Polyphasic examination of microbial communities in soils contaminated with organic pollutants.

Short Title:
Polyphasic examination of soil microbial communities

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Abstract

A polyphasic approach was used to examine the impact of contamination on soil microbial community structure. Two systems were examined using a combined biochemical and molecular biological approach. Petroleum hydrocarbon contaminated soils from two Northern Canadian sites, representing long-term contamination, were examined using Biolog GN plates and PCR-denaturing gradient gel electrophoresis (DGGE) analysis of total community 16S rDNA. Results obtained using both methods demonstrated a positive correlation between samples that was based on the geographical origin of the samples, not on contamination level. In the second system, non-contaminated soil was contaminated with the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) to monitor the effect of short- to medium- term contamination. Changes in the soil microbial community were examined using PCR-DGGE of total community 16S rDNA combined with RDX mineralization and chemical analysis of intermediates. The non-contaminated loam soil had an inherent RDX degradative capability and contamination of soil columns with 1000 mg RDX/kg soil did not significantly change the 16S rDNA bacterial community profile. The bacterial diversity remained high as estimated by the number of bands present in the DGGE and by

statistical rarefaction analysis of 16S rDNA clone RFLPs. The same soil, used in 10% soil slurries (w/v), demonstrated two apparently different RDX degradation mechanisms based on mineralization and chemical analysis. The differences were based on aerobic versus anaerobic conditions and the presence/absence of Na_3-citrate. PCR-DGGE performed on 16S rDNA from aerobic slurries amended with Na_3-citrate detected the stimulation of 3 operational taxonomic units, identified as *Stenotrophomonas* sp., *Sphingomonas* sp. and a member of the Alcaligenaceae. The results from the two systems examined (short- to medium-term and long-term contamination) demonstrated the utility of a polyphasic approach in the examination of bacterial communities impacted by anthropogenic organic contamination. Significant information about the effect of contamination on soil microbial communities was obtained which can be used to correlate *in situ* observations and contribute to an environmental effects monitoring strategy for regulatory bodies entrusted with environmental protection and conservation.

Résumé

Une approche polyphasique a été utilisée pour étudier l'impact d'une contamination sur la structure d'une communauté microbienne dans un sol. Deux systèmes ont été étudiés à l'aide d'une approche combinant la biologie moléculaire et la biochimie. Dans le premier système, des sols contaminés par des hydrocarbures pétroliers provenant de deux sites du nord canadien, représentant une contamination à long terme, ont été étudiés à l'aide de plaques Biolog GN et d'analyses de l'ADNr 16S de la communauté microbienne totale par la réaction en chaîne de la polymérase (PCR) et par électrophorèse sur gel à gradient dénaturant (DGGE). Les résultats des deux méthodes ont démontré une corrélation positive entre les échantillons qui était basée sur leur origine géographique plutôt que sur leur niveau de contamination. Dans le deuxième système, un sol sain a été contaminé avec l'explosif hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) pour suivre l'effet d'une contamination à court et moyen termes. Les changements dans la communauté microbienne du sol ont été étudiés a l'aide d'analyses par PCR-DGGE de l'ADNr 16S de la communauté totale en combinaison avec des essais de minéralisation du RDX et des analyses chimiques des intermédiaires de dégradation. Le terreau sain possédait une

capacité inhérente de dégradation du RDX alors que la contamination de colonnes de sol avec 1000 mg de RDX/kg de sol n'a pas affecté de façon significative le profil de l'ADNr 16S de la communauté bactérienne. La diversité bactérienne est demeurrée élevée tel qu'estimé par le nombre de bandes présentes dans la DGGE et par des analyses statistiques de raréfaction des polymorphismes dans la longueur des fragments de restriction des clones d'ADNr 16S. Le même sol, utilisé dans des suspensions à 10% (p/v), a démontré deux mécanismes apparemment différents de dégradation du RDX d'après les essais de minéralisation et les analyses chimiques. Ces différences étaitent basées sur les conditions aérobies versus anaérobies ainsi que sur l'absence ou la présence du citrate de sodium. Des analyses par PCR-DGGE de l'ADNr 16S de suspensions aérobies amendées avec du citrate de sodium ont détecté la stimulation de 3 unités taxonomiques opérationnelles, identifiées comme *Stenotrophomonas* sp., *Sphingomonas* sp., et un membre de Alcaligenaceae. Les résultats des deux systèmes examinés (contamination à court et moyen termes et contamination à long terme) ont démontré l'utilité d'une approche polyphasique lors de l'étude de l'influence d'une contamination organique d'origine anthropomorphique sur des communautés bactériennes.

Des informations significatives sur l'effet d'une contamination sur les communautés microbiennes d'un sol ont été obtenues et peuvent être utilisées pour mettre en corrélation des observations faites *in situ* et pour contribuer à une stratégie de suivi des effets environnementaux par les agences responsables de la protection et de la conservation de l'environnement.

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Contribution of Authors

In the two manuscripts contained within this thesis (Chapters 2 and 3) all of the experimental work, the accompanying analysis, and writing of the manuscripts/chapters was performed by David Juck. Drs. Charles W. Greer, Lyle G. Whyte, Brian Driscoll, and Trevor Charles provided critical reading of the manuscripts and suggested ways in which to improve the work.

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Chapter 1. Literature Review

1.1. Environmental Pollution

Pollution in the environment has become a major problem; one in which much time, effort, and resources have been invested. A large number of technologies have been developed and employed in the remediation of contaminated sites. The nature of each technology depends on the characteristics of the pollutant, the extent of contamination, and the nature of the matrix. Remediation technologies can be divided into two main approaches: *ex situ* and *in situ*. *Ex situ* technologies, which typically involve the physical removal of the contaminated matrix, include land farming, composting, land filling, and incineration. *In situ* technologies are generally designed to disrupt the environment as little as possible, and include pollutant immobilization, chemical destruction, soil washing, pumping and treating, phytoremediation, and bioremediation. Bioremediation, based on the exploitation of microorganisms in degrading contaminants, has proven to be a very efficient, cost-effective, versatile and environmentally friendly approach to cleaning polluted sites. The choice of bioremediation technology is based on several factors, including type of

pollutant, extent of pollution, proximity of contamination to sensitive receptors, and the time frame for clean up. Aggressive approaches, such as pumping out contaminated groundwater for bio-treatment or excavation of contaminated soil, are required when contamination is extensive, the clean up time frame is short, or sensitive receptors such as a potable water source are nearby. In cases where contamination levels are low, the pollutant is only slightly mobile, time is not a major concern, or no immediate receptors will be affected, natural attenuation of the pollutant has become an accepted option. This approach relies on demonstrating the intrinsic bioremediation potential of a site.

In most bioremediation cases, whether aggressive or not, biostimulation of the indigenous microbial population is required or advantageous for effective bioremediation. Large carbon inputs, in the form of the contaminant(s), often result in the depletion of bioavailable inorganic nutrient pools, limiting growth and biodegradative activities. The nutrients most often lacking in contaminated soils are nitrogen and phosphorus. Successful amendment of depleted soils, resulting in enhanced rates of pollutant degradation, can be accomplished by the addition of nitrogen-phosphorous-potassium, urea-based or oleophilic fertilizers.

Determination of the microbial species responsible for the *in situ* bioremediation of a target contaminant has been limited to environments comprised of only several member microorganisms. This is in part due to the extreme complexity of terrestrial systems, and the difficulty in following distinct microbial populations under normal field conditions. For this reason, most sites undergoing bioremediation are often looked upon as a 'black box'. The inputs (e.g. target contaminant, nutrient additions), and outputs (e.g. disappearance of the contaminant, expected metabolites) of these systems can be measured, but the actual mechanisms and microorganisms involved may not be known or are poorly understood. Other complicating factors at the meso- and micro-scale are the physico-chemical fluctuations experienced under normal field conditions. Examples of this include periods of drying/flooding, temperature fluctuation, predation, nutrient competition and bioavailability of the contaminant in question.

The isolation of microorganisms with the desired catabolic activity has contributed invaluable insight into the potential mechanisms operating in contaminated environments. Catabolic pathways, regulation of responsible enzymatic systems, co-metabolic processes, and microbe-microbe interactions are some examples of the important knowledge gleaned by studying lab isolates.

The difficulty is in translating activities observed in the lab to *in situ* mechanisms under field conditions. In the case of most lab isolates, they are often r-strategists (Andrews 1983), better able to take advantage of the high nutrient conditions typically used in the isolation of microorganisms. K-strategists, which are better suited to low nutrient conditions found in most terrestrial environments, are less often studied under laboratory conditions. In the last few years and with the development of more sensitive molecular methods (to be discussed below), it has become apparent that many of the microbial species (i.e. r-strategist) isolated and studied in the lab are only present at very low levels *in situ*.

The effect of chemical contamination on microbial community interactions and structure is poorly understood, yet it is an important aspect in determining the overall effect and subsequent consequences of pollution on an environment. In general, it has been observed that contamination, such as petroleum hydrocarbons, results in a decrease in the bacterial numbers and diversity of a system (Atlas 1983; Atlas et al. 1991; Lindstrom et al. 1999; Nyman 1999). Some exceptions to this 'rule' have been noted (Leahy and Colwell 1990; MacNaughton et al. 1999), including work to be presented in Chapter 2. These changes in the microbial community resulting from contamination have only

recently been examined, owing to the development and modification of molecular techniques that do not rely exclusively on the culturable component of the bacterial community.

1.2. Molecular Microbial Ecology

The field of microbial ecology has for many years relied upon bacterial cultivation to study the microorganisms of interest. The ability to differentiate between bacterial species based on cell and colony morphology has always been a problem due to the limited range of morphologies possible at such a small scale. The ability to culture potentially important microorganisms is also impaired by our limited knowledge of the appropriate conditions required for growth under laboratory conditions. To this effect, it has been estimated that in terrestrial systems, only 1% of the total bacterial species have been successfully cultivated (Ward et al. 1990).

Over the past decade, the application and refinement of molecular biological techniques, as applied to environmental problems, has resulted in a greater understanding of the complexity of ecosystems at the microbiological level. Some of the environments that have been examined include hot springs (Weller

et al. 1991; Ferris et al. 1996; Hugenholtz et al. 1998; Ramsing et al. 2000), Californian estuaries (Murray et al. 1996), bacterioplankton communities of a meromictic lake (Øvreås et al. 1997), coastal sand dunes (Kowalchuk et al. 1997), deep-sea hydrothermal vents (Moyer et al. 1994; Muyzer et al. 1995), biofilms growing in the mollusc *Montacuta ferruginosa* (Gillan et al. 1998), agricultural and forest soils (Felske and Akkermans 1998; Jensen et al. 1998; Bruns et al. 1999; Dunbar et al. 1999; El Fantroussi et al. 1999; Nüsslein and Tiedje 1999), Siberian premafrost (Zhou et al. 1997), biodegraded wall paintings (Rölleke et al. 1996), and northern environments (Juck et al. 2000).

1.2.1. 16S rDNA

In the majority of these studies, the gene of interest encodes the RNA backbone (16S) of the small subunit (SSU) or 30S portion of the procaryotic ribosome. There are several important reasons why 16S rDNA is used in the determination of isolate and/or clone phylogeny. Firstly, the 16S rDNA gene is found in all prokaryotes, making direct comparison of both closely and distantly related microorganisms possible. Secondly, the length of the complete 16S rDNA gene (approximately 1,500 bp) is easily sequenced. Each 16S rDNA gene

possesses regions of highly conserved and highly variable sequences. In the case of highly conserved regions, these sequences are important in determining rRNA-protein interactions as well as the secondary and tertiary folding structures crucial for the proper functioning of the ribosome. Due to the structural importance of these regions, variations are few, and are restricted to higher level phylogenetic classifications. The benefit of this higher level organization is that these regions can be used in the comparison of distantly related microorganisms, and for the development of broad-spectrum probes/primers (Amann et al. 1995) used to monitor target microorganisms in the environment. The highly variable regions found in 16S rDNA are not directly involved in determining the secondary and tertiary structure of the ribosome, and as such, are less constrained with respect to sequence variations. The availability of 8-9 highly variable regions interspersed throughout the 16S rDNA allows for the phylogenetic comparison of highly related microorganisms, as well as the development of probes and primers able to differentiate between related species and strains (Amann et al. 1995). These attributes of 16S rDNA have provided an important tool to significantly increase our understanding of environmental systems that have not been

previously studied, or have only been described based on the culturable portion of the bacterial population.

In almost all studies, 16S rDNA sequences have been identified which represent microorganisms not previously cultured. In some cases, these novel operational taxonomic units (OTUs) (Moyer et al. 1994) appear to comprise a significant portion of the microbial population where they were otherwise never observed. One such example stems from the work first performed on bacterioplankton 16S rRNA clones originating from the Sargasso Sea (Giovannoni et al. 1990), an area of the world's oceans typically under very low nutrient conditions. Sequencing of random clones revealed several novel microbial groups, none of which displayed significant similarity to marine bacterioplankton cultivated from similar habitats. One group of clones, the SAR11 cluster which belongs to the alpha Proteobacteria, constituted 12.5% of the total bacterioplankton population in the Sargasso Sea based on oligonucleotide probing. Subsequent studies have also found 16S rDNA sequences similar to the SAR11 cluster in free-living bacterial assemblages from ocean surface environments around the globe. They include the Western Mediterranean (Acinas et al. 1999), Columbia River Estuary (Crump et al. 1999),

North Atlantic algal blooms (Gonzalez et al. 2000), and the coastal region of the U.S. western continental shelf in the Pacific Ocean (Rappé et al. 2000).

1.2.2. Analysis of 16S rDNA

1.2.2.1. Clone Analysis

The analysis of amplified 16S rDNA is based on two approaches, both involving, as final steps, nucleotide sequencing of the selected bands and phylogenetic comparison against current databases. The main difference between the two approaches is in the method of fragment separation from the bulk amplification mixture. The first approach is based on cloning of the amplification products followed by restriction enzyme digestion, creating restriction fragment length polymorphism (RFLP) patterns. These patterns are then used in the selection of unique clones for sequencing, or they can be examined by rarefaction analysis. Rarefaction is a statistical method (Simberloff 1978) which provides an estimation of the number of random clones needed to obtain a representative sampling of the diversity in a sample (Dunbar et al. 1999; Ravenschlag et al. 1999; Eilers et al. 2000). This is performed by randomly

sampling a set number of clones and determining how many unique patterns are within this group. This is repeated with an increasingly larger number of random clones, and the values are then plotted, i.e. number of random clones sampled vs. number of unique patterns. When the number of unique patterns begins to plateau, the corresponding number of random clones is considered to be sufficiently large enough so that it represents the majority of the diversity present within the sample. The number of unique patterns at the plateau is an estimation of the level of diversity found within the sample being examined. Although limited to studies having only a few samples, the main advantage of a cloning based approach is that microorganisms that represent a small fraction of the total population can be identified.

1.2.2.2. Denaturing Gradient Gel Electrophoresis

The second, more versatile approach, is based on denaturing gradient gel electrophoresis (DGGE). It is a powerful technique used to separate DNA fragments of similar size based on nucleotide sequence differences. It was initially developed for the detection of sequence differences and point mutations (Fischer and Lerman 1983; Myers et al. 1985; Myers et al. 1987; Abrams and

Stanton 1992). Amplified DNA fragments of similar size are electrophoresed on a polyacrylamide gel containing an increasing linear concentration gradient of denaturants (i.e. urea and formamide) (Muyzer et al. 1993). As the amplicons move through the gradient, localized regions of the fragment, defined by the sequence, denature, creating a single stranded 'bubble' of increased size. This retards the fragment's movement through the gel, and separates the amplicons based on sequence differences, not size differences (Lerman et al. 1984). The addition of a 'GC clamp' (a stretch of dGTPs and dCTPs 40-50 bp in length) at one end of the amplicon during amplification, prevents complete separation of the strands. If complete separation of the strands were to occur, the resulting single strands would no longer be differentially retarded within the gel, and no banding patterns would be observed.

DGGE bands generated using 16S rDNA specific primers and total community DNA extracted from the environment under study can be excised from the gel and sequenced. These sequences can then be compared to the rapidly expanding molecular databases such as the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). This provides an indication of the potential phylogenetic relationship between bands of interest (OTUs), and

known microorganisms. The detection limit of an OTU, using PCR combined with DGGE, is 1 % of the total population (Muyzer et al. 1993; Stephen et al. 1999).

The significant advantage DGGE has over cloning approaches is the ability to examine and compare many samples simultaneously. Dynamic experimental systems, in which shifts in microbial population composition are important, can be monitored over time. Examples include temporal-spatial variation and disturbances of hot spring microbial mat samples originating from Octopus Spring, Yellowstone Park (Ferris et al. 1996; Santegoeds et al. 1996; Ferris et al. 1997; Ferris and Ward 1997), methane enrichments from agricultural soils (Jensen et al. 1998), depth dependent variation of aquatic bacteria from a stratified meromictic lake (Øvreås et al. 1997) or stratified fjord (Teske et al. 1996), comparison of bacterioplankton from two oceanic bays in California (Murray et al. 1996), and temporal-spatial comparison of ammonia-oxidizing bacterial communities from Dutch coastal sand dunes (Kowalchuk et al. 1997) and root and bulk sediment from a shallow lake (Kowalchuk et al. 1998).

1.2.2.3. PCR Induced Bias

One caveat to PCR based analyses such as cloning and DGGE, is potential bias causing unequal amplification of different 16S rDNA fragments. The result of this may be an under or over representation, based on band intensity, of the OTU's original level within the environment under investigation. In two studies examining PCR bias of two and three membered DNA template mixtures (Suzuki and Giovannoni 1996; Polz and Cavanaugh 1998), it was determined that the concentration of template DNA, the length of amplicon produced, and the number of amplification cycles were all important factors influencing the amount of amplification bias observed. To combat the observed bias, DNA template concentrations were increased, the number of amplification cycles decreased, and replicate reactions were pooled for analysis.

1.2.2.4. Disadvantage of Molecular Approaches

One drawback to strictly molecular approaches relying on 16S rDNA in describing members of a microbial community is the inability to determine the metabolic potential of respective OTUs. A phylogenetic relationship can be established and the metabolic potential postulated, but this cannot be verified

without having the OTU in pure culture (Dojka et al. 1998; Hugenholtz et al. 1998). To this end, isolation and study of ecologically important species remains an important complementary approach to molecular biologically based microbial ecology.

1.3. Biolog GN Plates

An alternative to describing a microbial population at the molecular level is to examine the metabolic potential of the community as a whole. One way that this has been accomplished has been through the use of the Biolog system. The Biolog plate system was originally developed for the identification of medically important bacterial species (Bochner 1989). Each 96 well plate contains 95 different carbon substrates which include carbohydrates, esters, polymers, carboxylic acids, alcohols, amides, amines, amino acids, aromatics, phosphorylated, and brominated chemicals (Garland and Mills 1991). The principal of the Biolog system is based on the transfer of electrons during microbial oxidation of a carbon substrate. Electrons donated to an electron transport chain may be transferred to a tetrazolium dye acting as an artificial electron acceptor. The reduction of the tetrazolium dye, tetrazolium purple in the

case of the Biolog system, results in the production of an insoluble formazan that can be monitored visually or spectrophotometrically.

1.3.1. Biolog in Microbial Community Analysis

The use of Gram-negative specific Biolog plates (Biolog GN) in describing the metabolic potential of a microbial community was pioneered by Garland and Mills (Garland and Mills 1991). Biolog plates are inoculated with a dilution of the environment under study, and the plates are then incubated at an appropriate temperature. The level of formazan formation is monitored at regular intervals and analyzed statistically to provide a community-level physiological profile (CLPP) (Lehman et al. 1995). The utility of the Biolog system in investigating sample differences or changes in a community over time has been demonstrated by the varied number of environments examined. These include shifts in compost microbial communities (Insam et al. 1996), differences in waste water treatment systems (Victorio et al. 1996), effect of hydrocarbon contamination in soil (Wünsche et al. 1995), impact of carbon and water inputs in soil (Bossio and Scow 1998), addition of a genetically modified and parent strain of *Corynebacterium glutamicum* to agricultural soil (Vahjen et al. 1995), and

changes in river bacterial communities following the addition of *E. coli* and chlorine (Lawley and Bell 1998).

1.3.2. Analysis of Biolog Patterns

Several different statistical approaches have been employed for analyzing the oxidation patterns generated in Biolog plates. One of the more commonly used methods involves calculation of the average well color development (AWCD) (Garland and Mills 1991), (Garland and Mills 1999) followed by principal component analysis (PCA). AWCD is calculated using the following formula: $[\Sigma(R-C)]/95$, where R is the measured response for each well containing a carbon source, and C is the measured response in the control well without a carbon source (note - the above formula has been corrected from the original publication (Garland and Mills 1991) according to Garland and Mills (1999)). The blanked value of each well (i.e. R-C) is then scaled by dividing it by the AWCD. The reason for this data transformation is to reduce the influence of inoculum density which directly affects the rate at which color develops. This is particularly important when inoculum density is not known. Different samples can then be compared by using time points in which the AWCD are similar.

Principal component analysis (PCA) is a commonly employed statistical tool for analyzing CLPP data. PCA is a multivariate statistical method used to reduce large data sets into more manageable sizes while retaining the information contained within the set. In the case of CLPP analysis, for a single time point, there are 95 variables, i.e. one color measurement per carbon substrate. Comparison of several samples or experimental conditions results in a very large data set. PCA is able to reduce the data set from each sample (i.e. 95 carbon sources) into a discrete point on a graph. The axes, or principal components (PC), of the graph are structured so that the first PC retains the majority of information, the second PC retains slightly less, and so on. In most cases, the vast majority of information contained within the data set is retained in the first 2-3 PCs. Plotting of the points in a 2 or 3 dimensional space provides a graphical illustration of the measured relationship between the samples.

A second method used in interpreting Biolog data is to retain the inherent kinetic information contained within the data (Lawley and Bell 1998), (Lindstrom et al. 1998). This involves calculation of the rate of carbon source oxidation, the lag before oxidation commences, and the overall extent of oxidation. These calculations are similar to those used when analyzing bacterial growth curves due

to the sigmoidal nature of formazan formation (Haak et al. 1995). One caveat to this approach is that inoculum densities must be comparable, as this plays an important role in color development. Although not as widely used, this approach may retain additional information that would be lost upon transformation of the data using AWCD. The debate as to which is the most appropriate method for analysis of Biolog generated CLPP is ongoing (Howard 1997; Garland and Mills 1999; Garland and Mills 1999; Howard 1999), and to date, no clear consensus has been reached.

A second, more important question, concerns the interpretation of CLPP patterns. The Biolog system is culture based, subject to the same limitations as other culture-based systems. Growth of the bacterial inoculum is often required to reach sufficiently high levels (~10^8 CFU/mL) for a positive utilization response (Schutter and Dick 2001). In an attempt to understand how changes in bacterial populations affect the resulting CLPP, research has been performed using defined bacterial communities consisting of two or three members (Verschuere et al. 1997). The primary conclusion of this work was that the profiles generated by mixed populations were not simply a combination of the individual profiles, but rather that the profiles most resembled that of the fastest growing member of the

community. The patterns of slower growing bacteria were partially masked, even when they represented 90% of the initial inoculum. Another study, using potato rhizosphere or activated sludge as inocula, examined this phenomenon in more detail (Smalla et al. 1998). Inoculation of Biolog GN plates and subsequent examination of positive wells using PCR and temperature gradient gel electrophoresis (TGGE), a molecular method similar to DGGE, revealed a decrease in bacterial diversity. The predominant bands observed could be assigned to the gamma Proteobacteria. This suggests that the CLPP patterns generated from mixed bacterial communities do not necessarily reveal the physiological potential of the numerically dominant members of the population, but rather that of the aerobic fast growers, more suited to growth under nutrient rich conditions. As the question of what information CLPP provides is studied and debated, the usefulness of culture based approaches remains high, especially when considering that only a small fraction (~1%) of all bacterial species have been cultured in the lab (Ward et al. 1990).

1.4. Polyphasic Characterization of Environments

As previously stated, each approach to describing and studying a bacterial community has both benefits and drawbacks. In using a polyphasic approach, which incorporates multiple strategies such as molecular and biochemical analyses, a broader range of parameters may be examined. In doing so, more detail, combined with a broader context, will be provided. An example of such an approach would use molecular biological techniques (PCR-DGGE) to postulate the phylogenetic affiliation of a microorganism or OTU, which could then be supported using the appropriate biochemical methods.

Examples of environments examined using a polyphasic approach includes agricultural soils (Øvreås et al. 1998; Øvreås and Torsvik 1998), cyanobacterial mats (Teske et al. 1996; Ferris et al. 1997), fresh water sediments (Straub and Buchholz-Cleven 1998), and sulfate-reducing bacteria in a stratified marine water column (Teske et al. 1996). In all but one of these studies, DGGE of amplified 16S rRNA/rDNA was the preferred molecular approach. The physiological techniques employed included most-probable-number culturing (MPN), enrichment cultures, the Biolog system, monitoring of O_2 consumption, $^{14}CO_2$ incorporation, and the oxidation of both methane and methanol.

