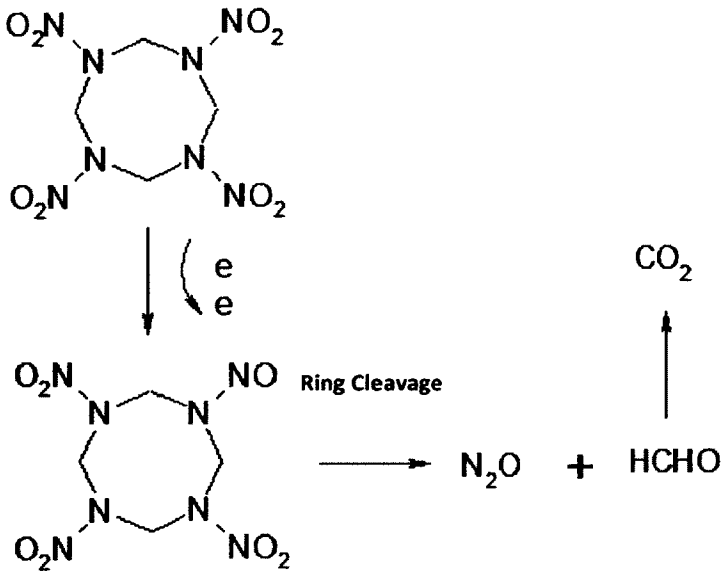

Figure 1.9: Metabolism of HMX by reduction of a nitro group followed by ring cleavage (Zhao *et al.*, 2007).

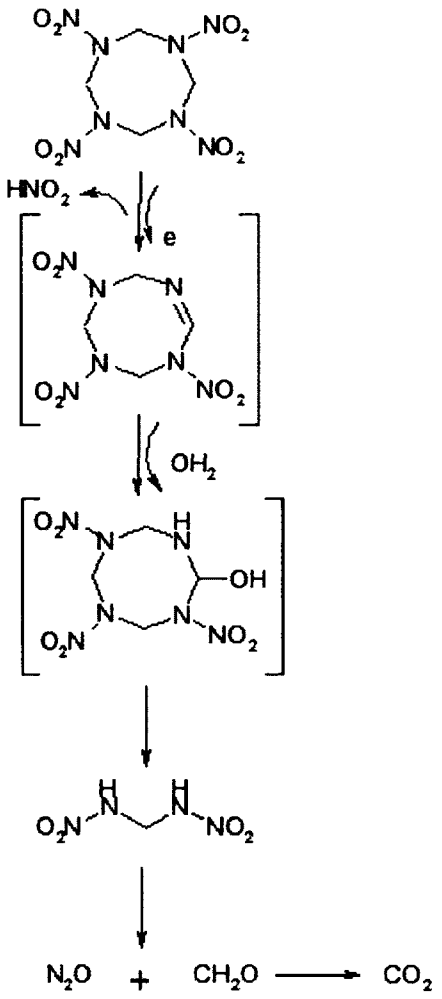

Figure 1.10: Anaerobic metabolism of HMX through denitrition (Zhao *et al.*, 2007).

The first step in the aerobic biodegradation pathway of HMX is the reduction to the mono-nitroso derivative 1-NO-HMX (Fournier *et al.,* 2004). This metabolite is then degraded either by denitration or alpha-hydroxylation followed by hydrolytic ring cleavage to produce 4-nitro-2,4-diazabutanal (NDAB), nitrite, nitrous oxide and formaldehyde. This biodegradative pathway was modeled based on transformation by the fungus *Phanerochaete chrysosporium*. Strict aerobic biodegradation of HMX has only been observed in fungi, and has not yet to have been reported in bacteria.

1.7 BIOREMEDIATION OF MIXTURES OF TNT, RDX, & HMX

There are examples of microbes able to transform more than one of the principle explosives described above. For example, a photosymbiotic bacterium, *Methylobacterium sp.*, isolated from poplar tissues and identified through 16S rRNA and 16S-23S sequence analysis, was shown to transform TNT and mineralize RDX and HMX in pure cultures (Van Aken *et al.,* 2004). Due to the similar chemistries of RDX and HMX (both are non-aromatic, cyclic, nitramine derivatives), there are many instances in which a bacterial species is able to degrade both of these compounds (Crocker *et al.,* 2006). Examples of bacterial species able to catabolise both RDX and HMX include *Morganella morganii* B2, *Citrobacter freundii* NS2 *Providencia rettgeri* B1 (Kitts *et al.,* 1994) and several species of the genus *Clostridium* (Zhao *et al.,* 2003).

Biodegradation of a mixture of TNT, RDX and HMX may take place differently than the degradation of each nitro-organic individually. The degradation of a mixture would

be more relevant as these three explosives are often utilized in various combinations together. The presence of another explosive can have an effect on the metabolism of another due to factors such as competitive biodegradation, by a metabolite inhibiting biodegradation of another compound by preventing enzymatic activity in the other's catabolic pathway, or as the result of toxicity of one explosive or its metabolite (Sagi-Ben Moshe *et al.,* 2009). In a soil slurry spiked with a mixture of TNT, RDX, and HMX with concentrations of 16, 20 and 5 mg/L respectively it was observed that the explosives were degraded sequentially by indigenous microorganisms (Sagi-Ben Moshe *et al.,* 2009). TNT was degraded first and when it disappeared from the slurry then RDX began to be degraded. HMX degradation only began following a significant decrease in the RDX concentration (Sagi-Ben Moshe *et al.,* 2009). The degradation of RDX and HMX was observed to be much faster separately than in a mixture containing RDX, HMX, and TNT. It can thereby be inferred that TNT inhibits the degradation of RDX and HMX. The complete biodegradation inhibition of the latter two explosives when a high concentration of 90 mg/L of TNT was added to the slurry is consistent with toxicity of TNT and/or its metabolites on the microbes that degrade RDX and HMX (Sagi-Ben Moshe *et al.,* 2009).

There are many soil microorganisms that may adapt to degrade TNT, RDX and HMX upon the addition of a nutrient growth substrate to the bioreactor treatment. For example, in soil contaminated with all three explosives, addition of growth supplement resulted in reduction in the concentrations of TNT, HMX, and RDX from 283 ±100, 67± 20 and 144 ± 50 mg/kg soil to 10 ± 10, 34 ± 20 and 12 ± 10 mg/kg of soil respectively

(Axtell *et al.,* 2000). The addition of growth supplement allows for the growth of bacteria that cannot grow solely by degrading the nitro-explosives by providing alternative sources of carbon and nitrogen. A summary of microbes known to degrade the nitro-organic explosives is provided in Table 1.2.

Table 1.2: Summary of bacteria capable of metabolising the nitro-organic explosives TNT, RDX and HMX.

Nitroaromatic Explosive	Genus	Species	Identification Method	Reference
TNT	*Pseudomonas*	*N/A*	DGGE	Georges *et al.*, 1998
		aeruginosa	Culturing, fatty acid assay	Martin *et al.*, 1997
		savastanoi		
		fluorescens		
		chlororaphis		
		putida		
		marginalis		
	Alcaligenes	*xylosoxydans*		
		piechaudii		
	Brevundimonas	N/A	DGGE	Georges *et al.*, 1998
	Acidovorax			
	Rhodanobacter			
RDX	*Pseudomonas*	N/A	DGGE	Fuller *et al.*, 2010
	Rhodococcus			
	Enterobacter			
	Shewanella			
	Clostridium			
	Rhodococcus	*rhodochrous*	Aerobic enrichment of soil, 16S sequence alignment	Seth-Smith *et al.*, 2002
	Williamsia	*sp. KTR4*	Culture enrichment, 16S rRNA alignments	Thompson *et al.*, 2005
	Gordonia	*sp. KTR9*		
	Providencia	*rettgeri B1*	Dilution plating, Biolog system, API20E system	Kitts *et al.*, 1994
	Morganella	*morganii*		
	Citrobacter	*freundii NS2*		
	Serratia	*marcescens*	Liquid culturing from horse manure sample spiked with RDX.	Young *et al.*, 1997
	Clostridium	*bifermentans*	Enrichment culturing, 16S sequence alignments	Zhao *et al.*, 2003.
		Celerecrescens		
		saccharolyticum		
		butyricum		
	Desulfovibrio	*Desulfuricans*		
HMX	*Clostridiales, Paenibacillus, Tepidibacter* and *Desulfovibrio*	N/A	16S sequence alignment from unexploded marine ordinance site	Zhao *et al.*, 2007
TNB	*Pseudomonas*	*vesicularis*	Enrichment culturing	Davis *et al.*, 1997
TNT, RDX, HMX	*Methylobacterium*	N/A	16S-23S sequence analysis	Van Aken *et al.*, 2004

1.8 METAGENOMIC ANALYSIS

One of the major hurdles in the study of microbial composition from soil is the fact that over 99% of microorganisms are not amenable to culturing (Suenaga, 2011). It has been reported that 1 g of soil can contain up to 10 billion microorganisms and thousands of different species (Knietch *et al.,* 2003). Culturing would thereby fail to detect a large quantity of bacteria in the soil. Inability to culture in a laboratory setting often arises from an extremely high substrate concentration or a lack of specific nutrients (Mocali & Benedetti, 2010). It also arises from the difficulty in precisely mimicking environmental conditions in a laboratory culture. In order to obtain a more accurate description of environmental microbial populations a variety of culture-independent DNA- and RNA-based analysis techniques have been established that are collectively referred to as metagenomics. For example, when estimating the community genome size of a heavily polluted site using culturing methods, less than 40 genomes were recovered. However, using culture independent methods on the same sample a genome that was 300-1500 times the size of the *Escherichia coli* genome was obtained (Torsvik *et al.,* 2002). This emphasizes how community structure can be described in much detail using DNA- and RNA-based analysis techniques.

1.8.1 16S rRNA GENE FOR MICROBIAL IDENTIFICATION

Metagenomic analysis begins with the extraction of nucleic acids from soil microbes; in many applications the subsequent amplification of a gene of interest for microbial identification. A common target gene for this purpose is the 1.5 kb 16S rRNA gene. The 16S rRNA gene is ideal for the establishment for phylogenetic relationships as it does not undergo genetic transfer between species, its function is constant throughout, and it contains a sufficient number of residues that change at a rate that is proportional to evolutionary distance between microbes (Pace *et al.,* 1986). Another rRNA gene that also meets these requirements and is another common target gene is the 23S rRNA. The 16S rRNA gene is an ideal marker due to its high overall conservation among prokaryotes with several sites of heterogeneity that allow for taxonomic relationships to be inferred (Case *et al.,* 2007). The 16S rRNA gene was first targeted for microbial identification in 1990 as a culture-independent fingerprinting method (Giovannoni *et al.,* 1990; Ward *et al.,* 1990). Universal primers are made to bind to the conserved regions of the 16S gene for its amplification as shown in Table 1.3 (Marchesi *et al.,* 1998). Several pairs of universal primers have been used with nomenclature of the primer based on its annealing site on the 16S rRNA gene. Examples of commonly used primers include 8F, 27F, 63F, 1387R, and 1389R. Primer 63F has been reported to show mismatching in its annealing to the 5' end of some sequences of *Bacillus,* making it non-ideal (Sipos *et al.,* 2007). Primers can also be designed to target variable regions of a specific species in order amplify the one specific 16S gene of interest (Hiorns *et al.,* 1995). The 16SrRNA molecule is depicted in Figure 1.11.

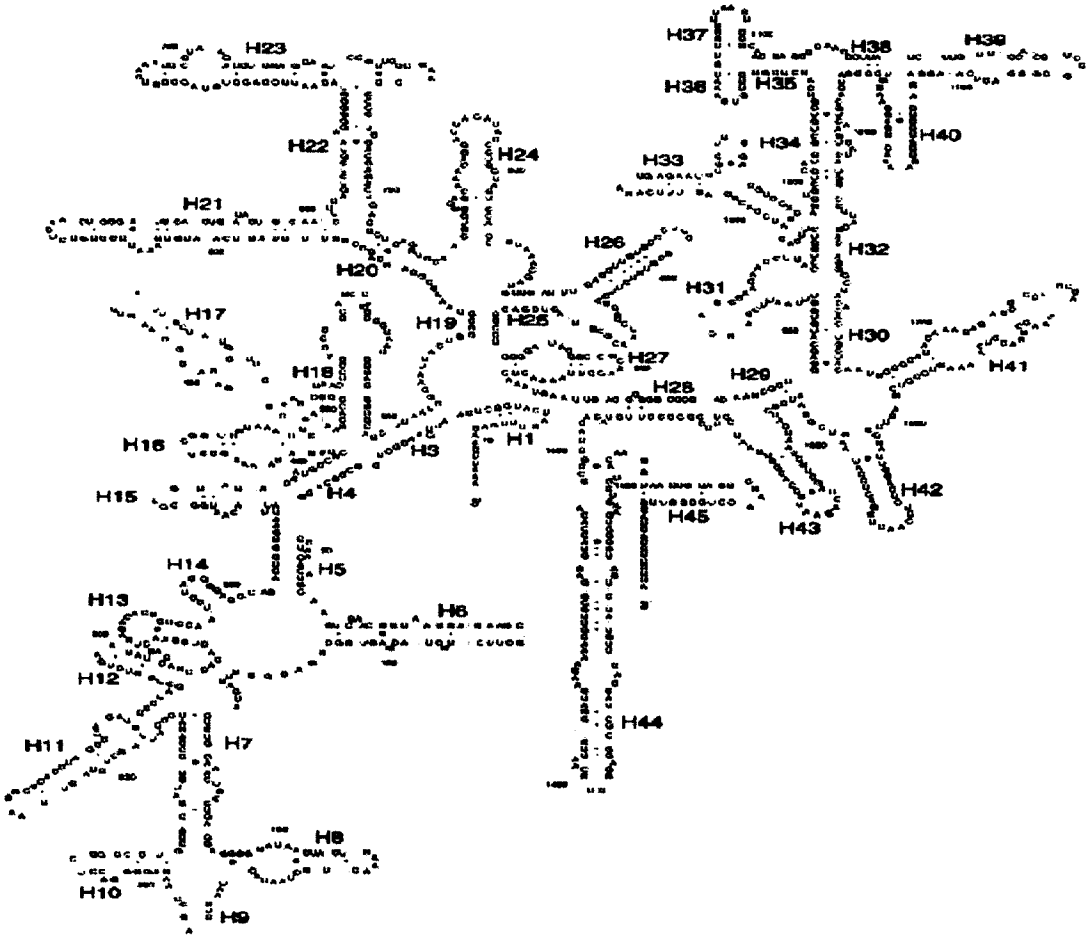

Figure 1.11: Structure of the 16S rRNA molecule based upon *E. coli* K-12 showing areas of intergenomic heterogeneity. Blue bases indicate heterogeneity observed in one other species while red bases are heterogeneous in two or more of the 111 species analyzed (Case *et al.*, 2007).

Table 1.3: Alignment of primers 27f[b], 63f[c], 1387r[d], and 1392r[e] to the 16S rRNA gene sequences of various species of *Proteobacteria*. Underligned bases indicate a mismatch between the primer and gene sequence (Marchesi *et al.*, 1998).

Strain	Woese group	Sequence vs primers	
		27f and 63f	1387r and 1392r
		AGAGTTTGATCMTGGCTC[b] **CAGGCCTAACACATGCAA–GTC**[c]	**CRTGTGTGGCGGGCA**[d] **CGGAACATGTGMGGCGGG**[e]
Rhodobacter sphaeroides ATH 2.4.1	α-Proteobacteria	GAGAGTTTGATCCTGGCTCAGAATGAACGCTGGCGGCAGGCCTAACACATGCAA–GTC	GCCTTGTACACACCGCCCGT
Alcaligenes eutrophus	β-Proteobacteria	AAGAGTTTGATCCTGGCTCAGATTnAACGCTGGCGGCATGCCTTACACATGCAA–GTC	GTCTTGTACACACCGCCCGT
Campylobacter concisus (FDC 288)	ε-Proteobacteria	GAGAGTTTGATCCTGGCTCAGAGTGAACGCTGGTGGCGTGCCTAATACATGCAA–GTC	GTCTTGTACTCACCGCCCGT
Campylobacter curvus (ATCC 35224[T]; VPI 9584)	ε-Proteobacteria	GAGAGTTTGATCCTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAA–GTC	GTCTTGTACTCACNGCCCGT
Campylobacter rectus (CCUG 19168)	ε-Proteobacteria	GAGAGTTTGATCCTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAA–GTC	GTCTTGTACTCACCGCCCGT
Campylobacter showae (CCUG 3054)	ε-Proteobacteria	GAGAGTTTGATCCTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAA–GTC	GTCTTGTACTCACCGCCCGT
Desulfobacter postgatei	δ-Proteobacteria	GAGAGTTTGATCCTGGCTCAGAATGAACGCTGGCGGCGTGCTTAACACATGCAA–GTC	GNNTTGTACACACCGCCNGT
Desulfovibrio desulfuricans (ATCC 27774)	δ-Proteobacteria	GAGAGTNTGATTCTGGCTCAGATTGAACGCTGGCGGCGTGCTTAACACATGCAA–GTC	GCCTTGTACACACCGCCCGT
Myxococcus xanthus DK1622 (ATCC null)	δ-Proteobacteria	GAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCGTGCCTAACACATGCAA–GTC	GCCTTGTACACACCGCCCGT
Acinetobacter calcoaceticus	γ-Proteobacteria	AAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAA–GTC	GCCTTGTACACACCGCCCGT
Escherichia coli	γ-Proteobacteria	AAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAA–GTC	CCTTGTACACACCGCCCGTC
Pseudoramibacter alactolyticus DSM 3980, ATCC 23263	γ-Proteobacteria	-AGAGTTTGATYMTGGCTCAGGACGAACGCTGGCGGTATGCTTAACACATGCAA–GTC	GTCTTGTACACACCGCCCGT

Sources of bias may result in differential PCR amplification of the 16S gene that would have an impact on subsequent community analysis. A major source of bias in the amplification of a complex mixture of different 16S genes is the preferential amplification of one copy of the gene over another. Reasons for preferential amplification of certain gene copies include easier denaturation due to a lower GC content, higher binding-efficiency of degenerate primers onto annealing sites with higher GC content, variable accessibility of the 16S genes within different genomes and a higher copy number of the gene. These biases are collectively referred to as *PCR selection* (Wagner *et al.,* 1994). The other type of bias is the inability to replicate amplification conditions in early cycles when the genomic template is still being amplified. This is known as *PCR drift*. In order to limit all of these biases, it is recommended to avoid primers that anneal to sites that are observed to show degeneracy to avoid preferential annealing to GC rich sites. Also, high concentrations of template should be used and replicate PCR products should be pooled together to limit PCR drift. To diminish the effects of PCR selection, the minimum number of cycles to adequately amplify the gene of interest should be used (Polz & Cavanaugh, 1998).

Another limitation to identifying microbial diversity through amplification of the 16S gene is the potential for the production of chimeric sequences. This phenomenon is characterized by the coamplification of two separate 16S sequences from a mixed genome population producing a composite sequence (Wang & Wang, 1997). The proposed mechanism for the formation of chimeric sequences is that an aborted extension product from a previous cycle of the PCR acts as a mis-primer in a subsequent

cycle (Haas *et al.,* 2011). The abundance of aborted extended sequences increases as the number of PCR cycles increases (Wang & Wang, 1997). In a model study to determine the frequency of chimeras using specific oligonucleotide probes at the ends of sequences it was found that the proportion of chimeras increased from 11.1% after 30 cycles to 15.5% after 35 cycles (Wang & Wang, 1997). Therefore, in order to limit this concern only the minimum number of cycles to obtain successful clone libraries should be employed when amplifying the 16S gene.

Additional problems that may arise through the use of the 16S gene for identification is that some microbes have been shown to have more than one copy of this gene. An example of this phenomenon is seen in *Nocardia nova* (Conville & Witebsky, 2005). Presence of more than one copy of the 16S gene would produce additional bands in fingerprinting analysis, which may lead to difficulties in microbial identification.

1.8.2 FINGERPRINTING

Fingerprinting is the term applied to a variety of laboratory techniques employed to visually detect genetic variation. Cloning is often performed through ligation of the 16S rRNA amplicon into a vector, such as the pCR 2.1-TOPO vector (Invitrogen), containing a selectable trait in order to identify successful 16S clones. Different copies of the 16S rRNA gene can then be observed through restriction fragment length polymorphisms (RFLP). Other fingerprinting methods commonly used include denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP), and terminal-restriction fragment length polymorphism (t-RFLP).

1.8.3 RESTRICTION FRAGMENT LENGTH POLYMORPHISM

RFLP is commonly used to look for variation between different species within one gene. For example, copies of the 16S rRNA gene derived from different bacteria will often contain different restriction sites. The variation between different copies of the gene can be visualized by comparing the different banding patterns from the digestion as a result of the varied restriction sites. The choice of restriction enzyme for the digestion is the preference of the experimenter, but the same enzyme is generally used for the entire fingerprinting experiment. Also, a four base pair cutter is optimal as it increases the probability that the gene will be cut at multiple sites, and this facilitates the acquisition of more detailed patterns needed for identification. Each unique banding pattern should correspond to a different sequence of the 16S rRNA gene. There is a

slight possibility that two digestions may exhibit the same RFLP pattern even though they have different sequences if their restriction sites are separated by the same number of base pairs along the gene. Qualitative measurements of microbial diversity can be inferred by observing the number of different restriction patterns on an agarose gel. Quantitative measurements of diversity can be made by cataloguing the number of different patterns as well as their frequencies.

A study to measure the diversity of soybean-nodulating rhizobia in soil from Japan was conducted using 16S rRNA gene amplification followed by RFLP (Suzuki *et al.*, 2008). Reference strains previously reported to have been found in similar soils were used in conjunction to the strains isolated from the sample sites. If a banding pattern from an isolated strain matched the banding pattern of the reference strain they were concluded to be the same and sequencing of the isolated strain would not be necessary. Three different restriction enzymes were used in the study to ensure results were consistent throughout. The RFLP method in this study allowed for quick identification of isolated strains based on previous data, but this would not be possible in previously uncharacterized soils with no known reference strains.

1.8.4 DENATURING GRADIENT GEL ELECTROPHORESIS

DGGE is an alternative fingerprinting method for measuring microbial diversity. This method was first utilized for microbial environmental fingerprinting by Muyzer *et al.* in 1993. In this protocol, the gene of interest for identification is amplified from microbial DNA and the PCR products are collectively run on a polyacrylamide gel that has a denaturation gradient increasing from the cathode to the anode. The distance travelled by a certain copy of the gene along the gel is determined by its GC-content. Sequences with a higher proportion of GC will require a higher concentration of denaturants to partially unwind the gene and halt its progress on the gel. Incorporation of a GC-ladder in the PCR primers ensures that only partial unwinding occurs as complete unwinding of the genes will cause diffusion off of the gel (Muyzer *et al.,* 1993). This technique allows for immediate differentiation of multiple copies of the same gene and bands of interest can be excised for sequencing. However, diverse microbial samples will produce a plethora of bands that may cause difficulties in identifying individual bands. Furthermore, additional bands on a DGGE gel may appear as a result of the abortion of the elongation reaction during PCR due to the formation of a hairpin loop in the GC-clamp (Nübel *et al.,* 1996). These aborted sequences would produce additional bands on the gel that would cause an overestimation in microbial diversity.