In our lab, a combination of molecular biological techniques (PCR and colony hybridization) combined with biochemical methods (mineralization of a radiolabeled test compound) has been routinely employed in analyzing petroleum hydrocarbon contaminated psychrotrophic environments (Whyte et al. 1996) or microorganisms (Whyte et al. 1999). The molecular techniques, targeting genes identified in hydrocarbon degradation, provide an estimation of the genetic potential present in the environment, while the mineralization assays provide concrete evidence that these or related genes are active under the experimental conditions.

1.5. Petroleum Hydrocarbon Contamination in Polar Regions

Expansion of human activities into polar regions of the globe has resulted in increasing levels of petroleum hydrocarbon (PHC) contamination in these fragile environments. Due to the low average temperatures experienced at these sites and the limited number of days above freezing, PHC contamination remains a problem for a longer period of time than it would be under mesophilic conditions. Low temperatures influence several factors in the bioremediation of PHCs. The most important of these include decreased degradation rates with lower

temperatures (Huddleston and Cresswell 1976), decreased volatilization of short-chain alkanes which increases their water solubility resulting in higher toxicity to microorganisms (Atlas 1981), and decreased bioavailability of long-chain alkanes due to melting temperatures above 10°C. Despite these factors, adaptation of indigenous psychrophilic and psychrotrophic microorganisms has resulted in the observed biodegradation of PHC contamination in many polar regions (Atlas and Bartha 1972; Atlas et al. 1978; Whyte et al. 1996; Whyte et al. 1997; Whyte et al. 1999).

The effect of terrestrial PHC contamination on microbial communities has been the focus of several studies. In general, microbial diversity decreases after contamination with PHC, although total heterotrophic numbers may not be negatively affected (Atlas 1983; Atlas et al. 1991; Lindstrom et al. 1999; Nyman 1999). This can be explained by an increase in bacterial species able to use PHCs as a source of carbon. As well, these microorganisms may not be significantly affected by the toxicity of lower molecular weight PHC components. In several studies, including work to be presented in Chapter 2, contamination of soil with PHC resulted in the maintenance (Pinholt et al. 1979; Holloway et al.

1980; Olsen and Sizemore 1981), or even increase in microbial diversity (Hood et al. 1975; MacNaughton et al. 1999).

1.6. RDX

Hexahydro-1,3,5-trinitro-1,3,5-triazine is an important energetic nitramine compound used in the manufacture of high explosives and propellants. It is commonly known as RDX (the British code name for Research Department Explosive or Royal Demolition Explosive) and has been utilized since World War II. The six membered heterocyclic triazine backbone (alternating carbon and nitrogen atoms) is substituted at the nitrogens by nitrate groups. Contamination of terrestrial environments with RDX is the result of many activities including production, waste water disposal, military activities, and munitions destruction. Detonation of munitions results in partial oxidation of RDX and causes wide dispersal of the uncombusted material. The failure of munitions to detonate creates additional hazards, at the same time increasing contamination levels through slow leaching of explosive materials into the water table. Despite RDX being only slightly soluble in aqueous solutions, ~42 mg/L, (Gorontzy et al. 1994), it travels easily through terrestrial systems due to its low sorption onto soil

matrices (Singh et al. 1998; Pennington et al. 1999; Sheremata et al. 2001). The toxicity of RDX has been demonstrated in humans, mammals, aquatic and terrestrial organisms and several bacterial species (McLellan et al. 1992; Talmage et al. 1999; Robidoux et al. 2000). This combination of toxicity and easy movement through the environment makes RDX remediation an important problem.

There have been significant advances in the development of RDX bioremediation strategies. The majority of *ex situ* technologies incorporate an anaerobic phase under slurry conditions (Greer et al. 1997; Boopathy et al. 1998; Boopathy et al. 1998; Freedman and Sutherland 1998; Ronen et al. 1998; Hawari et al. 2000; Shen et al. 2000). The bioremediation activities utilized within these processes were carried out either by indigenous soil microbial populations, or amendments with municipal anaerobic sludge. The ability of indigenous soil bacterial populations to degrade and mineralize RDX appears in several studies to be lower than that of anaerobic municipal sludge microorganisms (Shen et al. 2000), but this is not always the case (Greer et al. 1997; Boopathy et al. 1998). Examination of the RDX biodegradation process recently has resulted in new insights as to the possible mechanism(s) involved. The initial postulated

degradation pathway (McCormick et al. 1981) involved many intermediates, at least half of which where never observed. More recent work (Hawari et al. 2000; Halasz et al. 2001) has suggested a more streamlined mechanism resulting in the intermediate methylenedinitramine with the final production of N_2O and CO_2 (Figure 1.1.). The most important step in the degradation of RDX appears to be cleavage of the ring structure. Once this occurs, the resulting intermediates, including methylenedinitramine, decompose quite rapidly. As suggested in Figure 1.1., there are three potential enzyme candidates, C-N hydrolase, hydroxylase (path A) and nitroreductase (path C) and one abiotic mechanism functioning under high pH (path B). At this time, the only enzyme to have been studied in any detail with respect to RDX degradation is nitroreductase (Kitts et al. 2000). This group was working with a previously isolated RDX degrading enterobacterium, *Morganella morganii* (Kitts et al. 1994) and their only conclusion was that RDX reduction by this microorganism occurred via an O_2- insensitive type I nitroreductase(s).

As well as isolating an RDX degrading strain of *Morganella morganii,* Kitts et al. (1994) also isolated two other enterobacteria able to degrade RDX, *Citrobacter freundii,* and *Providencia rettgeri.* Other groups have also been able

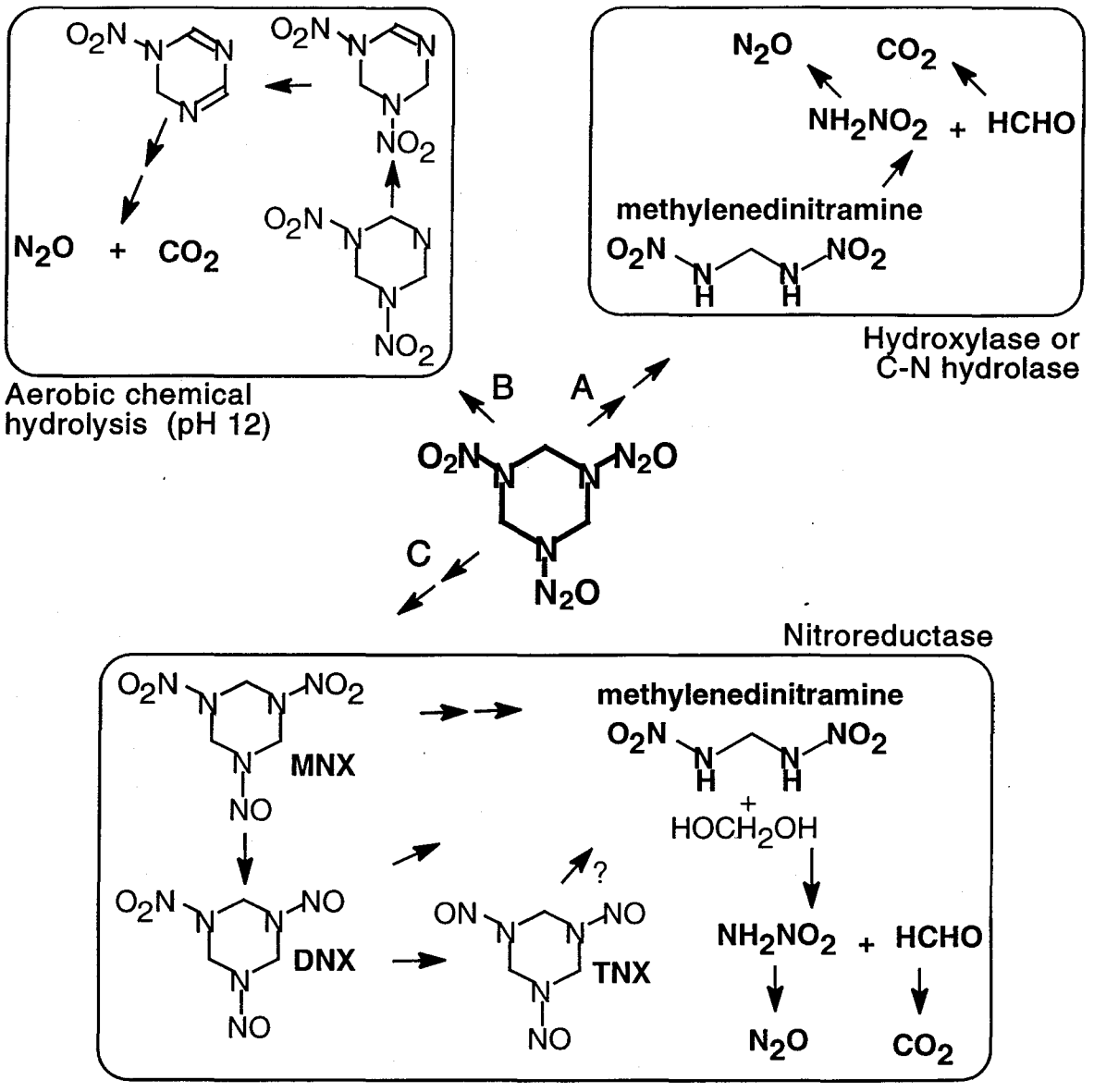

Figure 1.1. Postulated degradation pathways of RDX. Schematic representation of potential RDX degradation pathways, all producing N_2O and CO_2 as the final observed intermediates. Intermediates in bold text have been identified, single arrows represent single chemical reactions and double arrows represent multiple chemical reactions. Figure compiled from Hawari (2000).

to isolate microorganisms which are able to transform and degrade RDX. These include *Stenotrophomonas maltophilia* PB1 (Binks et al. 1995), *Serratia marcescens* (Young et al. 1997), and *Rhodococcus* sp. strain DN22 (Coleman et al. 1998). Several bacterial consortia from different sources have also been identified as possessing RDX degradation capabilities. This includes inocula from horse manure (Young et al. 1997), explosives contaminated creek sediment (Boopathy et al. 1998), and municipal anaerobic sludge (Shen et al. 2000).

In all of the above mentioned studies looking at *ex situ* and *in situ* bioremediation processes, isolation of RDX degraders, and RDX toxicity, no work has been performed on the effect RDX contamination with respect to bacterial community structure and potential shifts in population dynamics after contamination occurs.

1.7. Goals of this Study

The primary goal of this research was to characterize changes in soil microbial communities following contamination. Two different experimental systems were employed, the first was to examine the feasibility of a combined PCR-DGGE and Biolog approach in a previously contaminated environment, and

the second system to examine in greater detail the changes that occur in soil microbial populations after contamination.

The first system, PHC contaminated soil from Northern Canadian settlements, was chosen for the preliminary studies as there has been significant work performed on PHC contaminated sites and polar soil microbial populations, but not on a combination of the two. The examination of environments subjected to long term contamination provides a certain level of community stability enabling for a better evaluation of combined approaches and their suitability for examination of more dynamic environments.

The second system, constituting the bulk of the work to be presented, consisted of soil experimentally contaminated with the explosive RDX. The primary approach employed, in studying this system, was molecular biological (PCR-DGGE) for several reasons, including: the ease of sampling, storing and comparing samples of the microbial population over time, the ability to monitor small shifts in the microbial population, and the ability to determine a phylogenetic relationship to potentially important OTUs. The Biolog system was not used in this portion of the work due to the uncertainty of the information provided by the CLPPs, the enrichment nature of the assay limiting responses to

fast growing microorganisms, and the continued debate as to the most appropriate method for the processing and examination of CLPP data. Biochemically based experiments were limited to mineralization assays to monitor the breakdown of RDX and the attempted isolation of bacterial species able to degrade RDX.

In general, the approaches applied to the study of the above mentioned systems provided a broad overview of the bacterial community structure and the effect of contamination on its structure. Examination of changes in community structure at a more detailed level, or in systems where contaminant perturbations are at a small(er) scale, will require refinements of the present techniques or development of more sensitive detection methods.

Connecting Text

The work performed in Chapter 2 was designed to examine the utility of combining molecular biological techniques with culture based methods in the characterization of soil microbial communities in a contaminated environment. The system studied – petroleum hydrocarbon contaminated soils from Northern Canadian settlements – represented a case of long term contamination. David Juck performed all of the experimental work, accompanying analysis, and writing of the manuscript. Drs. Trevor Charles, Lyle G. Whyte, and Charles W. Greer provided critical reading of the manuscript and suggested ways in which to improve the work.

Chapter 2. PHC Contamination in Northern Environments

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2.1. Abstract

The cold-adapted bacterial communities in petroleum hydrocarbon contaminated and non-impacted soils from two Northern Canadian environments, Kuujjuaq, Québec, and Alert, Nunavut, were analyzed using a polyphasic approach. Denaturing gradient gel electrophoresis (DGGE) separation of 16S rDNA PCR fragments from soil total community DNA revealed a high level of bacterial diversity, as estimated by the total number of bands visualized. Dendrogram analysis clustered the sample sites on the basis of geographical location. Comparison of the overall microbial molecular diversity suggested that in the Kuujjuaq sites, contamination negatively impacted diversity whereas in the Alert samples, diversity was maintained or increased as compared to uncontaminated controls. Extraction and sequencing analysis of selected 16S rDNA bands demonstrated a range of similarity between 86% to 100%, to reference microorganisms, with 63.6% of the bands representing high G+C Gram-positive microorganisms in the order Actinomycetales and 36.4% in the

class Proteobacteria. Community level physiological profiles generated using Biolog GN plates were analyzed by cluster analysis. Based on substrate oxidation rates, the samples clustered into groups similar to those of the DGGE dendrograms, i.e. separation based upon geographic origin. The coinciding results reached using culture-independent and -dependent analyses reinforces the conclusion that geographical origin of the samples, rather than petroleum contamination level, was more important in determining species diversity within these cold-adapted bacterial communities.

2.2. Introduction

The expansion of human settlements to remote areas of the globe brings with it potential pollution problems. In the Canadian North, a large source of environmental contamination results from oil exploration and the spillage during transfer of petroleum hydrocarbons needed to generate heat and electricity. Temperature is one of the most important factors influencing the metabolic activity of microorganisms (Huddleston and Cresswell 1976), directly affecting rates of degradation in polar regions. Several groups have observed that petroleum hydrocarbons can be biodegraded in cold environments (Atlas and

Bartha 1972; Atlas et al. 1978; Whyte et al. 1996; Whyte et al. 1997; Whyte et al. 1999), and that the application of oil to previously uncontaminated soil results in an increase in total bacterial numbers under near Arctic conditions (Cook and Westlake 1974; Jobson et al. 1974), and cold climate regions of the Southern hemisphere (Colwell et al. 1978).

Estimates of microbial diversity within an environment can be divided into two broad categories, culture-dependent or -independent methods. Analysis of total community nucleic acids extracted from an environment using culture-independent methods permits the detection and phylogenetic identification of fastidious or as yet uncultured microorganisms. Denaturing gradient gel electrophoresis (DGGE) separation of 16S rDNA amplification products has been largely responsible for the description of many environments at the molecular level (Muyzer et al. 1993; Ferris and Ward 1997; Heuer et al. 1997). The total number of bands visualized in a DGGE gel also provides an estimate of the genetic diversity found within a given environment (Muyzer et al. 1993), and sequencing of selected DGGE bands enables phylogenetic relationships to be inferred (Ferris and Ward 1997; Heuer et al. 1997). The assignment of a metabolic capacity to a particular band from a DGGE gel, as inferred from

phylogenetic analysis, has been postulated, although this remains to be demonstrated (Dojka et al. 1998; Hugenholtz et al. 1998).

Culture-dependent techniques, such as Biolog generated community level physiological profiles (CLPP) (Garland and Mills 1991), have been used to estimate the *ex situ* metabolic potential of members of the microbial community from a variety of environments (see review by Konopka et al., 1998). CLPP provides an indication of the metabolic diversity present in an environment with respect to the number of defined substrates that can be oxidized (Garland and Mills 1991). Recent studies have suggested that the faster growing species, such as the pseudomonads, produce the generated patterns, and not the numerically dominant members of the microbial community (Garland 1997; Konopka et al. 1998).

The combination of culture-dependent and -independent approaches in the analysis of an environment may result in a more detailed description of the experimental system. Biolog analysis combined with either enterobacterial repetitive intergenic PCR (ERIC-PCR) (Di Giovanni et al. 1999) or DGGE separation of 16S rRNA amplification products (El Fantroussi et al. 1999) were used to monitor soil microbial community responses to different alfalfa strains and

to the application of urea herbicides, respectively. In both studies, consistent differences between control and test systems were identified with the culture-dependent and -independent techniques.

In this study, a similar approach combining culture-dependent and -independent techniques was used to evaluate the cold-adapted microbial population differences of soils impacted by petroleum hydrocarbons originating from two different communities in northern Canada. CLPP analysis of the soil microbial communities and DGGE analysis of 16S rDNA amplification products from total community DNA demonstrated that geographical separation between the 2 communities and differences in the location of sampling sites were more important than the petroleum hydrocarbon contamination levels in describing the microbial diversity.

2.4. Materials and Methods

2.4.1 Soils

A total of eight samples, four each from Kuujjuaq, Québec (58°06N) and from Alert, Nunavut (82°30N) were taken in July, 1997. The Kuujjuaq samples

(labeled K1, K2, K3, and K4) and the Alert samples (labeled Ac, A1, A2, and A3, which are equivalent to Pristine, I.D. #1, I.D. #2, and I.D. #4, respectively, in Whyte et al, 1999) were sampled from just below the surface to a depth of approximately 10 cm, sealed in plastic bags and transported on ice to the lab, where they were kept at 4°C until analyzed. Contaminated samples contained petroleum hydrocarbons originating from jet and/or diesel fuel spills. Total petroleum hydrocarbons (concentrations published in Whyte et al. (1999)) were extracted from the soil samples by sonication in hexane and quantified by gas chromatography/mass spectrometry. Total C, N, and extractable P concentrations were also published previously in Whyte et al. (1999). Total viable bacteria in the samples were enumerated after two weeks growth at 5°C, on mineral salts medium (Greer et al. 1990) containing 250 mg/l each of yeast extract, tryptone, and starch.

2.4.2 DGGE Analysis of Total Community DNA

Total DNA was extracted and purified using a beadbeater method and polyvinylpolypyrrolidone (PVPP) spin columns according to Berthelet et al. (1996) (see appendix 8.1.). A region of approximately 500 bp from the 16S rDNA gene

(341-758, E. coli numbering (Brosius et al. 1981)) was amplified using the primers b341GC (5'-*CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACG GGGGG*CCTACGGGAGGCAGCAG-3') (Rölleke et al. 1996) and b758 (5'-CTAC CAGGGTATCTAATCC-3') (Woese 1987; Lee et al. 1993) which are specific for almost all eubacterial 16S rDNA sequences. The italicized sequence in b341GC indicates the GC clamp required for DGGE analysis. Each 200 µl PCR mixture contained 4 µl of PVPP column cleaned soil DNA extracts, 25 pmol of each primer, 200 µM of each deoxyribonucleoside triphosphate, and 1 mM $MgCl_2$. The tubes were placed into a Perkin Elmer-Cetus thermal cycler 480 (Perkin Elmer-Cetus, Mississauga, Ontario, Canada) and heated to 96°C for 5 minutes. The temperature was then brought down to 80°C and 10 U Taq DNA polymerase (Pharmacia Biotech, Baie d'Urfé, Québec, Canada) in 20 µl of 10 X PCR buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$) were added. Touchdown PCR, essentially as described by Rölleke et al. (1996), was used to reduce non-specific fragment amplification (Don et al. 1991; Muyzer et al. 1993) with the following modification; 20 cycles were performed at the final annealing temperature of 55°C. Amplification products were analyzed on 1.2% agarose gels followed by ethidium bromide staining to determine if the amplification was

successful. PCR products were prepared for DGGE analysis as described by Teske et al. (Teske et al. 1996) with the following modifications; precipitation was performed at -20°C and the final pellet was resuspended in 10 µl dH_2O and 4 µl of 6x loading buffer III (Sambrook et al. 1989).

DGGE was performed using a Bio-Rad DCode system (Bio-Rad, Mississauga, Ontario, Canada), as described by the manufacturer. An 8% acrylamide gel with a 40-60% denaturant gradient was used (where 100% denaturant contained 7 M urea and 40% deionized formamide) and electrophoresis was performed at 60°C and 90 volts for 16 hours. The gels were then stained with ethidium bromide, destained in dH_2O, and photographed.

2.4.3 DGGE Fragment Isolation and Sequencing

DNA fragments from the DGGE were isolated as described by Rölleke et al. (1996) and then re-amplified under the following conditions; 50 µl PCR mixture containing 3 µl of precipitated DGGE band, 25 pmol of each primer (b341 [underlined sequence in b341GC] and b758), 200 µM of each deoxyribonucleoside triphosphate, and 1 mM $MgCl_2$, overlaid with mineral oil, and heated for 5 minutes at 96°C in the thermal cycler. The temperature was then

brought down to 80°C and 2.5 U Taq DNA polymerase in 5 µl 10 X PCR buffer was added. The cycling conditions were 1 minute at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C for 30 cycles. The single re-amplified bands were then purified using the QIAQuick PCR purification kit (QIAGEN, Mississauga, Ontario, Canada).

Sequencing of the re-amplified fragments was performed using the ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (Perkin Elmer, Montréal, Québec, Canada) and the ABI Prism 377 automated fluorescence sequencer (Applied Biosystems, Foster City, CA, USA) as specified by the manufacturers. Sequences were submitted for comparison to the GenBank databases using the BLAST algorithm (Altschul et al. 1997), the EMBL databases using the FASTA algorithm (Pearson and Lipman 1988), or the Ribosomal Database Project (RDP) (Maidak et al. 1999) using Sequence Match. Sequences which demonstrated strong similarity were then aligned to reference sequences using the Geneworks II software (Intelligenetics, Mountain View, CA, USA). The PHYLIP package (Felsenstein 1993) was used to generate phylogenetic trees. Distance matrices were produced using the DNADIST program and unrooted phylogenetic trees were constructed using the maximum

likelihood method of Felsenstein (Felsenstein 1993) in the NEIGHBOR program. Bootstrapping with the SEQBOOT program was then employed to determine the statistical significance of the groupings. Sequences which demonstrated uncertain alignments were checked for chimeras using the Chimera function at the RDP site. Sequences were submitted to Genbank and assigned accession numbers AF143747 to AF143770.

Dendrogram analysis of DGGE banding patterns was performed using the Dendron 2.2 software package (Solltech Inc., Oakdale, LA, USA). The dendrogram was created using unweighted pair groupings of a similarity coefficient (S_{AB}) matrix. The stability of the resulting dendrogram was tested by randomizing the sample order 100 times and recalculating the dendrogram with 95% background noise.

2.4.4 CLPP Analysis

Ten-fold dilutions used for bacterial enumeration were used to inoculate Biolog GN plates (150 µl per well) and incubated at 10°C. The plates were read every 12 hours ($O.D._{590}$) for the first 48 hours, and then readings were taken every 24 hours for the remainder of the incubation. All wells were blanked to the

water control well A1. The rate of substrate oxidation by each sample for individual carbon sources was calculated as the first linear portion of the plotted curve change in $O.D._{590}$ over time (Lawley and Bell 1998). Rates were analyzed by cluster analysis (CA) using the statistical program Minitab (Mintab Inc., State College, PA, USA).

2.5. Results

2.5.1 Site Characterization

Chemical analysis of the samples (Table 2.1.) revealed that the total petroleum hydrocarbon (TPH) contamination levels varied from <100-200 ppm (uncontaminated sites) to 26,900 ppm (heavily contaminated). Samples from Alert, which were contaminated primarily by jet and diesel fuels, possessed similar total carbon and total nitrogen levels, despite the large range of petroleum hydrocarbon concentrations present at the sites. The total carbon and total nitrogen values from the Kuujjuaq sites differed significantly between the K1 and K2 sites and the K3 and K4 sites. The K1 and K2 samples came from a sandy area contaminated with jet fuel whereas the K3 and K4 samples originated from

Table 2.1. Biological and physico-chemical characteristics of the sampled Arctic sites.

Sample	Bacterial Counts[a] (CFU/g)	Carbon (%)	Nitrogen (%)	C:N[b]	TPH[c] (ppm)	soil pH
Ac	1.25×10^6 (2.01×10^5)	3.79	0.06	63.2	<100	8.35
A1	1.39×10^8 (7.84×10^6)	4.26	0.07	60.9	26,900	8.26
A2	1.55×10^7 (2.28×10^6)	4.04	0.08	50.5	200	7.94
A3	2.61×10^7 (7.01×10^6)	4.28	0.02	214.0	8,100	8.90
K1	5.99×10^7 (1.12×10^7)	0.62	0.02	31.0	600	7.68
K2	7.72×10^6 (8.77×10^5)	0.69	0.03	23.0	1,400	7.50
K3	5.20×10^6 (1.68×10^6)	9.43	0.40	23.6	200	7.21
K4	5.27×10^6 (1.08×10^6)	7.75	0.35	22.1	<100	6.55

[a] Means of triplicate bacterial counts, standard deviation in brackets. Alert results previously published (Whyte et al. 1999).