DGGE was employed in a study measuring the methanotroph diversity in landfill soil (Wise *et al.,* 1999). DGGE band analysis revealed the presence of 12 distinct bands that were amplified through a 16S primer pair specific to type I methanotrophs. Two diffuse bands were observed at low denaturant concentration and it was determined that these

bands resulted from heteroduplex formations between two similar, but non-identical strands of DNA annealing together. These heteroduplex molecules have a lower melting temperature due to the various mismatches and therefore melt at a lower denaturant concentration. Heteroduplex molecules such as these can complicate a DGGE banding profile (Wise *et al.,* 1999). The other bands observed at larger denaturant concentrations were from methantrophs present in the soil sample and most were matched to isolates obtained from the soil sample based on culturing and cloning techniques.

1.8.5 SINGLE STRAND CONFORMATION POLYMORPHISM

Like DGGE, Single strand conformation polymorphism (SSCP) also involves the amplification of extracted nucleic acids; however one of the primers is phosphorylated at the 5' end. PCR products are then digested with lambda exonuclease that will selectively degrade the phosphorylated strand of double stranded DNA (dsDNA) leaving singly stranded DNA (ssDNA) which is then run on a polyacrylamide gel under non-denaturing conditions (Schwiger & Tebbe, 1998). Separation of the strands may also be accomplished through the use of an alkali denaturing buffer followed by heating and then an immediate cooling to prevent re-annealing (Stach *et al.,* 2001). Each ssDNA fragment will assume a unique conformation based on its sequence, and its progression along the gel will be determined by this conformation. SSCP allows for the differentiation of sequences that vary by as little as one base, but it is labour intensive to produce the single strands and ensure that there is no re-annealing.

SSCP was used to identify the microbial diversity of the same soil sample when different DNA extraction techniques were employed (Stach *et al.,* 2001). Whole community profiles using SSCP were too complex to allow for phylogenetic distinction. Possible re-annealing and the formation of heteroduplex strands were hypothesized to cause the complexity. Furthermore, there were a large number of target sites for the primers employed. To simplify the profile, primers specific for the genus *Pseudomonas* were employed. *Pseudomonas*-specific PCR-SSCP allowed for clear profiles to be observed and the quality of each extraction technique was assessed by the bands present and their quality.

1.8.6 TERMINAL-RESTRICTION FRAGMENT LENGTH POLYMORPHISM

T-RFLP is another commonly used fingerprinting technique first used to measure microbial diversity in 1997 (Liu *et al.,* 1997). A fluorescence tag is added at the 5' end of PCR primers for marker gene amplification. PCR products are then digested with restriction enzymes and loaded onto a non-denaturing polyacrylamide gel. Gels are then analyzed with a DNA sequencer equipped with a fluorescence detector. Only the ends of each gene containing the fluorescent marker will be visualized with each band theoretically corresponding to a different marker gene sequence (e.g. 16S). The intensity of the fluorescence will correspond to the frequency of the microbe producing that terminal sequence. An electropherogram is produced, which is a measurement of fragment size in base pairs corresponding to the intensity of the fluorescence. Since it is

only the ends of the sequences being analyzed, this method increases the possibility that a single band may correspond to more than one microbe.

T-RFLP was employed in a study measuring the microbial community structure in soil used for agriculture (Hartmann *et al.,* 2006). 16S rDNA was amplified using a primer pair with the 27F primer tagged with 6-carboxyfluorescein (FAM). PCR products were then digested with *MspI* and run on a polyacrylamide gel. A total of 79 terminal restriction fragments were observed. T-RFLP allowed for a qualitative measurement of diversity through the banding patterns obtained. Sequence analysis of the fragments allowed for further detection of various microbes, but identification could only be made at the genus level.

Each of the fingerprinting methods described above may be used for measuring microbial soil diversity. The choice of method is often dependent on the investigator's preference and the resources available. In this study, RFLP will be used to generate a catalogue of different banding patterns to reflect the microbial diversity in the soil. Prominent clones will then be sent for sequencing and aligned to provide their identification.

1.9 OBJECTIVES AND HYPOTHESIS

A soil sample from a munitions firing range in Petawawa was obtained. This soil is contaminated with various concentrations of the nitro-organic explosives TNT, RDX, and HMX. The bacterial community structure of the soil as a result of the explosives contamination will be determined through RFLP and sequence analysis of the 16S rRNA gene from nucleic acids extracted from bacteria in the soil. The soil is mixed under controlled conditions in a bioreactor and samples are taken at various time points. Analysis of the 16S gene will determine the effects of the munitions on the community structure dependant on the duration of the bioslurry treatment and the addition or absence of a nutrient supplement to the sample.

OBJECTIVES

- To examine the microbial diversity in a soil sample derived from a munitions site using RFLP and DNA sequence analysis of the 16S rRNA genes. This will be achieved by classifying the various Operational Taxonomic Units (OTUs) using RFLP analysis, and identifying microbes producing the most frequently observed OTUs through sequencing of their 16S rRNA gene.
- Using carefully controlled conditions in a bioreactor, to examine changes in microbial diversity in a munitions contaminated soil over a 90 day period, with and without the addition of nutrients.

HYPOTHESIS

- Bacterial 16S rRNA analysis from bioreactor treated explosive contaminated soil will identify microbes that may have the capacity to degrade nitrosubstituted munitions.
- Temporal changes to the microbial community, with and without the addition of nutrients, will arise during a bioslurry treatment that reflect the preferential ability of particular bacteria to degrade munitions.

2.0 METHODS AND MATERIALS

2.1 SOIL SAMPLES

Soil samples that were examined in this thesis were obtained from military training ranges at CFB Petawawa (Petawawa, Ontario) and were generously donated by Dr. Sylvie Brochu (Defence Scientist, Life Cycle of Munitions Group, Energetic Materials Section, Defence Research and Development Canada (DRDC), Valcartier, Quebec). Three separate sites were sampled. The first sample site, denoted as PET 1, was collected from an anti-tank firing position. The second site, denoted as PET 2, was collected from an anti-tank target area. Both of these sites were part of the A Range on the firing test facility, which is a part of the Impact Area A training area located at CFB Petawawa. The majority of weapons deployed at these two locations were M72 Light Anti-tank Weapon (LAW) rockets (Brochu, 2008). These rockets contain M7 Double-Base Propellant which has a mixture of nitrocellulose (NC), nitroglycerin (NG) and potassium perchlorate in a ratio of 55:36:8. The LAWs also contain an explosive composition known as Octol, which has a mixture of HMX and TNT at an approximate ratio of 70:30 and a booster of either tetryl or RDX (Jenkins *et al.,* 2005). The third sample site, denoted as PET 3, was collected from the Delta Tower firing point located within the Direct Fire Target (DFT) Area 2, which was a small arms training facility that would use the explosives NG and 2,4-DNT most extensively. All samples were collected at a depth of ≤2.5 cm.

2.2 CHEMICAL ANALYSIS AND AMES MUTAGENICITY ANALYSIS

HPLC analysis to determine the identities and concentrations of residual explosives and Ames testing of the soil extract mutagenicity was performed by and described by Jennifer McAllister (Department of Biology, University of Ottawa, 2011). It was determined that the soil sample PET 2 contained the highest concentration of nitro-organic explosives contamination. Soil from this site was henceforth referred to as High Contamination Soil and was subsequently used for microbial identification analysis through bioreactor treatments.

2.3 BIOREACTOR OPERATION

2.3.1 PREPARATION

Bioreactor treatment and the collection of soil samples during the course of the treatment were conducted by Matt Meier and Caroline Rose. Soil slurries were allowed to aerate and mix in two BioFlo benchtop bioreactors obtained from New Brunswick Scientific, Edison, New Jersey. The reactors were first disassembled and washed in a Lancer automatic dishwasher (Model# 1B014066) using an acidic rinsing agent consisting of 25% acetic acid and laboratory detergent (Deacon Laboratories Inc.). Internal components of the bioreactor were rinsed in hexane and acetone in a fume hood followed by thorough rinsing with Sterile Milli-Q® water (Millipore Corp., Bedford, MA). The bioreactors were subsequently reassembled and 5L of Sterile Milli-Q® water was added. In order to ensure a closed system, all seals were coated with vacuum grease prior to the reassembly. In addition, sterile PVC tubing (Fisher Scientific Ltd.) was

prepared and added to all addition ports using no-leak quick connectors (TekniScience Inc.). Probes were added into the appropriate ports to monitor pH and dissolved oxygen and the entire system was sterilized for 30 minutes at 121°C in a Steris SV-120 Scientific Prevacuum sterilizer. The bioreactors were then allowed to cool and equilibrate to operating conditions. Air flow to the system was provided by a Gas and Cryo Equipment Zero Air Generator (Model# ZAG-150-50) through a 0.2µm milipore filter. The air flow was set at 10 L/min and was directed into the slurry through an aspirator located at the bottom of the vessel. Exhaust from the bioreactors was first passed through a cold water condenser and then a 0.2 µm Millipore™ Millex™ filter (Fisher Scientific Ltd.), and finally onto a container of activated charcoal. Mixing of soil slurries was provided by internal propellers set 7.5 cm apart on the agitator's rotating shaft, spinning at 400 rpm for the entirety of both treatments. Internal temperature of the bioreactor vessels was maintained at 25 °C through the combination of an internal cold water cooling coil and external heating from an electric heating blanket. Upon temperature stabilization as indicated by the temperature probe, the pH meter was connected and calibrated using an external pH standard. The pH was adjusted using 0.5 M HCl and/or 0.5 M NaOH which were added to the system using a peristaltic pump. Once a steady temperature of 25 °C and pH of 7 were obtained, the dissolved oxygen probe (Mettler Toledo InPro 6800 series O_2 sensor) was connected. The system was allowed to equilibrate for 24 hr at which point saturation was set (100 % dissolved oxygen). The probe was then disconnected from the system until a steady input value was seen on the calibration

screen to produce a span value (0 % dissolved oxygen). The level probe was then inserted into the correct port and set to the "wet off" mode.

All input tubes providing either sterile Milli-Q water, 0.5 M HCl, or 0.5 M NaOH were primed until drops were observed entering the bioreactor vessels. The soil samples were then introduced via the access port and the level controller was adjusted to the top of the slurry. In order to minimize light penetration the bioreactors were covered with four layers of black landscaping fabric.

A software program was created for all treatments to control the physical parameters of the two bioreactors. These parameters consisted of a temperature of 25 °C, a pH range between 6.5 and 8.0 and an agitation rate of 400 rpm. Over the course of the 90 day treatment, the software recorded dissolved oxygen, pH, temperature and agitation rate every 6 minutes.

2.3.2 OPERATION

2.3.2.1 BIOREACTOR TREATMENTS

Soil taken from the CFB Petawawa site was subjected to a 90 day remediation run using the duplicate BioFlo 110 modular benchtop bioreactors. Samples of the soil slurry were taken every 15 days beginning at day 0. Two different treatments were examined. The first treatment, known as control treatment conditions, consisted of constant volume, aeration, pH, temperature, light and agitation. A second treatment known as

nutrient addition, consisted of the same aforementioned conditions with the addition of a nutrient and micro-nutrient supplement that was added to the slurry every 30 days, commencing at day 0. The reagents for the nutrient addition treatment were obtained from a Medina contaminated soil clean up kit (Medina Agricultural Products Co. Inc., Hondo, Texas) and it included Bio-D and a microbial activator.

The Bio-D solution maintained a constant ratio of C/N/P of approximately 100/10/1 and it consisted of 21.0 mg/g ammonia-N, 21.0 mg/g nitrate-N, 144.3 mg/g organic-N, 28.0 mg/g ortho phosphate, 18.3 mg/g potassium and 31.4 mg/g humic acid. The microbial activator contains essential micronutrients provided from an algae-based fermentation extract. The addition of 3.1 mL of Bio-D and 1.55 mL of microbial activator to the nutrient addition treatment was performed at days 0, 30 and 60.

2.3.2.2 SAMPLE COLLECTION

Samples of the soil slurry were taken every 15 days during the treatment, commencing at day 0. At each sampling, 4 x 25 mL samples were collected into sterile glass BioFlo 110 sample vials (Fisher Scientific Ltd.). The contents of one vial from each sampling were transferred under a laminar flow hood to a sterile conical plastic Falcon tube with 7.5mL of 50% glycerol solution. The Falcon tube was than thoroughly vortexed and stored at -80°C for use in microbial community analysis. The other three vials from each sampling were stored in the dark a 4°C until needed for chemical analysis or determination of mutagenic activity.

2.4 NUCLEIC ACID EXTRACTION

Soil slurries were allowed to thaw at room temperature and were then homogenized using an Analog Vortex Mixer (VWR). Two aliquots of 1 mL soil mixture were placed into separate, sterile 1.5 mL graduated flat top microtubes (Diamed Tec Choice) and centrifuged at 14 600 x g for five minutes in a Sorvall Legend Micro 21 bench top centrifuge (Thermo Scientific). Microbial nucleic acids were then extracted using the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc.). A summary of the solutions used in this kit is provided in Table 2.1.

Bead solution solvent from the DNA Isolation Kit was transferred to the two soil pellets and the pellets were dissolved by vortexing in an Analog Vortex Mixer (VWR). The mixtures from both tubes were transferred into the bead solution tube and gently vortexed. Sixty µL of Solution S1 was added to the bead solution tube, which was then inverted several times. Next, 200 µL of Solution IRS (Inhibitor Removal Solution) was added and the tubes were vortexed horizontally at maximum speed for 10 minutes. The tube was then centrifuged at 10 000 x g for 30 seconds, the supernatant was then transferred to a sterile 1.5 mL microcentrifuge tube, and the pellet was discarded. 250 µL of Solution S2 were then added, the tube was vortexed for 5 seconds and placed on ice for 5 minutes. The tube was then centrifuged for 1 minute at 10 000 x g and the entire supernatant was transferred to another sterile 1.5 mL microcentrifuge tube. Subsequently, 1.3 mL of Solution S3 was added and the tube was vortexed for 5 seconds. A 700 µL aliquot of this solution was added to the centre of a sterile spin filter tube. The spin filter tube was then centrifuged at 10 000 x g for 1 minute and the flow-

through was discarded. Additional 700 µL aliquots of the solution were spun through the filter until all of the mixture was filtered. Next, 300 µL of Solution S4 was added to the filter and the filter was centrifuged for 30 seconds at 10 000 x g. The flow-through was discarded and the spin filter was centrifuged again for 1 minute at 10 000 x g. The filter was then transferred to a new sterile 1.5 mL microcentrifuge tube and 50 µL of Solution S5 was added to the centre of the white filter. The tube was then centrifuged for 30 seconds at 10 000 x g. The flow-through then consisted of purified nucleic acids. A small sample of the flow through was run on a 0.7 % w/v agarose gel to confirm the presence of a DNA band. The DNA extract was stored indefinitely at -20 °C. Extractions were performed in duplicate for each soil sample and denoted either as Extraction A or Extraction B. No further purification of the DNA was needed in order to perform subsequent procedures.

Table 2.1: Summary of reagents used in the UltraClean Soil DNA Isolation Kit (MoBio).

Solution	*Function*
S1	SDS detergent solution that aids in cellular lysis by breaking down fatty acids and lipids associated with the cell membrane.
IRS	Inhibitory Removal Solution. Proprietary reagent that causes the precipitation of humic acids and other PCR inhibitors.
S2	Protein precipitation reagent that removes contaminating proteins that may reduce DNA purity and inhibit PCRs.
S3	DNA salt binding solution that allows for the DNA to bind to the silica in the spin filter membrane.
S4	Ethanol based wash solution that further cleans the silica-bound DNA from contaminating salts, humic acids, and other contaminants while allowing the DNA to remain bound to the filter membrane.
S5	Elution buffer that dilutes out the salt that binds the DNA to the silica filter membrane allowing for the collection of purified DNA.

2.5 0.7% W/V AGAROSE GEL ELECTROPHORESIS

Extracted nucleic acids were electrophoresed on a 0.7% w/v Ultrapure agarose gel. The gel was made by dissolving 0.525 g of agarose (Invitrogen) in 75 mL of 1X TAE buffer solution (20X: 96.8 g of 0.8 M Tris base, 23 mL of 0.4 M acetic acid, 11.68 g of 0.04 M EDTA (free acid), deionized water to 1 L). The mixture was heated in a Kenmore Kitchen Microwave for approximately 2 minutes and allowed to solidify in a gel caster (BioRad) with combs placed to provide wells for DNA to be run in the gel. Once solidified, the gel was placed in an electrophoresis tank (BioRad) and TAE buffer was poured in the tank to just completely cover the gel.

Five µL of each DNA sample was combined with 2 µL of loading buffer (Stop) (3 mL glycerol, 25 mg bromophenonl blue, deionized water to 10 mL) and added to separate lanes of the gel. A 2 µL aliquot of 1 kb DNA ladder (0.1 µg/µL, Invitrogen) was loaded alongside the DNA to serve as a size marker. The top of the electrophoresis tank was then added and connected to an EC135 electrophoresis apparatus (E-C Apparatus Corporation). Samples were run at 80 V for approximately 30 minutes to ensure adequate migration of the DNA bands. The gel was then removed from the tank and stained in a 0.05 % ethidium bromide solution for approximately 10 minutes. The gel was then removed from the ethidium bromide and destained in tap water for about 30 minutes. DNA bands were visualized and recorded under UV light using a FluorChem®HD2 UV Transluminator (Alpha Innotech).

2.6 16S rRNA GENE AMPLIFICATION

Genomic DNA was diluted by factors of 100, 200 and 500. A master mix was produced containing 164 µL of sterile dH_2O, 30 µL of 10X PCR buffer (Invitrogen), 12 µL of 50 mM $MgCl_2$ (Invitrogen), 6 µL of 10 mM dNTP solutions, 9 µL of each 10 mM primer (8F and 1389R), and 0.02 units of *Taq* polymerase (Invitrogen). Primer sequences are provided in Table 2.2. Fifteen µL of master mix was placed into PCR compatible tubes and 5 µL of either the 100 X, 200 X or 500 X genomic DNA dilutions was added onto the inside of the tubes above the master mixture. A positive control was produced by adding 5 µL of *Escherichia coli* purified genomic DNA into one reaction. A negative control was obtained by adding 5 µL of dH_2O into a separate reaction tube with 15 µL of the master mix. The tubes were spun down in a micro-centrifuge (Sorvall Legend Micro 21) to introduce the DNA to the master mix. The tubes were then placed in a thermocycler (BioRad iCycler). Amplification of the 16S rRNA gene was performed using the following program: denaturation at 94 °C for 1 minute; 30 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 56 °C for 30 seconds, extension at 72 °C for 3 minutes; a final extension at 72 °C for 10 minutes and hold at 4 °C. Successful amplification was confirmed by running PCR products on a 0.7 % agarose gel as described in Section 2.5. PCR products were stored at -20 °C until needed.

Table 2.2: Primer sequences used in PCR amplification over the course of the study.

Primer	*Sequence*	*Melting Temperature(°C)*
8F	5'-AGAGTTTGATCCTGGCTCAG-3'	51.8
1389R	5'-ACGGGCGGTGTGTACAAG-3'	52.6
TOPO-For	5'-CTCACTATAGGGCGAATTGGG-3'	54.4
TOPO-Rev	5'-GCTATGACCATGATTACGCCA-3'	52.4

2.7 CLONING OF 16S rRNA GENE: OVERVIEW

Cloning of the 16S rRNA gene was achieved through the use of the TOPO TA Cloning kit from Invitrogen. This method allows for effective ligations without the use of ligase. *Taq* polymerase that is used in the PCR amplification of the 16S rRNA genes has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine to the 3' ends of PCR products producing sticky ends (Invitrogen). The TOPO TA vector provided was linearized and has a single overhanging 3' deoxythymidine (T) that is complementary to the overhang in the PCR product. Attached to the T overhang on the vector is a topoisomerase I molecule isolated from the *Vaccinia* virus that has the unique ability to bind specifically to deoxythymidine at the end of the sequence 5'CCCTT. A bond is formed between the 3' T phosphate and the tyrosine 274 residue of the topoisomerase. The electrophilic phosphate in this bond can subsequently be attacked by the 5' hydroxyl group on the A overhang of the PCR product when in the correct orientation. This causes the release of the topoisomerase molecule and ligation of the PCR product into the vector. This reaction takes place within five minutes with incubation at room temperature.

The amplified 16S genes were ligated into the pCR 2.1-TOPO vector (Invitrogen). This vector contains a multicloning site along the *lacZ* gene. TOP 10 OneShot Chemical Transformation cells (Invitrogen) were transformed with the ligated vectors, and colonies containing ligated vectors were detected by plating in the presence of ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). The insertion site on the TOPO TA vector is within lacZα and downstream of a T7 RNA polymerase promoter.

The lacZ gene codes for β-galactosidase, which catalyzes the hydrolysis of X-gal to produce a blue product. Ligation of an insert within the β-galactosidase gene produces a malfunctioning β-galactosidase enzyme. This prevents the catabolism of X-gal and causes colonies with inserts to appear white instead of blue.

Colonies containing clones of the 16S rRNA samples were picked and the inserted DNA was further amplified through colony PCR. To qualitatively distinguish between different 16S rRNA sequences, PCR products were digested with *MspI* restriction enzyme which cuts at 5'-CCGG-3' (New England Biolabs). This produced unique banding patterns for individual species, and greatly reduced the amount of transformants needed to be sent for sequencing. It also provides a census of how often a specific species is detected based on how often the unique banding pattern is observed. This method is known as restriction fragment length polymorphism (RFLP).

2.7.1 LIGATION

Three PCR products, derived from the lowest template DNA concentration that produced visible bands, were pooled together and then spun down in a microcentrifuge tube (3 µL from each reaction). Two µL of the PCR mixture was pipetted into another sterile microcentrifuge tube. To this mixture was added 0.5 µL of salt solution (1.2 M NaCl; 0.06 M $MgCl_2$) and 0.5 µL of pCR 2.1-TOPO vector, which are half the recommended volumes from the manufacturer (Invitrogen). The tube was gently flicked and spun down in a bench top centrifuge (Themo Scientific). The mixture was then

allowed to incubate at room temperature for thirty minutes. Ligation reactions were stored at -20 °C until needed for cloning.

2.7.2 TRANSFORMATION

TOP 10 OneShot Chemical transformation cells (Invitrogen) were allowed to thaw on ice and 1.5 µL of the ligation reaction was added to 25 µL of thawed cells. Tubes were gently flicked and the mixture was held on ice for 30 minutes. The transformation was heat shocked for 30 seconds at 42 °C in a Canlab heat block (American Hospital Supply Canada Inc.) and then put back on ice for an additional 10 minutes. A 200 µL volume of S.O.C. solution (Super Optimal broth with Catabolite repression) was then added to the cells in a glass culture tube. The transformation mixture was shaken at maximum rotation speed in a 37 °C water bath for 1 hour. Cells were plated at various dilutions with S.O.C. solution on solid media plates, as described in section 2.7.2.1. The plates were inverted and incubated at 37 °C overnight.