[b] Total % carbon : total % nitrogen ratio

[c] Total petroleum hydrocarbons as measured in Whyte et al. (1999).

adjacent non-impacted areas. Despite the contamination of the K1 and K2 samples, the total carbon measured was still 10-15 times lower than in the K3 and K4 samples. The total nitrogen in the samples was also 10-fold lower in the K1 and K2 soils than in the K3 and K4 soils. The ratio of total carbon to total nitrogen (C:N) in the Kuujjuaq samples were all very similar (between 22.1 to 31.0) suggesting a balanced C:N ratio. The Alert samples by contrast were somewhat nitrogen deficient based on the observed C:N ratios (50.5 to 214.0).

To provide an indication of the relative abundance of total heterotrophic cold-adapted microorganisms present in each soil, a viable plate count was performed on nutrient-limited medium. The culturable counts, determined at 5°C, ranged from 10^6 to 10^8 CFU g soil-1 (Table 2.1.). The levels of contamination present at the Alert and Kuujjuaq sites did not negatively affect the viable microbial population levels. Differences within the Kuujjuaq samples were only one order of magnitude (10^6 to 10^7), with the highest counts originating from sample K1 (600 ppm TPH). The uncontaminated Alert sites Ac and A2 demonstrated one to two orders of magnitude lower total heterotroph counts (10^6-10^7, respectively) versus the contaminated sites A3 and A1 (10^7-10^8, respectively).

2.5.2 DGGE Analysis

The expected 500 bp PCR product specified by the bacterial 16S rDNA primers was amplified in all of the soils. Separation of these fragments by DGGE produced distinct and reproducible patterns comprising 20-35 distinct bands for each of the soil samples (Figure 2.1.A). Several of the observed bands appeared to be shared between samples, but the majority were unique for each sample site. Bands which were clearly separated from neighboring bands and were easily visualized were isolated for sequencing (Figure 2.1.B). Sequences of between 300 to 450 bp were obtained from all of the isolated bands. The highest similarity for each of the 22 sequenced bands, obtained by BLAST and FASTA comparisons, is shown in Table 2.2. The range of similarity to reference sequences contained in the databases was 86.1% (A11) to 100.0% (A2). The majority of the isolated bands were sequenced with relative ease and minimal background interference, suggesting that each band represented a unique microorganism. Several isolated bands were not amenable to sequencing due to high levels of background in the sequencing reaction. This result suggests that these bands are actually composites, made up of two or more distinct sequences

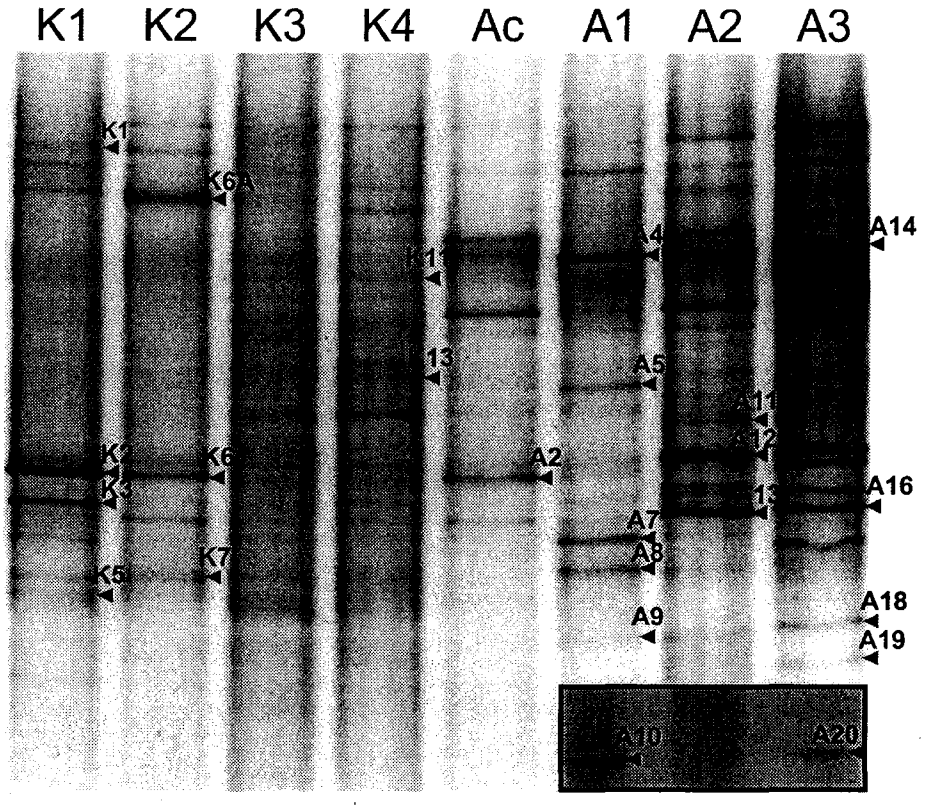

Figure 2.1. DGGE gel of 16S rDNA amplification fragments and corresponding bands isolated for sequencing. Separation of PCR fragments on a DGGE gel using a denaturant gradient of 40-60%. Inset at the bottom of lanes A1, A2, and A3 demonstrate bands A10 and A20 which were difficult to photograph. Bands which were successfully isolated and sequenced are shown in their corresponding positions.

Table 2.2. Nucleotide sequence similarity of sequenced DGGE bands.

DGGE Band[a]	Accession Number	Putative Division[b]	Database Similarity	% Similarity
A2	AF143747	High G+C	*Arthrobacter globiformis*	100.0%
A4	AF143748	gamma	*Xanthomonas axonopodis*	96.5%
A5	AF143749	gamma	*Xanthomonas axonopodis*	96.8%
A7	AF143750	High G+C	*Williamsia murale*	98.7%
A8	AF143751	High G+C	*Clavibacter xyli*	94.8%
A9	AF143752	High G+C	*Nocardioides* sp. OS4	96.6%
A10	AF143753	High G+C	*Bogoriella caseolytica*	96.4%
A11	AF143754	gamma	*Variovorax paradoxus*	86.1%
A12	AF143755	alpha	Unidentified alpha Proteobacteria	97.0%
A13	AF143756	High G+C	*Nocardioides jensenii*	97.0%
A14	AF143757	gamma	*Xanthomonas axonopodis*	96.2%
A16	AF143758	High G+C	*Nocardioides jensenii*	97.4%
A18	AF143759	High G+C	*Nocardioides* sp. OS4	97.7%
A19	AF143760	High G+C	*Nocardioides* sp. OS4	92.7%
A20	AF143761	High G+C	*Cellulomonas* sp.	95.7%
K1	AF143762	alpha	*Afipia* genospecies 13	93.8%
K2	AF143763	High G+C	*Arthrobacter globiformis*	98.9%
K3	AF143764	High G+C	*Arthrobacter* sp.	98.4%
K5	AF143765	High G+C	*Arthrobacter globiformis*	98.7%
K6	AF143770	High G+C	*Arthrobacter globiformis*	97.6%
K6A	AF143766	delta	*Pelobacter propionicus*	97.9%
K7	AF143767	High G+C	*Terrebacter* sp.	96.3%
K11	AF143768	delta	*Pelobacter carbinolicus*	88.6%
K13	AF143769	gamma	*Halomonas pantelleriense*	95.6%

[a] The bands are designated as shown in Figure 2.1. A# originating from Alert, K# originating from Kuujjuaq

[b] Putative division designations are as follows: High G+C, High G+C gram positive; alpha, alpha Proteobacteria; delta, delta Proteobacteria; gamma, gamma Proteobacteria

which possess similar electrophoretic mobility under the conditions used for DGGE (Muyzer et al. 1993; Rölleke et al. 1996). Assuming that several of the bands visualized in each sample are composites of distinct 16S rDNA fragments originating from different bacterial species, the bacterial diversity of the samples, as estimated by the total number of discernible bands in the DGGE gels for each site, is underestimated.

Phylogenetic analysis of the sequences (Figure 2.2.) revealed that the bacterial diversity present in the different soils was broad, being distributed between the Actinomycetales (63.6%) (Stackebrandt et al. 1997) and Proteobacteria (36.4%) (Woese 1987). Within each of these groups, the distribution was also widespread. Within the Actinomycetales, 33.3% of the sequences belonged to the Micrococcineae suborder, and included in part, the genera *Arthrobacter*, *Cellulomonas*, and *Clavibacter*. The second largest group was formed by the Nocardioidaceae (25%). There was a single sequence found within the Corynebacterineae, which was positioned between the genera *Rhodococcus* and *Williamsia*.

Within the Proteobacteria, the gamma subdivision was predominant (62.5%), composed in part by the genera *Xanthomonas*, *Halomonas*, and

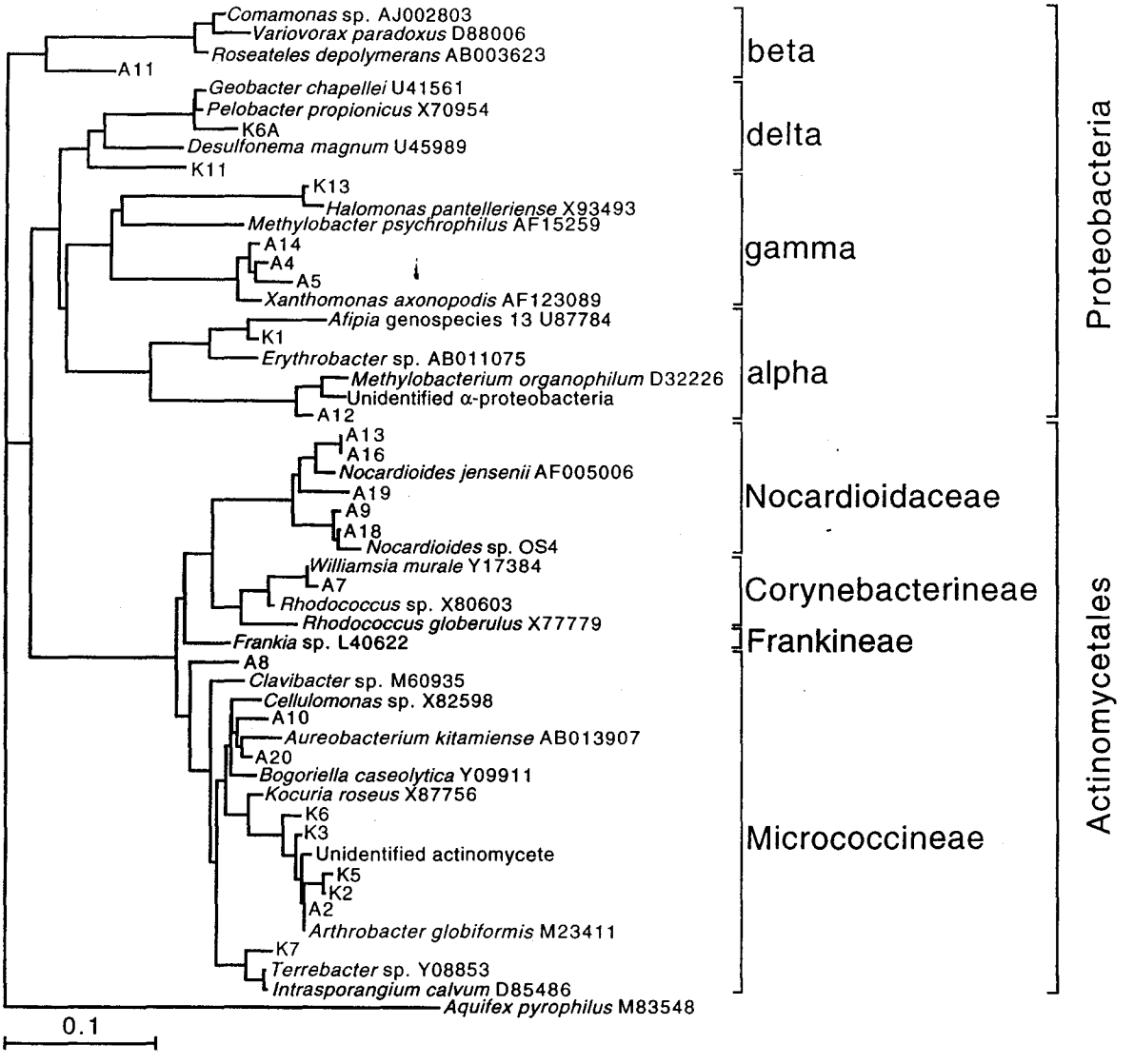

Figure 2.2. Phylogenetic tree of sequences obtained from 16S rDNA amplification fragments separated by DGGE gel. All of the sequences grouped into either the High G+C Gram-positives or Gram-negative Proteobacteria. DGGE bands are designated as shown in Figure 2.1. and reference sequences used are shown with their respective GenBank accession numbers. Bar, sequence dissimilarity.

Methylobacter. The alpha subdivision comprised 25% of the Proteobacteria observed, and included the genera *Methylobacterium*, *Sphingomonas*, and *Erythrobacter*. Two other sequences (K6A and K11) possessed significant sequence similarity to the delta subdivision genera *Geobacter* and *Pelobacter*. Sequence A11 was the only sequence to show similarity to the beta group, although it was somewhat distant at only 86%.

Several of the sequences possessed significant similarity to one another. Within the gamma subdivision of the Proteobacteria, grouping with *Xanthomonas*, sequences A4, A5, and A14 showed approximately 97% similarity to one another, with A4 and A14 differing by only 3 nucleotides. In the group of sequences clustering with *Arthrobacter* (A2, K2, K3, K5 and K6), similarity of the complete cluster was greater than 95%. Nucleotide comparison within this cluster revealed that no two sequences were less than 97% homologous. There were two distinct groups within the *Nocardioides* cluster; A13 and A16, and A9, A18, and A19 which demonstrated similarity within the cluster of almost 100% and greater than 93%, respectively. Although both of these groupings showed the strongest matches to *Nocardioides*, there was a notable difference between the two clusters in the form of a 10-bp deletion in the A13-A16 grouping.

The two sequences A11 and K11 demonstrated similarity to the beta and delta subdivisions of the Proteobacteria, respectively. Construction of phylogenetic trees consistently grouped these two sequences near the node of their respective branches, suggesting that they possessed only a distant relationship to other members of their group and may represent novel cold-adapted microorganisms.

2.5.3 Dendrogram Analysis of DGGE

Banding patterns generated by DGGE for each site were used for cluster analysis and the creation of a dendrogram (Figure 2.3.). Stability of the dendrogram was tested (as described in the Materials and Methods), and consistently resulted in the groupings shown in Figure 2.3. Sample sites divided into two clusters based on their geographic origin, i.e. Alert and Kuujjuaq. The Kuujjuaq samples were clustered amongst themselves based upon contamination level; K3 and K4 came from nearby tundra areas uncontaminated by petroleum hydrocarbons, whereas K1 and K2 came from contaminated areas adjacent to the refueling station. The Alert samples grouped together in a slightly different fashion. The Ac (uncontaminated) and A1 (heavily contaminated) soils

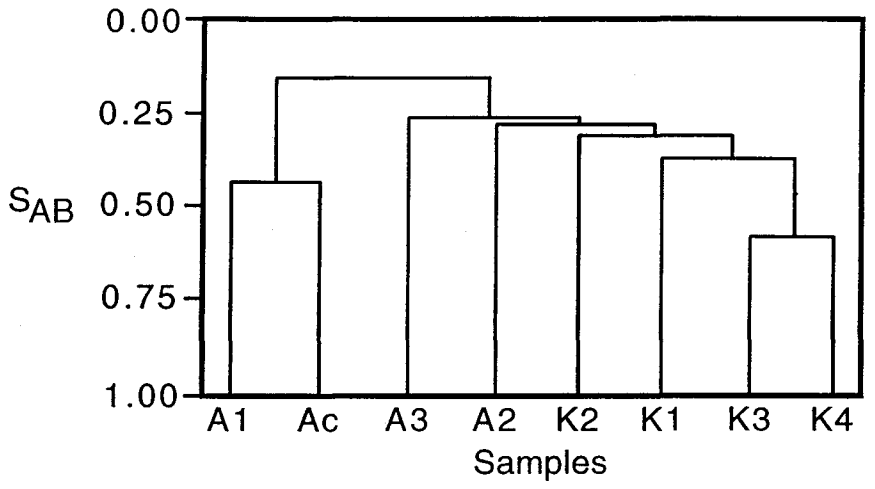

Figure 2.3. Cluster analysis of DGGE banding patterns based on position and intensity of bands. Samples are clustered geographically, with contamination levels having no apparent influence. S_{AB}, similarity coefficient.

clustered together within the Alert grouping. The A2 and A3 sites (uncontaminated and moderately contaminated, respectively) were grouped at an intermediate position between the other Alert samples and the Kuujjuaq sample cluster K3 and K4.

2.5.4 CLPP Analysis

The metabolic diversity of the cold-adapted soil microbial communities was estimated by the total number of substrates oxidized in the Biolog GN plates at 10°C. The samples divided into four groups (number of substrates oxidized out of 95 shown in brackets); A2 (87) and A3 (86); Ac (61), K1 (66), and K3 (70); K4 (46) and K2 (49); and A1 (26). There was no observable pattern in the groupings with respect to geographic origin of the samples, contamination levels, or any of the other measured parameters.

The rate of oxidation of each substrate was calculated (data not shown) and similarity between samples was assessed using cluster analysis. Dendrograms created using both squared Euclidian and Manhattan similarity indices generated identical groupings (Figure 2.4.) which were very similar to those of the DGGE dendrograms, i.e. clustering based upon geographic origin of the samples.

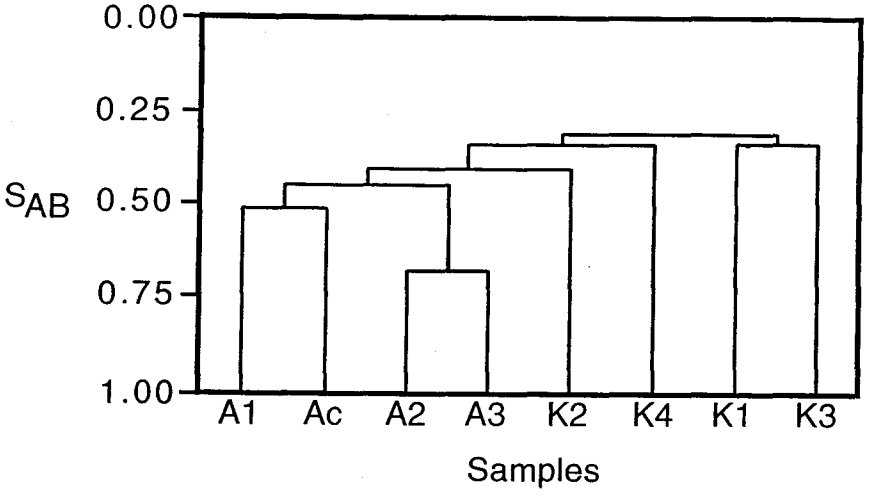

Figure 2.4. Cluster analysis of Biolog GN substrate oxidation rates analyzed using Manhattan and Squared Euclidian algorithms. Samples cluster geographically, with contamination levels having no apparent influence. S_{AB}, similarity coefficient.

Sample divisions within the two clusters could not be described by any of the measured parameters including contamination level and total carbon or nitrogen levels.

Sample A1 demonstrated a reduced ability to oxidize the Biolog GN substrates with respect to both the total number and the rates at which oxidation occurred. A1 was also the most severely contaminated sample at 26,900 ppm TPH.

2.6. Discussion

The soils examined in this study represent two different Northern Canadian communities, both subjected to relatively long term petroleum hydrocarbon contamination. The total heterotrophic cold-adapted population, determined at 5°C, ranged from 10^6 to 10^8 CFU g soil-1 (Table 2.1.). These values are comparable to results obtained from other petroleum hydrocarbon contaminated sites in the Far North, such as Baffin Island (10^5) (Berthelet et al. 1996) and Alaska (10^5 to 10^7) (Braddock et al. 1997). Previous studies have documented an increase in the hydrocarbon-degrading population after contamination (Sexstone and Atlas 1977; Leahy and Colwell 1990; Whyte et al. 1999).

Although changes in the total hydrocarbon tolerant/degrading population were not measured in this study, a similar increase in the total heterotrophic population was observed at the contaminated Alert and Kuujjuaq sites.

The bacterial molecular diversity in the soil samples was estimated by the number of amplified 16S rDNA bands following DGGE separation, as performed by Muyzer et al. (1993). Each band was assumed to represent a single operational taxonomic unit (OTU) (Moyer et al. 1994). Several interesting differences between the Alert and Kuujjuaq sites were noted. Banding patterns within the Kuujjuaq sites could be grouped into two categories, K1 - K2 (contaminated) and K3 - K4 (uncontaminated). Kuujjuaq samples K1 and K2 displayed a moderate number of bands, whereas samples K3 and K4 produced a greater number of distinct bands, all of equal intensity. This difference in the number of bands suggests that the microbial community within the contaminated samples K1 and K2 underwent a decrease in species diversity as members of the bacterial population were subjected to selective enrichment. The apparent decrease in diversity of a microbial community following contamination by petroleum hydrocarbons has also been observed by others (Atlas 1983; Atlas et al. 1991; Lindstrom et al. 1999; Nyman 1999). The greater number of bands

observed in the K3 and K4 samples suggested a greater diversity in the bacterial species present. The relatively equal intensities of the bands within the K3 and K4 samples is interesting, although the importance of band intensity remains to be determined.

The response of the Alert microbial communities to hydrocarbon contamination, as observed using DGGE, appeared to be different from that of the Kuujjuaq sites. Samples A2 (uncontaminated) and A3 (contaminated) produced very similar patterns with respect to the number of bands visualized, yet contamination levels were significantly different. A1, the most heavily contaminated sample, produced a similar number of bands as the uncontaminated site Ac, yet both of these produced significantly fewer bands than either A2 or A3. These results do not follow the observed decrease in species diversity following contamination, as in the Kuujjuaq samples or by other groups (Atlas 1983; Atlas et al. 1991; Lindstrom et al. 1999; Nyman 1999). In the case of A3, it appears that molecular diversity is maintained or increased in comparison to the uncontaminated controls A2 and Ac, respectively. Similar observations of stable or increased microbial diversity after contamination have been noted by other researchers (Leahy and Colwell 1990; MacNaughton et al.

1999). Alert, due to its latitude, has fewer plant species, which would limit the variety and complexity of plant derived organic substrates for indigenous bacteria. Following contamination at Alert, the number and variety of potential carbon substrates increased, resulting in an increase in bacterial activity, which could ultimately increase bacterial diversity. Local environmental and physico-chemical conditions would also play a role in the selective enrichment of the bacterial population (Leahy and Colwell 1990; Atlas et al. 1991). Kuujjuaq experiences above freezing temperatures at least 4 months of the year, whereas Alert is characterized by extremely cold temperatures with only 6-8 weeks per year at, or above, the freezing point.

Sequencing of selected bands isolated from the DGGE gels revealed a broad spectrum of microorganisms. The majority of sequences (greater than 63%) represented high G+C Gram-positive microorganisms from the Actinomycetales. Many microorganisms able to degrade petroleum hydrocarbons are found in this group, including *Rhodococcus*, *Corynebacterium*, and *Nocardia* (Stephens and Dalton 1987; Warhurst and Fewson 1994; Whyte et al. 1998). The examination of Siberian soils using culturing (Shi et al. 1997) and molecular methods (Zhou et al. 1997), identified the prevalence of the same

groups of microorganisms, although some significant differences were noted. Many members of the low G+C Gram-positives were identified using culturing methods (Shi et al. 1997), whereas in this study, none of the sequenced bands showed similarity to members of the low G+C Gram-positive microorganisms. The percentages of isolated Gram-positive and Gram-negative culturable microorganisms isolated were 65.5% and 35.5%, respectively (Shi et al. 1997), which were almost identical to the values observed in this study, despite the difference in approaches, i.e. culture-dependent vs. -independent methods. In the study using molecular methods (Zhou et al. 1997), Proteobacteria made up 60.5% of all sequenced clones, while Gram-positive microorganisms were less than 12%. None of the samples used in the Siberian studies were contaminated, suggesting that these surveys may represent typical bacterial populations found in tundra environments.

Comparison of the sequences obtained from bands K11 and A11 revealed 89% and 86% similarity, respectively, to reference sequences making it difficult to determine more than a general phylogenetic relationship. Sequence K11, consistently grouped with the delta Proteobacteria whereas sequence A11 grouped with the beta Proteobacteria. Despite these consistent groupings, both

of these sequences most likely represent two novel microorganisms within the class Proteobacteria.