2.7.2.1 MEDIA

Plates for colony growth were made by adding 0.5% LB (Luria-Bertani) and 0.75% agar in deionized H_2O. The media solution was autoclaved for 20 minutes at approximately 121 °C. Media was cooled in a 60 °C water bath after which 50 µg/mL ampicillin (Sigma-Aldrich) was added. Plates were poured by hand under a laminar flow hood and allowed to solidify. Once solid, 40 µL of X-gal (40 mg/mL in N,N-dimethyformamide) were spread over the plates for use in the X-gal assay.

2.7.2.2 SELECTION OF COLONIES

White colonies that were inferred to contain an insert based on the X-gal assay were picked using sterile toothpicks and transferred into 50 µL of sterile dH_2O in a 96-well plate (Falcon). The plates were taped shut and stored at 4 °C until needed for further analysis. Toothpicks used to pick colonies were streaked onto solid media plates which were incubated as described above. Once the colonies in a plate were successfully amplified through colony PCR (Section 2.8), 40 µL of sterile 50% UltraPure glycerol (Invitrogen) was added to the wells of the plate. These plates were then stored indefinitely at -80 °C.

2.8 COLONY PCR

Colonies containing clones of the 16S rRNA samples were picked and the inserted DNA was further amplified through colony PCR. Master mix containing 30 µL of 10X PCR buffer (Invitrogen), 12 µL of 50mM $MgCl_2$ (Invitrogen), 12 µL DMSO, 6µL of 10 mM dNTPs, 9 µL of each 10 mM primer (TOPO-For and TOPO-Rev), 0.02 units of *Taq* polymerase, and 200 µL of sterile deionzied H_2O was made. Nine µL of this master mix was put into each of 24 wells on a PCR-compatible plate (Falcon) as well as three separate PCR tubes. One µL of picked colony stock was added to the plate wells. One µL of purified ligated plasmid DNA was added to two of the PCR tubes to serve as positive controls and 1 µL of dH_2O was added to the third tube as a negative control.

Amplification of the 16S rRNA gene was performed using the following program: denaturation at 94 °C for 1 minute; 35 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 54 °C for 30 seconds, extension at 72 °C for 3 minutes; a final extension at 72 °C for 10 minutes; and hold at 4 °C. Amplified DNA was stored at -20 °C until needed.

2.9 RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS

To qualitatively distinguish between different 16S rRNA sequences, PCR products were digested with MspI restriction enzyme which cuts at 5'-CCGG-3' (New England Biolabs) sequences. This produced unique banding patterns for individual species, and greatly reduced the number of transformants that required sequencing.

A reaction master mix was prepared containing 32.4 µL of 10X NEBuffer 4 restriction buffer (1X NEBuffer 4 contains 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM Dithiothretiol, pH 7.9), 8.1 µL sterile deionized H_2O and 13.5 µL of MspI (10 U/µL). The reaction mixture was spun down briefly in a Sorvall Legend Micro 21 centrifuge. Two µL of the mixture was added to the entire colony PCR product. The reactions were then allowed to float in a 37°C water bath for 2 hours.

2.10 4 % W/V AGAROSE GEL ELECTROPHORESIS

The entire volume of digested samples with 2 µL of Stop added were loaded on a 4 % agarose gel (4 g Ultrapure agarose in 100 mL 1X TAE buffer) and placed in an EC135

electrophoresis apparatus system. Samples were run at 80 V for approximately 1 hour to ensure adequate separation of DNA bands. The gel was stained and visualized as described in Section 2.5.

2.11 OTU BANDING PATTERN ANALYSIS

Banding patterns were assigned an identification number in the order that they were observed. The sizes of the bands were determined by measuring band positions on the gel in relation to the DNA ladder using a ruler. A catalogue of all of the banding patterns was made to track all of the different patterns as well as the number of times each pattern was observed.

2.11.1 OTU SEQUENCING

Representatives of the most abundant bands were amplified as described in Section 2.8 and thermocycler conditions as described in Section 2.6. Amplification was confirmed by running PCR products on a 0.7% w/v agarose gel as described in Section 2.5. Crude PCR products were then shipped to BioBasic Inc. (Markham, ON) for sequencing.

2.11.2 ANALYSIS OF SEQUENCING RESULTS

Sequencing results were supplied in FASTA format, which were then uploaded to the Ribosomal Database Project (RDP) for bacterial identification through the Seqmatch

tool. Matches were made at least on the phylum level, and in some cases could be made up to the genus level.

2.12 DIVERSITY ANALYSIS

Sequence data was analyzed using the software MOTHUR (Schloss *et al.,* 2009). Rarefaction curves were made from total sequence data, control slurry sequences, and nutrient addition sequences using the rarefaction.single command and an OTU definition of 95% genetic dissimilarity. The boneh command was used to estimate how many more additional OTUs would be detected in double the sample sized sequenced in this study.

Diversity of the RFLP fingerprints was measured by calculating several diversity indicators. Each banding pattern was assumed to belong to a different OTU at a genetic distance of 0.03. Simpson index values and Shannon index values were determined using the online calculator http://www.alyoung.com/labs/biodiversity_calculator.html (Young, 2012). Chao1 and Abundance Based Coverage Estimators (ACE) values were calculated using the formulae described by Hughes *et al.* (2001).

3.0 RESULTS

3.1 CHARACTERIZATION OF SOIL SAMPLE

Concentrations of the different explosive compounds in the PET2 soil sample were determined by Jennifer McAllister using HPLC. Compounds detected, and their concentrations, are shown in Table 3.1. HMX was detected at the highest concentration and it was detected at levels that were two orders of magnitude higher than the next most concentrated explosive, TNT. RDX was detected at three orders of magnitude less than HMX. TNT derivatives 1,3,5-TNB, 4a-DNT, and 2a-DNT were also detected in the soil.

Table 3.1: Initial concentrations of explosives detected in the soil sample analyzed in this study.

Compound	*Concentration ppm*
HMX	1145.4
RDX	0.651
TNT	5.88
1,3,5-TNB	0.702
4a-DNT	1.74
2a-DNT	0.336

3.2 OTU PATTERN ANALYSIS

The community structure of the PET2 soil contaminated with the explosives, as described in Table 3.1, was determined through a bioreactor study to stimulate growth of potential bioremediators. PET2 soil was processed in two bioreactors per treatment condition under control conditions with no nutrient addition, and following treatment with the addition of a nutrient supplement. The soil was constantly mixed and provided with constant controlled conditions such as dissolved oxygen and pH. Samples of the bioreacted soil were taken at days 0, 45, and 90. The 16S rRNA gene, used in this study as a microbial identifying gene, was amplified and cloned. Fingerprinting was performed by digesting the cloned 16S rRNA genes with *MspI*. Different sequences of the 16S rRNA gene would correspond to a different banding pattern obtained upon digestion. A catalogue of the different banding patterns was collected, in which each unique banding pattern was assigned an identification number and the amount of times that pattern was observed was recorded. This was performed with a bioreactor-treated PET2 soil from control conditions with no nutrient addition and a treatment with the addition of a nutrient supplement. An example of an RFLP agarose gel is provided in Figure 3.1.

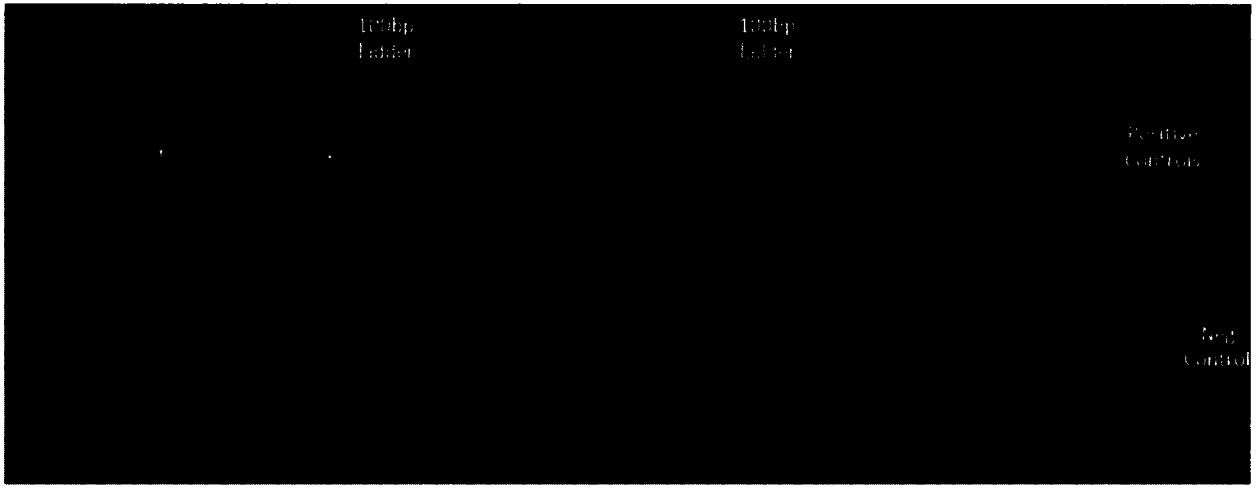

Figure 3.1: 4% w/v agarose gel of 16S rRNA gene clones digested with *MspI* from nutrient treated soil, bioreactor 2, day 45, DNA extraction A, clones 49-72.

3.2.1 CONTROL SLURRY

3.2.1.1 DAY 0

Two separate nucleic acid extractions were performed for the control slurry treatment at day 0 from both bioreactors. Sampling of bioreactor 1 resulted in 36 different banding patterns from 49 clones from the nucleic acid extraction A, and 56 different banding patterns from 72 amplified clones in the B nucleic acid extraction. Many of the banding patterns were unique as they only appeared once. The most abundant patterns observed from nucleic acid extraction A were assigned numbers 25 and 113, and these patterns were observed in three separate A extraction clones. In the B extraction, patterns 25 and 113 were observed in one clone each. The most abundant patterns in the B extraction were assigned identification numbers 53 and 111. Both of these banding patterns were observed in four clones. In the A extraction, pattern 53 was observed in two clones and pattern 111 was only seen once. A summary of all of the observed banding patterns from the control slurry, day 0, bioreactor 1 is provided in Figure 3.2.

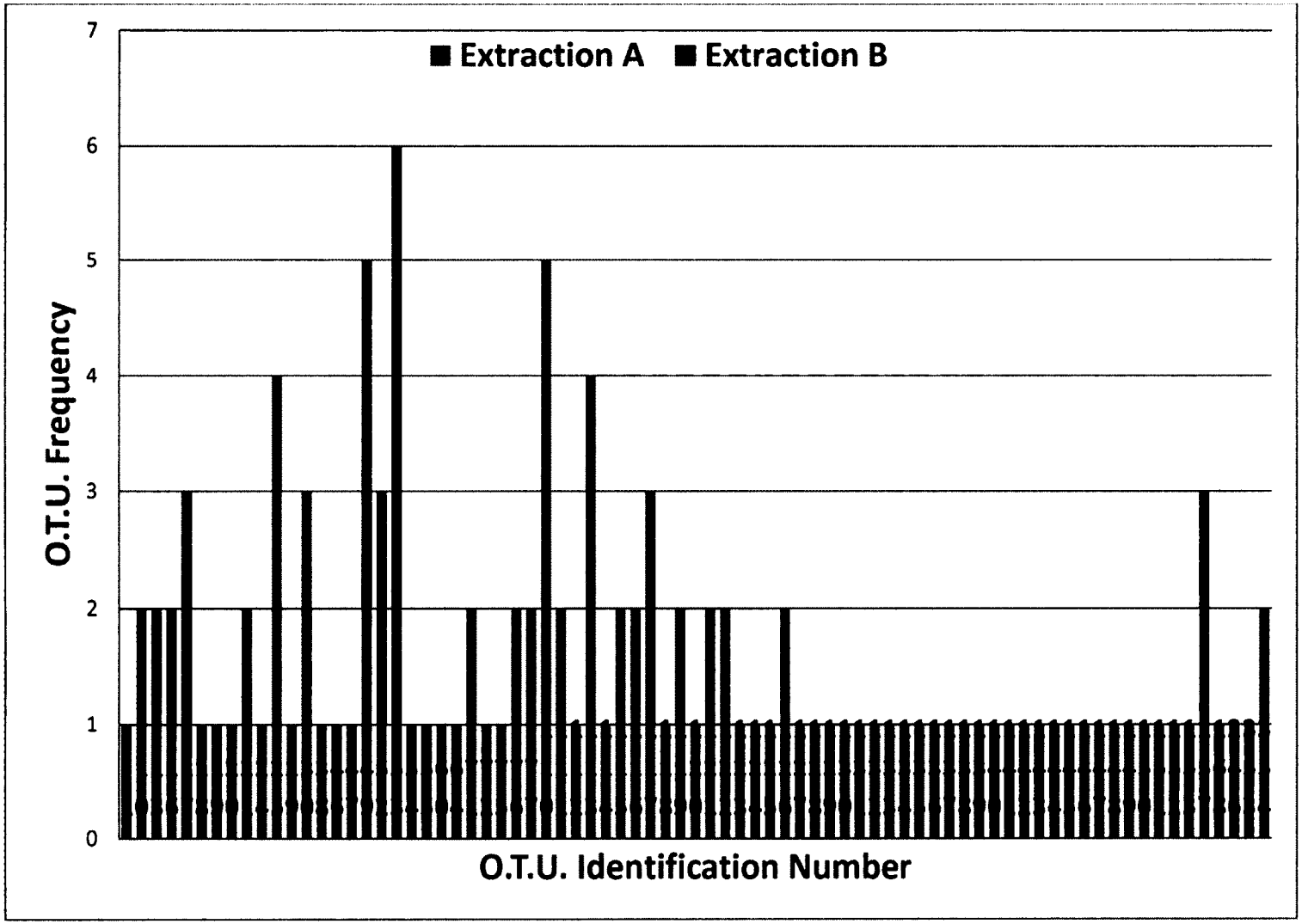

Figure 3.2: OTU banding patterns observed for control treatment soil, bioreactor 1, day 0.

Sampling of the control slurry soil at day 0 was also performed from bioreactor 2. Nucleic acid extraction A produced 41 different banding patterns resulting from 56 analyzed clones. The B extraction produced 54 different banding patterns from 75 analyzed clones. Many of the banding patterns from both extractions were only observed once. The most abundant patterns from extraction A were assigned to numbers 49 and 115, and each of these patterns were observed in 5 clones. From the B extraction pattern 49 was seen in 3 clones and pattern 115 was observed in 1 clone of the 16S gene. The most abundant pattern from the B extraction was assigned number 15, and this pattern was absent in the A extraction. A summary of all of the banding patterns observed from bioreactor 2, control slurry, day 0 clones is provided in Figure 3.3.

Sampling from both control slurry bioreactors at day zero produced many singularly occurring banding patterns. Patterns most abundant from samples taken from one bioreactor were not observed as abundantly from samples analyzed from the other bioreactor.

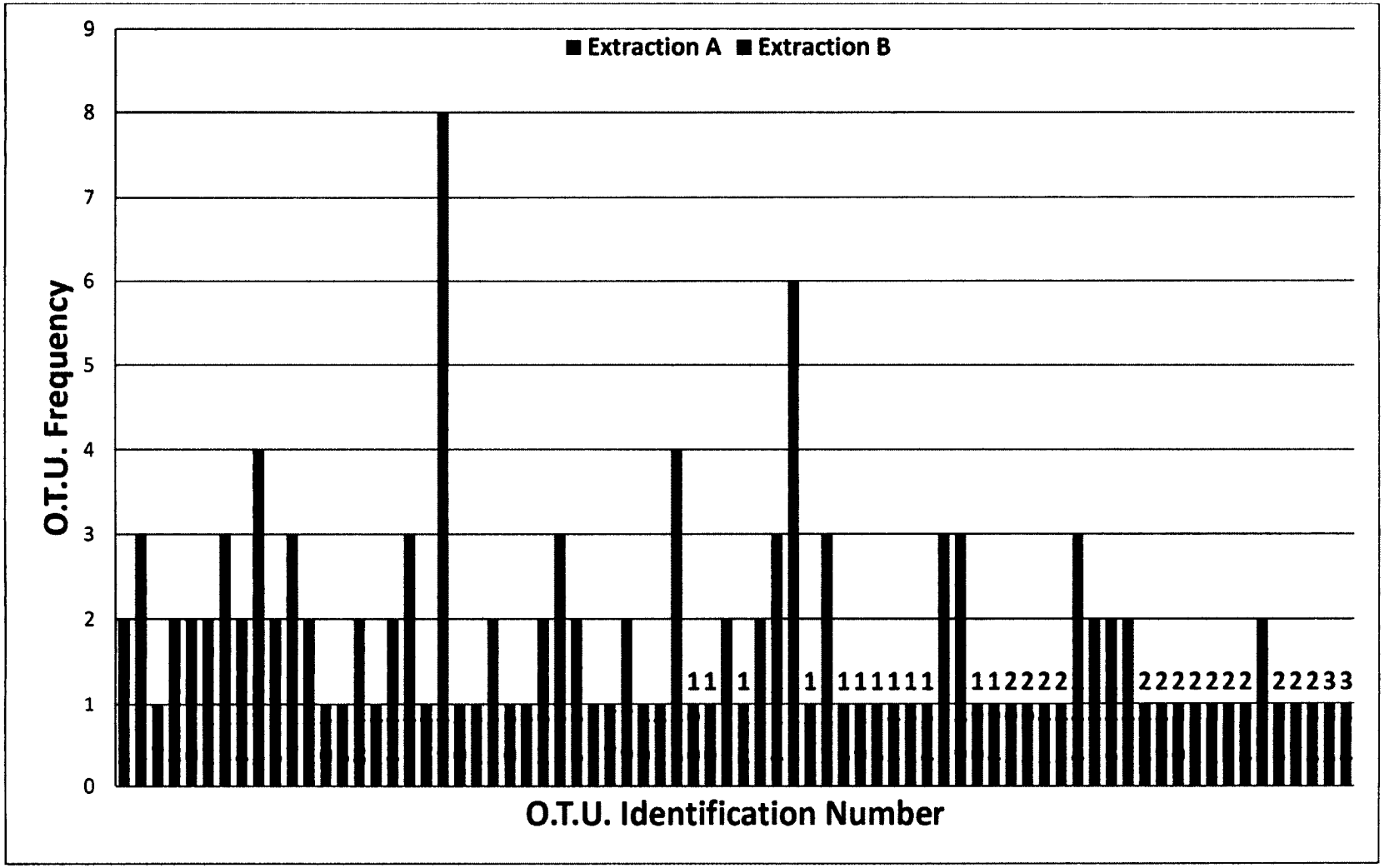

Figure 3.3: OTU banding patterns observed for control treatment soil, bioreactor 2, day 0.

3.2.1.2 DAY 45

Sampling of the microbial community was also performed at day 45 from bioreactors 1 and 2. Extraction A from bioreactor 1 produced 40 different OTU patterns from 62 analyzed clones. The B extraction resulted in 33 different banding patterns from 50 clones. There were 28 banding patterns observed only once in clones from extraction A. The most abundant pattern from extraction A was assigned identification number 27 and was documented in 8 clones. In the B extraction, pattern 27 was observed in 4 clones, but was not the most abundant. The most abundant pattern in extraction B was assigned to number 354 and it was observed in 6 clones. In the A extraction pattern 354 was also observed in 6 clones. A summary of the banding patterns documented in clones from the control slurry in bioreactor 1 at day 45 is provided in Figure 3.4.

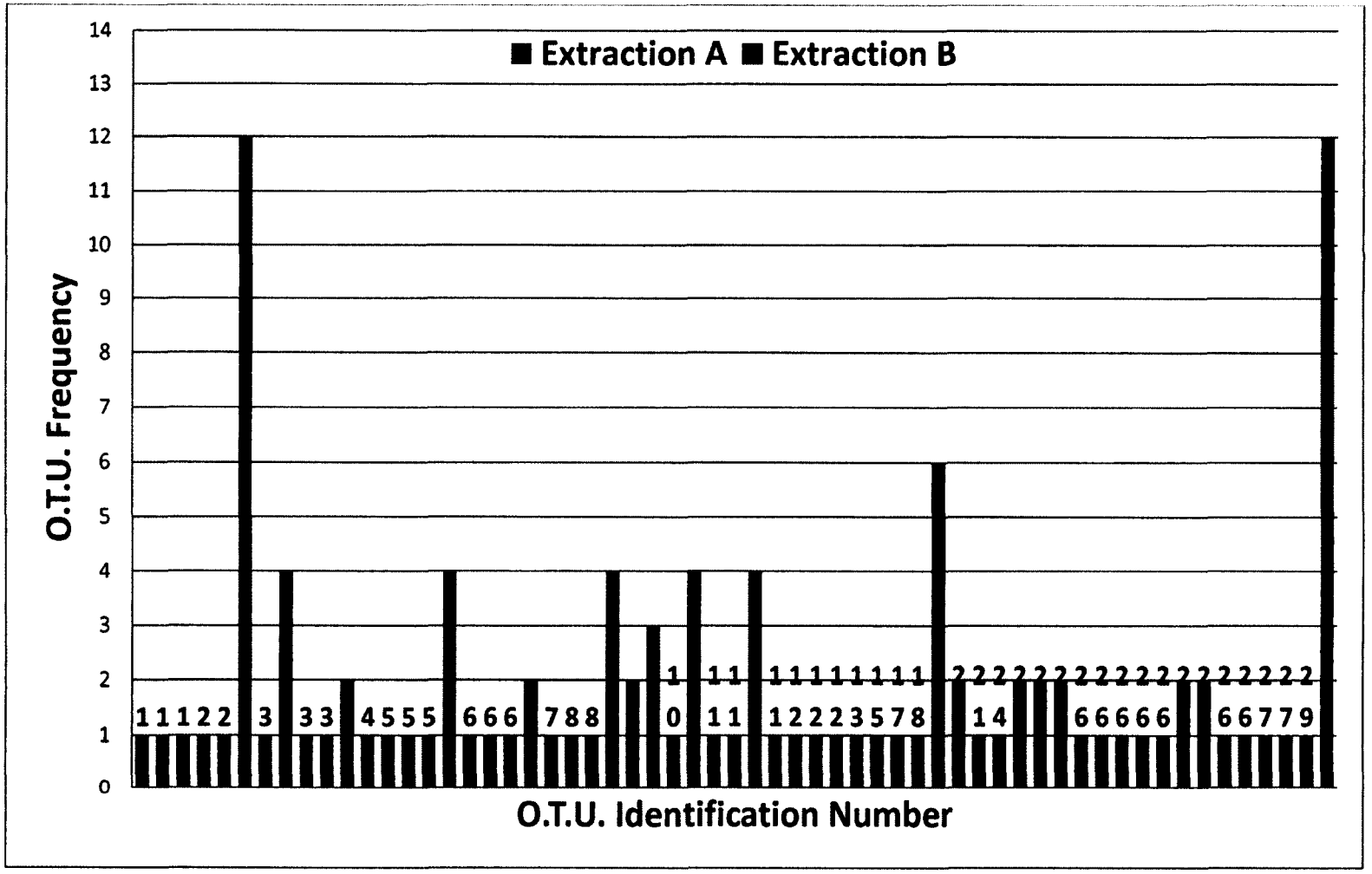

Figure 3.4: OTU banding patterns observed for control treatment soil, bioreactor 1, day 45.

Sampling of the control slurry at day 45 was also conducted from bioreactor 2. The A extraction produced 26 different banding patterns from 69 analyzed clones. The B extraction resulted in 33 different banding patterns from 79 clones. The most abundant pattern by far in both extractions was assigned number 259, and it was documented in 25 clones from the A extraction and 30 clones in the B extraction. The second most abundant banding pattern from both extractions was assigned identification number 354, and it was documented in 10 and 6 clones from the A and B extractions respectively. A summary of all of the banding patterns observed from control slurry samples in bioreactor 2 at day 45 is provided in Figure 3.5.

Pattern 259, which was documented in large abundance from bioreactor 2 samples was only observed in 2 clones from samples taken from bioreactor 1. Sampling from both bioreactors resulted in similar levels of detection for pattern 354.

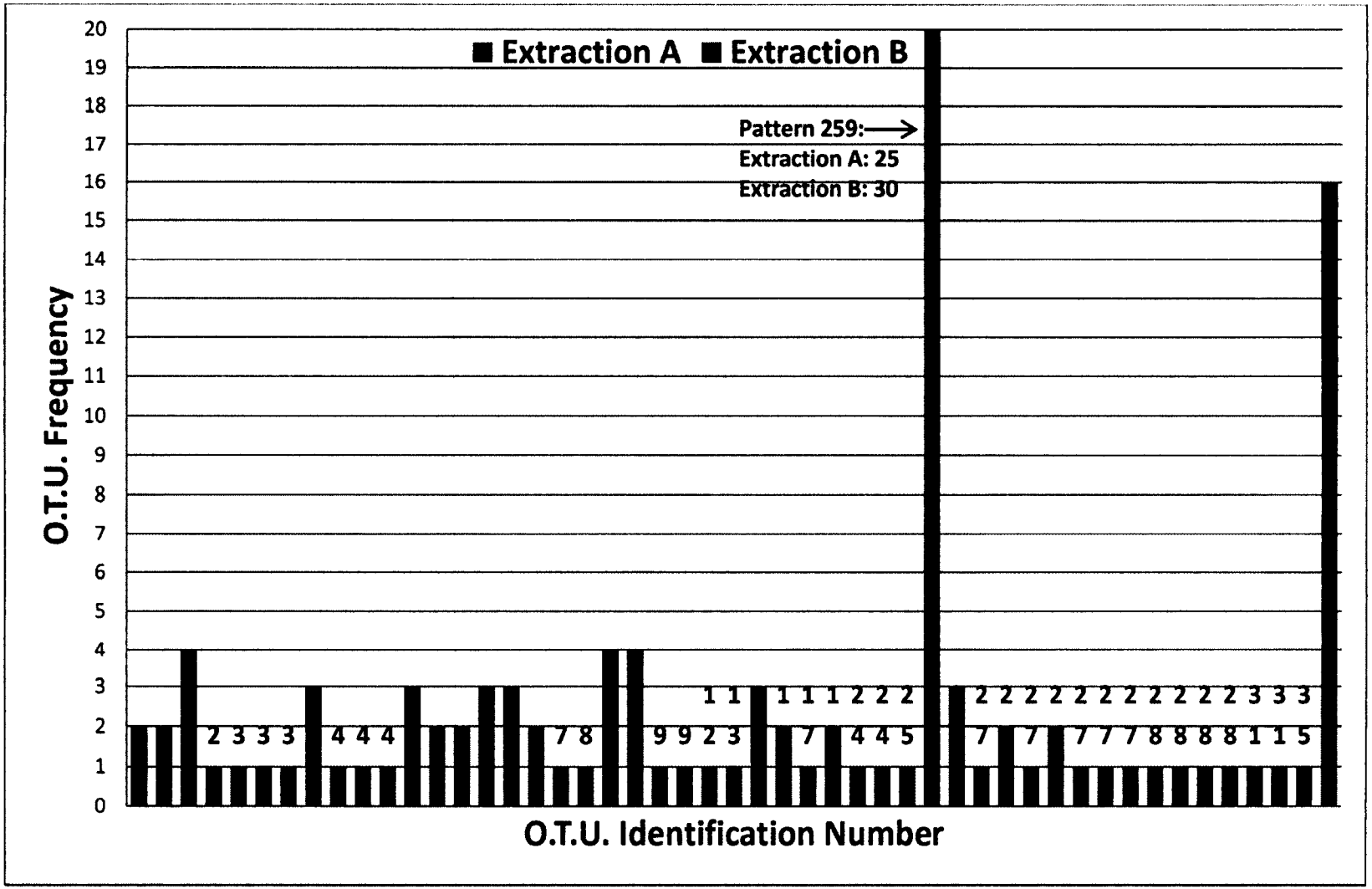

Figure 3.5: OTU banding patterns observed for control treatment soil, bioreactor 2, day 45.

3.2.1.3 DAY 90

The community fingerprint from the control slurry at day 90 was also analyzed from both bioreactors. The A extraction from bioreactor 1 produced 24 different restriction patterns from a total of 45 clones. The B extraction resulted in 40 different patterns from 74 clones. The most abundantly detected pattern from nucleic acid extraction A was number 83, which was observed in 7 clones. This restriction pattern was not documented in the B extraction. The most abundant pattern from the B extraction was assigned to pattern 189 and it was seen in 10 clones. Pattern 189 was documented in 3 clones from extraction A. Pattern 65 was also relatively abundant from the B extraction and it was detected in 8 clones, but was absent in clones from the A extraction. A summary of the restriction patterns detected in bioreactor 1 for the control slurry at day 90 is provided in Figure 3.6.

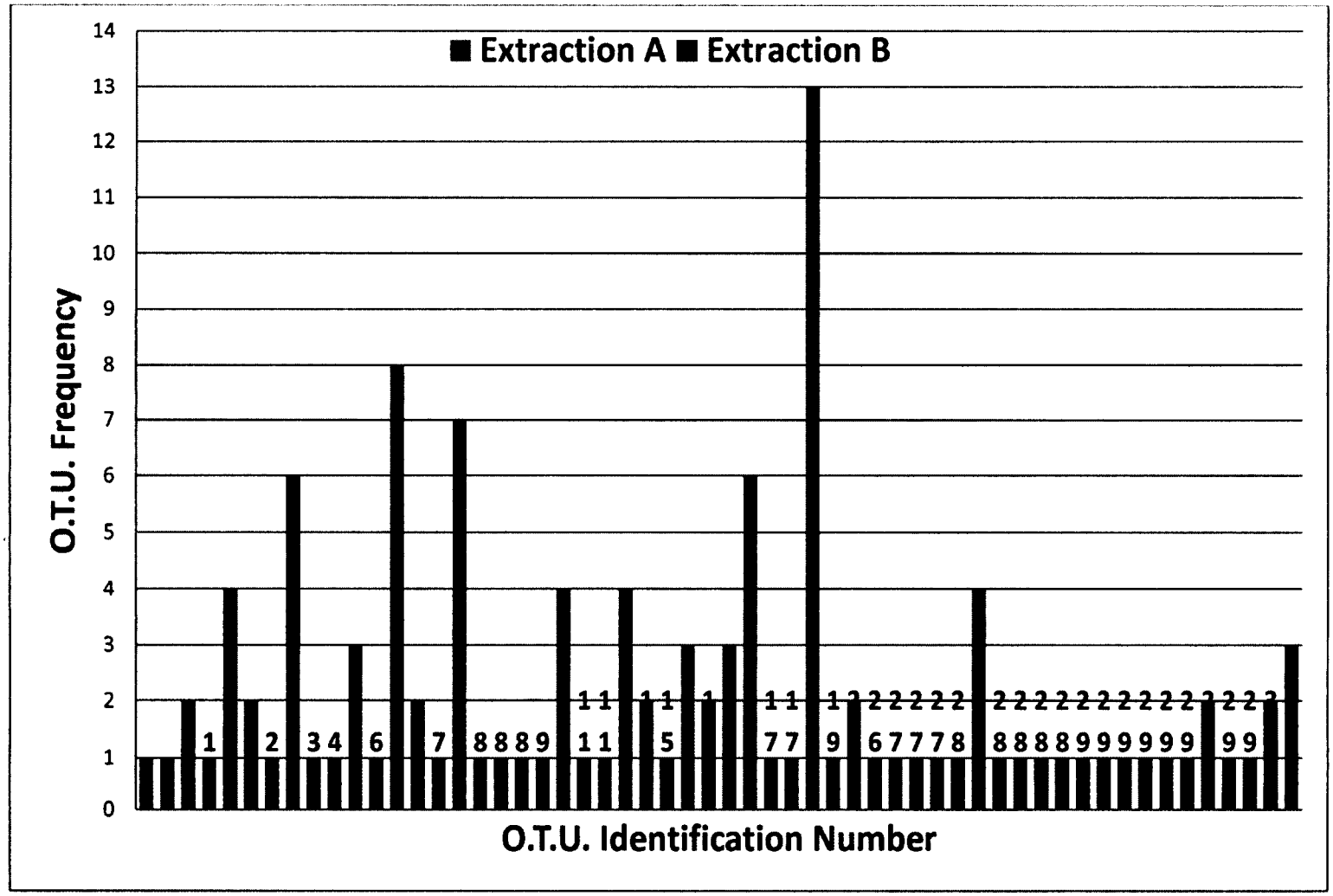

Figure 3.6: OTU banding patterns observed for control treatment soil, bioreactor 1, day 90.

Day 90 sampling from the control treatment bioreactor 2 was also performed. The A extraction produced 25 different banding patterns from 62 analyzed clones. From the B extraction there were 38 different banding patterns documented from 63 clones. The most abundant pattern from the A extraction was assigned to number 321 and it was detected in 9 clones. This banding pattern was undetected in extraction B clones. Additionally, patterns 297, 100, and 36 were detected in 7, 6, and 6 clones respectively from the A extraction. Pattern 36 was the only one of these patterns that was also detected in the B extraction, and it was observed in 3 clones. The B extraction produced 18 patterns that were only detected in one clone. The most abundant pattern from the B extraction was pattern 18, which was documented in 5 clones. This pattern was absent in samples taken from the A extraction. Pattern 259 which was present in high proportions from bioreactor 2 samples taken at day 45 was greatly reduced in bioreactor 2 samples taken at day 90 and was only detected in 3 clones from extraction A. A summary of the banding patterns documented in the control slurry, day 90, bioreactor 2 is provided in Figure 3.7.

There were patterns detected in a high proportion of clones from one bioreactor at day 90, but not in the other. Patterns 27, 65, 83, 170, and 182 were detected in 5 or more clones in bioreactor 1, but were absent or present in 2 or fewer clones from the bioreactor 2 sample at day 90. Similarly, restriction patterns 18, 36, 100, 297, and 321 were detected in 5 or more clones in bioreactor 2, but were nearly absent in samples taken from bioreactor 1.

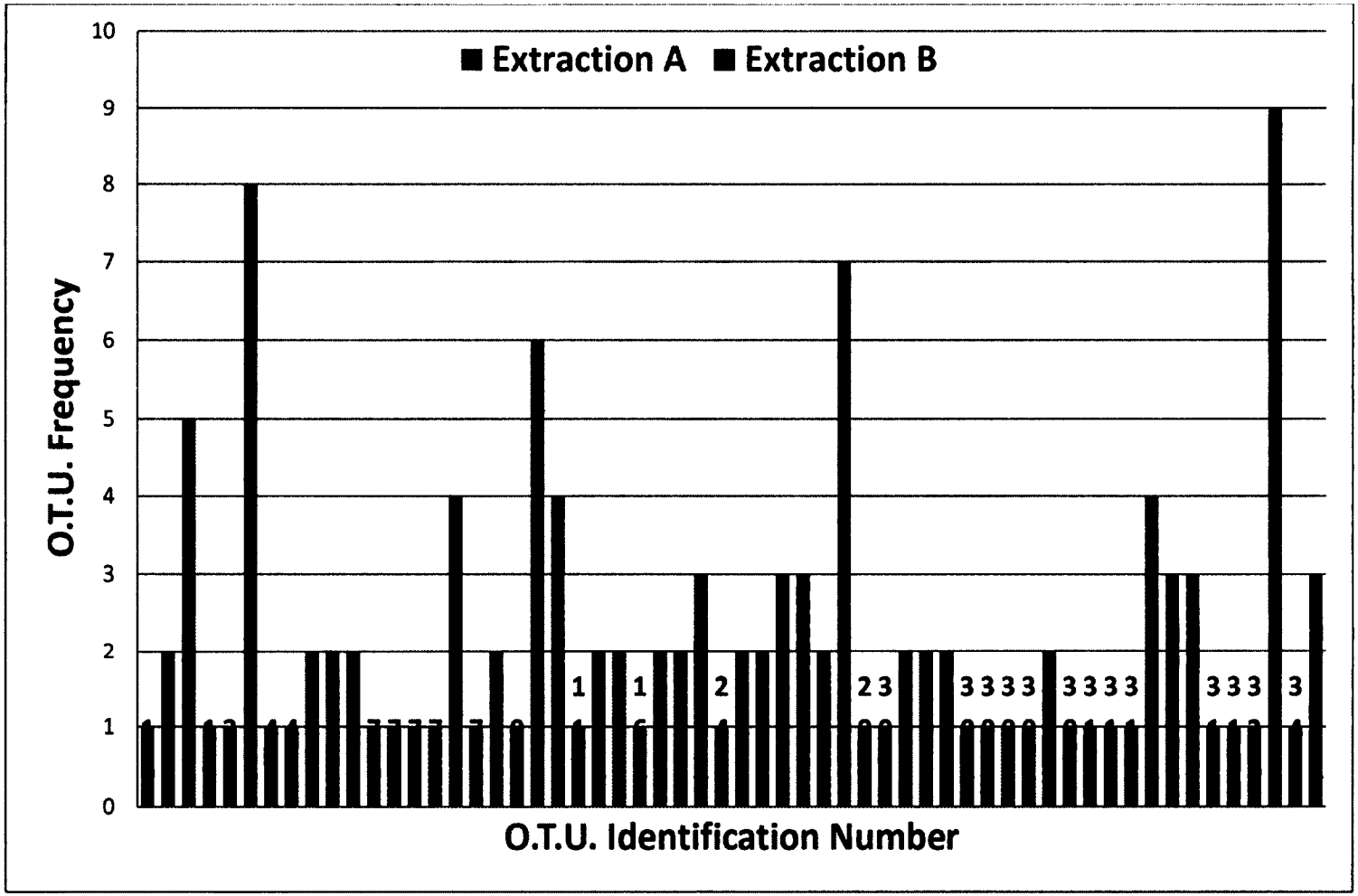

Figure 3.7: OTU banding patterns observed for control treatment soil, bioreactor 2, day 90.

3.2.2 NUTRIENT ADDITION SOIL

3.2.2.1 DAY 0

Microbial community fingerprinting was performed for nutrient addition soil run in both bioreactors. At day 0, bioreactor 1, the A nucleic acid extraction produced 12 different restriction patterns from 18 analyzed clones. The B extraction produced 68 different restriction patterns from 90 clones. The most abundant pattern detected from extraction A was number 65 and it was observed in 7 clones. This pattern was not detected in the B extraction. The rest of the patterns observed from the A extraction were only detected once each. The most abundant pattern from the B extraction was number 25 and it was detected in 4 clones. This pattern was not reported in clones from the A extraction. There were a total of 52 of the 68 patterns from the B extraction that were detected only once. A summary of the different restriction banding patterns is provided in Figure 3.8.

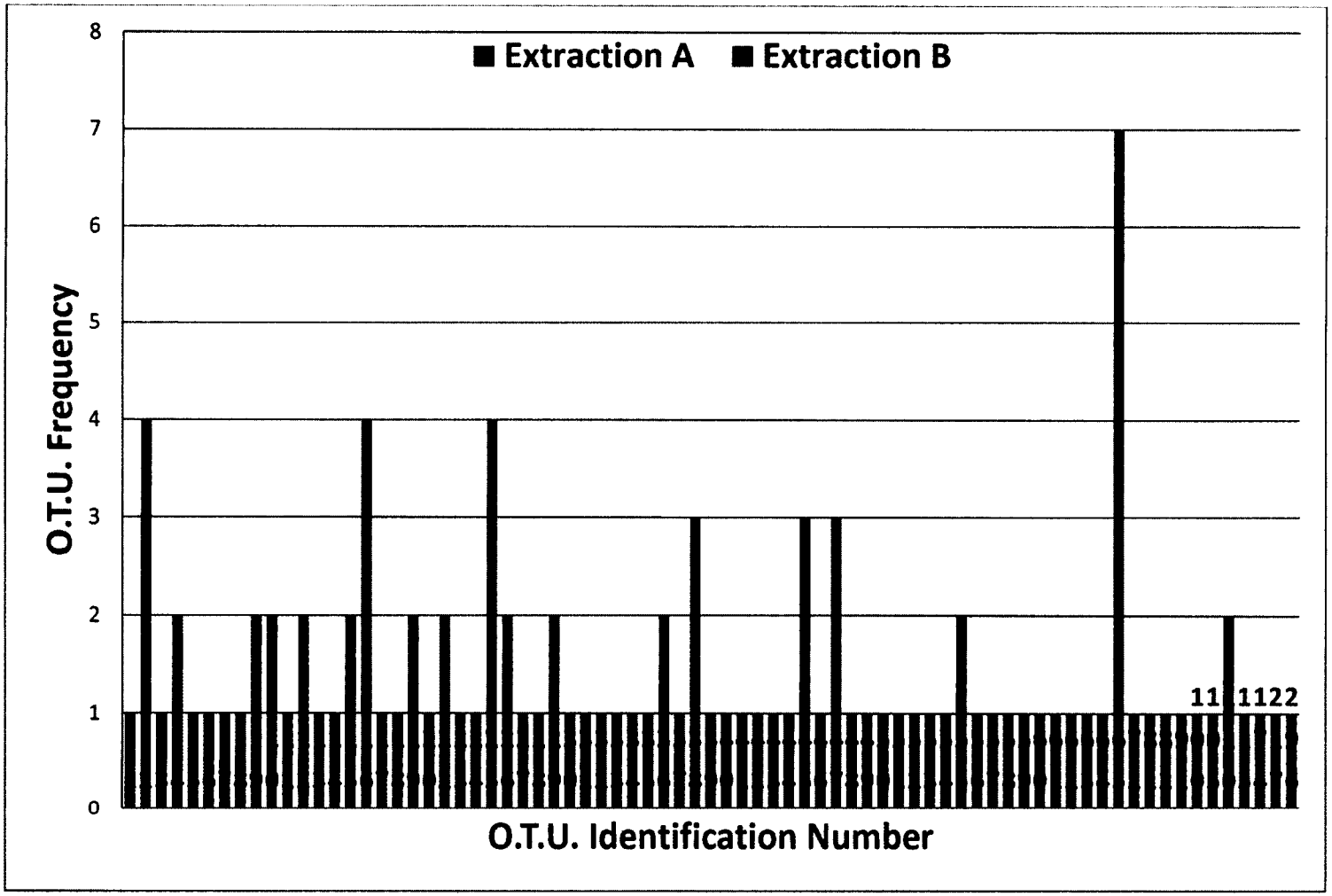

Figure 3.8: OTU banding patterns observed for nutrient addition treatment soil, bioreactor 1, day 0.

Soil was also sampled from the nutrient addition at day 0 from bioreactor 2. The A extraction produced 56 different restriction patterns from 74 successful clones of the 16S gene. The B extraction produced 48 different patterns from a total of 61 clones. Both extractions had a large proportion of clones appear only once. The most abundant pattern from the A extraction was pattern 173, which was detected in 4 clones. This pattern was also observed in 2 clones from the B extraction. The most abundant OTU from the B extraction was assigned identification number 15 and it was detected in 4 clones. This pattern was also detected in 3 clones from the A extraction. A summary of the banding patterns observed at day 0 from bioreactor 2 is presented in Figure 3.9.

The community fingerprints from both bioreactors were similar in that many unique patterns appearing only once were detected. The most abundant pattern from both reactors was number 65 detected in 7 clones.

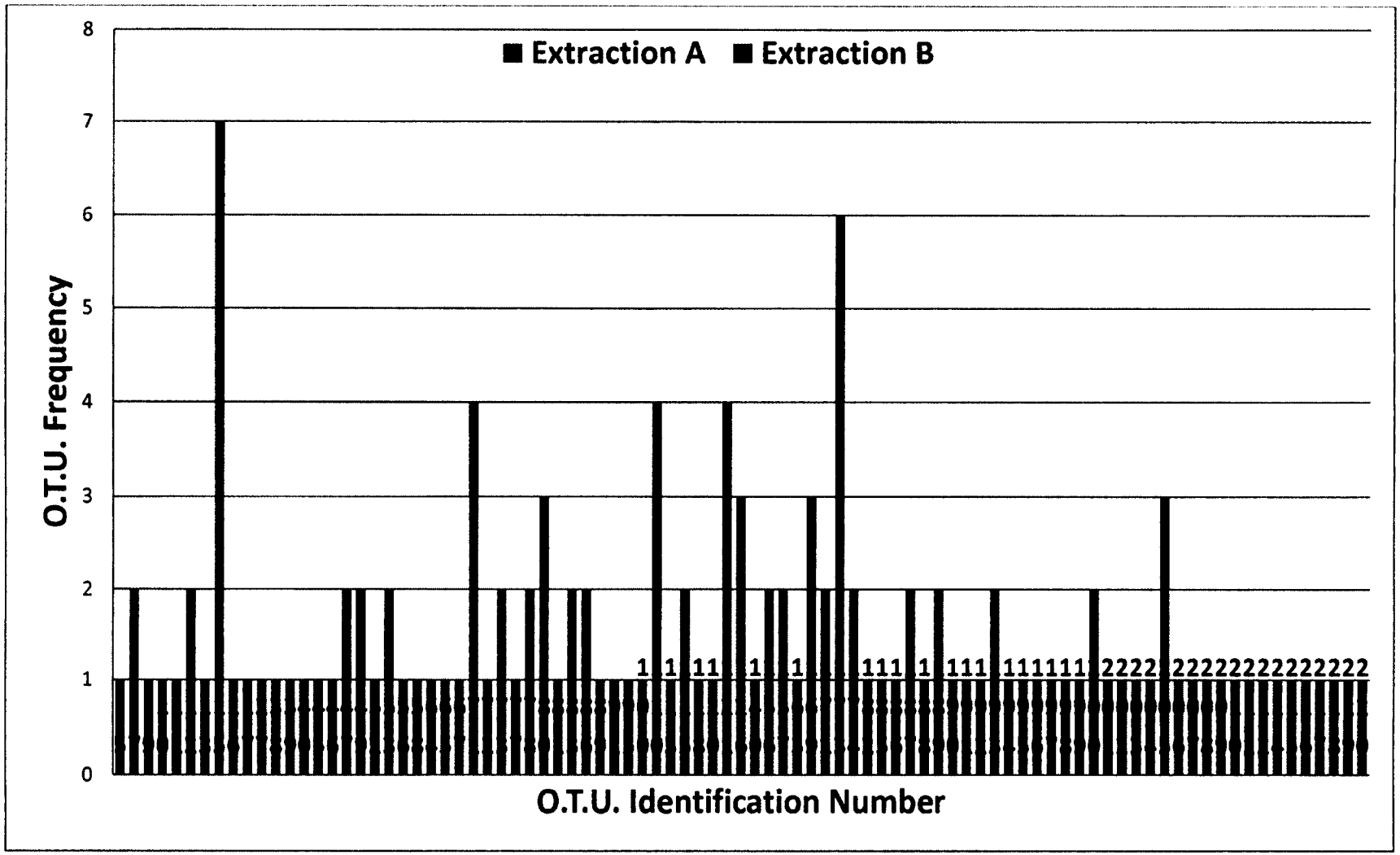

Figure 3.9: OTU banding patterns observed for nutrient addition treatment soil, bioreactor 2, day 0.