Metabolic diversity of the cold-adapted microbial communities was estimated using Biolog GN plates incubated at 10°C. Clustering of the samples into four groups, based on the total number of substrates oxidized, suggested that the TPH levels were not consequential in influencing the overall metabolic diversity. All of these clusters possessed both uncontaminated and contaminated samples. None of the other measured physico-chemical parameters (see Table 2.1.) provided an explanation for these divisions, suggesting that other environmental factors were important in influencing the metabolic diversity of the microbial communities. Cluster analysis, using the rates of oxidation for each substrate, suggested some unique relationships between the samples and the rates at which substrates were oxidized. Three of the four clusters on the dendrogram followed the trends observed in the total number of substrates oxidized. The three clusters included samples A2 and A3, K2 and K4, and K1 and K3. These groupings were not completely unexpected as cluster analysis of the rates of oxidation are also influenced by the total number of substrates oxidized. The most interesting cluster was the fourth one composed of samples

A1 and Ac. These samples were grouped together based on rates of oxidation, yet the total number of substrates oxidized by each of the samples was significantly different (61 and 26 for Ac and A1, respectively). All of the clusters observed were also independent of the level of TPH contamination and were primarily based on the geographic origin of the samples, i.e. Alert samples grouped separately from the Kuujjuaq samples. A1 was the only sample to demonstrate a significant inhibition of metabolic activity, probably due to the level of TPH contamination. TPH contamination above 25,000 ppm has been shown to be inhibitory to soil microbial populations (E.P.A. 1995; Long et al. 1995). In addition, it was observed that despite several different treatment protocols, the rates and levels of hexadecane mineralization in soil A1 were markedly decreased compared with the moderately contaminated soil A3 (Whyte et al. 1999).

In conclusion, the combination of culture -dependent and -independent methods to characterize the cold-adapted soil microbial population of two Northern Canadian communities impacted by petroleum hydrocarbons suggests that the influence of contamination is only one of several factors that determines the selective pressures on indigenous microbial communities. The only trend

consistently observed between the culture -dependent and -independent approaches, with respect to the level of microbial diversity, was that of a geographical separation between the Alert and Kuujjuaq samples. CLPP results were not able to differentiate samples based on level of contamination. In the Alert soils, hydrocarbon contamination increased the apparent microbial diversity, while in Kuujjuaq soils, the bacterial diversity was decreased. 16S rDNA sequencing of selected DGGE bands identified high G+C Gram-positive microorganisms belonging to the Actinomycetales as the predominant microorganisms, which are known to be important in the biodegradation of petroleum hydrocarbons.

Acknowledgments

The authors thank Steve Siciliano and Réal Roy for invaluable help with the Biolog analysis, Hélène Bergeron for DNA sequencing, and Major C. Bellerose (RMC, National Defense, Kingston, Ontario) for access to CFS-Alert soil samples. D.J. was funded by Fonds pour la formation de chercheurs et l'aide à la recherche, Québec.

Connecting Text

The results of comparing culture –dependent and –independent techniques from Chapter 2 were applied to the examination of the short term effect of RDX contamination on soil microbial communities in Chapter 3. This included the contiuned use of 16S rDNA amplification and DGGE analysis, and the replacement of Biolog plates by mineralization analysis. David Juck performed all of the experimental work, accompanying analysis, and writing of the manuscript. Drs. Charles W. Greer and Brian Driscoll provided critical reading of the manuscript and suggested ways in which to improve the work.

Chapter 3. RDX Columns

Submitted to FEMS Microbiology Ecology January, 2002 under the title: Effect of Experimental Contamination with the Explosive RDX on Soil Microbial Communities.

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3.1. Abstract

The effect of contamination with the highly energetic compound hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) on an indigenous soil microbial population was examined. Two glass columns containing approximately 3 kg each of an uncontaminated loam soil, along with indigenous grasses, were assembled. The columns were watered weekly and incubated under a diurnal cycle of 16 hours light, 8 hours dark at 24°C and 18°C, respectively. One column was spiked twice on the soil surface with 1.45 g RDX crystals, for a total RDX load of 1000 mg/kg soil. The RDX concentration in the soil leachate correlated with the fluctuating volume of leachate removed from the contaminated column. The reduced metabolite of RDX degradation, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), was observed in the column leachate, suggesting anaerobic degradation of RDX. Denaturing gradient gel electrophoresis of PCR amplified 16S rDNA from soil depths of 7 and 35cm indicated that the microbial communities within the soil columns were highly diverse and did not change over time with or without RDX contamination. This result was supported by restriction fragment length polymorphism and rarefaction analyses of random 16S rDNA clones from the two depths in the contaminated column. Therefore, it appears long term exposure to

1000 mg RDX/kg soil did not significantly impact the indigenous soil microbial population diversity.

3.2. Introduction

The explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a commonly used component of conventional munitions. Release of RDX into the environment can be attributed to manufacturing processes and waste disposal, military testing and training, and ordnance detonation. It is estimated that there are more than 100 sites in Canada contaminated with RDX and two other commonly used explosives, 2,4,6-trinitrotoluene (TNT) and tetrahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Thiboutot et al. 1998). RDX toxicity has been demonstrated in humans (McLellan et al. 1992) and affects the central nervous system, gastro-intestinal tract and kidneys (Etnier 1989). The toxicity of RDX has also been demonstrated in various aquatic (Cook and Westlake 1974; Jobson et al. 1974) and terrestrial organisms (Simini et al. 1992; Winfield et al. 1999; Robidoux et al. 2000). RDX toxicity, combined with its weak sorption to soil matrices (Singh et al. 1998; Pennington et al. 1999; Sheremata et al. 2001), and the wide distribution of contaminated sites, makes its movement to

groundwater and susceptible downstream receptors an important environmental problem.

Several RDX bioremediation technologies have been developed, including composting (Jarvis et al. 1998), soil slurry bioreactors (Boopathy et al. 1998; Shen et al. 2000), and flooded biopiles (Greer et al. 1997). In the majority of these processes, an anaerobic component such as sulfate-reducing conditions (Boopathy et al. 1998), nitrate-reducing conditions (Freedman and Sutherland 1998) or a two-stage anoxic-aerobic system (Ronen et al. 1998) was included to optimize RDX degradation. A recent study (Price et al. 2001) determined that a redox potential below -150 mV greatly increased the rate of RDX mineralization in soil slurries.

There have been several reports of microbial isolates with RDX-degrading activity, including *Citrobacter freundii, Morganella morganii,* and *Providencia rettgeri* (Kitts et al. 1994), *Stenotrophomonas maltophilia* PB1 (Binks et al. 1995), *Serratia marcescens* (Young et al. 1997), and *Rhodococcus* sp. strain DN22 (Coleman et al. 1998). In addition to pure cultures, several bacterial consortia possessing RDX-degrading activity have been identified from different sources, including horse manure (Young et al. 1997), explosives contaminated creek

sediment (Boopathy et al. 1998), and municipal anaerobic sludge (Shen et al. 2000). The microorganisms responsible for the *in situ* anaerobic biodegradation of RDX have not yet been identified, nor have the proposed *in situ* enzymatic systems been confirmed.

The effect of RDX contamination on soil microbial processes has only been examined in one previous study (Gong et al. 2001). RDX contaminated soil was monitored for soil dehydrogenase activity, potential nitrification, nitrogen fixation, basal respiration, and substrate-induced respiration. The lowest concentration at which RDX had observable negative effects on the measured parameters was 1235 mg RDX/kg soil. In contrast, another widely used explosive, TNT, inhibited the same metabolic activities at only 1-2 mg TNT/kg soil (Gong et al. 1999).

The goal of this study was to re-create the conditions of an accidental RDX spill in a terrestrial environment and to identify the impact on the soil microbial populations. To the best of our knowledge, this is the first such study looking at the changes affected by RDX contamination in soil microbial populations at a molecular level.

3.4. Materials and Methods

3.4.1. Soil characteristics

The soil used in this study originated from the top 30 cm of a grassy field in southern Québec. The soil was classified as a loam (21% clay, 46% silt, and 33% sand), contained 8.9% organic matter, had a pH of 7.8 and 7.1 (before and after addition of $CaCl_2$, respectively) as determined by the method of McLean, (McLean 1982), and a cationic exchange capacity (CEC) of 32.5 meq/100g. Large rocks and other debris were removed and an effort was made to preserve the natural soil aggregate structure.

3.4.2. RDX mineralization in soil microcosms

Mineralization of RDX was performed in triplicate using 20 g of 2.0 mm sieved soil per serum bottle amended with a total of 50 mg/kg of a mixture of [U-^{14}C] labeled and unlabeled RDX dissolved in acetone. Both the labeled and unlabeled RDX were supplied by the Defence Research Establishment Valcartier, Val Bélair, Québec, Canada, and the specific activity of the labeled RDX was 28.7 μCi/mmol (>99% purity). The serum bottles were incubated statically at

room temperature in the dark and the rate of $^{14}CO_2$ evolution was monitored by liquid scintillation spectrometry as previously described (Whyte et al. 1996). Control microcosms were the same as above, but were autoclaved twice for 20 minutes with a 24 hour incubation at room temperature between autoclavings.

3.4.3. Soil column construction

Two glass columns (85 cm x 9.25 cm) were each loaded with 3 kg of the above loam soil, on top of a layer of glass wool. The soil was lightly compacted to a final height of approximately 35 cm, and a root plug of indigenous grasses (primarily *Echinichloa crus-galli*) from the site was placed onto each soil surface. The columns had six sampling ports at 7 cm intervals, numbered from top to bottom, with the first port initially below the soil surface. The columns were incubated under a diurnal cycle of 16 hours light at 24°C and 8 hours dark at 18°C. The columns were incubated for 59 days prior to RDX application and sampling to allow the system to stabilize.

3.4.4. Column treatment and leachate analysis

Crystalline RDX (1.45 g) was twice added (day 0 and 166) to column A on the surface for a calculated load of 1000 mg RDX/kg soil. RDX was not added to column B, the control system. The columns were watered once weekly with 150 mL distilled water until day 276, and with 200 mL from then on. Samples for chemical and molecular analysis were taken every 2-3 weeks, 24 hours after watering. At each sampling interval, the columns were drained of leachate via the drainage port and the collected volume was recorded.

Samples were mixed 1:1 with acetonitrile and analyzed by HPLC for RDX, and its potential metabolites hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) as previously described (Sheremata et al. 2001) (see Appendix 8.2. for a detailed description). As standards for the determination of MNX, DNX, and TNX concentrations were not available, the abundance of these compounds was expressed as the area under each respective peak. The MNX, DNX, and TNX peaks were identified by liquid chromatography/mass spectrometry and comparison to reference compound simulation as previously described (Sheremata et al. 2001).

3.4.5. DNA extraction, PCR, and DGGE analysis

Duplicate horizontal cores of bulk soil (0.2 g) were taken from ports 2 and 6, at depths of 7 and 35 cm, respectively, from both columns. Samples were placed in 2 mL screw cap tubes and immediately frozen at -80°C until DNA was extracted. Total community DNA was extracted from the soil samples using a beadbeater method and purified using polyvinylpolypyrrolidone (PVPP) spin columns as previously described (Berthelet et al. 1996).

A 500 bp fragment of 16S rDNA was amplified from the cleaned community DNA using two eubacterial primer pairs, 8FGC-519R and b341GC-b758. Primer sequences are as follows: 8FGC (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCGAGAGTTTGATCCTGGTCCAG-3') (Eden et al. 1991), 519R (5'-GTATTACCGCGGCTGCTGG-3') (Coates et al. 1998), b341GC (5'-CGCCCG CCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGCCTACGGGAGGCAG CAG-3') (Rölleke et al. 1996) and b758 (5'-CTACCAGGGTATCTAATCC-3') (Woese 1987; Lee et al. 1993). The underlined sequences represent the GC clamps required for DGGE analysis (Muyzer et al. 1993). Each 50 µL PCR mixture contained 1-3 µL of cleaned soil DNA extract, 200 µM of each dNTP, 20

pmol of each primer, and 1 mM $MgCl_2$. A modified hot start and touchdown combination was employed to reduce non-specific fragment amplification as previously described (Juck et al. 2000). After reducing the temperature to 80°C, 5 µL of 10x buffer (15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl (pH 9.0) mixed with 1.25 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Inc., Baie d'Urfé, Québec, Canada) was added to each tube and touchdown PCR was started. A portion of the amplified DNA was visualized on 1.2% agarose gels stained with ethidium bromide, following standard protocols (Sambrook et al. 1989).

DGGE was performed as previously described (Juck et al. 2000) with several modifications. A 6% acrylamide stacking gel (no denaturant) was used, and the remaining amplification reaction (45 µL) was mixed with 10 µL of 6x loading dye III (Sambrook et al. 1989) before loading. DGGE gels were stained using Vistra green (Amersham Pharmacia Biotech, Inc., Baie d'Urfé, Québec, Canada) and visualized using a FluorImager 595 (Molecular Dynamics, Amersham Pharmacia Biotech, Inc., Baie d'Urfé, Québec, Canada) with a 488 nm excitation filter and a 530 nm emission filter.

3.4.6. Cloning of 16S rDNA

Fragments of 16S rDNA from a subset of the above DNA samples (contaminated column, ports 2 and 6, day 343), were amplified by PCR and cloned into a plasmid vector. Primer pair 8F (same as 8FGC, without the underlined GC clamp) and 758R was used to generate a fragment of approximately 800 bp. The PCR protocol was the same as above, except that 15 cycles at 55°C were performed, and a 10 minute extension at 72°C was added after the last round of amplification. The PCR products were purified using QIAQuick Spin Columns (Qiagen, Mississauga, Ontario, Canada) and resuspended in 30 µL of Elution Buffer (10 mM Tris-HCl, pH 8.5). DNA concentrations were estimated by comparing staining intensity with a 100 bp DNA molecular weight standard (MBI Fermentas, Burlington, Ontario, Canada) on 1.2% agarose gels. The fragments were ligated to *Eco RV* cleaved pBluescript KS (Stratagene, La Jolla, California, U.S.A.) DNA to which a 3' T overhang had been added, and the ligations were transformed into *E. coli* DH5∝, following standard methods (Sambrook et al. 1989). Transformants were selected on LB agar plates supplemented with ampicillin (100 mg/L), X-gal (20

mg/L), and IPTG (100 µM), following standard methods (Sambrook et al. 1989). Inserts were screened using the protocol of Sekar (Sekar 1987).

3.4.7. 16S rDNA RFLP fingerprints

Two hundred random clones from each sample were analysed. A small portion of each colony was suspended in 50 µL sterile dH_2O, heated to 96°C for 5 minutes, then placed on ice. The cellular debris was pelleted at 1,300 x g for 10 minutes at 4°C, and 5 µL of the supernatant was used as DNA template for PCR. The PCR mixtures were the same as described above but were scaled down to a 25 µL volume, and used custom primer pairs KS (5'-CGAGGTCGACGGTATCG-3') and SK (5'-TCTAGAACTAGTGGATC-3'), specific for regions of the vector adjacent to the insert. The PCR program was 5 minutes at 96°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at 54°C, and 1 minute at 72°C and then a final 4°C hold.

Restriction endonuclease digestion was performed directly in the PCR tubes by adding 1.7 µL ddH_2O, 1.5 U each of *MspI* and *HhaI*, and 3 µL of 10x One-Phor-All-Plus buffer (Amersham Pharmacia Biotech, Inc., Baie d'Urfé, Québec, Canada). The restrictions were mixed, and then incubated at 37°C for 3 hours.

The reactions were stopped by addition of 6 µL loading dye III, and then 10 µL was loaded onto the gel. The gel was 4% NuSieve 3:1 agarose (FMC BioProducts, Rockland, Maine, U.S.A.) in TBE buffer (Sambrook et al. 1989). The samples were electrophoresed using a Bio-Rad Sub-Cell model 96 electrophoresis apparatus (Bio-Rad, Mississauga, Ontario, Canada) at 3.6 volts/cm for 5 hours. The RFLP patterns were visualized and photographed following staining with ethidium bromide.

3.4.8. RFLP analysis and rarefaction

RFLP photographs were scanned and then analysed using the Dendron 2.4 software package (Solltech Inc., Oakdale, LA, USA). The patterns were compared according to manufacturer's instructions, and dendrograms were created based on band position. Clone clusters were visually verified and then tabulated for input into the program Analytical Rarefaction 1.3 (Version 1.3, distributed by S.M. Holland, Stratigraphy Lab, Universty of Georgia, Georgia).

3.5. Results

3.5.1. Mineralization of RDX by soil microcosms

Prior to performing the soil column study, the ability of the indigenous soil microbial population to mineralize RDX was tested using aerobic microcosms. After a lag of approximately 2 weeks, RDX was mineralized at a rate of 0.585 mg/kg/day. Over the course of 94 days, approximately 90% of the added ^{14}C-RDX was recovered as ^{14}C-CO_2 (Figure 3.1.).

3.5.2. Column contamination and leachate analysis

Once the RDX mineralization potential of the soil community was established, the first application of RDX to the experimental column was performed. A second addition of RDX was made at day 166, to bring the total RDX load to 1000 mg/kg soil. RDX had no apparent effect on plant growth until several months into the experiment. At that time, the predominant grass (*Echinichloa crus-galli)*, died off, and was replaced by the vine-like *Stellaria* sp. The plant community in the control column, to which no RDX had been added, appeared unchanged over the course of the experiment.

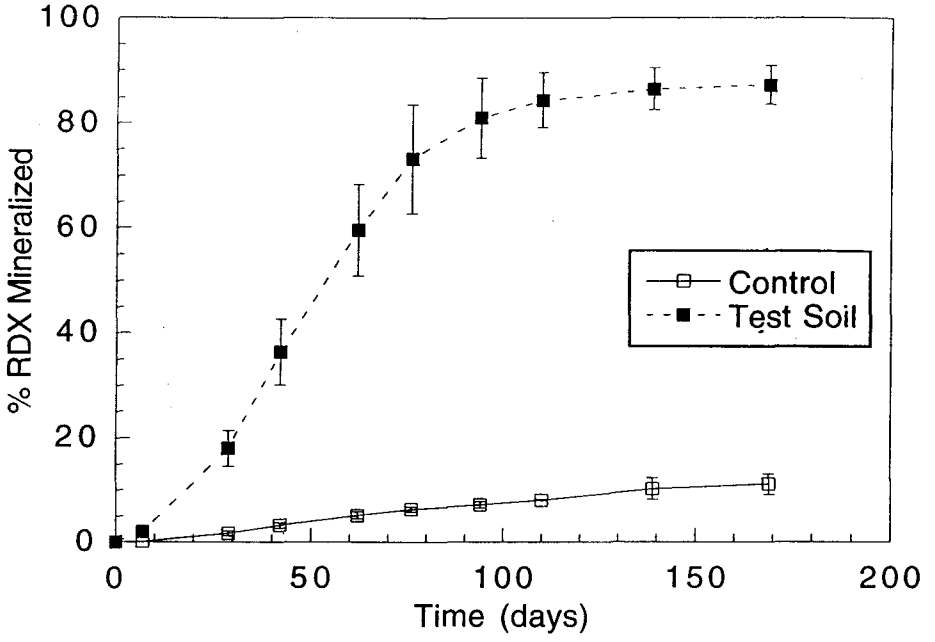

Figure 3.1. Mineralization of ^{14}C-RDX in soil microcosms. Mineralization was monitored by the production of $^{14}CO_2$ from 20 g of soil in sealed serum bottles. The control microcosms were identical to the test soil microcosms, but were autoclaved twice before spiking with ^{14}C-RDX. Data points are the mean cumulative amount of $^{14}CO_2$ produced from triplicate microcosms and error bars represent the standard deviation.

Soil column leachates were tested for the presence of RDX and metabolic intermediates indicative of RDX degradation (MNX, DNX, and TNX). RDX was detected in the leachate from the first sampling, 19 days after the first addition (Figure 3.2.). The concentration of RDX in the leachate decreased over the next 90 days to below 1 mg/L. The pattern of appearance of MNX in the leachate wassimilar to that of RDX, and was undetectable by 145 days. Neither DNX nor TNX were detected in the experimental system leachate, and none of the compounds were detected in the leachates of the control column, over the course of the experiment.

As most of the RDX and MNX had disappeared from the leachates by day 147, a second spike of 1.45 g RDX crystals was applied to the upper surface of the experimental column on day 166. A slight increase in the leachate RDX concentration (from 1.3 mg/L to 2.1 mg/L) in the 5 subsequent samplings following the second RDX addition (days 187 to 243) was observed (Figure 3.2.). The leachate RDX concentration increased again on day 258, with a second peak of 8.8 mg/L being detected on day 278. Although the appearance of MNX in the leachate mirrored that of RDX, MNX quickly decreased to undetectable

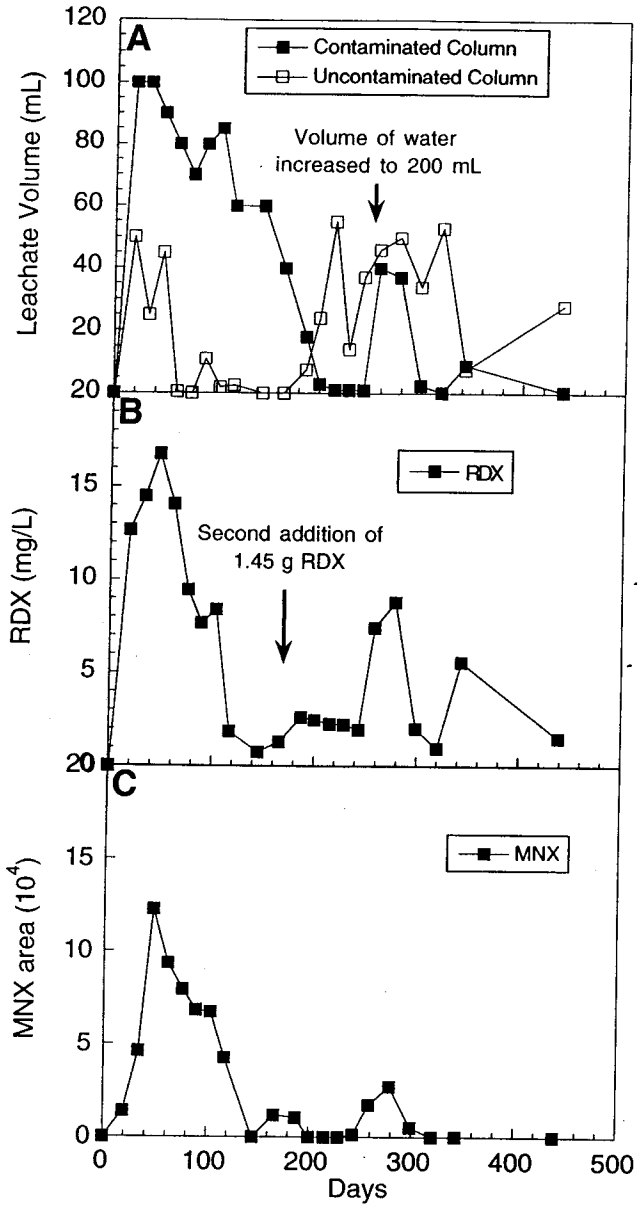

Figure 3.2. Analysis of soil column leachate. A) Volume of leachate removed from contaminated and uncontaminated columns. The volume of water was increased from 150 mL to 200 mL at day 277, as indicated. B) RDX concentration measured in the contaminated column leachate. RDX (1.45 g) was added at day 0 and 166. C) Level of MNX in contaminated column leachate. As an analytical standard was available for MNX quantification, the area under the curve is presented. Metabolites DNX and TNX were not detected in the leachate.

levels. The RDX concentration in the final leachate sample at day 439 (273 days following the second addition of RDX), was 1.5 mg/L.