3.2.2.2 DAY 45

Nutrient addition soil slurries were sampled and fingerprinted at day 45 from both bioreactors. The A extraction from bioreactor 1 produced 37 different restriction fragment patterns from a total of 91 16S rRNA gene clones. The B nucleic acid extraction resulted in 45 different OTUs from a total of 88 clones. The most abundant banding pattern from both extractions was assigned to number 65. The A extraction had 25 clones displaying this banding pattern and the B extraction had 27 clones. The second most abundant banding pattern in the A extraction was assigned identification number 67 and it was observed in 13 clones. This pattern was reported in 3 clones from the B extraction. The second most common patterns from the B extraction were numbers 13 and 46 and each pattern was observed in 5 clones. Pattern 13 was absent in clones from nucleic acid extraction A, and pattern 46 was detected in 2 clones. A summary of all of the reported banding patterns from the nutrient addition soil sample at day 45 from bioreactor 1 is provided in Figure 3.10.

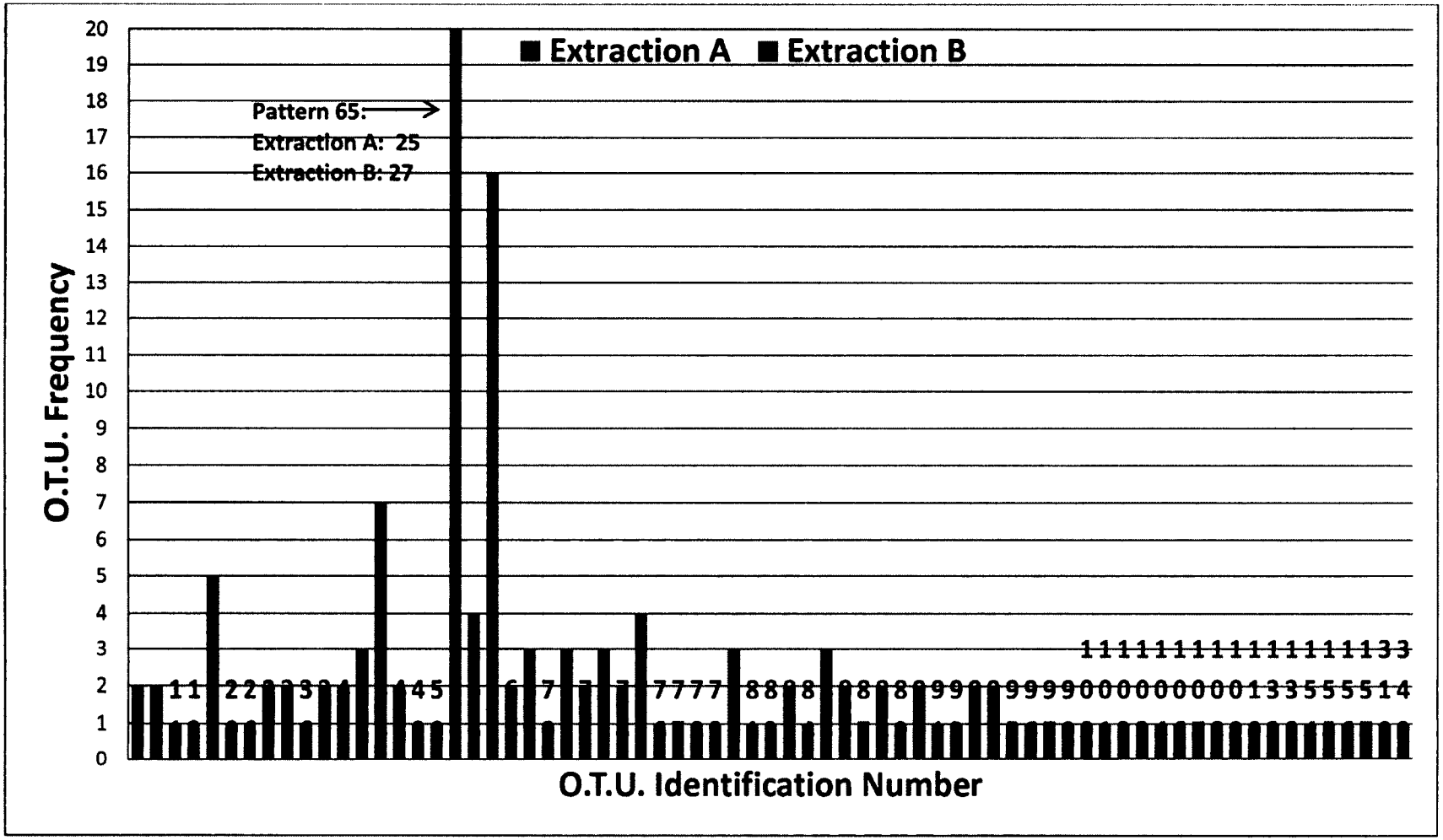

Figure 3.10: OTU banding patterns observed for nutrient addition treatment soil, bioreactor 1, day 45.

Sampling at day 45 from bioreactor 2 was also performed. The A extraction produced 38 different banding patterns from a total of 89 clones. The B extraction produced 38 different patters from 80 analyzed clones. The most abundant pattern from each extraction was number 65. Pattern 65 was detected in 31 clones from the A extraction and 24 clones from the B extraction. The second most abundantly occurring pattern from the A extraction was number 100, and it was observed in 9 clones. This pattern was absent in the B extraction. The second most abundant pattern from the B extraction was assigned to number 125 and it was detected in 5 clones. Pattern 125 was observed in 1 clone from the A extraction. A summary of the banding patterns obtained from the nutrient addition soil at day 45 from bioreactor 2 is provided in Figure 3.11.

Samples from both bioreactors and each nucleic acid extraction had a high proportion of pattern 65 compared to the rest of the patterns detected. Pattern 67 was detected in 16 clones from bioreactor 1, but it was detected in only 2 clones from bioreactor 2 samples.

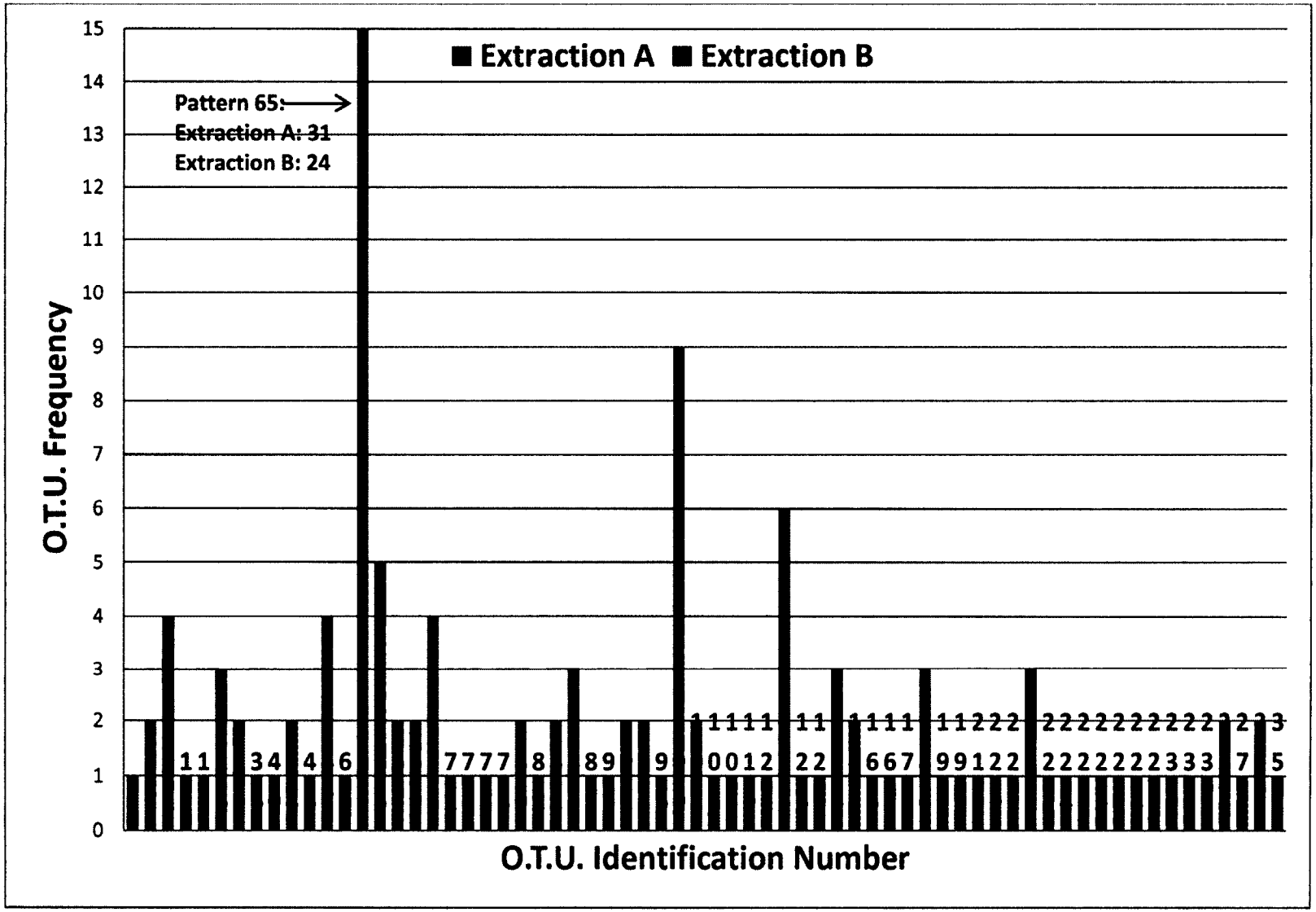

Figure 3.11: OTU banding patterns observed for nutrient addition treatment soil, bioreactor 2, day 45.

3.2.2.3 DAY 90

Fingerprinting of the microbial community structure at day 90 was also performed for the two bioreactors. From the A extraction of bioreactor 1 there were 42 banding patterns from 84 clones. The B extraction had 50 different restriction fragment patterns from 90 clones. The most abundant pattern from both the A and B extractions was pattern 65, and it was observed in 15 clones from A and 13 clones from B. The amount of clones belonging to OTU 65 in bioreactor 1 at day 90 greatly reduced from clones obtained at day 45 from the same bioreactor. The second most abundant patterns from the A extraction were 13, 46, 69, and 245 and each were detected in 4 clones. Pattern 46 was also the second most abundant pattern from extraction B and it was detected in 5 clones. A summary of the restriction patterns observed from bioreactor 1 nutrient addition at day 90 is provided in Figure 3.12.

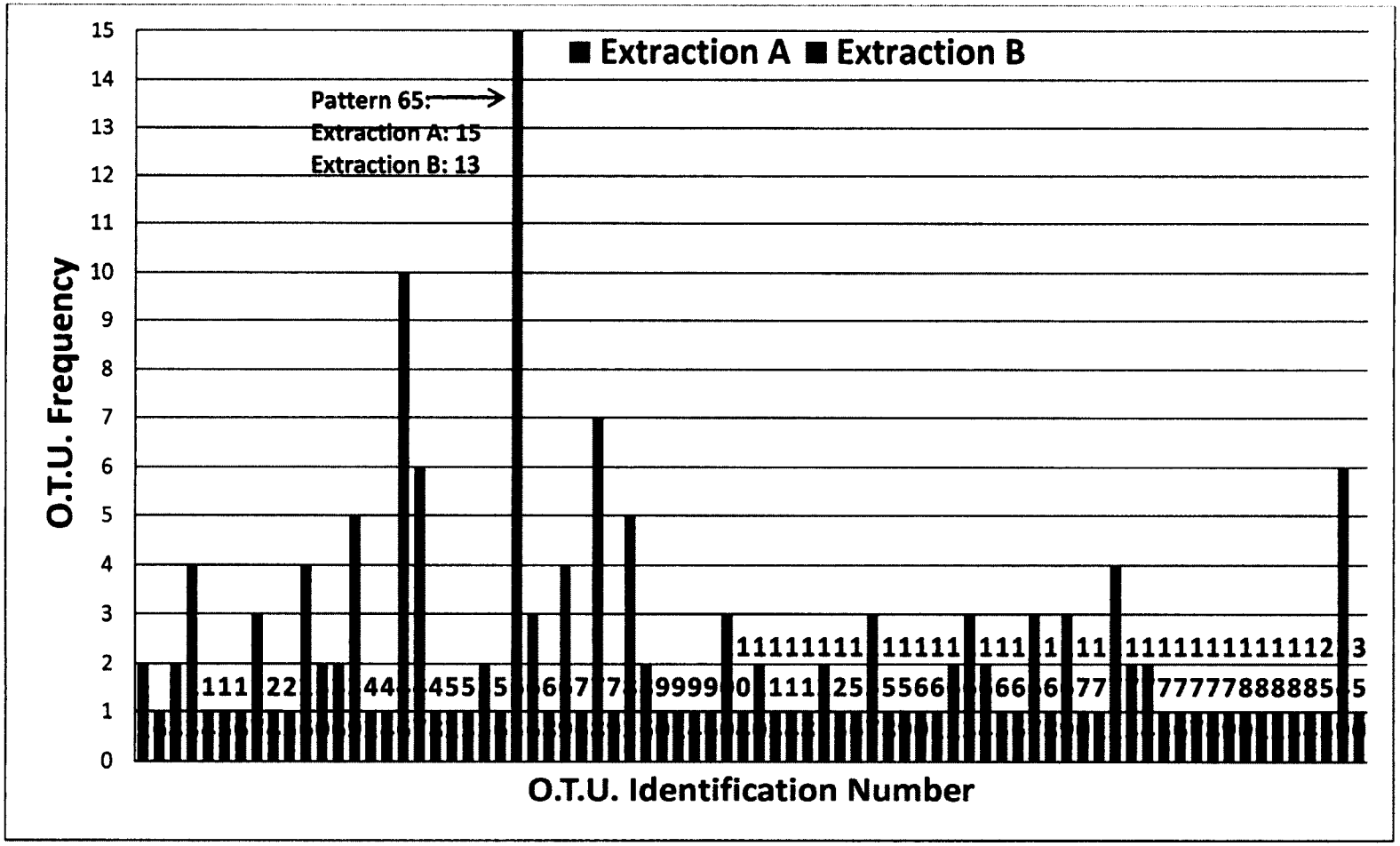

Figure 3.12: OTU banding patterns observed for nutrient addition treatment soil, bioreactor 1, day 90.

The A extraction from bioreactor 2 samples produced 34 different restriction patterns from 81 analyzed clones. The B extraction produced 30 different patterns from 87 clones. The most abundant pattern from both extractions was number 65. Pattern 65 was detected in 25 clones in extraction A and 26 clones in extraction B. The abundance of pattern 65 clones was approximately double that from day 90 samples from bioreactor 1. Six patterns in extraction A were observed in 3 clones each. The second most abundant pattern in extraction B clones was pattern 96 and it was observed in 8 clones from B and 3 clones from extraction A. Extraction B also produced 7 clones assigned to identification number 83. Pattern 83 was detected in 3 clones from extraction A. A summary of the restriction patterns obtained from nutrient addition in bioreactor 2 at day 90 is provided in Figure 3.13.

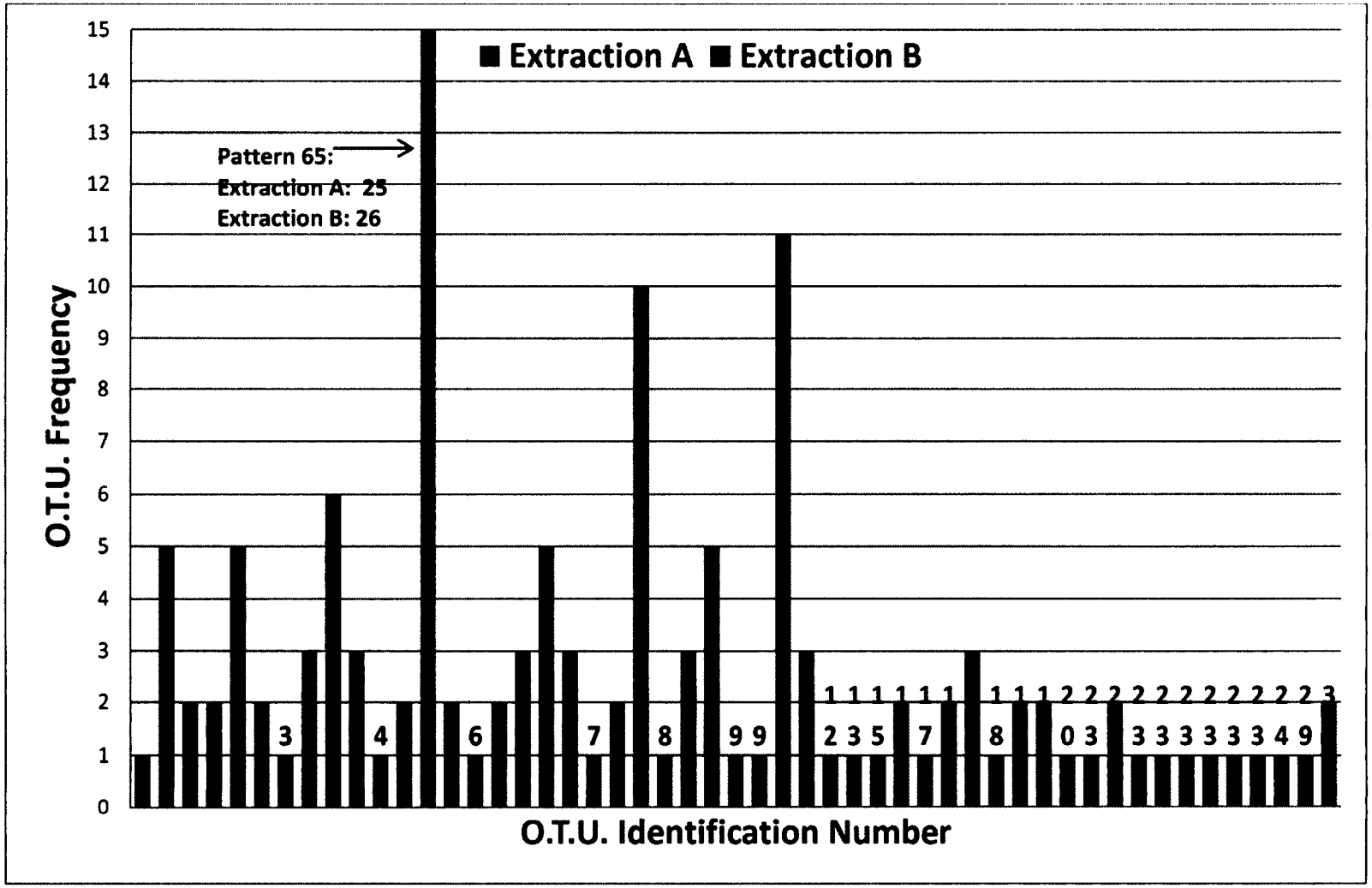

Figure 3.13: OTU banding patterns observed for nutrient addition treatment soil, bioreactor 2, day 90.

3.3 OTU SEQUENCE ALIGNMENT ANALYSIS

A representative colony of the most abundantly observed patterns from the restriction digest analysis was amplified as described in Section 2.6 and the crude PCR product was sent to the company BioBasic for sequencing using fluorescent dye-terminator method that may obtain a read length of over 650 base pairs. A total of 130 clones were sent for sequencing. The average read length obtained from the sequencing procedure was approximately 1300 base pairs.

Obtained sequences were uploaded to the Ribosomal Database (www.rdp.cme.msu.edu/) where they were aligned with previously archived microbial 16S rRNA gene sequences. In total, 91 experimental sequences were uploaded to the databank of which 82 sequences were able to be aligned to a minimum of the phylum level. A summary of clones uploaded to the Ribosomal Databank is provided in Table 3.2. Using the chimera detection program on the sequence alignment software Mothur, no chimeric sequences were found using submitted sequences to the Ribosomal Databank Project as a template against the experimental sequences.

Table 3.2: Summary of 16S rRNA gene clone samples submitted for sequence alignment.

Soil Sample	*Number of samples sequenced*
Bioreactor 1, Control slurry, Day 0	7
Bioreactor 2, Control slurry, Day 0	1
Bioreactor 1, Control slurry, Day 45	2
Bioreactor 2, Control slurry, Day 45	14
Bioreactor 1, Control slurry, Day 90	7
Bioreactor 2, Control slurry, Day 90	3
Bioreactor 1, Nutrient addition, Day 0	14
Bioreactor 2, Nutrient addition, Day 0	1
Bioreactor 1, Nutrient addition, Day 45	7
Bioreactor 2, Nutrient addition, Day 45	9
Bioreactor 1, Nutrient addition, Day 90	17
Bioreactor 2, Nutrient addition, Day 90	1
Positive Control	8

3.3.1 CONTROL SLURRY

3.3.1.1 BIOREACTOR 1

The most prominent OTUs at day 0 in the control slurry samples from bioreactor 1 belonged to the phylum *Proteobacterium*. The A extraction at day 0 had 6.1% of bacteria belonging to the family *Sphingomonadaceae*, 4.1% bacteria belonging to an undetermined order of *Gammaproteobacteria,* 4.1% classified to an undetermined genus belonging to the family *Xanthomonadaceae,* and 2.0% to an undetermined genus of the family *Legionellaceae*. The next most prominent sequences were an undetermined genus of the family *Chitinophagaceae,* an determined genus of the family *Corynebacterineae*, and the genus *Sphaerobacter* and each of these were observed in 2.0% of the clones.

The most prominent sequenced phylum from the B extraction was also *Proteobacteria.* The most abundant sequenced OTU was the undetermined order of *Gammaproteobacteria* assigned identification number 49 and it was detected in 4.2% of clones. The bacterium belonging to an undetermined genus of the family *Sphingomonadaceae* that was detected in 6.1% of clones in the A extraction was only present at a proportion of 1.4% in the B extraction. The undetermined genus of the family *Legionellaceae* was detected in 1.4% of clones. There were 1.4% clones detected belonging to the phylum *Bacteroidetes*. There was one clone belonging to an unknown genus of the family *Chitinophagaceae* and another clone aligned to the genus *Ferruginibacter* of the same family. The order *Solirubrobacterales* of the phylum

Actinobacteria was detected in 1.4% in the B extraction as was the genus *Sphaerobacter* and the family *Corynebacterineae*.

At day 45, the most prominent sequenced OTU from both extractions was from the undetermined genus from the family *Chitinophagaceae* that was detected in 9.7% and 12.0% of the clones from the A and B nucleic acid extractions respectively. The proportion of this bacterium decreased to 4.4% and 1.4%, respectively, in the A and B extractions at day 90. The genus *Nitrosospira* was detected in 4.8% of clones in the A extraction at day 45 and 2.0% in the B extraction. Genus *Nitrospira* of the phylum *Nitrospirae* was detected at similar proportions of 3.2% and 2.0% of clones from the two extractions. Genus *Gemmatimonas* was observed in 1.6% of clones from the A extraction and at 6.0% of clones from the B extraction.

At day 90, the sequenced microbial population varied significantly between the A and B extractions. For example, from the A extraction there was 11.1% of *Acidobacteria Gp4* and 15.6% *Acidobacteria Gp7* and in the B extraction the proportion of these bacteria detected decreased to 1.4% and 0% respectively. The B extraction consisted of 5.4% *Acidobacteria Gp6* and this group was absent in the A extraction. The genus *Nitrosospira* was present most frequently in the B extraction in 10.8% of clones but was not detected from the A extraction.

A summary table of the proportion of the sequenced OTUs present in the control slurry reactor 1 samples at days 0, 45, and 90 is provided in Table 3.3.

Table 3.3: Proportion of the sequenced restriction patterns present in the Control Treatment, Reactor 1 at days 0, 45 and 90 and for each DNA extraction (A or B). Shading denotes the most abundant of the sequenced OTU detected in an extraction.

OTU #	Phylum	Class	Order	Family	Genus	% Day 0		% Day 45		% Day 90	
						A	B	A	B	A	B
85	*Proteobacteria*	*α-proteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingopyxis*	0	0	0	2.0	2.2	0
113					-	6.1	1.4	1.6	0	0	0
174			*Caulobacterales*	*Caulbacteraceae*	*Phenylobacterium*	0	0	0	0	0	0
173			*Rhizobiales*	*Hyphomicrobiaceae*	*Pedomicrobium*	0	0	0	0	2.2	0
43				*Phyllobacteriaceae*	*Mesorhizobium*	0	0	0	0	0	0
65		*β-proteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*	*Nitrosospira*	0	0	4.8	2.0	0	10.8
259			*Methylophilales*	*Methylophiaceae*	*Methylotenera*	0	0	3.2	0	0	0
68			*Burkholderiales*	*Comamonadaceae*	*Hydrogenophaga*	0	0	0	2.0	0	2.7
49		*γ-proteobacteria*	-	-	-	4.1	4.2	0	0	2.2	2.7
71			*Legionellales*	*Legionellaceae*	-	2.0	1.4	1.6	2.0	2.2	0
15					*Legionella*	0	0	0	0	0	0
126			*Xanthomonadales*	*Xanthomonadaceae*	-	4.1	0	1.6	0	0	0
285				*Sinobacteraceae*	*Steroidobacter*	0	0	0	0	6.7	1.4
7		*Δ-proteobacteria*	*Bdellovibrionales*	*Bdellovibrionaceae*	*Vampirovibria*	0	0	0	0	0	0
170	*Acidobacteria*	*Gp4*	-	-	-	0	0	0	0	11.1	1.4
100		*Gp6*	-	-	-	0	0	1.6	0	0	5.4
83		*Gp7*	-	-	-	0	0	0	0	8.9	0
96	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*	0	0	3.2	2.0	0	0
26	*Bacteroidetes*	-	-	-	-	0	0	0	0	0	0

OTU #	Phylum	Class	Order	Family	Genus	% Day 0		% Day 45		% Day 90	
						A	B	A	B	A	B
276	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Cytophagacea*	*Dyadobacter*	0	0	0	0	2.2	0
354				*Chitinophagaceae*	-	2.0	1.4			4.4	1.4
46					*Ferruginibacter*	0	1.4	0	0	0	0
86					*Terrimonas*	0	0	1.6	0	0	1.4
91	*Verrucomicrobia*	*Subdivision3*	-	-	-	0	0	0	0	0	0
36	*Gemmatimonadetes*	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*	0	0	1.6	6.0	0	0
121	*Chloroflexi*	*Thermomicrobia*	*Sphaerobacteridae*	*Sphaerobacteraceae*	*Sphaerobacter*	2.0	1.4	1.6	0	0	0
351	*Actinobacteria*	*Actinomycetales*	*Pseudoncardineae*	*Pseudonocardiaceae*	*Pseudonocardia*	0	0	0	0	0	0
353		*Rubrobacteridae*	*Solirubrobacterales*	-	-	0	1.4	0	0	0	0
16		*Actinobacteridae*	*Actinomycetales*	*Corynebacterineae*	-	2.0	1.4	1.6	0	2.2	0
Number of clones						49	72	62	50	45	74
Proportion of sample sequenced (%)						22.3	14.0	33.7	28.0	51.0	27.2

3.3.1.2 BIOREACTOR 2

At day 0 from the A extraction the most abundant sequence belonged to an undetermined order of *Gammaproteobacteria* at a proportion of 8.9%. This same order was detected in 3.9% of the B extraction clones at day 0. There were small proportions of the genera *Pedomicrobium* and *Nitrosospira* detected in both extractions. The genus *Legionella* was detected most frequently in the B extraction at a proportion of 5.2% of clones.

At day 45 there was a significant proportion of the genus *Methylotenera* detected in both nucleic acid extractions. In the A extraction this genus was observed in 36.2% of clones and in the B extraction it was detected in 38.0% of clones. The prominence of this genus was greatly reduced in day 90 samples as it was completely absent from the A extraction and was detected in only 4.8% of clones in the B extraction. The second most abundant sequence in both extractions at day 45 belonged to an undetermined genus of the family *Chitinophagaceae* at proportions of 14.5% and 7.6% in the A and B extractions respectively.

The day 90 samples showed variation between the two extractions. The genus *Gemmatimonas* was detected in 9.7% of clones in extraction A and 3.2% of clones in extraction B. *Gemmatimonas* was detected in 9.7% of clones in the A extraction, but was absent in the B extraction at day 90. Group 6 of the phylum *Acidobacteria* was also detected in 9.7% of clones in the A extraction, but was only detected in 3.2% of clones in the B extraction. The most abundant clones in the B extraction at day 90 were the genus

Methylotenera and an undetermined genus of the family *Chitinophagaceae* which were detected each in 4.8% of clones. A summary of the presence of the sequenced OTUs sampled from control conditions in bioreactor 2 is provided in Table 3.4.

Table 3.4: Proportion of the sequenced restriction patterns present in the Control Treatment, Reactor 2 at days 0, 45 and 90 and for each DNA extraction (A or B). Shading denotes the most abundant of the sequenced OTU detected in an extraction.

OTU #	Phylum	Class	Order	Family	Genus	% Day 0		% Day 45		% Day 90	
						A	B	A	B	A	B
85	*Proteobacteria*	*α-proteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingopyxis*	0	0	4.3	1.3	0	0
113					-	0	2.7	0	0	4.8	1.6
174			*Caulobacterales*	*Caulbacteraceae*	*Phenylobacterium*	0	0	0	0	0	0
173			*Rhizobiales*	*Hyphomicrobiaceae*	*Pedomicrobium*	1.8	2.7	0	0	0	3.2
43				*Phyllobacteriaceae*	*Mesorhizobium*	0	0	2.9	1.3	0	0
65		*β-proteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*	*Nitrosospira*	1.8	2.7	1.4	1.3	3.2	0
259			*Methylophilales*	*Methylophiaceae*	*Methylotenera*	0	0			0	
68			*Burkholderiales*	*Comamonadaceae*	*Hydrogenophaga*	0	0	2.9	1.3	0	3.2
49		*γ-proteobacteria*	-	-	-		3.9	0	3.8	0	3.2
71			*Legionellales*	*Legionellaceae*	-	0	1.3	0	0	0	1.6
15					*Legionella*	0		0	0	0	0
126			*Xanthomonadales*	*Xanthomonadaceae*	-	0	1.3	0	0	0	3.2
285				*Sinobacteraceae*	*Steroidobacter*	0	0	0	0	0	0
7		*Δ-proteobacteria*	*Bdellovibrionales*	*Bdellovibrionaceae*	*Vampirovibria*	1.8	0	0	0	0	0
170	*Acidobacteria*	*Gp4*	-	-	-	0	0	0	0	0	3.2
100		*Gp6*	-	-	-	0	1.3	0	0	9.7	0
83		*Gp7*	-	-	-	0	0	0	0	0	0
96	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*	0	0	0	0	0	1.6
26	*Bacteroidetes*	-	-	-	-	0	0	0	0	0	0

OTU #	Phylum	Class	Order	Family	Genus	% Day 0		% Day 45		% Day 90	
						A	B	A	B	A	B
276	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Cytophagacea*	*Dyadobacter*	0	0	1.4	1.3	0	3.2
354				*Chitinophagaceae*	-	0	1.3	14.5	7.6	0	[illegible]
46					*Ferruginibacter*	0	0	0	1.3	0	1.4
86					*Terrimonas*	0	0	1.4	0	0	1.4
91	*Verrucomicrobia*	*Subdivision3*	-	-	-	0	0	0	0	0	0
36	*Gemmatimonadetes*	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*	0	1.3	0	1.3	[illegible]	3.2
121	*Chloroflexi*	*Thermomicrobia*	*Sphaerobacteridae*	*Sphaerobacteraceae*	*Sphaerobacter*	0	1.3	0	0	0	0
351	*Actinobacteria*	*Actinomycetales*	*Pseudoncardineae*	*Pseudonocardiaceae*	*Pseudonocardia*	0	0	0	0	0	0
353		*Rubrobacteridae*	*Solirubrobacterales*	-	-	1.8	0	1.4	0	0	0
16		*Actinobacteridae*	*Actinomycetales*	*Corynebacterineae*	-	0	2.7	0	0	0	0
Number of clones						56	75	69	79	62	63
Proportion of sample sequenced (%)						16.1	27.7	66.4	58.5	27.4	39.6

3.3.1.3 COMPARISON OF CONTROL SAMPLES BETWEEN BIOREACTORS

At day 45 in the second bioreactor a large proportion of the genus *Methylotenera* was detected. This genus was mostly absent in samples taken from bioreactor 1. At day 0, an undetermined order of *Gammaproteobacteria* was detected in all extractions from the two bioreactors. In addition, the unidentified class of *Bacteroidetes* was present in more than 7% of clones in both bioreactors at day 45 and decreased at day 90. In both reactors the proportion of clones corresponding to the phylum *Acidobacteria* was increased at day 90 as compared to earlier time points.

3.3.2 NUTRIENT ADDITION SLURRY

3.3.2.1 BIOREACTOR 1

At day 0 of the bioreactor 1 nutrient supplementation sample, the genus *Nitrosospira* was represented in 38.9% of the clones from nucleic acid extraction A. This genus was absent in extraction B. At day 45, *Nitrosospira* was detected in 27.5% of clones from extraction A and 30.7% of clones from extraction B. The next most abundant genus detected at day 45 was *Ferruginibacter* and it was detected in 5.7% of clones in the B extraction. This genus was detected in 2.2% of clones in extraction A.

At day 90, *Nitrosospira* was still represented in a large proportion of clones at 17.9% and 14.4% in the A and B extractions, respectively. The genus *Ferruginibacter* was detected in 4.8% of A extraction clones and 6.7% of B extraction clones. 10.8% of the bacteria in extraction A and 10% of the bacteria in extraction B belonged to groups in the phylum *Acidobacteria*. A summary of all sequenced OTUs present from nutrient addition samples in bioreactor 1 is provided in Table 3.5.

Table 3.5: Proportion of the sequenced restriction patterns present in the Nutrient addition Treatment, Reactor 1 at days 0, 45 and 90 and for each DNA extraction (A or B). Shading denotes the most abundant of the sequenced OTU detected in an extraction.

OTU #	Phylum	Class	Order	Family	Genus	% Day 0		% Day 45		% Day 90	
						A	B	A	B	A	B
85	*Proteobacteria*	*α-proteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingopyxis*	0	0	2.2	1.1	0	0
113					-	0	0	0	0	1.2	0
174			*Caulobacterales*	*Caulbacteraceae*	*Phenylobacterium*	5.6	0	0	0	0	2.2
173			*Rhizobiales*	*Hyphomicrobiaceae*	*Pedomicrobium*	0	0	0	0	0	2.2
43				*Phyllobacteriaceae*	*Mesorhizobium*	0	1.1	0	0	0	0
65		*β-proteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*	*Nitrosospira*	38.9	0	27.5	30.7	17.9	14.4
259			*Methylophilales*	*Methylophiaceae*	*Methylotenera*	0	0	0	0	0	0
68			*Burkholderiales*	*Comamonadaceae*	*Hydrogenophaga*	0	0	2.2	0	1.2	0
49		*γ-proteobacteria*	-	-	-	0	1.1	0	1.1	0	0
71			*Legionellales*	*Legionellaceae*	-	0	0	2.2	1.1	0	0
15					*Legionella*	0	2.2	0	0	1.2	0
126			*Xanthomonadales*	*Xanthomonadaceae*	-	0	0	0	0	0	0
285				*Sinobacteraceae*	*Steroidobacter*	0	0	0	0	0	0
7		*Δ-proteobacteria*	*Bdellovibrionales*	*Bdellovibrionaceae*	*Vampirovibria*	0	1.1	0	0	0	0
170	*Acidobacteria*	*Gp4*	-	-	-	0	0	0	0	0	1.1
100		*Gp6*	-	-	-	5.6	0	0	1.1	1.2	2.2
83		*Gp7*	-	-	-	0	0	2.2	0	4.8	1.1
96	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*	0	0	0	1.1	0	1.1
26	*Bacteroidetes*	-	-	-	-	5.6	1.1	1.1	0	2.4	2.2

OTU #	Phylum	Class	Order	Family	Genus	% Day 0		% Day 45		% Day 90	
						A	B	A	B	A	B
276	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Cytophagacea*	*Dyadobacter*	0	0	0	0	0	0
354				*Chitinophagaceae*	-	0	0	0	0	0	0
46					*Ferruginibacter*	0	1.1	2.2	5.7	4.8	6.7
86					*Terrimonas*	0	0	1.1	0	0	1.1
91	*Verrucomicrobia*	*Subdivision3*	-	-	-	5.6	0	1.1	0	0	1.1
36	*Gemmatimonadetes*	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*	0	2.2	0	1.1	0	2.2
121	*Chloroflexi*	*Thermomicrobia*	*Sphaerobacteridae*	*Sphaerobacteraceae*	*Sphaerobacter*	0	0	0	0	0	2.2
351	*Actinobacteria*	*Actinomycetales*	*Pseudoncardineae*	*Pseudonocardiaceae*	*Pseudonocardia*	0	0	0	0	0	0
353		*Rubrobacteridae*	*Solirubrobacterales*	-	-	0	0	0	0	0	0
16		*Actinobacteridae*	*Actinomycetales*	*Corynebacterineae*	-	5.6	3.3	0	0	1.2	0
Number of clones						18	90	91	88	84	90
Proportion of sample sequenced (%)						66.9	13.2	41.8	43	35.6	39.8

3.3.2.2 BIOREACTOR 2

In bioreactor 2 at day 0 the most abundant sequenced genus characterized from the A extraction was *Pedomicrobium*, which was detected in 5.4% of clones in extraction A and 3.3% of clones in extraction B. The most abundant genus detected in the B extraction was *Legionella* which was detected in 6.6% of clones and was also present in 4.1% of clones in the A extraction.

At day 0, there were only 1.4% *Nitrosospira* in extraction A and 0% in extraction B. However, at day 45, the proportion of this genus increased to 34.8% and 30.0% in the A extraction and B extraction respectively. At day 90, the proportion of *Nitrosospira* was similar at 30.9% and 29.9%. From day 45 onwards it was the most abundant genus detected.

At day 45, the second most abundant OTU from the A extraction belonged to *Acidobacteria Gp6* and it was detected in 10.1% of clones but was not detected in the B extraction. At day 90, the A extraction consisted of 7.4% of bacteria belonging to the phylum *Acidobacteria.* This percentage was 12.5% in the B extraction. The genus *Nitrospira* was detected in 9.2% of clones from the B extraction at day 90 and at a reduced proportion of 3.2% in the B extraction. A summary of the sequenced microbial community structure from nutrient addition samples taken from bioreactor 2 is provided in Table 3.6.

Table 3.6: Proportion of the sequenced restriction patterns present in the Nutrient addition Treatment, Reactor 2 at days 0, 45 and 90 and for each DNA extraction (A or B). Shading denotes the most abundant of the sequenced OTU detected in an extraction.

OTU #	Phylum	Class	Order	Family	Genus	% Day 0		% Day 45		% Day 90	
						A	B	A	B	A	B
85	*Proteobacteria*	*α-proteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingopyxis*	0	0	0	1.3	1.2	0
113					-	0	1.6	0	0	0	0
174			*Caulobacterales*	*Caulbacteraceae*	*Phenylobacterium*	2.7	0	0	0	2.5	0
173			*Rhizobiales*	*Hyphomicrobiaceae*	*Pedomicrobium*	4.8	3.3	0	1.3	0	0
43				*Phyllobacteriaceae*	*Mesorhizobium*	0	0	0	0	3.7	3.4
65		*β-proteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*	*Nitrosospira*	1.4	0	34.8	30.0	30.9	29.5
259			*Methylophilales*	*Methylophiaceae*	*Methylotenera*	0	0	0	0	0	0
68			*Burkholderiales*	*Comamonadaceae*	*Hydrogenophaga*	0	0	0	0	1.2	0
49		*γ-proteobacteria*	-	-	-	1.4	1.6	0	0	0	0
71			*Legionellales*	*Legionellaceae*	-	4.1	1.6	1.1	3.8	2.5	3.4
15					*Legionella*	4.1	6.6	1.1	0	0	0
126			*Xanthomonadales*	*Xanthomonadaceae*	-	1.4	3.3	0	1.3	0	0
285				*Sinobacteraceae*	*Steroidobacter*	0	0	0	0	0	0
7		*Δ-proteobacteria*	*Bdellovibrionales*	*Bdellovibrionaceae*	*Vampirovibria*	2.7	0	2.2	0	0	0
170	*Acidobacteria*	*Gp4*	-	-	-	0	0	0	0	2.5	0
100		*Gp6*	-	-	-	1.4	0	10.1	0	0	3.4
83		*Gp7*	-	-	-	2.7	0	0	3.8	3.7	8.0
96	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*	0	0	1.1	0	3.7	9.2
26	*Bacteroidetes*	-	-	-	-	0	0	2.2	1.3	3.7	2.3

OTU #	Phylum	Class	Order	Family	Genus	% Day 0		% Day 45		% Day 90	
						A	B	A	B	A	B
276	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Cytophagacea*	*Dyadobacter*	0	0	0	0	0	0
354				*Chitinophagaceae*	-	0	0	0	0	0	0
46					*Ferruginibacter*	1.4	0	1.1	0	1.2	0
86					*Terrimonas*	0	0	0	0	0	3.4
91	*Verrucomicrobia*	*Subdivision3*	-	-	-	0	1.6	0	0	1.2	0
36	*Gemmatimonadetes*	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*	0	0	1.1	0	2.5	0
121	*Chloroflexi*	*Thermomicrobia*	*Sphaerobacteridae*	*Sphaerobacteraceae*	*Sphaerobacter*	0	0	0	0	1.2	0
351	*Actinobacteria*	*Actinomycetales*	*Pseudoncardineae*	*Pseudonocardiaceae*	*Pseudonocardia*	0	0	0	2.5	0	0
353		*Rubrobacteridae*	*Solirubrobacterales*	-	-	0	0	0	0	0	2.3
16		*Actinobacteridae*	*Actinomycetales*	*Corynebacterineae*	-	1.4	0	0	0	0	0
Number of clones						74	61	89	80	81	87
Proportion of sample sequenced (%)						30.1	19.6	54.8	45.3	61.7	65.3

3.3.2.3 COMPARISON BETWEEN NUTRIENT ADDITION BIOREACTORS

The most prominent genus detected from samples taken from both bioreactors for days 45 and 90 was *Nitrosospira.* In bioreactor 1, the proportion of this genus dropped by nearly 50% by day 90 as compared to day 45, but this decrease was not evident in samples taken from bioreactor 2. *Nitrosospira* was detected in day 0 samples from bioreactor 1, but only slightly in samples from bioreactor 2. The genus *Pedomicrobium* was detected most frequently at day 0 in bioreactor 2 samples, but was not detected in soil from bioreactor 1. *Nitrosospira* was observed more frequently in day 90 samples from bioreactor 2 than day 90 samples from bioreactor 1. At day 90 from bioreactor 2 *Nitrospira* was detected in 3.2% of clones from the A extraction and 9.2% from the B extraction. In bioreactor 1 samples taken at day 90, the genus *Nitrospira* was not detected in any clones from the A extraction and only 1.1% of clones in the B extraction. A similar difference between genera detected in different bioreactors was observed when the genus *Ferruginibacter* was detected in almost all bioreactor 1 samples but was detected infrequently in bioreactor 2 samples.

3.3.3 COMPARISON BETWEEN CONTROL AND NUTRIENT ADDITION

Nitrosospira was the most prominent genus from day 45 onwards in nutrient addition samples but was detected far less frequently in control samples. The genus *Methylotenera* was detected frequently in control samples from bioreactor 2 at day 45, but was not detected in sampling from the nutrient addition treatments. Over the duration of the treatment, the phylum *Acidobacteria* gradually increased in abundance in both control and nutrient addition samples. Day 0 samples from all soil treatment samples had many fingerprints that were observed infrequently.

3.4 SAMPLING ANALYSIS

The thoroughness of the sampling conducted in this experiment was analyzed using the software MOTHUR. The boneh value to estimate the number of additional OTUs that would be detected through sending an additional 91 clones for sequencing was determined and is shown in Table 3.7. An additional 20.1 unique sequences forming OTUs would be expected. If OTUs varied by a genetic difference of 0.10 or less, an additional 16.2 OTUs would be expected.

Rarefaction curves of the DNA sequences sampled are provided in Figure 3.14. The line for the nutrient slurry is less steep than the control slurry line, which is less steep than the line for the total soil sample, indicating that further sampling would yield fewer new OTUs for the nutrient sample compared to the control.

Table 3.7: Estimation of additional OTUs detected by doubling the sample size sequenced.