The concentration of RDX in the leachate appeared to be related to the volume of leachate removed from the contaminated column. A decreasing trend in leachate volume, despite the consistent application of 150 mL water per week, was observed for the first 200 days (Figure 3.2.). Leachate volumes decreased from approximately 100 mL to 1 mL over this period. At sampling day 258, there was a spike in the volume of recovered leachate (40 mL). At this point, the weekly volume of water was increased from 150 mL to 200 mL. At the next sampling point (day 278), 37 mL of leachate was removed. This was followed by several fluctuations in leachate volume (from approximately 1 to 10 mL) until sampling was stopped at day 439. The control column also demonstrated an initial decrease in leachate volume over the first 200 days. Fluctuation in leachate volume was also observed from the control column after the weekly water addition was increased to 200 mL. The relationship between RDX concentration and leachate volume was examined by plotting RDX concentration versus volume of leachate (Figure 3.3.). There was a significant correlation

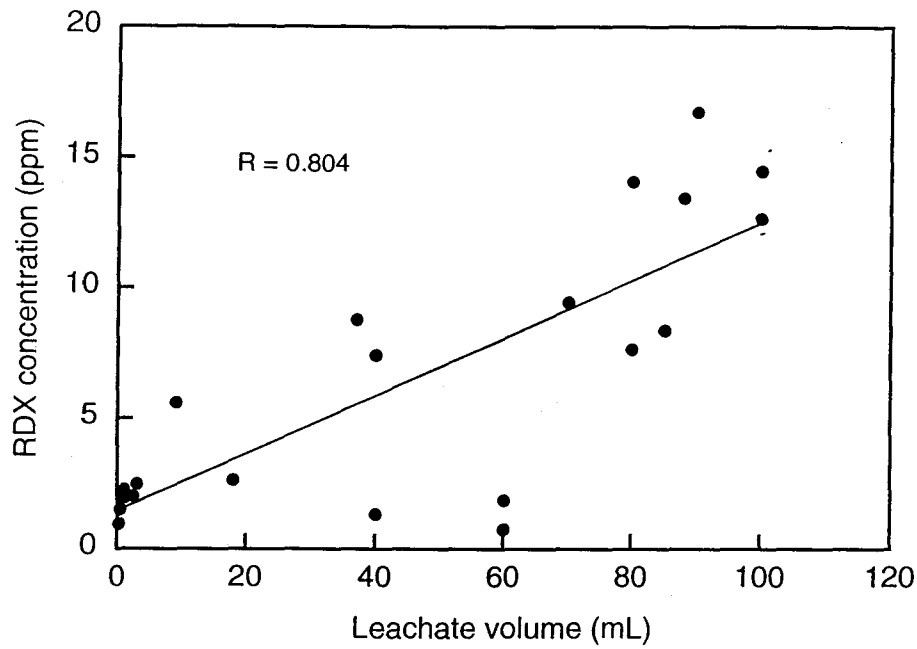

Figure 3.3. Leachate RDX concentration versus volume of leachate removed from the RDX contaminated column. Linear regression revealed a significant correlation (R value = 0.804) between the volume of leachate removed and the concentration of RDX in the leachate.

between RDX concentration and leachate volume (R value of 0.804 at the 95% confidence level).

3.5.3. DGGE and rarefaction analysis of 16S rDNA fragments

Fragments of 16S rDNA were amplified using total community DNA extracts from ports 2 and 6 in both columns on days 0, 103, 258, and 343 and analyzed by DGGE. The analysis revealed the presence of a large number of bands, all of roughly equal intensity (Figure 3.4.). The patterns were almost identical between samples. Neither the appearance of new bands nor the disappearance of observed bands occurred, with respect to sample depth, RDX treatment, or time. The intensity of several bands changed over time, suggesting minor shifts in microbial population over the course of the experiment. These shifts did not appear to be related to RDX contamination, since they also occurred in the uncontaminated control column. Due to this observation, no bands were isolated for sequencing. The results were the same for both of the primer pairs used (i.e. 8FGC-519R and b341GC-b758).

The number of bands observed suggested a very high level of bacterial diversity in the bulk soil. To determine if random cloning might be useful in

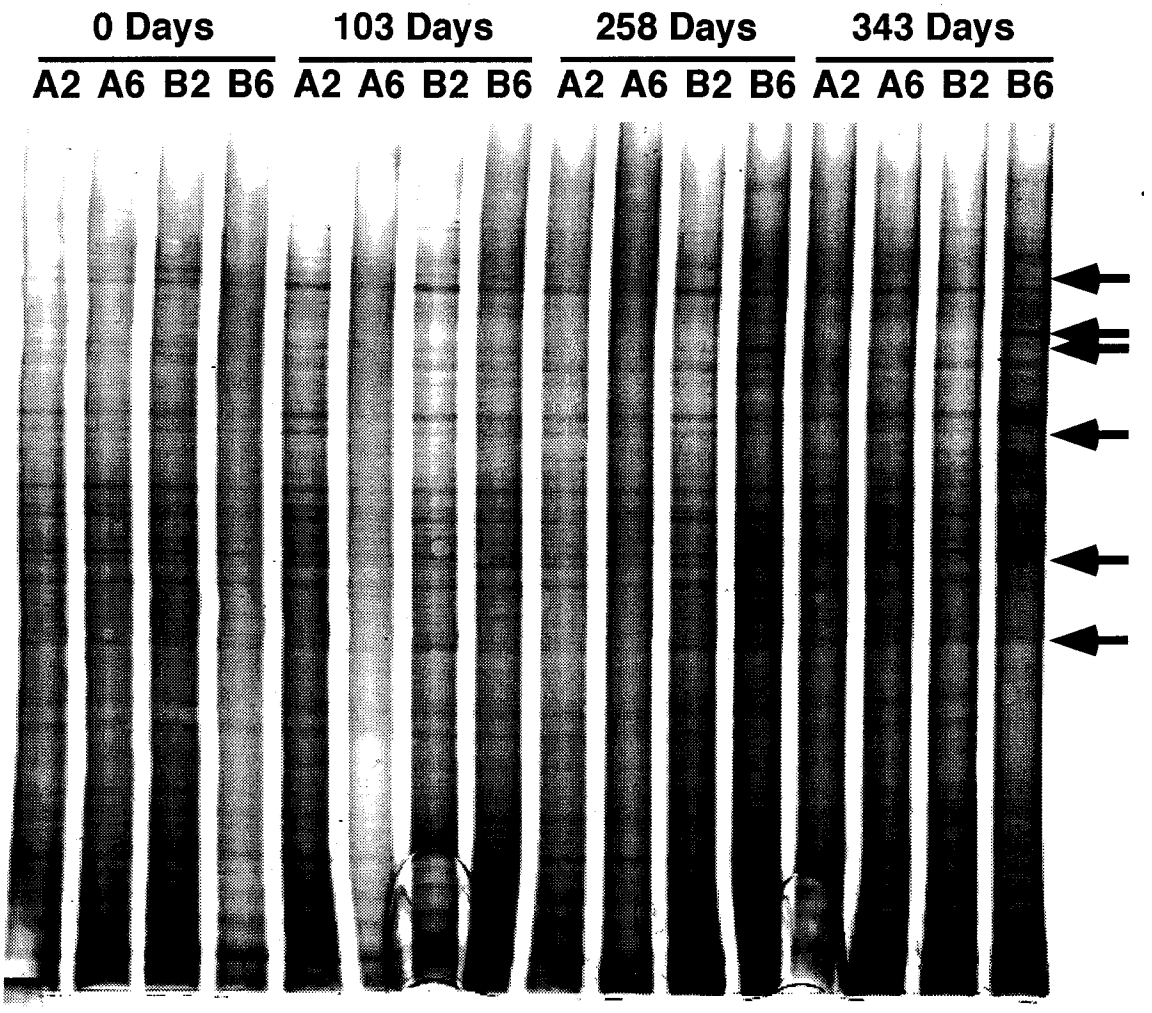

Figure 3.4. DGGE of 16S rDNA amplification fragments generated using primer pair 8FGC-519R. PCR fragments were separated on an 8% acrylamide DGGE gel with a denaturant gradient of 40-60%. Bands indicated by arrows demonstrated fluctuation in band intensity over the course of the experiment. Similar results for primer pair b341GC-b758 were observed (not shown). Sample designation: A, RDX contaminated column; B, uncontaminated control column; 2, upper sampling port (7 cm depth); 6, lowest sampling port (35 cm depth).

surveying the bacterial diversity, rarefaction analysis of RFLP patterns from 16S rDNA clones was performed. Total community 16S rDNA, amplified and cloned, from ports 2 and 6 of the contaminated column at 343 days were examined. Plasmid DNA from 196 random clones, from each port, was digested with the restriction endonucleases *Msp*I and *Hha*I. Each of the two samples (i.e. from ports 2 and 6) contained approximately 160 unique RFLP patterns. Comparison of the patterns between the sample ports revealed very little similarity. This high level of diversity is in accordance with the results observed using DGGE. Rarefaction analysis on the RFLP patterns was performed to determine the minimum number of clones needed for an estimation of the bacterial molecular diversity. The resulting linear plot of unique RFLP patterns versus number of clones sampled (Figure 3.5.), suggests that a sample size of 196 clones per port is considerably lower than that required for an accurate estimation of diversity.

3.6. Discussion

Preliminary mineralization assays demonstrated the inherent RDX degrading capacity of the indigenous soil microbial population in the soil used in this study. This degradation ability has been observed in previous work in

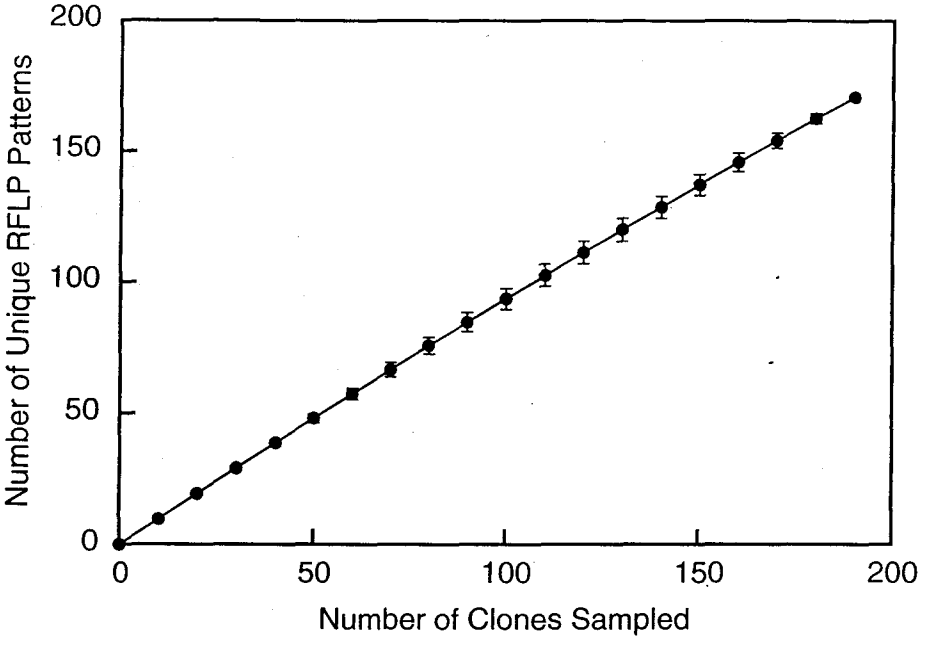

Figure 3.5. Rarefaction analysis of cloned 16S rDNA RFLP patterns from the upper sample port (port 2) of the RDX contaminated column. The linear trend of the plot indicates that 196 clones do not adequately represent the total microbial community. The plot of cloned 16S rDNA RFLP patterns from sample port 6 of the same column is identical in nature. The plots were generated using the program Analytical Rarefaction 1.3.

contaminated soils (Greer et al. 1997; Axtell et al. 2000; Groom et al. 2001), soil slurries, (Shen et al. 2000; Price et al. 2001), municipal anaerobic sludge (Hawari et al. 2000) and experimentally contaminated clean soil (data not shown). The observed 2 week lag period prior to the appearance of $^{14}CO_2$ may be explained by mass transfer of crystalline RDX into a bioavailable form. This is affected by the low aqueous solubility (42 mg/L) of RDX, which limits microbial exposure, and would therefore increase the time required for microbial adaptation to occur. This includes growth of stimulated populations, increases in enzyme activity and positive up-regulation of enzymatic systems. Once the appropriate enzymatic systems/microbial populations are activated, biotransformation can proceed.

The toxicity of RDX to terrestrial plants (Simini et al. 1992; Winfield et al. 1999) was demonstrated by the kill-off of the original plant species *Echinichloa crus-galli*. The triazine backbone of RDX is identical to several commonly used herbicides such as atrazine and simazine. Although the mode of RDX toxicity to terrestrial plants has not been established, it may possess a similar photosystem II inhibitory effect as the triazine based herbicides, which bind to the D-1 quinone binding protein (Oettmeier 1992).

Dissolution of surface crystals of RDX and movement through the soil column was swift, as evidenced by RDX detection in the first leachate sample. The relatively rapid movement of RDX through the soil column, despite its low aqueous solubility, was likely due to its low sorption capacity (Pennington et al. 1999; Sheremata et al. 2001). The published sorption capacity constant for RDX in soil matrices is low and ranges from 0.21 to 0.83 L/kg (Pennington et al. 1999; Sheremata et al. 2001). It has been previously reported that long-term exposure of soil matrices to RDX does not significantly increase the non-extractable sorbed RDX (Singh et al. 1998; Sheremata et al. 2001). In these studies, a large percentage of the RDX sorbed to the soil matrix remained easily recoverable, resulting in a potential source of long term leachable RDX.

The trend of decreasing RDX concentration in the contaminated column leachate suggested that, as exposure to RDX continued, a subset of the microbial population was able to adapt and more readily biotransform RDX. Evidence of this is the slightly delayed appearance of MNX in the leachate during the first sampling period. The presence of MNX in the leachate is consistent with the postulated anaerobic degradation of RDX (McCormick et al. 1981; Hawari et al. 2000), the first steps being the reduction of the RDX nitro groups, leading to

the production of MNX, DNX, and TNX. The slight delay in the appearance of MNX is most likely due to the importance of establishing reducing redox conditions within the soil column. Previous studies have observed increased rates and extents of RDX mineralization under various anaerobic and reduced redox conditions (McCormick et al. 1981; Boopathy et al. 1998; Freedman and Sutherland 1998; Ronen et al. 1998; Shen et al. 1998; Shen et al. 2000; Price et al. 2001). The further reduction products DNX and TNX were never observed in contaminated column leachates. This suggested that either the reduction of MNX was not occurring in the columns and that the MNX ring was being cleaved, or that the further reduction through DNX and TNX was very rapid. The metabolites of a proposed alternate anaerobic biotransformation pathway, namely methylenedinitramine and bis(hydroxymethyl)nitramine (Hawari et al. 2000) were not examined for since no standards were available.

The observed relationship between RDX concentration and leachate volume can best be explained by adsorption/desorption phenomena associated with preferential flow paths (Flury et al. 1994; Bundt et al. 2001). In the RDX contaminated column, organic matter released from the dead *Echinichloa crus-galli* plant material would increase the water holding capacity of the soil, as

evidenced by the decreasing leachate volume. Heterogeneity of the soil matrix resulted in the occasional saturation of preferential flow paths. This in turn increased wetting of non-preferential flow areas, released RDX loosely sorbed to the soil matrix, and resulted in the observed correlation between leachate volume and RDX concentration.

Over the course of monitoring, no DGGE bands were significantly impacted by RDX contamination. The use of two different primer pairs, as a means of reducing potential amplification bias (Suzuki and Giovannoni 1996), did not change this observation. In fact, the bands that demonstrated the most variation in intensity, were those from the uncontaminated control column. Previous studies have determined the detection limit of DGGE to be 1% of the total bacterial population under study (Muyzer et al. 1993; Stephen et al. 1999). Based on this, and the lack of any significant observable changes in the microbial community after RDX contamination, the soil bacterial communities may be considered very stable and not significantly affected by RDX contamination. The lack of any observable positive stimulation by RDX contamination (i.e. appearance or significant increase in the intensity of a DGGE band) made it

impossible to determine which microorganisms may have been responsible for the observed transformation of RDX.

An alternative approach was used to estimate the level of bacterial molecular diversity in the columns. Rarefaction analysis of restriction digested 16S rDNA clone patterns has been previously used to estimate diversity levels (Dunbar et al. 1999; Ravenschlag et al. 1999; Eilers et al. 2000). Analysis of RFLP patterns generated from clones at the top and bottom of the contaminated column at sampling day 343 revealed that a high level of diversity was present. Of the 392 clones randomly chosen (196 clones per sample port), over 80% possessed unique RFLP patterns. Of the clones demonstrating similar RFLP patterns, the distribution was equal in terms of shared patterns within and between the top and bottom ports. Rarefaction analysis (Figure 3.5.) indicated that the number of clones examined was not sufficiently large to accurately estimate the level of diversity of present. This high level of diversity in the contaminated column, after almost one year of exposure to RDX, suggested that the soil microbial community was not negatively impacted by RDX, which agrees with the DGGE results.

In conclusion, contamination of soil with 1000 mg RDX/kg soil and maintaining it for more than one year had no detectable impact on the soil microbial community structure. Microbial communities were stable, maintained a high level of diversity, and based on the disappearance of RDX with the concomitant appearance of MNX, actively transformed RDX. These results indicate that the soil possessed active RDX-degrading bacteria and that passive bioremediation of RDX contaminated soil is a viable remediation approach.

Acknowledgments

The authors wish to thank Louise Paquet for analytical services, Diane Labbé for technical advice and primer pair KS-SK, Alan Watson and Marcia Waterway of the Plant Sciences Dept. of Macdonald Campus, McGill University, for plant identification, and Jalal Hawari for critical discussions. DJ was funded by Fonds pour la formation de chercheurs et l'aide à la recherche, Québec, and McGill University.

Connecting Text

The results from Chapter 3 suggested that within the soil column there was an active RDX degrading bacterial population. The molecular biological techniques used (16S rDNA amplification combined with DGGE analysis) were not able to discern the microorganisms potentially responsible for this activity, so the isolation of RDX degrading axenic cultures was performed. David Juck performed all of the experimental work, accompanying analysis, and writing of the chapter.

Chapter 4. Isolation of Axenic RDX Degrading Cultures

4.1. Abstract

Several attempts were made to isolate RDX degrading bacteria. The source of potential isolates was a soil exposed to RDX for almost 2 years, and which displayed an inherent ability to mineralize RDX. Isolation conditions included enrichment and spread plating using a defined minimal medium with RDX as the sole source of nitrogen, inclusion of alternate nitrogen sources along with RDX, enrichment and plating using a rich complex medium, and both aerobic and anaerobic conditions. Microcosm studies on several randomly selected isolates demonstrated possible RDX mineralization although levels were too low to be considered significant. No definitive RDX degraders, based on RDX crystal clearance on solid media, were isolated, suggesting that proper enrichment conditions were not provided and/or degradation of RDX in the soil under study is performed by a bacterial consortium.

4.2. Introduction

In trying to elucidate the catabolic pathway(s) of *in situ* RDX biodegradation, isolation and examination of RDX degraders is an important step. Several studies have been successful in the isolation of pure bacterial strains or consortia able to biotransform RDX. These include *Stenotrophomonas maltophilia* PB1 (Binks et al. 1995), *Rhodococcus* sp. strain DN22 (Coleman et al. 1998), *Rhodococcus* strain A (Jones et al. 1995), *Morganella morganii, Providencia rettgeri*, and *Citrobacter freundii* (Kitts et al. 1994), a defined consortium of sulfate-reducing bacteria including *Desulfovibrio desulfuricans* A, *D. desulfuricans* B, *D. gigas*, and *D. vulgaris* (Boopathy et al. 1998), and *Serratia marcescens* isolated from an RDX degrading consortium (Young et al. 1997). In all but two of these studies (Kitts et al. 1994 and Young et al. 1997), RDX was used as the sole source of nitrogen. RDX could not be used as a sole source of carbon by any of the isolates or consortia. Another unifying factor in all but one of these studies was that bacterial isolates all originated from environments with a previous history of long-term RDX contamination and a variety of other explosives.

In an effort to identify the microorganisms responsible for RDX biodegradation in the uncontaminated soil used for previous work (Chapter 3),

isolation of RDX degraders was attempted. Various approaches were used, all based on enriching for microorganisms able to use RDX as the sole source of nitrogen.

4.4. Materials and Methods

4.4.1. Method 1 for RDX Degrader Isolation

The first attempt at isolating an RDX degrader used uncontaminated column soil in a modified liquid minimal salts medium (MSM) (Greer et al. 1990) free of nitrogen (MSM-N) (Figure 4.1.). Stock solution 3 was changed from 1M $(NH_4)_2SO_4$ to 1M Na_2SO_4 to eliminate the major source of nitrogen in the medium, and stock solution 1 was changed from 1M NaH_2PO_4 to 1M KH_2PO_4 to reduce the amount of sodium present. RDX, at a final concentration of 100 mg/L, was added to the MSM-N (MSM-N+R) by first placing 250 µL of an RDX stock solution (40,000 mg/L in acetone) into sterile 100 mL Erlenmeyer flasks and allowing the acetone to evaporate leaving the RDX crystals. Yeast extract was also added to the MSM-N+R (MSM-N+RY) at a concentration of 5 mg/L to aid in the establishment of an active culture. Inoculation of 100 mL of medium was

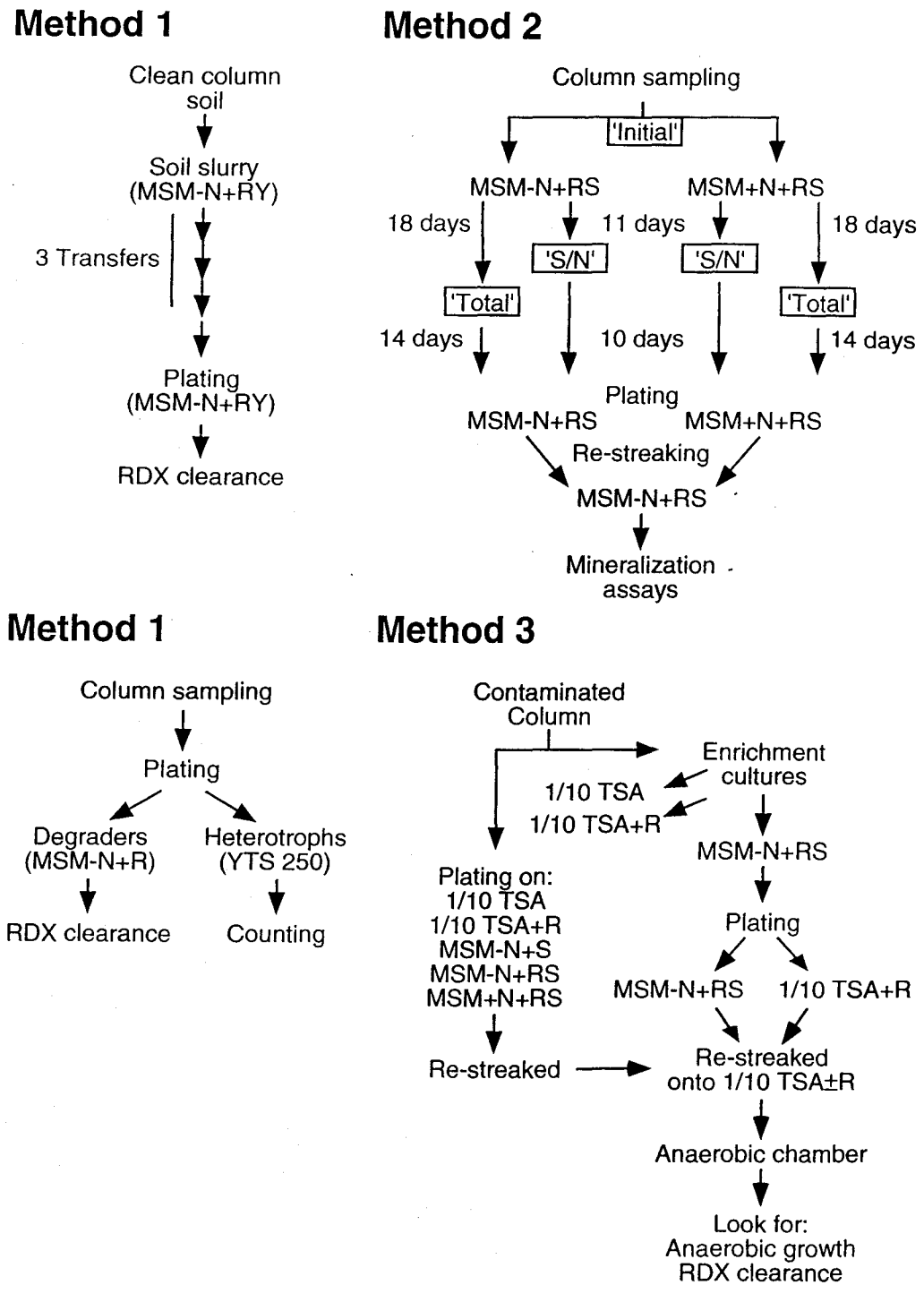

Figure 4.1. Flow chart for the enrichment of RDX degrading bacteria. Abbreviations are described in the text.

performed in duplicate with 10 g of sieved (2.0 mm) subsurface column soil previously stored at 4°C for 2 months. The flasks were incubated at room temperature in the dark and rotated at 150 rpm.

A total of 3 transfers were performed at 2 week intervals, each consisting of a 10% inoculum into a total of 100 mL of MSM-N+RY. The third transfer flask, two weeks after inoculation, was plated out to identify RDX degraders. A dilution series of 10^{-2} to 10^{-4} was prepared using 0.1% tetrasodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$), and 100 µL per plate was plated out in triplicate on solid MSM-N+RY (1.5% agar present). RDX was applied to the surface of cooled plates by spreading 62.6 µL of an RDX stock solution (40,000 mg/L in acetone) until the acetone was evaporated and only RDX crystals were remaining. The bacterial dilution series was then spread on the dried plates. Plates were incubated in the dark at room temperature and periodically monitored for zones of RDX crystal clearance, indicating transformation of RDX.

Direct plating of RDX degraders and total heterotrophs from soil columns was performed using 0.5 g soil taken from sample ports 2, 4, and 6 (top, middle and bottom, respectively) from both the contaminated and uncontaminated columns. A ten fold dilution series, as described above, was created and 100 µL

was plated. To isolate RDX degrading colonies, dilutions 10^{-2} to 10^{-3} were plated on MSM-N+R (100 mg/L RDX). For total heterotrophs, dilutions 10^{-5} to 10^{-7} were plated on 250 mg yeast extract, 250 mg tryptone, 250 mg starch, and 15 g agar per liter (YTS250). Plates were incubated at room temperature in the dark.

4.4.2. Method 2

A second method was attempted to isolate RDX degraders using MSM-N+R, amended with 10 mM (unless otherwise stated) succinate (MSM-N+RS) as a carbon source, with or without the addition of 50 mg/L $(NH_4)_2SO_4$ (MSM±N+RS) as a source of nitrogen. Soil from column sample ports A2, A6, B2, and B6 (2g) was used as inoculum for either 20 mL of MSM-N+RS or MSM+N+RS (labeled 'initial'). The 50 mL Erlenmeyer flasks were incubated at room temperature in the dark with shaking at 150 rpm. After 11 days, 2 mL of culture supernatant (S/N) per flask (flasks were held stationary for 1 hour and the surface was sampled) was transferred to a second Erlenmeyer containing (labeled 'S/N') 18 mL of the same medium (i.e. MSM±N+RS). After 18 days, a second set of enrichment cultures were started using 5 mL of the 'initial' cultures transferred to 45 mL of the same media (i.e. MSM±N+RS). The original flasks were not allowed to settle

during sampling, and the subsequent enrichment cultures were labeled 'total'. Both the s/n and total enrichments were incubated in the dark at room temperature with shaking at 150 rpm.

RDX concentrations were monitored in the enrichments (i.e. s/n and total) by sampling 800 µL of culture fluid (while mixing), addition of 800 µL of acetonitrile and incubation at room temperature for 1 hour. The solution was then filtered (0.2 µm sterile Millex-GP syringe filters, Millipore, Mississauga, Ont.) and RDX concentration was determined as described in Chapter 3. Since standards for RDX reduction products MNX, DNX, and TNX were not available at the time of enrichment culture sampling, no attempt was made at monitoring their levels. Plating of s/n and total enrichments was performed 10 and 14 days, respectively, after starting the cultures. A ten-fold dilution series was plated in triplicate for each sample on the same media as originally used. Plates were incubated in the dark at room temperature and periodically examined for zones of RDX clearance surrounding colonies. Re-streaking of selected colonies was performed on MSM-N+R plates plus 10 mM or 100 mM succinate and incubated at room temperature in the dark. As a positive control for RDX degradation (i.e. a zone of clearing on

solid media), *Rhodococcus* strain A (Jones et al. 1995) was also plated on media containing RDX.

Colonies selected for mineralization studies were grown in 5 mL liquid MSM-N+RS overnight at room temperature with shaking at 150 rpm and the $O.D._{600}$ was determined. Serum microcosm bottles containing MSM-N were amended with 17 mM or 170 mM succinate and 5 mg/L yeast extract and inoculated with pure culture to a final $O.D._{600}$ between 0.02-0.05 (at a final total volume of 20 mL). Microcosms were set up in triplicate and the negative control was identical but had no bacterial culture. Radio labeled (^{14}C) and unlabeled RDX was added as an acetone solution to a final concentration of 50 mg/L and 100,000 dpm. Both the [U-^{14}C] labeled RDX (specific activity of 28.7 µCi/mmol and >99% purity) and unlabeled RDX were supplied by the Defence Research Establishment Valcartier, Val Bélair, Québec, Canada. The serum bottles were incubated at room temperature with shaking at 150 rpm and sampled for production of $^{14}CO_2$ as described in Chapter 3.

4.4.3. Method 3

Isolation of RDX degraders was also attempted 644 days after the columns were spiked with RDX. A combination of 1/10 strength tryptic soy agar (1/10 TSA) with or without 200 mg/L RDX (1/10 TSA±R) and MSM±N±R+S (i.e. MSM-N+S, MSM-N+RS, MSM+N+RS) was used. A tenfold dilution series was prepared from a 3 g soil sample originating from sample port 2 of the RDX contaminated soil column. Dilutions 10^{-4} to 10^{-6} were plated out and incubated at room temperature in the dark. Parallel enrichment cultures were also started at the same time by inoculation of 45 mL media with 5 mL of dilutions 10^{-1} to 10^{-6}. The liquid media used were 1/10 tryptic soy broth with and without 100 mg/L RDX and MSM-N+RS and incubation conditions were 24°C with shaking at 150 rpm in the dark.

Selected colonies of the directly plated dilutions were re-streaked on the same media as the initial plating, with incubation under the same conditions. After one week, enrichment cultures grown in MSM-N+RS originally inoculated with dilutions 10^{-1} to 10^{-3}, were plated (dilutions 10^{-1} to 10^{-6} of the enrichments plated) on MSM-N+RS and 1/10 TSA+R and incubated at room temperature in the dark. One week later, a total of 66 colonies were streaked onto 1/10 TSA±R.

The selected colonies were from the re-streaks of the original direct plating and from the dilution series originating from the enrichments. All plates were incubated in anaerobic chambers (Difco Laboratories, Detroit, Michigan, USA) under a CO_2 + H_2 atmosphere at room temperature in the dark. The chamber was opened once a week to check the plates for zones of RDX clearance at which time the CO_2 + H_2 atmosphere was replenished. An anaerobic indicator strip supplied with the reaction pouches was always included in the anaerobic chambers and indicated that the chambers remained anaerobic.

4.5. Results

4.5.1. Method 1

Previous mineralization studies on the uncontaminated soil (see Chapter 3) indicated the presence of an indigenous RDX degrading bacterial population. The first attempt at isolation of RDX degraders was based on bacterial enrichment from this uncontaminated soil. After three subcultures in liquid MSM-N+RY, the final enrichment plated on solid MSM-N+RY possessed a wide range

of colony morphologies. After several weeks of incubation, no obvious zones of RDX clearing were observed around any of the colonies.

Direct plating of RDX degraders from the RDX contaminated column at day 61 also resulted in no obvious zones of RDX clearing. Total heterotroph counts for both columns at 1 and 2 weeks are presented in Table 4.1.

4.5.2. Method 2

The second method to isolate RDX degraders included the addition of succinate in the enrichment medium as an additional carbon source. Ammonium sulfate was also provided at low levels to stimulate the initial enrichment culture before RDX degradation could commence. The 'initial' enrichment cultures were inoculated with soil originating from the top and bottom (samples 2 and 6, respectively) of the contaminated and uncontaminated (A and B, respectively) soil columns from Chapter 3. Disappearance of RDX in the 'initial' culture, as measured in the enrichment culture supernatants, suggested that RDX biotransformation had occurred (Table 4.2.). After eight days, the slightly higher level of RDX in the A2 and A6 inoculated enrichments, in comparison to the B2 and B6 inoculated samples, suggests different rates of RDX degradation were

Table 4.1. Total heterotrophic plate counts.

	Sample Ports[a]					
Weeks	A2	A4	A6	B2	B4	B6
1	1.15×10^8	8.93×10^7	6.27×10^7	1.24×10^8	1.60×10^8	1.45×10^8
2	1.57×10^8	1.39×10^8	1.01×10^8	1.71×10^8	2.08×10^8	1.99×10^8

[a] Contaminated (A samples) and uncontaminated (B samples) soil columns. Sample ports 2, 4, and 6 are from the top, middle and bottom of the column, respectively. Values represent plating of triplicate samples for each dilution.

Table 4.2. Decrease of RDX concentration in enrichment cultures

Culture Conditions[b]	RDX Concentration (mg/L)[a] Initial		S/N
	8 days[c]	11 days	10 days
A2-N	79.6	49.0	51.2
A6-N	66.0	47.2	57.2
B2-N	59.2	47.0	50.0
B6-N	59.0	48.2	55.0
A2+N	75.6	49.2	63.7
A6+N	62.0	48.0	67.9
B2+N	57.6	47.4	60.4
B6+N	58.2	47.4	65.2

[a] RDX concentration at time 0 was 100 mg/L.

[b] Contaminated column (A), uncontaminated column (B), top of columns (2), bottom of columns (6), no additional nitrogen source (-N), nitrogen amended (as 50 mg $(NH_2)_4SO_4$/L) (+N)

[c] Days after inoculation

occurring. These levels were similar after eleven days of incubation, when subculturing was performed. RDX levels in the 'S/N' enrichments also demonstrated a decreasing trend, but the enrichments containing no additional nitrogen demonstrated a higher level of RDX removal than those cultures amended with ammonium sulfate.

As was observed during the first attempt at isolating RDX degraders, a large variety of colony morphologies were present after plating of the enrichments. Approximately 65-70 colonies were re-streaked, along with the known RDX degrader *Rhodococcus* strain A. The only colony type to produce a zone of RDX clearing was *Rhodococcus* strain A4. Due to contamination on several plates, all colonies were re-streaked at least once more. This resulted in the loss of several colonies. A rapid examination of the RDX mineralization capacity and effect of two different carbon source concentrations was performed with 3 of the more robust colonies, (i.e. 6, 11, and 23) (Figure 4.2.). These isolates, along with *Rhodococcus* strain A, were used to inoculate MSM-N+ yeast extract (50 mg/L) microcosms containing either 17 mM or 170 mM succinate. After 30 days of mineralization, the microcosms containing 17 mM succinate appeared to

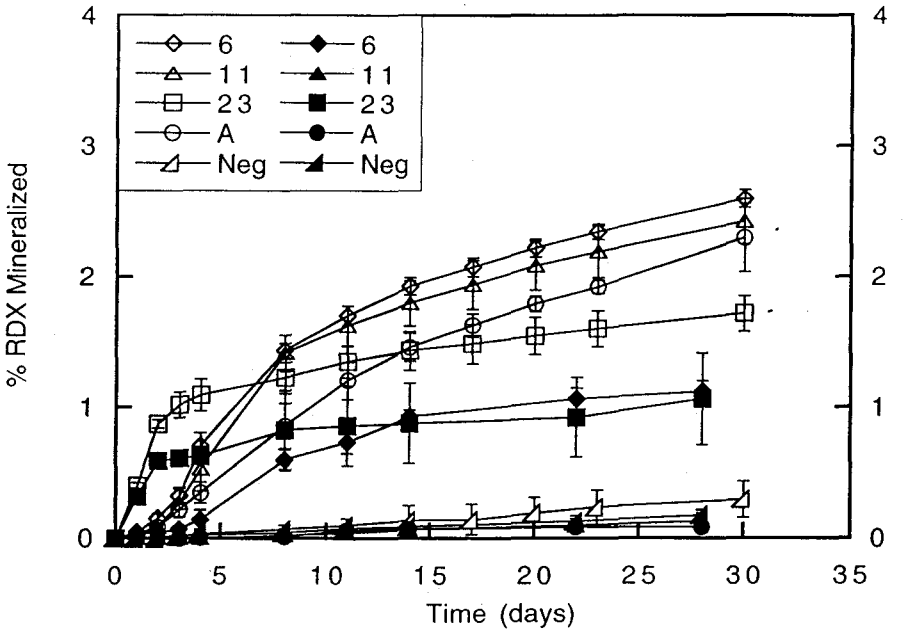

Figure 4.2. Mineralization of RDX by soil isolates. Mineralization of RDX in MSM-N+ yeast extract (50 mg/L) with the addition of 17 mM and 170 mM succinate (open and closed symbols, respectively). Soil bacterial isolates were labeled 6, 11, and 23, and the positive control *Rhodococcus* strain A was labeled as A. Microcosms incubated at room temperature with shaking at 150 rpm. Error bars represent the calculated standard deviation for triplicate samples.

mineralize more RDX than those containing 170 mM succinate. The range of RDX mineralization observed was 0.1% to 2.6%.

4.5.3. Method 3

In the third attempt at isolation of RDX degraders, a richer medium (1/10 TSA) was included so that potential degraders requiring additional sources of carbon could be accommodated. Examination of the direct platings demonstrated a wide variety of colony morphologies, but did not reveal any zones of RDX clearance. In the case of the enrichment cultures, after one week of incubation, all of the 1/10 TSA±R cultures appeared to be heavily overgrown so only the MSM-N+RS cultures were used for plating. A wide diversity of colony morphologies was also observed from the enrichment culture plating, although no zones of clearance were observed.

Growth of the re-streaked colonies from the direct plating and enrichment series under anaerobic conditions was performed to determine if RDX degradation might be limited to anaerobic microorganisms or conditions. After 3-4 weeks of incubation, approximately two thirds of the colonies grew under the anaerobic conditions. No zones of RDX clearance were observed under

anaerobic atmosphere. One additional observation made was that of all the microorganisms which grew on the 1/10 TSA plates also grew on the 1/10 TSA+R plates, suggesting that the toxicity of RDX on solid media was not significant for these colony types.

4.6. Discussion

The initial selective enrichment of RDX degrading bacteria from an uncontaminated soil was based on previous studies demonstrating the use of RDX as a sole nitrogen source (Binks et al. 1995; Jones et al. 1995; Boopathy et al. 1998; Coleman et al. 1998; Ronen et al. 1998). For this reason, the medium used was modified to significantly reduce the amount of available nitrogen (MSM-N). The soil used for inoculation of the enrichment cultures had been previously demonstrated to posses an indigenous bacterial population with an inherent RDX mineralization capacity (Chapter 3). Selective plating of the enrichment cultures on MSM-N, with RDX as the sole nitrogen source, resulted in the growth of a large number of distinct colony morphologies. Despite this apparent diversity, no colonies appeared to degrade RDX, as determined by the lack of zones of RDX clearance. This method of colony screening was chosen as it has proven

effective for the selection of microorganisms possessing biodegradative capabilities against compounds having low aqueous solubilities such as phenanthrene (Greer et al. 1993) and the observed clearance of RDX by *Rhodococcus* strain A, previously isolated in our lab (Jones et al. 1995).

The second method for the enrichment and isolation of RDX degraders used experimentally contaminated soil from the columns in Chapter 3. In addition to this, succinate was included in the MSM-N medium as an alternative carbon source. This was based on observations that RDX degraders can not use RDX as a sole source of carbon (Binks et al. 1995; Jones et al. 1995; Boopathy et al. 1998; Coleman et al. 1998; Ronen et al. 1998) and that indigenous sources of carbon might be limiting. Measured RDX concentrations in the culture supernatants decreased over time, suggesting the presence of an active RDX degrading bacterial species or population. The slightly higher level of RDX observed after eight days in the 'initial' enrichment supernatants originating from the A column (contaminated) versus the B column inoculated enrichment samples is most likely due to carry over of RDX from the contaminated soil. The concentration of RDX was measured 120 days later from ports 2 and 6 and was found to be 636 mg/kg and 14 mg/kg, respectively. With the 10 % inoculum used

for the 'initial' cultures, the A2 enrichments potentially contained 160 mg/L RDX. The levels actually transferred were most likely substantially higher as there were 120 days between inoculation of the enrichments and measurement of RDX concentration within the column. Because the levels of RDX removal were the same after eleven days, the rate of RDX degradation in the A enrichments must be higher than those from the uncontaminated B enrichments. The 'S/N' enrichments on the other hand demonstrated a clear distinction based on the presence or absence of ammonium sulfate. Enrichments without the additional nitrogen source reduced RDX to lower levels than the amended enrichments. This agrees with other studies observing that RDX will be used as a source of nitrogen only when other sources have been exhausted (Binks et al. 1995; Coleman et al. 1998; Freedman and Sutherland 1998).

Mineralization of RDX by three isolates was observed, but at very low levels. The known RDX degrader *Rhodococcus* strain A also demonstrated reduced mineralization rates compared to previous work in bioaugmented soil (Jones et al. 1995). This suggests that conditions suitable for significant mineralization of RDX were not provided. Despite this fact, the three isolates

were not studied further as they did not demonstrate RDX clearance on solid media, which was observed with *Rhodococcus* strain A.

The first two methods relied on aerobic degradation of RDX, as has been demonstrated by other groups (Binks et al. 1995; Jones et al. 1995; Coleman et al. 1998). The third isolation method included a rich medium and anaerobic conditions due to observations by other groups that faster rates of RDX degradation and mineralization can occur under O_2 limited or anaerobic conditions (McCormick et al. 1981; Funk et al. 1993; Kitts et al. 1994; Young et al. 1997; Boopathy et al. 1998; Freedman and Sutherland 1998; Shen et al. 2000). As observed with the first two methods, a wide diversity of colony morphologies was observed, with a significant proportion facultatively anaerobic. Despite the high degree of diversity observed, combined with the longer exposure to RDX (columns sampled after 644 days), no RDX degraders were isolated from the contaminated soil column.

The inability to isolate RDX degrading pure cultures was likely due to several factors. These include; failure to provide the proper growth conditions for RDX degraders in the contaminated column, the inability for RDX clearance on solid media to adequately detect RDX degraders or that RDX degradation and

mineralization in this particular soil may be due to a bacterial consortium. The degradation of RDX by bacterial consortia has been observed by others (Young et al. 1997; Boopathy et al. 1998; Boopathy et al. 1998). The conditions used to isolate a pure bacterial culture, capable of degrading RDX, does not provide the appropriate conditions for isolation of bacterial consortia. Additional experiments focusing on bacterial consortia may have successfully isolated an active RDX degrading consortium, although time became a limiting factor during this stage of work.

Another potentially important factor explaining why no RDX degrading isolates were obtained may be that in almost all other studies, the environments used to inoculate enrichments had histories of long-term RDX contamination. The soil and soil bacteria in this study were exposed to RDX for under 2 years. The lack of obvious changes in the bacterial populations related to RDX exposure observed in Chapter 3, i.e. less than the detection limit of 1% of the total population, suggested that no significant enrichment of RDX degraders occurred in the soil columns. This suggests that the concentration of RDX used was below a stimulatory threshold necessary for selective enrichment of RDX degraders. This may be related to the low aqueous solubility of RDX and the periodic nature

of RDX exposure to the bacterial community. This suggests that the biodegradation of RDX in the contaminated soil column, under *in situ* field conditions, is due primarily to the action of a bacterial consortium and not a single species.

Connecting Text

The soil used in the column experiments (Chapters 3 and 4) had demonstrated RDX mineralization under non-saturated conditions. The degradation of RDX under saturated conditions in soil columns was examined. The column set up was modified to enable buffer re-circulation and simulate previously proven RDX biodegradation technologies. David Juck performed all of the experimental work, accompanying analysis, and writing of the chapter.

Chapter 5. Flooding of Soil Columns

5.1. Abstract

Flooding of soil columns previously contaminated with RDX (1000 mg/kg soil) was performed in an attempt to stimulate the biodegradation of RDX under low oxygen and/or anaerobic conditions. The complete removal of leachable RDX was accomplished in 18 days by the recirculation of a citrate amended phosphate buffer, and was accompanied by the transient appearance of reduced RDX intermediates MNX, DNX, and TNX. Measured leachate oxygen levels in both the control and contaminated columns decreased to 1.0 mg/L within 1 day, and remained at that level for the remainder of the experiment.

5.2. Introduction

Removal of RDX from terrestrial systems and waste water streams is important in the remediation of contaminated sites and the prevention of further contamination, respectively. To this end, several approaches have been studied.

Although the biodegradation of RDX has been observed under aerobic conditions (Binks et al. 1995; Jones et al. 1995; Coleman et al. 1998), the

preponderance of studies suggest that the most efficient method of treating large scale RDX contamination or industrial RDX waste is through the use of anaerobic systems. This includes nitrate and sulfate reducing conditions in soil slurries or waste water streams (Light et al. 1997; Boopathy et al. 1998; Freedman and Sutherland 1998), inoculation of anaerobic soil slurries with municipal anaerobic sludge (Hawari et al. 2000; Shen et al. 2000; Halasz et al. 2001), or the use of a combined oxic-anoxic system under nitrogen-limiting conditions in waste water streams (Ronen et al. 1998).

Detailed work on the (bio)degradation of RDX has revealed that the initial attack on RDX appears to be biologically mediated (Halasz et al. 2001), the direct or indirect RDX cleavage product methylenedinitramine, in aqueous solution, spontaneously decomposes to N_2O and formaldehyde (Hawari 2000; Halasz et al. 2001), and the resulting formaldehyde can be transformed to CO_2 through formate by methanogens and acetogens, respectively (Hawari 2000). Several potential pathways that lead to the formation of methylenedinitramine are postulated, and were presented in Chapter 1 (Figure 1.6.1.).

Previous work has demonstrated that flooding of contaminated soil and provision of organic substrates provided the proper physico-chemical conditions

for the rapid removal of RDX (Greer et al. 1997). Based on these results, and other work performed in our lab on a second nitramine explosive octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in contaminated soil, flooding of the columns was performed using a phosphate buffered citrate solution. The observed rapid disappearance of RDX, the transient appearance of RDX reduction intermediates MNX, DNX, and TNX, and the low oxygen concentration measured in the leachate, suggested the presence of an RDX degrading population which functioned at significant rates under low oxygen/anoxic conditions.

5.4. Materials and Methods

5.4.1. Column Flooding

The RDX contaminated and uncontaminated soil columns described in Chapter 3 were flooded at day 476 (after the first RDX spiking) with approximately 1.3 L and 1.0 L of a 100 mM phosphate/citrate buffer (29.41 g/L tri-sodium citrate, 8.19 g/L di-basic sodium phosphate, 5.84 g/L mono-basic sodium phosphate, pH 7.0), respectively. Flooding was performed from the bottom up

using a peristaltic pump, as shown in Figure 5.1. All of the tubing used was Teflon except for the connections (Tygon), and through the peristaltic pump head (Pharmid). The two T junctions used (manometer-peristaltic pump splitter (b) and sampling port (c)) were made of glass. The T connector for the sampling port possessed one threaded end, upon which was installed a Teflon lined septa held in place by a threaded cap with no center section. The columns were flooded so that there was approximately 5-7 cm of buffer on top of the soil surface, and this level was marked on the side of each column. At day 14, several mL of buffer was added to both columns to return liquid levels to the original levels. The flow rate for the two columns (in a downward direction) was set at 2 mL/minute. The installed manometer was regularly inspected to ensure that a vacuum was not created within the column due to the action of the peristaltic pump. Creation of a vacuum was indicated by the buffer level in the manometer falling below the bottom of the column.

5.4.2. Column Sampling

Sampling of the column leachate for the determination of RDX concentration and O_2 concentration was performed in the following manner: the clamp located

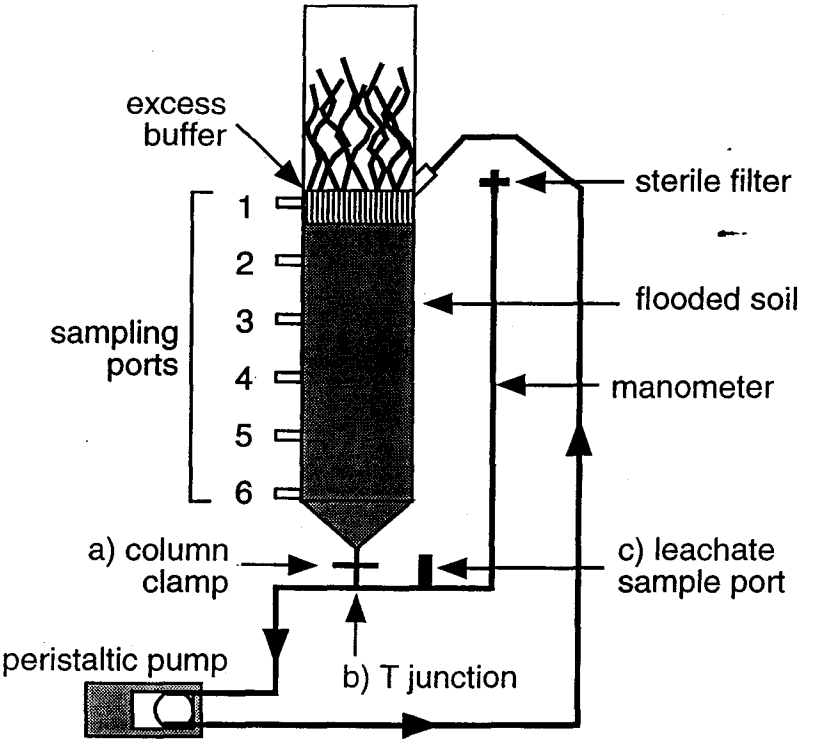

Figure 5.1. Schematic of flooded soil columns. Buffer was circulated at 2 mL/minute in the direction indicated. The portion of the columns containing the flooded soil was covered with aluminum foil.

at the base of the column (a) was closed, the peristaltic pump was increased to maximum speed and the liquid present in the manometer was evacuated past the T junction at the base of the column (b). The clamp was then opened, and leachate was allowed to flow freely until reaching the sampling port (c), at which point a 5 mL sample was taken and the peristaltic pump re-started.