Genetic Difference Defining an OTU	*Additional OTUs Expected*
Unique	20.102581
0.05	19.393309
0.06	18.688868
0.07	17.974847
0.08	17.399976
0.09	17.224263
0.10	16.179430
0.11	14.761714
0.12	14.085356

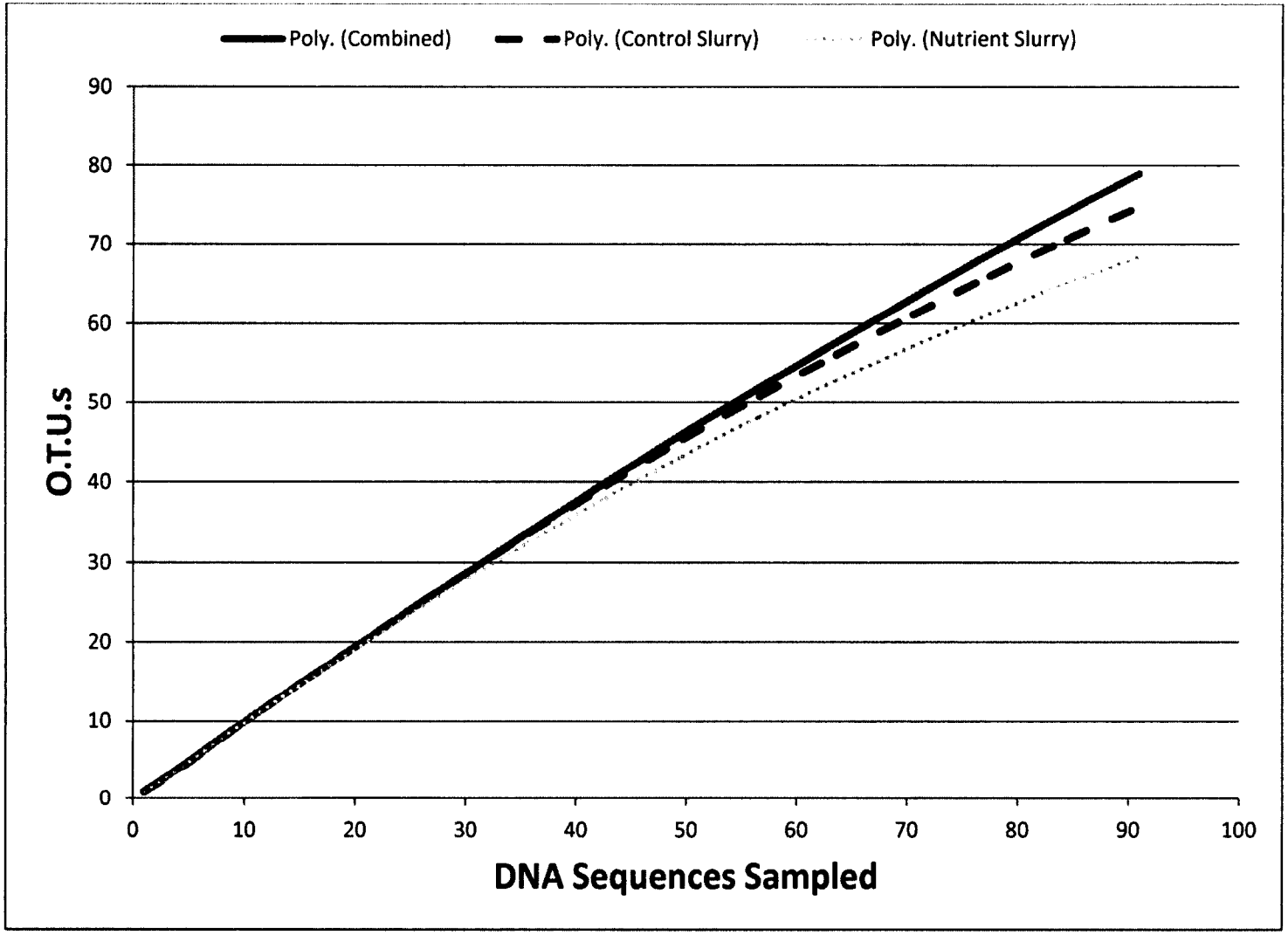

Figure 3.14: Rarefaction curves of the control slurry, nutrient slurry, and total samples combined with OTUs defined at a 0.05 distance cutoff.

3.5 DIVERSITY ANALYSIS OF RFLP FINGERPRINTS

Each restriction fragment pattern obtained was assumed to belong to a unique OTU at a distance cutoff of 0.03. Diversity and richness indices were calculated using the frequency of each pattern obtained from each sample. Richness was evaluated using the Chao1 and the abundance based coverage estimator (ACE). The Simpson and Shannon diversity indices were used to evaluate diversity. These indices are presented in Table 3.8.

The Simpson index values for the control slurry soil at day zero from both bioreactors are 94.251 and 81.900. At day 45 from the control slurry the Simpson index value decreased to 32.884 and 6.592 in bioreactors 1 and 2 respectively. The Simpson index values increased in day 90 control samples compared to day 45 to 34.928 and 47.847. The Simpson index values displayed a similar pattern in the nutrient samples where the day 0 values were 96.339 and 107.677 for bioreactors 1 and 2, respectively and the values decreased to 10.460 in bioreactor 1 and 8.921 in bioreactor 2 at day 45. At day 90 the Simpson values increased from day 45 levels to 27.315 and 9.579 from bioreactors 1 and 2 respectively (Table 3.8).

Shannon index values from control slurry samples at day zero were about 4.1 for both bioreactors. Shannon index values decreased to 3.702 and 2.878 in control slurry day 45 samples. The Shannon index value from bioreactor 1 decreased from its day 45 value to 3.674 at day 90 while the bioreactor 2 value at day 90 increased from its day 45 level to 3.809. Shannon index values from nutrient slurry samples at day 0 were 4.156

and 4.319 from bioreactors 1 and 2, respectively. At day 45 Shannon values from the nutrient soil were 3.374 and 3.295. The Shannon index value from the nutrient addition soil bioreactor 1 increased from its day 45 value at day 90 where it was 3.840. The bioreactor 2 value decreased to 3.136 at day 90 compared to day 45. The Simpson index values and the Shannon index patterns displayed a near identical pattern of increasing and decreasing over the course of the bioreactor treatments.

For both the reciprocal Simpson index and the Shannon index, larger values indicate greater diversity. The values were largest at day 0 for both the control and nutrient supplement and therefore day 0 samples showed the greatest diversity. After 45 days selectable pressure from the nitro-organic explosives would start to be observed and diversity would decrease as shown through a decrease in the Simpson and Shannon index values as shown in Table 3.8. After 90 days of treatment the concentration of explosives in the soil would have decreased and present a less selectable advantage for bacteria that utilize the contaminants as a substrate and the diversity would increase. This is evident in the increase in most of the Simpson and Shannon index values from day 45 to day 90 (Table 3.8).

The largest Chao1 value from the control slurry was from bioreactor 1 at day 0. Chao1 values from the same bioreactor decreased over time, and the smallest Chao1 value was detected from bioreactor 2 at day 90. A similar trend was observed in ACE values from the control slurry except in bioreactor 2 the ACE value increased from day 45 where it was 84.81 to 106.44 at day 90 (Table 3.8).

In the nutrient addition soil samples, the largest Chao1 and ACE values were observed at day 0 for both bioreactors. In bioreactor 2 samples, Chao1 and ACE values at day 45 decreased from the day 0 values, and decreased further at day 90. In samples taken from bioreactor 1, Chao1 and ACE values at day 45 were less than day 0 values, but then increased from day 45 to day 90 (Table 3.8). From these values it can be concluded that the samples were richest at day 0. Richness generally decreased over the duration of the treatment for both treatments as indicated in the decrease in Chao1 and ACE values (Table 3.8).

Table 3.8: Diversity index values for RFLP fingerprints obtained from bioreactor treated soil analyzed in this study.

Soil Treatment	*Bioreactor*	*Day*	*Reciprocal Simpson Index*	*Shannon Index*	*Chao1*	*ACE*
Control	1	0	94.251	4.174	167.13	174.43
	2		81.900	4.129	118.24	145.78
	1	45	32.884	3.702	152.39	153.95
	2		6.592	2.878	95.72	84.81
	1	90	34.928	3.674	132.56	125.70
	2		47.847	3.809	82.5	106.44
Nutrient	1	0	96.339	4.156	205.67	227.58
	2		107.677	4.319	195.78	225.21
	1	45	10.460	3.374	121.53	130.36
	2		8.921	3.295	130.65	146.43
	1	90	27.315	3.840	164.17	178.11
	2		9.579	3.136	71.35	79.47

4.0 DISCUSSION

There were two objectives in this study as outlined in Section 1.9. The first objective was to measure the microbial diversity in the soil sample derived from a munitions testing site in Petawawa using RFLP and DNA sequencing of the 16S rRNA gene. RFLP was employed to classify various copies of the 16S rRNA gene into OTUs and the microbes producing the most frequently observed OTUs were identified through DNA sequencing. The second objective of this study was to examine changes in the microbial community structure under controlled conditions over a 90 day period with and without the addition of a nutrient supplement. This was accomplished by incubating the soil in a bioreactor and sampling the community fingerprint at days 0, 45, and 90. It was hypothesized that bacterial 16S rRNA analysis from the bioreactor treated soil would identify microbes that have the capacity to degrade nitro-substituted munitions. In addition, temporal changes in the microbial community, with and without the addition of nutrients, would arise during the bioreactor treatment that would reflect the preferential ability of certain bacteria to degrade the explosive contaminants. In total, RFLP analysis yielded a total of 326 different OTUs from all of the various samples. A total of 29 different OTUs were sequenced and aligned to at least the phylum level. Differences in the microbial community structure were observed at different time points, and following the addition of the nutrient supplement.

4.1 NITRO-ORGANIC EXPLOSIVE CONTAMINATION IN SOIL

Initial concentrations of explosive compounds in the soil showed relatively low levels of contamination (Table 3.1). The largest contaminant was HMX at concentrations

of 1154.4 ppm. HMX-microbial bioremediators would theoretically be most abundant in the soil samples. However, HMX bioremediation occurs most efficiently under anaerobic conditions. A study of HMX-bioremediation in soil sediments from unexploded ordinance sites revealed greater removal of HMX when air was removed from the reaction vessels through the addition of Argon gas (Zhao *et al.,* 2007). Although some HMX was removed under aerobic conditions, the removal rate under anaerobic conditions was much greater. Since the bioreactors were run in an aerobic environment, the extent of HMX bioremediation is expected to be lower. There is the possibility that anaerobic pockets may arise within the soil that would allow for anaerobic bioremediation to occur, but this likelihood is decreased through the constant aeration provided by the steady rotation in the reaction vessel.

The next most concentrated nitro-organic explosive in the soil was TNT. It was found at an initial concentration of 5.88 ppm (Table 3.1). Possible TNT-metabolites 1,3,5-TNB, 4a-DNT and 2a-DNT were also present in the soil at concentrations of 0.702 ppm, 1.74 ppm, and 0.336 ppm respectively. The presence of these metabolites may suggest that bioremediation was occurring in the soil before the bioreactor runs were initiated. The addition of 140 μg/mg TNT to a pristine soil sample was shown to cause a slight alteration in the 16S rRNA DGGE fingerprint compared to control conditions over time (Georges *et al.,* 2008). The TNT concentration in this study is much lower, so the extent of microbial community shift compared to pristine soil samples in this study due to TNT is expected to be minimal.

RDX has been reported to be biodegraded under both aerobic and anaerobic conditions. In this study, RDX was present at an initial concentration of 3×10^{-4} µg/mg, which was the least concentrated of the nitro-organic explosives excluding possible TNT metabolites (Table 3.1). It is therefore expected that RDX would play the smallest role in causing a shift in the bacterial community structure. The K*m* of the RDX-degrading enzyme XplA is reported to be 83.7 ± 17.8 µM (Jackson *et al.,* 2007). At the low reported RDX concentration, the enzyme would not be saturated with substrate.

4.2 CONTROL SLURRY DIVERSITY

The control slurry samples from bioreactor 1 showed great microbial richness with many different OTUs that appeared once or twice. Microbial richness is defined in this study as the amount of different microbes detected in the soil sample based on the amount of different 16S rRNA gene fingerprints. Diversity is defined as the relative abundance of each microbe detected. The more varied the fingerprints, the more diverse the sample is deemed to be. A score to quantify how often OTUs would only be observed once is provided through the Chao1 score and ACE value (Hughes *et al.*, 2001). The Chao1 value factors the abundance of singletons and doubletons observed to provide a relative score to indicate how much sampling is required to view an individual OTU more than once (Hughes *et al.,* 2001). The ACE value provides a similar indication, but factors in OTUs detected ten times or less (Hughes *et al.,* 2001). At day zero there were many OTUs detected only once from both bioreactors and the high Chao1 and ACE values reflect this as shown in Table 3.8. This shows that at day 0 for both control and

nutrient addition treatments that the soil was rich with many different bacteria. As time progressed in the treatments, the explosive contamination provided a selectable pressure for possible bioremediators and the richness of the community structure would decrease. This is evident in the decrease in the Chao1 and ACE values evident at day 45 and day 90 compared to day 0 values for all treatments (Table 3.8). Where there was dominance by an individual OTU as in the control treatment in bioreactor 2 and day 45 the richness and diversity of other microbes decreases (Table 3.8).

In this study there were 741 16S RFLP fingerprints obtained from the control sample that were assigned to 209 OTUs. The slope of the rarefaction curve for the control slurry with OTU cut-off distance defined at 0.05 is fairly steep, but begins to flatten out slightly towards the end (Figure 3.13). This shows that additional sampling would yield more OTUs, some of which may belong to previously reported nitro-organic explosive bioremediators.

Diversity index values at day 0 from the control slurry were similar as shown in Table 3.8. Simpson index values were approximately 0.01 and Shannon index values were approximately 4.1 from both reactors. The reciprocal Simpson scale is proportional to microbial richness (Hill, 1973). The more microbes present, the smaller the Simpson index value. Conversely, the Shannon scale is proportional to microbial richness (Hill, 1973). This would suggest that the nitro-organic explosives in the soil were not selecting a particular microbe over the other. At day 45 in samples from bioreactor 2, there was a significant proportion of the microbial community belonging to the genus

Methylotenera (Table 3.3). This large proportion (36.2% in the A extraction and 38.0% in the B extraction) was evident in both nucleic acid extractions. Since the proportion of *Methylotenera* was consistent in the two extractions, it would seem that this spike was not a result of a PCR "jackpotting" bias or a bias associated with the nucleic acid extraction procedure. As a result, the overall diversity of the sample collected at day 45 from bioreactor 2 decreased. This is evident in the larger Simpson index value of 0.1517 and smaller Shannon index value of 2.878 (Table 3.8). *Methylotenera* was not detected as abundantly in control day 45 samples from bioreactor 1 and samples from bioreactor 1 at day 45 were richer in microbes as shown in the diversity index values listed in Table 3.8. Day 90 samples from bioreactor 2 did not have a large abundance of *Methylotenera* and its overall microbial structure was richer (Table 3.8). Hypotheses to explain the prominence of *Methylotenera* is provided in Section 4.2.2.

4.2.1 POTENTIAL BIASES BETWEEN BIOREACTORS

The reason for the observed inconsistency between bioreactors could be a result in the difficulty in maintaining exact conditions between multiple reactors. Although conditions such as dissolved oxygen, pH and temperature were programmed to be constant and consistent between the two bioreactors, there would still be the possibility that other variables may have been consistent. These factors could be variation in the room temperature between the two locations of the reactors, differential exposure to light, and the possibility that there may have been contaminants in the bioreactors. These undetermined variables may allow for the selection of different bacteria in each

bioreactor. In order to increase the accuracy of the true microbial community structure, more bioreactors should be used.

Another source of inter-bioreactor differences could be due to a lack of homogeneity in the soil sampling, which is a more likely cause for the different community structures in control samples at day 45. Soil in the environment is very heterogeneous in its composition and soil used to seed the reactors as well as soil sampled from the bioreactors at different time points would be no exception. Different soil components such as sand, silt, clay, and organic matter are dispersed irregularly throughout the soil and provide myriads of microhabitats with the potential for different microbial niches to be established (Garbeva *et al.,* 2004). Soil microbial communities are randomly spread out to follow nutrient and moisture gradients producing what is known as a "hot-spot" distribution (Nunan, *et al.,* 2002). The variation in soil composition can even result in anoxic pockets that allow for anaerobic bioremediation. Therefore, despite a constant rotating of the soil that the bioreactors provide, it is unlikely that the soil composition will be homogenous and different micro-environments may be present in one sample versus another due to this heterogeneity.

4.2.2 METHYLOTENERA

The genus *Methylotenera* provided the most abundant OTU for the control soil. Its restriction fragment length polymorphism was first observed at day 45 for both reactors. This genus was observed far more frequently in Bioreactor 2 treated soil compared to Bioreactor 1.

The genus *Methylotenera* belongs to the family *Methylophilaceae*. This family is referred to as methylotrophs and they are able to utilize substrates containing no carbon-carbon bonds such as methane and methanol as their sole source of energy and carbon (Chistoserdova *et al.,* 2009). Mineralization of RDX and HMX produces single-carbon substrates for the growth of methylotrophs such as those belonging to the genus *Methylotenera*. In the stepwise reduction RDX-degenerative pathway described by McCormick *et al.* (1981) two of the end products are methanol and formaldehyde (Figure 1.8). Accumulation of methanol and formaldehyde in the bio-slurry following RDX reduction would allow for substrates needed for methylotrophic bacteria. This is a possible reason why the abundance of methyltrophs is first observed starting at day 45. Theoretically at this point sufficient RDX reduction may have taken place that could result in an accumulation of methanol and formaldehyde which serves as substrates for the genus *Methylotenera*. Methanol metabolism in this genus is accomplished through a methanol dehydrogenase that is encoded by the *mxaF* gene (McDonald & Murrell, 1997). Formaldehyde is also a reported byproduct in the direct ring-cleavage of RDX by methanogens (Hawari *et al.,* 2000; Figure 1.7). Formaldehyde is utilized to form cellular material by assimilating carbon in bacteria such as the genus *Methylotenera* through what is known as the ribulose monophosphate cycle (RuMP cycle), or formaldehyde assimilation. This pathway is controlled by an operon with a DNA-binding regulatory protein, *rmpR,* which regulates the *rmpA* gene encoding 3-hexulosephosphate synthase (HPS) and the *rmpB* gene encoding phospho-3-hexuloisomerase (PHI). HPS and PHI function together to produce D-fructose-6-phosphate from the condensation of

formaldehyde and D-ribulose-5-phosphate (Mitsui *et al.,* 2000). Formaldehyde which can be used in the RuMP pathway is also produced through the bioremediation of HMX through both ring cleavage and denitrition (Zhao *et al.,* 2007; Figure 1.9; Figure 1.10).

A near total reduction of the genus *Methylotenera* is observed at day 90. This may result from the depletion of methane and/or methanol needed for the survival of methylotrophs. This reduction may also arise from the heterogeneity of the soil sampling and a different pocket of microbes may have been sampled at day 90 compared to day 45.

In the nutrient addition samples, the presence of the genus *Methylotenera* is negligible compared to the control bioreactors. It is reported that methylotrophic bacteria in soil can be inhibited by high levels of ammonium (Meyers *et al.,* 2007). This is a result of more favourable conditions for denitrifying bacteria that out compete the methylotrophic bacteria (Veillette *et al.,* 2011). The nutrient supplementation contains significant concentrations of ammonium that provide favourable conditions for the denitrifying bacteria that would have a growth advantage as compared to methylotrophs.

4.3 NUTRIENT ADDITION SLURRY DIVERSITY

In total, there were 915 16S fingerprints obtained from the nutrient addition soil samples that were assigned to 217 OTUs. A measure of the diversity can be obtained by

determining the average number of clones representing each OTU and from the nutrient sample this value is 4.2 fingerprints/OTU. The slope of the rarefaction curve for the nutrient supplemented soil flattens out more than the curve for the control soil (Figure 3.13). This suggests that additional sampling of the control slurry soil would yield fewer additional OTUs, possibly suggesting that the nutrient supplementation reduced the diversity of the bacterial composition. The addition of a nutrient supplement may allow for the growth of bioremediating bacteria that are unable to utilize the contaminant as a sole source of energy or only partially degrade the contaminant. The nutrient solution provides alternative carbon, nitrogen, and phosphorus sources for bacterial growth. Zhao *et al.* (2007) showed that the rate of HMX and RDX removal greatly increased in soil isolates when a glucose supplement or a yeast extract was added to the growth media. Therefore, the decrease in microbial diversity observed in the nutrient addition soil samples may be a result of certain bioremediators being able to function in the presence of the nutrient supplement, which would provide for a selectable advantage in the soil environment. This decreased diversity is evident by comparing the Simpson and Shannon index values between control and nutrient samples. At day 0 from the nutrient addition there were many OTUs observed only once or twice, resulting in similar diversity index scores than the control samples (Table 3.8). However, in all nutrient addition soil samples from day 45 onwards there was a large proportion (≥ 14.4%) of clones assigned to OTU 65. Sequence alignment revealed that this 16S rRNA gene belonged to the genus *Nitrosospira.* This genus was very prominent from day 45 and the diversity of the other microbes decreased, suggesting that this genus had a selectable

advantage in the soil. Simpson scores were higher and Shannon scores were lower in the nutrient addition soil at day 45 compared to control samples at the same time point, indicating lower diversity in the nutrient sample due to the high proportion of *Nitrosospira*. In bioreactor 2 Simpson and Shannon scores are similar in control and nutrient addition samples at day 45 due to the control sample having a large proportion on an individual OTU as well. At day 90, the proportion of *Nitrosospira* dropped down significantly in bioreactor 1, result in larger diversity indicators (Table 3.8). *Nitrosospira* proportion at day 90 from bioreactor 2 was similar to its day 45 levels, resulting in lower microbial richness.

4.3.1 NITROSOSPIRA

The genus *Nitrosospira* is classified as an ammonia-oxidizing bacteria (AOB) genus and has previously been detected in different soil composts using culture-independent (Innerebner *et al.,* 2006) and culture-dependent methods (Tiquia *et al.,* 2002). It is of the class β-proteobacteria.

Ammonium, ammonia, and nitrate in soil and water environments can arise from undentonated explosives such as ammonium nitrate fuel oil (ANFO) and other nitrogen containing blasting agents. This could provide favourable conditions for AOB genera such as *Nitrosospira* (Karkman *et al.,* 2011). However, if the nitro-organic explosives present in the soil were releasing ammonium and nitrate into the soil we would expect to see a similar proportion of *Nitrosospira* in the control soil as well. It is likely that the thriving of *Nitrosospira* in the nutrient addition soil was related to the nutrient

supplement itself. The nutrient addition included a solution known as Bio-D, which among other compounds included 21.0 mg/g ammonia-N, 21.0 mg/g nitrate-N and 144.3 mg/g organic-N. This would add more nitrogen to the soil through various forms compared to the control treatment and would allow for the high proportion of *Nitrosospira* that was evident in the nutrient addition. The fact that *Nitrosospira* was shown to spike at day 45 supports this hypothesis. Sampling immediately after the addition of the nutrient supplement at day 0 would show no effects of the nutrient supplement on the microbial structure. After 45 days, any selection resulting from the nutrient supplementation may be observed. The nutrient supplement was added to the bioreactor at days 0, 30, and 60 during the treatment. At day 90 the nutrient supplement in the soil may be near depletion, resulting in less favourable conditions for the microbes utilizing the nutrients. This may have resulted in the drop in *Nitrosospira* proportion at day 90 samples taken from bioreactor 1.

AOB have the unique ability to convert ammonia to nitrite as their sole energy source (Kowalchuk & Stephen, 2001). This conversion is a two step reaction that first involves catabolism by a membrane-bound, multisubunit enzyme known as ammonia monooxygenase (AMO), followed by catabolism by a periplasm-associated enzyme known as hydroxylamine oxioreductase (HAO) (Kowalchuk & Stephen, 2001). Ammonia oxidation is a central reaction in the nitrogen fixation cycle in soil as depicted in Figure 4.1, as well as in the biological removal of nitrogen in polluted sites. While it is probable that the high proportion of AOB in the nutrient addition soil was a result of the nitrogen supplementation, it is also possible that the nutrient addition allowed for the growth of

HMX degrading bacteria. As discussed in Section 1.6.2, HMX degradation pathways involve the production of nitrous oxide. This influx of nitrous oxide would be introduced back into the nitrogen fixation cycle in the soil, which may also lead to more ammonia in the soil to act as a substrate for AOB. No documented direct catabolism of TNT, RDX or HMX by AOB such as *Nitrosospira* has been reported. The shift in community structure to *Nitrosospira* may therefore be a result of bioremediation by other microbes that produce nitrogen containing substrates for *Nitrosospira*.

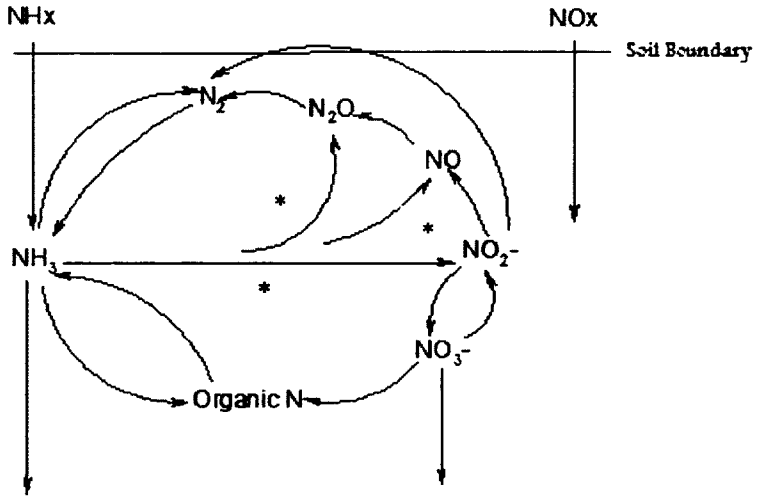

Figure 4.1: Nitrogen fixation cycle in a soil ecosystem. * denotes reactions involved in ammonia oxidation. Adapted from Kowalchuk *et al.* (2001).

4.4 *ACIDOBACTERIA*

Alignments of another abundant OTU revealed the presence of the phylum *Acidobacteria*. Surveyed members of this phylum clustered into four different groups. Molecular surveys of pristine soil ecosystems revealed that *Acidobacteria* is the second most common occurring phylum, at proportions in uncontaminated soil reported at 8-31% (Janssen, 2006). As a result, it would be expected to observe this phylum in any random soil community, including the soil in this study. However, in heavily contaminated sites of various pollutants this phylum is not mentioned as one of the dominant microbial taxa, suggesting a possible sensitivity to pollutants (George *et al.,* 2009). *Acidobacteria* populations in soil show a negative response to increasing TNT concentrations, with a proportion of only 1% in soil contaminated with 44 ppm (Georges *et al.,* 2009). Further increases in TNT concentration resulted in an increased reduction of *Acidobacteria* representation in the soil. The proportion of *Acidobacteria* in the soil can therefore serve as a biomarker for TNT pollution with an inverse relationship between *Acidobacteria* proportion and TNT concentration. The initial TNT concentration measured in the soil in this study was 5.88 ppm (Table 3.1), which is less than the value reported in the study by Georges *et al.* Initial *Acidobacteria* representation would thereby be expected to be larger than 1%. The average day 0 *Acidobacteria* proportion as seen in Table 4.1 was about 1.0%. No members of the *Acidobacteria* were observed in Control samples from bioreactor 1, and the largest proportion was observed from nutrient addition samples from bioreactor 2 with a proportion of 2.2%.

The abundance of *Acidobacteria* more than tripled at day 45 to 3.7%. At day 90 the abundance of *Acidobacteria* was about 10.6%, which was more than the *Acidobacteria* proportion at day 45. This trend may indicate that TNT is being degraded over the duration of the bioreactor runs due to the sensitivity of *Acidobacteria* to large TNT concentrations (Georges *et al.,* 2009).

Table 4.1: Proportion of the phylum *Acidobacteria* present in soil samples obtained throughout the duration of the study.

Soil Treatment	Bioreactor	% Day 0	% Day 45	% Day 90
Control	1	0.8	0.9	14.3
	2	0.8	0.7	7.2
Nutrient Addition	1	1.9	5.6	10.9
	2	3.0	7.7	10.1
Average		1.6	3.7	10.6

4.5 MICROBIAL COMMUNITY STRUCTURE AND ITS POTENTIAL FOR BIOREMEDIATION

Common genera reported in the degradation of one or more of the nitro-organic explosives include *Pseudomonas*, *Rhodococcus*, *Clostridium*, *Desulfovibrio*, and others as listed in Table 1.2. It was hypothesized that if bioremediation of the explosive contaminants was taking place then some of these genera would be well represented in the community structure. These genera were not observed, however this does not dismiss the possibility of bioremediation taking place in the soil.

Pseudomonas, *Rhodococcus*, *Clostridium*, *Desulfovibrio* and other previously reported nitroaromatic-degrading genera may have been present in the soil, but the level of contamination may have been too small to serve as a selectable pressure. RFLP allows for the identification of the most prevalent fingerprints and these genera may have been represented in the less commonly occurring fingerprints. Table 3.7 shows that doubling the sequencing sampling size would yield approximately 19.3 additional OTUs defined at a distance of 0.05 or less. The new OTUs discovered from this additional sampling may correspond to previously reported bioremediators.

TNT-degrading microbes for example have been found in both polluted and unpolluted sites (Klausmeier *et al.*, 1974). In soils more heavily contaminated with TNT, a larger proportion of TNT-degrading bacteria are observed compared to soil with low levels of contamination (Fuller & Manning, 1998). If the firing range soil was more

heavily contaminated with these explosives, their degraders would be expected to be more abundant.

TNT is toxic in large concentrations to many naturally occurring bacteria and TNT-degraders would be expected to be present in large proportions especially near the beginning of the bioreactor treatment. Although none of the TNT-degrading genera were present, the prevalence of the TNT-sensitive phylum *Acidobacteria* increased over time, which may be a result of TNT bioremediation throughout the experiment.

Bioremediation may be accomplished by the prevalent bacteria surveyed in this study although it was not previously documented. This potential may be provided by the phenomena of horizontal gene transfer (HGT). HGT occurs when DNA is released from a cell and transformed into another cell. DNA release may result after bacterial death following infection by bacteriophages, or through the release of plasmid and chromosomal DNA by living bacteria (Neilsen *et al.,* 2007). Released DNA binds to soil particles and humic substances in the soil and is protected from enzymatic degradation. Transformation of the DNA into another host occurs when a susceptible bacterium comes into contact with the soil-DNA complex (Nielsen *et al.,* 1997). Over a long period of time in the firing range soil community, HGT will enhance the microbial diversity through increased microbial fitness (Neilson *et al.,* 1994). This may allow bacteria to acquire the genes necessary for explosives remediation.

A significant proportion of sequences obtained in this study did not align with known genera. This is a common reported consequence of using the 16S rRNA gene as

an identifier. It is reported that 79-89% of 16S rRNA sequences archived to sequence libraries are not affiliated with known genera (Janssen, 2006). This is an indication of novel bacteria being identified through metagenomic characterization that may not have been characterized through culturing methods. It is possible that these members affiliated with no known genera may contribute to the nitro-organic explosive remediation but had not been previously identified.

4.6 BIASES IN NUCLEIC ACID EXTRACTION

Results based on metagenomic studies may show biases from various factors in the nucleic acid extraction. A major limitation in the extraction of microbial nucleic acids for metagenomic analysis is the lack of consistency from lab to lab and even between different individuals in the same lab. Variation in concentration and the purity of the nucleic acids, as reflected by the *A260/A280* ratio, has been observed the extraction of nucleic acids from the same soil sample using the same extraction kit (Pan *et al.,* 2010). As a result of this variation there were differing results in band intensities in denaturing gradient gel electrophoresis (DGGE) which would suggest differences in relative microbial abundance between different preparations of the same samples. The source of variation would presumably arise from different pipetting routines/techniques between investigators using the same equipment. An extraction method with internal standards to prevent this variation has not yet been developed. As a result, multiple extractions for each sample should be performed in metagenomic studies to limit bias from one extraction over another.

It has also been reported that nucleic acid extraction is more readily accomplished in Gram-negative bacteria as opposed to Gram-positive bacteria. This is due to the greater difficulty in achieving cellular lysis in Gram-positive bacteria that have a thicker peptidoglycan cell wall layer (Meyers *et al.,* 2007). Gram-positive bacteria belong to 2 phyla, *Firmicutes* and *Actinobacteria* (Sutcliffe, 2010). Sequence alignment revealed a small presence of bacteria belonging to the phylum *Actinobacteria,* while bacteria in the phylum *Firmicutes* were not detected. It is possible that these phyla were under-represented in this study through the cellular lysis bias associated with the nucleic acid extraction.

4.7 EVALUATION OF RFLP FINGERPRINTING

RFLP has been extensively used in genotyping since the discovery of restriction endonucleases in the 1970s (Hartl & Jones, 2001). Variations within the marker gene will cause alterations in restriction sites producing a different series of bands when the digests are run on an agarose gel. This produces what is known as the "fingerprint" in the metagenomic study. A qualitative measure of microbial diversity is obtained by observing and comparing all of the different restriction patterns while a quantitative measure of diversity is obtained by cataloguing all of the different patterns and recording how frequently each pattern appears.

There are several disadvantages of using RFLP fingerprinting for metagenomic analysis. Firstly, it may be difficult to detect small variations between different copies of

the 16S gene from two different genera. This is especially true if the variation does not alter any of the restriction sites along the gene (CCGG restriction site for the enzyme MspI used in this study). It is therefore possible that two different species may have produced the same restriction pattern. Furthermore, analysis via RFLP patterns allows for an increased possibility in interpretation error by the experimenter. Two patterns may look very similar and be assigned the same OTU identification number, but may have bands of slightly different sizes upon a closer inspection. Additionally, RFLP is a labour intensive and time consuming process. After amplifying the cloned sequences, digestions took two hours to perform, which was then followed by agarose gel electrophoresis that would take an additional hour to complete. Cataloguing of the patterns would take on average 5 minutes to analyze each individual pattern due to the large quantity of patterns to compare against. Clones producing the most frequent patterns were then amplified again and sent for sequence analysis. Sequence alignments reveal the identity of the clones producing these most frequent patterns. RFLP would theoretically save costs associated with sequencing as only the most abundant clones are analyzed. However, less abundant microbes that may play a role in the bioremediation process would not be detected. The big picture of the microbial community structure is obtained through RFLP, but not the total community structure.

An alternative to RFLP analysis would be the direct sequencing of each 16S rRNA gene clone. The advantages associated with this method would include better detection of slight differences between different sequences, results would be obtained much faster and the total community structure would be obtained. The major hurdle involving

direct sequencing of all clones would be the large associated costs. Assuming an average price of $6 (BioBasic) to sequence each clone, it would cost a total of $6912 for sequencing all of the clones in this study. However, direct sequencing provides results at a higher accuracy and greater sensitivity and takes much less time than performing the same study with RFLP and is highly recommended if sufficient resources are available (Davis *et al.*, 2007).

4.8 FUTURE DIRECTIONS

This survey of the microbial community structure of nitro-organic explosive contaminated soil provided an answer to what microorganisms are thriving in this soil, but the answer to why they are there is still unknown. There is no way to confirm that bioremediation of the soil is taking place unless the concentrations of the explosives in the soil are determined throughout the bioreactor run. A correlation between shifts in the microbial community structure and a decrease in nitro-organic explosive concentration would provide a good guide as to whether bioremediation by these microbes is taking place. One such correlation was provided in the study of polyaromatic hydrocarbon (PAH) contaminated soil from a former coal gasification site in Rock Bay, Victoria, British Columbia by Chris Whynot and Caroline Rose. The total PAH concentration was measured every 15 days for each treatment from both bioreactors. Shifts in the microbial community structure during the bioreactor treatment were then able to be correlated to an overall decrease in PAH concentration.

4.8.1 ALTERNATIVE METAGENOMIC METHODS

There are other ways to provide possible answers to explain the microbial community structure of the nitro-organic explosive contaminated soil. One possible method to determine the degree of bioremediation is Stable-Isotope Probing (SIP). In this method, a ^{13}C labelled substrate (ex. HMX) is added to the soil before the production of a soil slurry (Radajewski *et al.,* 2003). Bacteria that metabolise this labelled substrate will then incorporate the heavy carbon into its biomass in molecules such as phospholipid fatty acids, DNA, and RNA (Boschker *et al.,* 1998). Bacteria capable of incorporating the labelled substrate into their biomass would then be deemed capable of metabolising the substrate of interest. Heavy DNA could be separated from light DNA through density gradient ultracentrifugation that produces a clear separation between heavy and light nucleic acids (DeRito *et al.,* 2005). Fingerprinting analysis of the heavy DNA will determine the microbes involved with the substrate catabolism. One drawback of bioremediation analysis using SIP is for the possibility of cross-feeding, meaning the uptake of ^{13}C markers by bacteria utilizing metabolites produced by the primary metabolizers (Manefield *et al.,* 2002). In order to limit the occurrence of cross-feeding, shorter bioreactor treatment times should be used.

The 16S rRNA gene was used as an identifier in this experiment due to its conservation among microbes with heterogeneous sites for identifying different species. A multitude of universal primers have been developed for accurate amplification of a mixture of 16S rRNA genes. However, identifying soil bacteria based on their 16S gene

sequence provides no details towards their possible role in the bioremediation process. Alternatively, genes encoding enzymes involved in the bioremediation pathway being studied can be amplified and sequenced from a mixture of genomic nucleic acids. For example, the *amoA* (ammonia monooxygenase) and *nifH* (nitrogenase reductase) genes have been reported to have been targeted genes for metagenomic analysis of contaminated soils (Mocali & Benedetti, 2010). The latter would be especially useful for looking into bioremediation of nitro-organic explosive compounds.

4.8.2 MICROBIAL SEED STUDIES

The addition of a nutrient supplement was shown to have an impact on the microbial community structure. Another additive used in soil slurry studies is a microbial seed. Microbial seeds that contain bacteria previously shown to be capable of nitro-organic explosive bioremediation may be added to the biolurry. The theory behind the use of a microbial seed in bioslurry studies is that often the biological response to contamination lags behind by weeks or months in sites with no previous exposure history. By introducing bacteria that had already adapted to degrade the contamination, this lag period is greatly decreased and survival, persistence and degradative capabilities are increased, leading to enhanced remediation of the polluted site (Megharaj *et al.*, 1997). The seed is often incorporated from isolates taken from a similarly contaminated site. This is based on the principle of pollution-induced community tolerance (PICT), which states that communities exposed to a pollutant over a long period of time become tolerant to the contaminant due to individual acclimation, genetic or

physiological adaptation, and loss of sensitive species (Gong *et al.,* 2000). For example, increased microbial TNT-tolerance was observed in four soil sites that had long-term exposure to TNT compared to two soil sites with no exposure history (Gong *et al.,* 2000). The relative abundance of microbes from the seed compared to microbes native to the soil can be measured for the duration of the treatment. If native microbes to the soil adapt well to the contaminant over time they may be able to outcompete the members of the seed and a shift in the microbial structure would be observed.

4.8.3 PHYTOREMEDIATION

This study focussed on the characterization of microbial communities that may be associated with decontamination of soils containing nitro-organic explosives. A similar strategy for bioremediation involves the use of plants in what is known as phytoremediation. The advantages of phytoremediation over physical and chemical remediation techniques are similar to those of bioremediation: cost efficiency, less destruction to the environment, and greater practicality on a large scale. An additional advantage of the use of plants is an added aesthetic appeal to the contaminated site (Rylott & Bruce, 2009). Plants have been reported to naturally detoxify soils of nitro-aromatic explosives. For example, a gene cluster in *Arabidopsis thaliana* known as oxophytodienoate reductases (OPR) have been shown to transform TNT, and these genes are upregulated in response to TNT contamination in soil (Mezzari *et al.,* 2005). However, natural phytoremedation of explosives is not as common as bacterial remediation. This is due to the slow generation time of plants, as compared to

microorganisms, that causes plants to have less time to evolve efficient methods for explosive detoxification (Rylott & Bruce, 2009). A new direction in phytoremediation that overcomes this major hurdle and that links it greatly with bioremediation is the bioengineering of plants to metabolise explosives. Genetic engineering is used to transfer bacterial bioremediation genes to plants to increase their ability to detoxify explosives. Engineered plants would increase the total detoxifying biomass and associated metabolic activity that could significantly improve explosive decontamination. *Arabidopsis* plants have been bioengineered to express the RDX-degrading enzyme XplA cloned from *Rhodococcus rhodochrous* and these plants have been shown to remove all RDX from solution and are able to grow in sites contaminated with up to 2000 mg/kg of RDX in soil (Rylott *et al.,* 2006). A nitroreductase (NR) enzyme encoded by the *nsfI* gene cloned from *Enterobacter cloacae* was expressed in tobacco plants for the phytoremediation of TNT, with similar results seen in the XplA transformed plants (Hannink *et al.,* 2001). In areas contaminated by both RDX and TNT, as in this study, it would be possible to have plants express genes to detoxify both compounds. These plants can be planted along the border of the military training facility to prevent leaching of the explosive compounds to other sites. The application of phytoremediation would rely heavily on bacterial studies such as this to first establish what microbes are able to live in contaminated sites. The next step is to locate possible genes in those microbes that express detoxifying enzymes that may subsequently have the potential to be transformed into plants.

4.8.4 GENETICALLY ENGINEERED MICROORGANISMS

The use of genetically engineered microorganisms (GEMs) has also been explored for its applications in soil bioremediation. This allows for the manufacture of microbes capable of degrading contaminants in conditions where naturally occurring bacteria are unable to efficiently degrade those contaminants. For example, GEMs have been engineered with bacterial haemoglobin (VHb) to promote degradation of aromatic organic pollutants such as TNT under hypoxic conditions (Urgun-Demirtas *et al.,* 2006). This would allow for aerobic bioremediation through the use of oxygenases due to the affinity for the limited available oxygen provided by VHb. Other genes specific to explosive biodegradation may be inserted into GEMs for the removal of these contaminants.

There are drawbacks in the use of GEMs. The engineered bacteria have extra energy demands due to the presence of foreign genetic materials in the cell (Singh *et al.,* 2011) and may not compete effectively with native microbes. Furthermore, there is potential for the horizontal gene transfer from these GEMs into undesirable microorganisms (Saylor & Ripp, 2000). In principle, this detriment can be limited through the engineering of genetically engineered suicidal microorganisms that undergo programmed cell death after detoxification to prevent an uncontrolled release of these engineered genes into the environment (Pandey *et al.,* 2005).

4.9 ENVIRONMENTAL METAGENOMIC STUDIES USING NEXT-GENERATION SEQUENCING TECHNIQUES

The use of DNA sequencing in the study of environmental microbial diversity has allowed for a more thorough community structure than through cultivation techniques. Analysis of the 16S rRNA gene provides a DNA barcode for bacterial identification that can distinguish between closely related species. With the advent of nucleic acid identification of microbes, new DNA sequencing technologies are being developed that are faster, more accurate, lower costs, and can read DNA from multiple templates in parallel. These new DNA sequencing technologies are referred to as next-generation DNA sequencing.

Traditional DNA sequencing was accomplished using dideoxyribonucleoside triphosphates (ddNTP) as described by Sanger *et al.* (1977). Sanger sequencing has an accuracy of 99.999% per-base and on average costs $500 per megabase to perform (Shendure & Ji, 2008). Major limitations associated with Sanger sequencing are the need for a vector-based cloning procedure needed to amplify and separate different DNA templates (Shokralla *et al.*, 2012). Vector based cloning is relatively time consuming and cloning kits such as the TOPO cloning kit used in this study from Invitrogen can be quite expensive. Distinguishing between different clones before sequencing is also time consuming and additional resources are needed for restriction analysis. Next-generation sequencing methods such as 454 pyrosequencing, Solexa, SOLiD, single-molecule real time (SMRT) sequencing, and Heliscope allow for metagenomic sequencing from an

environmental sample without cloning. Additionally, minimal PCR amplification of the template nucleic acids is required which would limit "jackpot" biases. The devices necessary to perform next-generation sequencing can be expensive as shown in Table 4.2, but the costs to run the sequencing program can be as inexpensive as $1 per megabase. Samples for next-generation sequencing would therefore most likely be sent away to companies such as SeqWright to perform at their location and results would be sent back to the experimenter. There are many next-generation sequencing methods available, and the method chosen for sequencing would depend on criteria such as desired read length, template size, and available resources. Many of these methods are newly developed and had they been available at the start of the experimentation for this study would have been utilized in lieu of the cloning approach conducted. This would have facilitated the acquisition of more sequences in less time, allowing for a more complete community structure of the bacteria present in the nitro-aromatic explosives contaminated soil sample. Next-generation sequencing methods are developing and improving at a fast pace allowing for more robust applications towards environmental metagenomic studies in the future (Shokralla *et al.*, 2012). A summary of some next-generation sequencing methods currently employed is provided in Table 4.2.

Table 4.2: Summary of the read length obtained and the cost associated with some of the next-generation sequencing methods. Adapted from Shendure & Li, 2008.

Method	*Read Length (base pairs)*	*Cost per megabase ($)*	*Cost per instrument ($)*
454 pyrosequencing	250	60	500 000
Solexa	36	2	430 000
SOLiD	35	2	591 000
HeliScope	30	1	1 350 000

4.10 CONCLUSIONS

The microbial community fingerprints were determined in soil contaminated by various concentrations of nitro-organic explosives. Sequencing of the most abundant fingerprints did not reveal the presence of any previously reported genera capable of degrading TNT, RDX, or HMX. The community structure of nutrient supplement addition soil was dominated by the genus *Nitrosospira* beginning at day 45. This genus was far less evident in control condition soil. Potential bias between bioreactors was observed in the control condition soil as the genus *Methylotenera* was predominantly detected in bioreactor 2 at day 45, but was absent in samples taken from bioreactor 1. Control samples showed greater diversity than nutrient addition samples as indicated by the Simpson and Shannon indexes. Further studies are needed to examine the changes in nitro-organic explosives concentration in the soil over the duration of the treatment.

Microbes present in the soil were identified over the course of the treatment, but their bioremediation potential cannot be establish as hypothesized. Prominent genera discovered during the course of the treatment were *Methylotenera* and *Nitrosospira*. Bioremediation of the nitro-organic explosives analyzed in this study by these genera was not previously reported. The increase in proportion of the phylum *Acidobacteria* during the course of the treatment may serve as a biomarker for the transformation of TNT. This is due to the high toxicity of TNT towards the phylum *Acidobacteria* (Georges *et al.,* 2008). In order to better establish the bioremediation potential of the microbes in the soil, characterization of the nitro-organic concentrations

during the course of the entire treatment needs to be conducted through methods such as HPLC.

Further, changes in the diversity and richness of the bacterial community during the course of the treatment were observed as hypothesized. Samples taken during the course of the bioreactor treatment showed greatest diversity and richness at day 0 for both control and nutrient addition treatment soils. At day 45, the richness and diversity values decreased from day 0 levels for all treatments. Day 90 richness and diversity levels both increased and decreased in samples analyzed during the course of the experiment. Although changes in microbial diversity and richness were observed, it cannot be established whether this was a result of the nitro-organic explosives degradation. Decreases in richness and diversity may be correlated to the preferential ability of certain microbes to degrade the explosives through methods such as SIP.

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