5.4.3. Analytical Methods

Analysis of leachate samples included O_2 concentration, RDX and nitroso metabolites (MNX, DNX, and TNX), citrate, and anions (NO_2^-, NO_3^-, HPO_4^{2-}, and SO_4^{2-}). Measurement of leachate O_2 was performed using a YSI Model 5300 Biological Oxygen Monitor (YSI Inc., Yellow Springs, Ohio, USA) equipped with a YSI 5331 Standard Oxygen Probe. Measurement of O_2 was performed immediately after sampling using 3 mL of leachate, which was carefully injected into the measuring chamber through the side channel of the lucite plunger. The plunger was depressed until all remaining air was expelled, the stirrer was then started and the value recorded once the readings had stabilized. The probe was assembled according to the manufacturers instructions using 2 M KCl and high

sensitivity membranes and calibrated before use with O_2 saturated H_2O (and recorded as 100% saturation) which was equivalent to 8.5 mg O_2 /L H_2O.

Of the remaining 2 mL leachate sample, 1 mL was used to measure citrate and anion concentrations. The sample was diluted 1:10 using Zenopure water and anions were measured using a Hamilton PRP-X100 polymer-based chromatography column (250x41mm O.D.) on a high-performance liquid chromatograph Spectra-Physics model SP8800 & SP8760. The mobile phase consisted of 4.0 mM p-hydroxybenzoic acid (pH 8.5) with 2.5 % methanol at a flow rate of 2 mL/min and a column temperature of 40°C. Conductivity data was obtained by using a Waters Millipore detector model 431. Citrate was measured by capillary electrophoresis and UV detection using a Hewlett-Packard HP^{3D} CE System (Hewlett-Packard, Wilmington, Delaware, USA), consisting of a photodiode array detector and an HP fused silica bubble capillary (inside diameter, 50 µm; length, 56 cm) (Hawari et al. 2000).

The remaining 1 mL of leachate was used for RDX and its reduction intermediates. The sample was diluted 1:1 with acetonitrile, placed at 4°C for at least 30 minutes, and filtered using Millipore Millex-GP 0.2 µm syringe filters (Millipore, Mississauga, Ont.). Measurement of aqueous RDX and nitroso

metabolites was performed as described in Chapter 3. Quantitative standards for MNX, DNX, and TNX were not available at the time of sampling, so only the peak areas was monitored. Estimation of the total RDX concentration remaining in the column before flooding was performed on samples from ports 2, 4, and 6 using EPA method 8330. Approximately 1.5 g of soil/slurry was removed from each port, frozen at -80°C and then lyophilized overnight. Soil was then sieved (1.0 mm) and 1.0 g soil mixed with 5.0 mL of acetonitrile in a 15 mL screw cap glass tube. The sample was sonicated overnight at 23°C and then left to settle for 30 minutes. An aliquot of the sonicated slurry (2.5 mL) was transferred to a clean tube, mixed with freshly prepared $CaCl_2$ solution (5.0 g/L) and incubated statically for 15 minutes. The supernatant was then filtered (2-3 mL) using Millipore Millex-HV syringe filters (0.45 µm pore size) and analyzed for RDX using the above method.

5.5. Results

Prior to flooding of the columns, the concentration of RDX was determined at the top, middle and bottom of the contaminated column (port 2, 4, and 6, respectively). At port 2, where RDX was initially added (2.9 g total), it was

measured at 636 mg/kg. At ports 4 and 6, RDX concentrations were measured at 26 mg/kg and 14 mg/kg, respectively.

After flooding of the columns, RDX concentrations measured in the leachate (Figure 5.2.A.) reached a peak of 18 mg/L after 4 days, followed by a steady decrease until it was non-detectable at day 20. At no time did the leachate become saturated with RDX (~ 40 mg/L). The nitroso- intermediates of RDX, reduction, MNX, DNX, and TNX, were all transiently observed in the column leachate. MNX levels peaked at day 4 and were not detected by day 15. DNX was first detected on day 7 and was not detected after day 13 while TNX was only observed on day 7.

The level of O_2 observed throughout the duration of the experiment remained at a relatively stable level (Figure 5.2.B.). For the contaminated column, O_2 remained at approximately 1.0 mg/L, and only decreased transiently at day 15. The uncontaminated column demonstrated more fluctuation around the 1.0 mg/L level, reaching concentrations of 2.3 mg/L on day 21 and non-detect levels near the end of the experiment.

Citrate levels in both columns decreased rapidly, reaching non-detect levels after 1 and 4 days for the uncontaminated and contaminated columns,

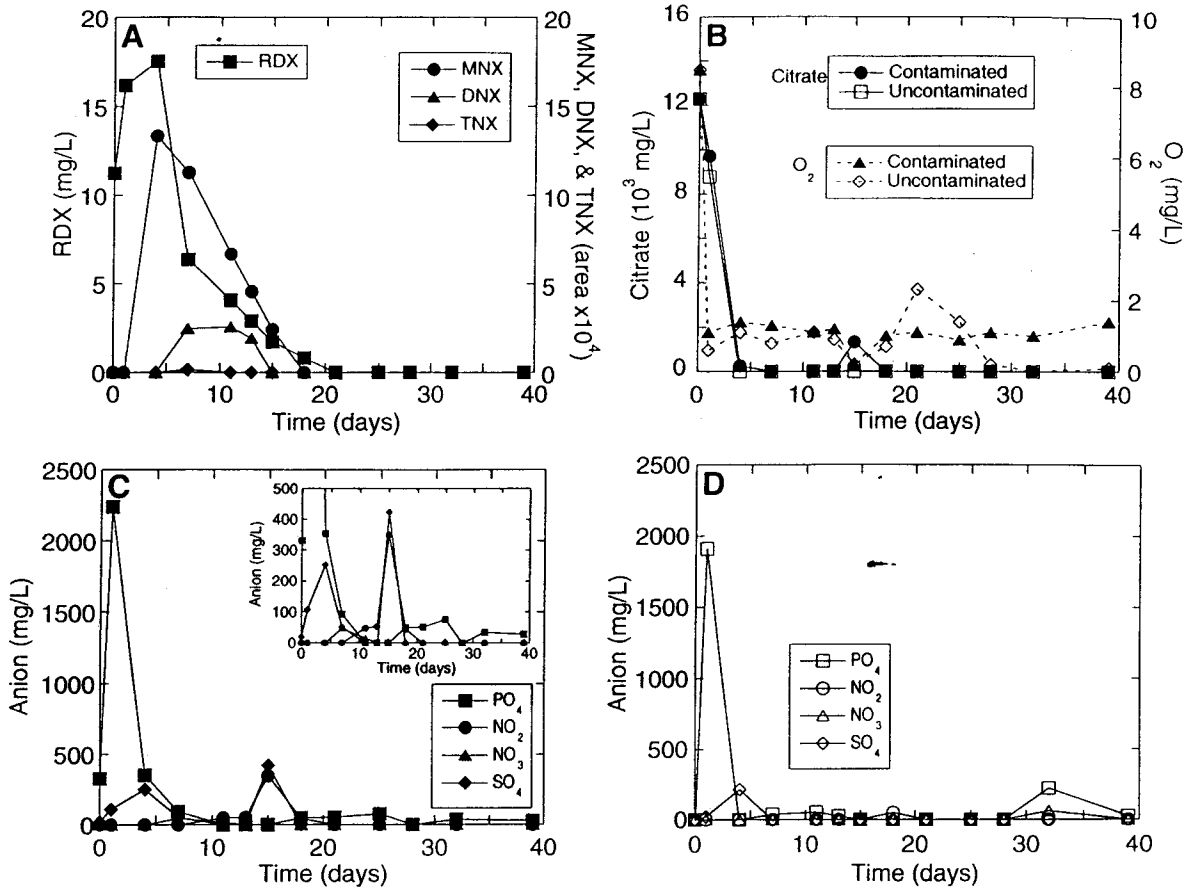

Figure 5.2. Chemical parameters measured in contaminated and uncontaminated leachates. A) RDX and RDX reduction products MNX, DNX, and TNX measured in the contaminated column leachate. Chemical standards for the reduction products were not available, so the area under the curve (x10^4) is displayed. B) Citrate and O_2 concentrations measured in both column leachates. C) Anion concentrations measured in the contaminated column leachate. Inset is an exploded view of the same graph showing anion fluctuations. D) Anion concentrations measured in the uncontaminated column leachate.

respectively (Figure 5.2.B.). In the contaminated column, a transient spike of 1333 mg citrate/L was detected in the leachate on day 15.

Anion levels in the column leachates remained stable throughout the experiment, with several exceptions (Figure 5.2.C, D.). Phosphate, which was present in the buffer, started at high levels (2236 mg/L and 1911 mg/L in the contaminated and uncontaminated columns, respectively) and decreased to levels between 0 mg/L and 75 mg/L after 1 week. In the uncontaminated column, a peak in phosphate levels of 222 mg/L was observed on day 32. Nitrite was only observed in the uncontaminated column at day 18, whereas in the contaminated column, it was present between day 11 and day 18, and reached a peak of 349 mg/L on day 15. Nitrate was also only observed at one time point in the uncontaminated column (62 mg/L on day 32) and on days 7 and 11 in the contaminated column (45 mg/L and 16 mg/L, respectively). Sulfate levels in both the contaminated and uncontaminated columns peaked on day 4 (252 mg/L and 217 mg/L, respectively), and decreased to non-detectable levels on day 11 and 7, respectively. In the contaminated soil column, there was a transient peak in leachate sulfate levels of 423 mg/L on day 15.

5.6. Discussion

Prior to flooding of the columns, RDX levels in the leachate (Chapter 3) reached a peak of 17 mg/L after 47 days, while after flooding, the peak RDX concentration observed was 18 mg/L at 4 days. The complete disappearance of RDX under flooded conditions occurred after 18 days, whereas the lowest RDX levels under non-saturated conditions occurred after 145 days (< 1mg/L). Flooded conditions resulted in a significant increase in the rate of RDX removal from the system. Explanation of this increased rate can be attributed to the combination of microbial adaptation to and increased bioavailability of RDX. Prior to flooding, the soil microbial population was exposed to RDX for 476 days. Under the previous non-saturated conditions, exposure of the soil microbial population to RDX would be periodic in nature as RDX was dissolved and transported from the surface downward. Flooding of the column likely resulted in desorption of loosely bound RDX (Pennington et al. 1999; Sheremata et al. 2001) resulting in increased bioavailability and sustained exposure of the soil microbial populations to RDX. This in turn resulted in the observed faster rates of biotransformation and removal.

The disappearance of RDX was also accompanied by the transient appearance of the RDX reduction intermediates MNX, DNX, and TNX. These intermediates are typically found under anaerobic degradation conditions (McCormick et al. 1981; Hawari et al. 2000) and when soil is used as a source of inoculum (Halasz et al. 2001; Sheremata et al. 2001), suggesting that conditions in the column were anaerobic. Anaerobic microenvironments within the soil matrix would be stimulated and/or augmented under the low oxygen concentrations measured in the leachate. Oxygen levels dropped to approximately 1 mg/L immediately after flooding of the columns and consistently remained at this level. The presence of citrate in the buffer provided a readily available carbon source for heterotrophs, which in turn would result in a rapid depletion of oxygen. The soil used in the column experiments had a relatively high in organic matter content (8.9%), which would sustain heterotrophic activity and deplete oxygen levels. The observed drop in oxygen levels at day 15 corresponds to the addition of fresh buffer at day 14. There was also a spike in the citrate level of the contaminated column leachate, but not in the uncontaminated column leachate. This may be due to a smaller addition of fresh buffer to the uncontaminated column.

The anion concentrations observed in the leachate showed several fluctuations, but remained at relatively constant levels throughout the flooding. The buffer phosphate concentrations decreased rapidly and remained at low levels throughout the experiment. Bioavailable phosphate in the column may have been limiting, and upon addition would have been readily utilized by the microbial population. The concentration of sulfate in both columns demonstrated a slight spike at 4 days, which may be attributable to anionic exchange of bound sulfate and the added phosphate. Under the low oxygen conditions present in the columns, sulfate would readily be used as an electron acceptor, resulting in its rapid disappearance. The second spike of sulfate observed in the contaminated leachate at day 15 may also be due to the addition of fresh buffer at day 14 and the resultant anionic exchange. The spike in nitrite concentration of the contaminated soil column at day 15 is not readily explainable, but may be associated with the addition of fresh buffer. The absence of a spike in sulfate and nitrite levels in the uncontaminated leachate may be due to the above-mentioned smaller addition of buffer resulting in a less pronounced affect.

The observed rapid rate of RDX removal from the contaminated column leachate is supported by other studies demonstrating significant rates of RDX

transformation under anaerobic treatment conditions (Light et al. 1997; Boopathy et al. 1998; Freedman and Sutherland 1998; Shen et al. 2000). The addition of citrate appeared to aid in the degradation of RDX by decreasing the oxygen concentration and/or providing an alternative carbon source that could be coupled to the utilization of RDX as a nitrogen source. This demonstrates that saturation of the experimental soil and provision of an alternative carbon source readily achieves the removal of RDX from terrestrial systems in a simple leachate recycling system.

Connecting Text

The demonstrated degradation of RDX in soil under saturated conditions (Chapter 5) was examined in more detail using a polyphasic approach similar to that employed in Chapter 3. Experiments were performed using 16S rDNA amplification and DGGE analysis combined with mineralization and chemical analysis of batch soil slurries contaminated with RDX. David Juck performed all of the experimental work, accompanying analysis, and writing of the chapter.

Chapter 6. RDX Removal in Soil Slurries

6.1. Abstract

The degradation of RDX under soil slurry conditions was examined using biochemical and molecular techniques. The addition of Na_3-citrate to aerobic slurries stimulated complete removal of 250 mg RDX in 300 days, whereas in unamended flasks more than 50% of an initial 50 mg RDX remained after the same time period. The effect of Na_3-citrate on anaerobic slurries was the opposite, as determined by RDX mineralization in microcosms. In the presence of Na_3-citrate, only 40% of the RDX was mineralized after 94 days, while in unamended slurries 90% mineralization was observed after 42 days. Mass balance analysis of the microcosms revealed that over 90% of the ^{14}C label was mineralized or remained in a water soluble (bioavailable) form. PCR-DGGE analysis of the aerobic slurries revealed the stimulation of several environmentally important bacteria, including *Sphingomonas* spp., members of the *Alcaligenaceae* group, and a *Stenotrophomonas* sp. These results suggested that a bacterial consortium is involved in the complete degradation of

RDX, and the different members/mechanisms involved are determined by the environmental conditions.

6.2. Introduction

The need to remediate RDX contaminated soils has led to the development of biologically based technologies. In most cases, these systems are contaminated soil slurries run under anaerobic conditions (Funk et al. 1993; Young et al. 1997; Boopathy et al. 1998; Freedman and Sutherland 1998; Shen et al. 2000) or combined oxic/anoxic stages (Ronen et al. 1998). Specific treatment conditions included nitrogen deficiency (Ronen et al. 1998), nitrate-reduction (Freedman and Sutherland 1998), amendment of soil slurries with anaerobic municipal sludge (Shen et al. 2000), sulfate-reduction (Boopathy et al. 1998), carbon source amendment (Funk et al. 1993) or inoculation with horse manure (Young et al. 1997).

Previous work based on flooding of contaminated soil columns (Chapter 5) demonstrated a significant increase in the rate of RDX degradation using a sodium phosphate-buffered Na_3-citrate solution. To further explore the effect of flooding and Na_3-citrate amendment on RDX degradation and the indigenous

bacterial community, a series of soil slurry batch flasks were set up. RDX and reduction intermediate levels were monitored, along with shifts in bacterial community structure as determined by PCR-DGGE analysis.

6.4. Materials and Methods

6.4.1. Soil Slurry Flasks

A total of eight flasks (labeled 1 to 8) were set up containing 50 g of 2.0 mm sieved soil (soil used in the soil columns and stored at 4°C) and 450 mL of sterile 20 mM sodium phosphate, pH 7.0, made with tap water. In half of the flasks (odd numbers), 50 mM Na_3-citrate was included. Flasks were paired (with or without Na_3-citrate addition) for the purpose of comparing the effect of Na_3-citrate on RDX degradation. To this end, 50 mg RDX was added (as crystals) to flasks 1 and 2, 125 mg RDX was added to flasks 3 and 4, and 250 mg RDX was added to flasks 5 and 6. Flasks 7 and 8 were control flasks and did not contain RDX. All flasks were incubated in the dark at 24°C with shaking at 150 rpm.

6.4.1.1 Sampling for RDX and PCR-DGGE

Periodically, 2 mL of slurry sample was removed from each flask immediately after shaking was stopped, placed in a 2 mL screw cap tube, spun for 20 minutes at 16,000 x g at 4°C. The supernatant was removed, filtered using 0.22 µm Millex-GP syringe filters (Millipore, Mississauga, Ont.), diluted 1:1 with acetonitrile and stored at 4°C until analysis, as described in Chapter 3. The remaining slurry pellet was frozen at -20°C until DNA extraction and purification was performed as described (Chapter 2) on samples taken day 1, 16, 29 and 64. Amplification of an approximately 500 bp fragment of 16S rDNA was performed using primer pairs 8FGC-519R and b341GC-u758 as described in Chapter 3. Amplicons were separated by DGGE using a gradient of 40 to 60% denaturant and selected bands were excised. Sequencing, raw sequence compilation and sequence comparison using the GenBank and EMBL databases was performed as described in Chapter 2.

6.4.2. Slurry Microcosms

Duplicate soil slurry microcosms were set up to determine if several treatment variables had a significant effect on RDX mineralization. The treatment

variables included citric acid versus Na_3-citrate and immediate anaerobic conditions (i.e. N_2 atmosphere) versus the heterotrophic depletion of oxygen, for a total of 6 different combinations. All microcosms contained 2 g of 2.0 mm sieved column soil (stored at 4°C) and 20 mM sodium phosphate buffer (pH 7.0) to a final volume of 20 mL. For microcosms containing either citric acid or Na_3-citrate, final concentrations were 100 mM.

All flasks were sealed with gas impermeable butyl rubber stoppers (Bellco Glass Inc, Vineland, New Jersey), and for anaerobic microcosms, serum bottles were completely assembled as described in Chapter 3, save for the radiolabeled ^{14}C-RDX. The anaerobic serum bottles went through 3 vacuum evacuation/flushing cycles consisting of 5 minutes vacuum evacuation followed by 5 minutes flushing with N_2 (Praxair Products Inc., Mississauga, Ontario), and leaving a final atmosphere of N_2. All microcosms were spiked with a total of 50 mg RDX/L, which was made up of ^{14}C-RDX and cold RDX, as described in Chapter 3. All microcosms were incubated with shaking at 150 rpm and at 25°C in the dark. The KOH traps contained in the microcosms were periodically sampled for $^{14}CO_2$ to monitor mineralization by removal of the total 1 mL of KOH solution to a scintillation vial, rinsing of the trap with an additional 1 mL of 0.5 M

KOH which was pooled with the initial KOH solution, and refilling of the trap with 1 mL of 0.5 M KOH. The pooled KOH solutions were then mixed with 18 mL of scintillation cocktail, and ^{14}C levels measured using a Tri-Carb 2100TR Liquid Scintillation Analyzer (Packard Instruments Co., Meriden, Connecticut, USA). All microcosms containing an N_2 headspace were sampled using Mininert valves (Supelco, Bellefonte, Pennsylvania, USA) and 0.5 M KOH flushed with N_2 as described above.

The oxygen levels within the air atmosphere microcosms were monitored using an SRI model 310 gas chromatograph (SRI Instruments, Torrance, California, USA), equipped with a 2 meter molecular sieve column (60°C) and a thermal conductivity detector (TCD) set at 100°C, using helium as the carrier gas (25 mL/minute). Before sampling of the microcosms for $^{14}CO_2$, 0.5 mL of headspace was sampled for oxygen levels.

The effect of the amendments on soil slurry pH was determined aerobically using 50 mL slurries set up identically to the microcosms above, but using autoclaved 50 mL Erlenmeyer flasks. The flasks were incubated in the dark at room temperature and pH was measured using an Orion model 720A pH meter

equipped with an Orion model 81-65 ROSS pH electrode (Orion Research Inc., Boston, Massachusetts, USA) at time 0 and 116 hours.

6.4.3. Microcosm Mass Balance

6.4.3.1. Carbonates and Water Soluble Phase

Microcosms were sacrificed after 94 days for mass balance using modified protocols (Allison 1960; Nelson and Sommers 1982; Nelson 1982). Following removal of $^{14}CO_2$ trapped in KOH, the microcosms were frozen at -80°C. Thawed microcosms, after the addition of 1.0 mL fresh 0.5 M KOH to the CO_2 traps, were acidified by the addition of 50 µL 0.05 N HCl and incubated at room temperature with shaking (150 rpm) for 24 hours. This acidification released $^{14}CO_2$, trapped as carbonates, which might have accumulated during mineralization of RDX. The KOH, containing any released $^{14}CO_2$, was sampled and measured as described above.

The microcosms were then opened, the soil slurry transferred to a 250 mL centrifuge tube and the serum bottle rinsed using distilled water, which was transferred to the centrifuge bottle. The bottles were centrifuged for 15 minutes

at 12,400 x g at room temperature and the supernatant carefully decanted into a 250 mL glass graduated cylinder. The pellets were then washed and centrifuged twice using 30 mL of distilled water. The washes were pooled with the initial supernatant, the water phase volume was recorded, and 2 mL was sampled for scintillation counting to determine the water soluble fraction. The soil pellet was transferred to a pre-weighed glass petri dish bottom, the volume of any rinse water required was recorded, the dish and soil were weighed and left to dry overnight. The dried soil and dish were re-weighed, and the difference (minus the volume of rinse water) represents the volume of water in the pellet, and was added to the above recorded water phase volume.

6.4.3.2. Humic and Fulvic Acid Fraction

The dried soil pellet was carefully crushed and transferred to a 50 mL Oak Ridge centrifuge tube (the weight of the transferred soil pellet was recorded), 30 mL of 0.1 N NaOH was added, and the tube was flushed with nitrogen for 5 minutes. The tubes were then tightly sealed, and shaken in a wrist action shaker (Burrell Corp., Pittsburgh, Pennsylvania, USA) for 24 hours at room temperature at 430 strokes/minute. The tubes were then centrifuged for 15 minutes at room

temperature at 12,400 x g, the supernatant was transferred to a 50 mL Falcon tube, and an additional 15 mL of 0.1 N NaOH was added to the soil pellet and mixed briefly. The resulting soil slurry was filtered using Whatman GF/F glass microfiber filters (0.7 µm pore size, 55 mm diameter) (Whatman International Ltd., Maiden, England) supported by a porcelain Buchner funnel, the filtrate was combined with the supernatant, and subsampled to obtain the humic acid/fulvic acid fraction.

6.4.3.3. Residual Organic Fraction

The filter and soil residue were carefully transferred to a 50 mL Falcon tube and frozen at -20°C until soil combustion was performed. Soil combustion was performed to determine the amount of ^{14}C present in the residual organic matter using the apparatus illustrated in Figure 6.1. (modified from Allison 1960). Cold water was circulated through the condenser column, 20 mL of fresh 1 M KOH was added to each of the KOH traps (50 mL vacuum flasks), and all connections were secured prior to combustion. The soil residue and filter were transferred to the combustion flask, 2 g $K_2Cr_2O_4$ crystals were added and the neck was rinsed with 3 mL distilled H_2O. The separatory funnel connection was tightened and

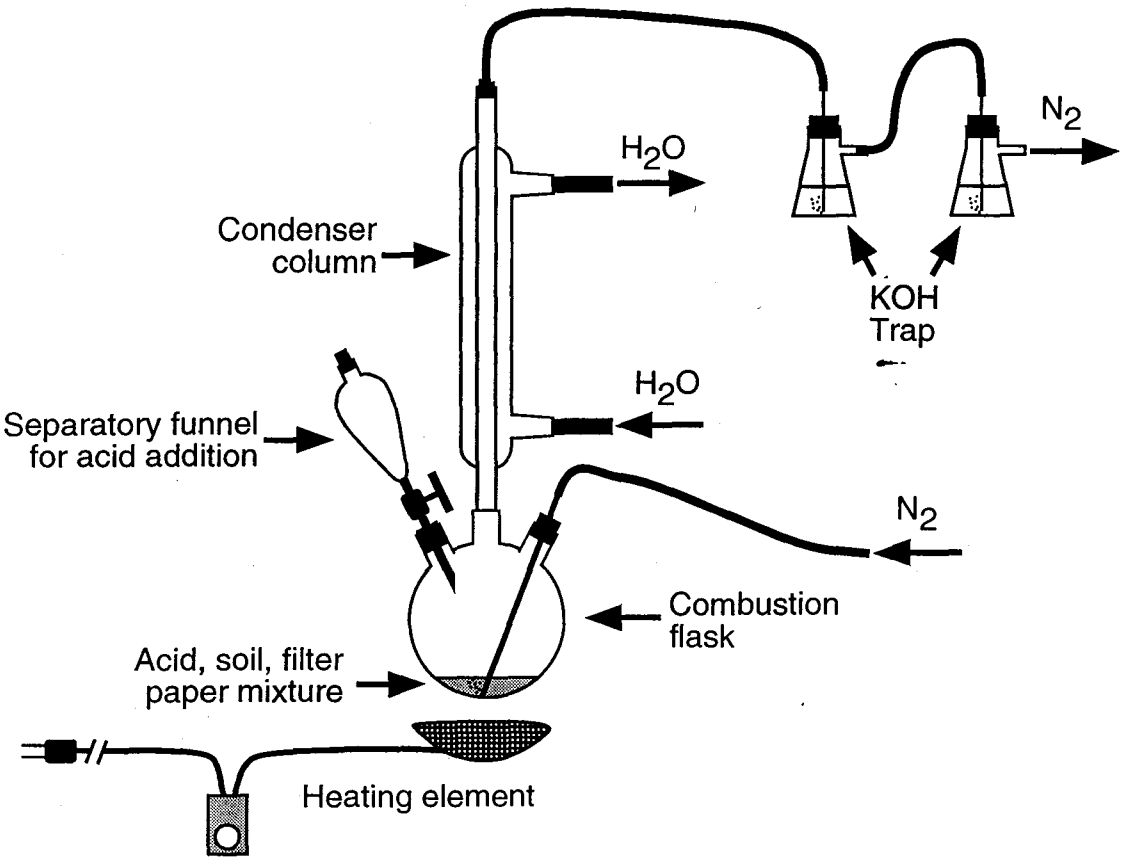

Figure 6.1. Schematic of the soil combustion apparatus.

25 mL of concentrated sulfuric acid:phosphoric acid (60:40) was added and the valve closed immediately after. Dinitrogen gas was then bubbled into the combustion mixture through a glass cannula (glass 2 mL pipette) at a rate of 2 bubbles /second, as measured in the KOH traps. The combustion flask was brought to boiling, at which point the heat was reduced and the mixture was boiled for 10 minutes. At this point, the heating element was turned off, removed, and the reaction flask was allowed to cool for 20 minutes with a bubbling rate of 6-8 bubbles/second. The gas was then turned off, the KOH were traps pooled and a 2 mL subsample was taken for scintillation counting.

6.5. Results

6.5.1. Soil Slurries

Addition of Na_3-citrate to the soil slurries resulted in an immediate change in appearance of the slurries. The larger aggregates seen in the slurries not amended with Na_3-citrate disappeared, and the color was a much darker brown. The non-amended slurries only reached this same appearance after several months of incubation.

RDX levels measured in all three unamended flasks (2, 4, and 6) reached saturation levels (~50 mg/L) after approximately 2-3 weeks (Fig. 6.2.A.). In the flasks containing Na_3-citrate (1, 3, and 5), saturation levels were never reached, and only in flask 5 (250 mg RDX) was a stable concentration plateau of 35 mg/L reached between days 17 and 25. In the remaining two Na_3-citrate flasks 1 and 3 (containing 50 mg RDX and 125 mg RDX, respectively), peak RDX concentrations of 18 mg/L and 31 mg/L were recorded between days 15 and 17 and 16 and 21, respectively.

MNX, the first reduction intermediate, was observed in all flasks containing RDX (Fig. 6.2.B.). In the flasks not amended with Na_3-citrate, the accumulation of MNX was linear and did not reach a plateau during the course of the experiment (301 days). Appearance of MNX in flask 6 (250 mg RDX) was observed on day 1, while in flasks 2 and 4 (50 mg RDX and 125 mg RDX, respectively), MNX was first detected on day 3. In the flasks containing Na_3-citrate, a similar trend was observed. Flask 5 (250 mg RDX) produced measurable MNX on day 1, while in flasks 1 and 3 MNX was observed on day 2. In the three Na_3-citrate amended flasks, the detection of MNX was transient.MNX peaks for flasks 1, 3, and 5 were observed at days 10, 10, and 8, respectively.

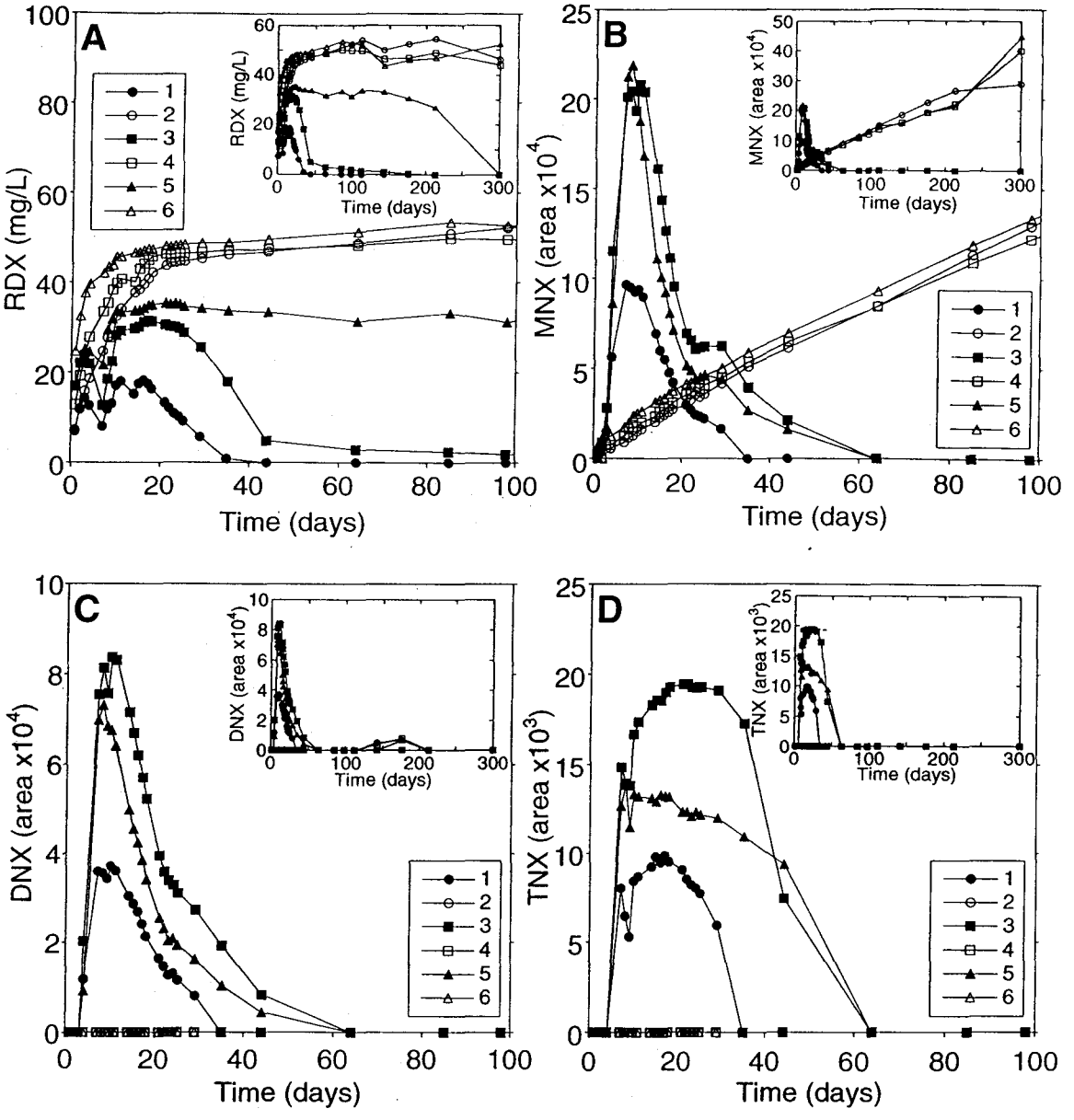

Figure 6.2. Monitoring of RDX and reduction intermediates in soil slurry flasks. Flasks 1, 3, and 5 amended with Na_3-citrate (solid symbols) and flasks 2, 4, and 6 unamended (open symbols). Amount of RDX added was 50 mg (flasks 1 and 2), 125 mg (flasks 3 and 4, and 250 mg (flasks 5 and 6). Insets display the complete experimental period of 301 days. Panels are (A) RDX concentration; (B) MNX level; (C) DNX level; (D) TNX level. Single samples taken at each time point.

In flasks 2 and 4, the second RDX reduction product, DNX, was observed transiently at days 142 and 177 whereas in flask 6, it was only observed on day 177 (Fig. 6.2.C). In all three of these flasks, the amount of DNX measured was significantly less than observed in flasks containing Na_3-citrate. Flasks 1, 3, and 5 also demonstrated peaks in DNX levels that coincided with MNX peaks, i.e. flasks 1 and 3 peaked on day 10 while flask 5 peaked on day 8. DNX was first detected in these flasks on day 4 and reached non-detectable levels at day 35 for flask 1 and day 64 for flasks 3 and 5.

TNX, the third reduction intermediate, was only detected in the flasks amended with Na_3-citrate and was first observed at day 7 (Fig. 6.2.D). In all three flasks, the TNX peak was also broader in nature as compared to RDX, MNX, and DNX. In flask 1, TNX peaked between days 15 and day 18, in flask 3 the peak was observed between day 18 and day 25, and in flask 5 the TNX peaked at day 8 (immediately after it first appeared on day 7) followed by a gradual decrease until day 44. TNX reached non-detectable levels in flask 1 at day 35, and in flasks 3 and 5 it was not detected by day 64.

6.5.2. Slurry Microcosms

In the microcosms possessing an air headspace, the disappearance of oxygen was rapid (Figure 6.3.). The Na_3-citrate amended microcosm decreased to 1% oxygen after 6 days, while the unamended and citric acid amended microcosms decreased to 1% oxygen after 9 and 13 days, respectively. Differences in RDX mineralization levels ranged from less than 1% to greater than 90%, depending on the amendment type (Figure 6.3.). The initial oxygen headspace microcosms all exhibited higher levels of mineralization (6%, 7% and 15% for unamended, citric acid amended and Na_3-citrate amended, respectively) than the nitrogen headspace microcosms, despite the type of amendment used. The slurries amended with citric acid showed very little mineralization, and after 94 days the nitrogen headspace microcosms only reached 1% mineralization while the oxygen headspace microcosms reached 8% mineralization. The Na_3-citrate amended microcosms with a nitrogen headspace reached 29% RDX mineralization, whereas the oxygen headspace samples reached 44% RDX mineralization. The unamended slurries reached the highest mineralization levels, 87% and 92% for the nitrogen and oxygen headspace microcosms, respectively.

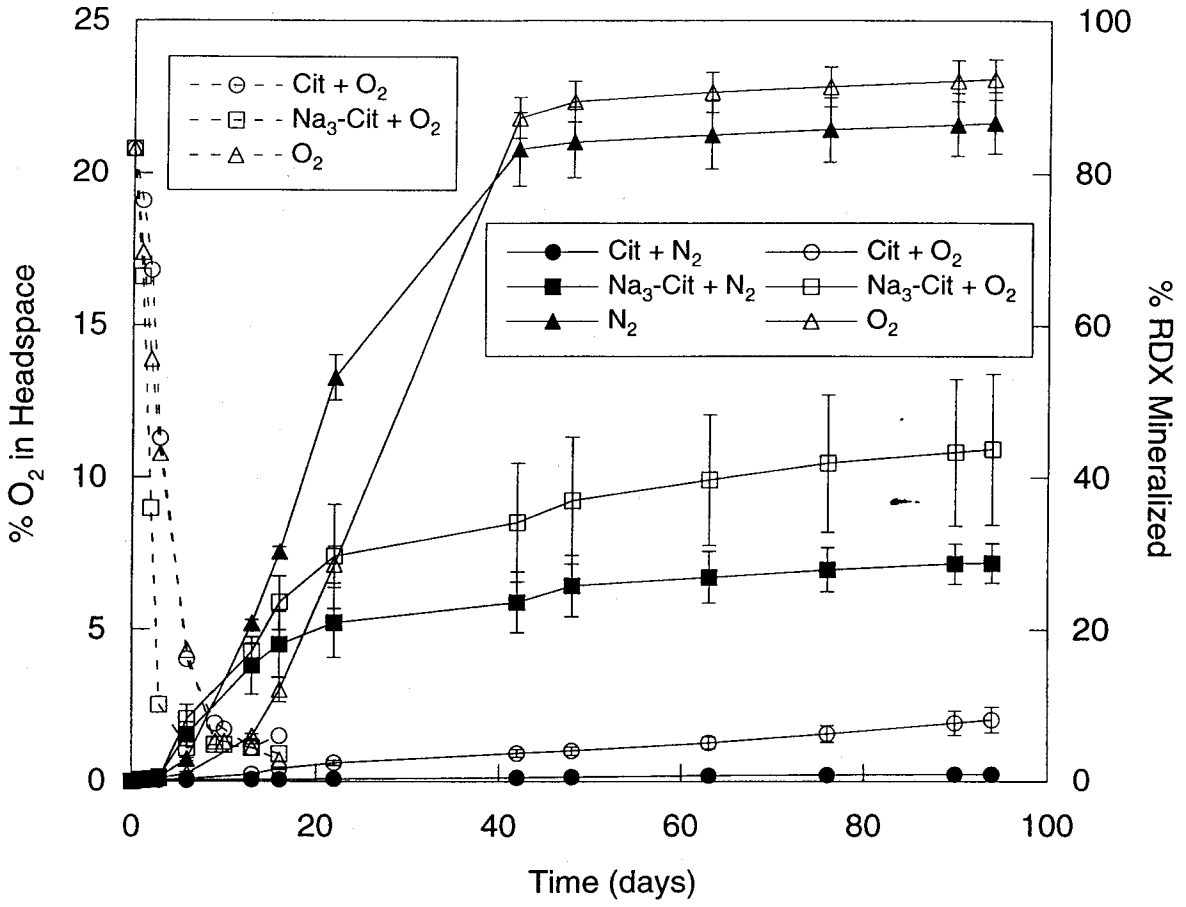

Figure 6.3. Oxygen concentration (broken lines) and RDX mineralization (solid lines) of citrate amended microcosms. Microcosms were incubated at 25°C with shaking (150 rpm) in the dark. Headspace atmosphere was either nitrogen (N_2), or air (O_2) and slurry amendments were 100 mM of either citric acid (Cit), Na_3-citrate (Na_3-Cit) or none. Oxygen was measured as percent O_2 present in the microcosm headspace and mineralization was calculated as percent of total ^{14}C added (as ^{14}C-RDX) recovered as $^{14}CO_2$. Sample points are the mean of triplicate samples, and error bars represent the standard deviation.

In terms of the start of RDX mineralization, there was less variation observed. The Na_3-citrate and unamended slurries first demonstrated RDX mineralization on day 6. The citric acid amended microcosms possessing an oxygen headspace first exhibited mineralization at day 13, whereas the citric acid amended nitrogen headspace microcosm did not demonstrate a clear start of mineralization.

Determination of soil slurry pH under the three condition used (unamended, citric acid amended and Na_3-citrate amended) revealed a significant difference between the citric acid amended slurry as compared to the unamended and Na_3-citrate amended slurries. The citric acid amended slurry pH was 2.85 and 3.65 at time 0 and 116 hours, respectively, as compared to the unamended (7.03 and 7.38 at time 0 and 116 hours, respectively) and Na_3-citrate amended slurries (7.29 and 8.97 at time 0 and 116 hours, respectively).

6.5.3. Mass Balance

Mass balance of the slurry microcosms resulted in recovery of 97% to 99% of the original amount of ^{14}C present in the labeled RDX (Figure 6.4.). The humic acid/fulvic acid fraction and the residual organic matter fraction combined to

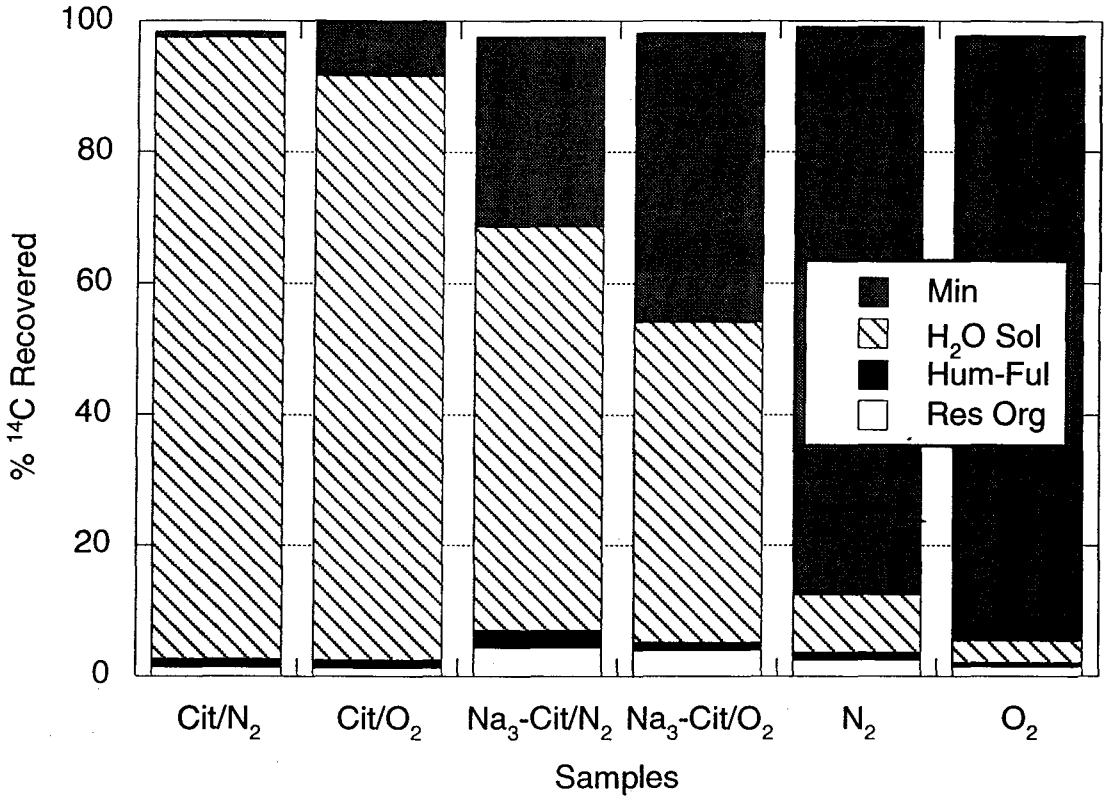

Figure 6.4. Mass balance of ^{14}C-RDX degradation. Mass balance performed on slurry microcosms, monitoring the amount of ^{14}C recovered from mineralization (Min), water soluble fraction (H_2O Sol), humic acid – fulvic acid fraction (Hum-Ful) and residual organic matter released during combustion (Res Org). Sample designation: cit, citric acid amendment; Na_3-cit, Na_3-citrate amendment; O_2, oxygen atmosphere; N_2, nitrogen atmosphere. Samples represent triplicates of each condition.

account for only 2% to 7% of the total ^{14}C recovered. The remaining 90% to 97% of the ^{14}C label was found in the mineralized and H_2O soluble fractions.

6.5.4. PCR-DGGE

Due to the disappearance of RDX in only the Na_3-citrate amended flasks after 64 days, sample points taken on day 1, 16, 29, and 64 days were analyzed using PCR-DGGE (Figure 6.5.). Amplification of 16S rDNA genes from the slurry samples proved to be difficult using both primer pairs (8FGC-519R and b341GC-u758). The presence of both RDX and Na_3-citrate affected a shift in the bacterial population. Most notably, the appearance of two distinct bands (A and B) in the RDX amended flasks (1, 3, and 5), and the appearance of a strong band (C) in all flasks. A fourth band (D) seen only in flask 7 (no RDX) was also observed. Direct sequencing of excised bands A-3, A-5, B-1, and B-5 was performed, revealing that B-1 and B-5 (294 nt and 341 nt, respectively) were 100% identical to *Sphingomonas* spp. Sequencing results for bands A-3 and A-5 revealed background interference, suggesting that multiple operational taxonomic units (OTUs) (Moyer et al. 1994) were present in each band. Although these sequences contained approximately 10 to 15% unreadable nucleotides, the

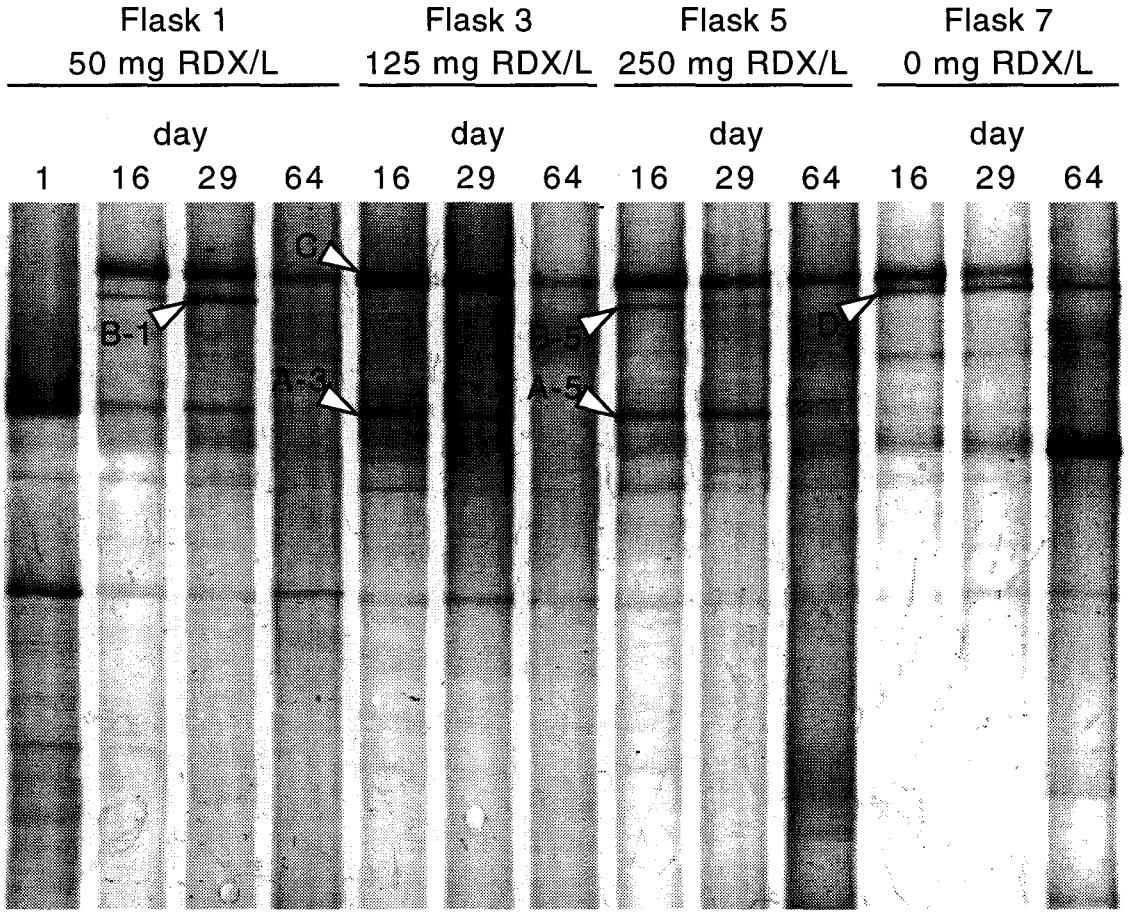

Figure 6.5. DGGE of Na_3-citrate amended soil slurries. PCR-DGGE performed using DNA templates from the indicated samples and amplification with primer pair b341GC-u758. A denaturing gradient of 40-60% was used and bands A-3, A-5, B-1, and B-5 were excised and directly sequenced.

closest database matches were over 95% similar for readable positions. The predominant sequence retrieved from A-3 (approximately 340 nt) was most similar to *Stenotrophomonas* sp., whereas the sequence for A-5 (approximately 290 nt) demonstrated similarity with *Alcaligenes/Achromobacter/Bordetella*, all members of the Alcaligenaceae group.

6.6. Discussion

The results of RDX degradation in soil slurry flasks were in sharp contrast to the results obtained in soil slurry microcosms, which appeared to be influenced by a different set of physico-chemical parameters. The disappearance of RDX in the soil slurry flasks combined with the transient appearance of the RDX reduction intermediates MNX, DNX, and TNX indicated that reductive degradation of RDX was occurring under aerobic conditions. Previous studies have also observed reduction intermediates under similar conditions (Young et al. 1997; Freedman and Sutherland 1998; Shen et al. 2000; Groom et al. 2001; Oh et al. 2001). The rapid disappearance of RDX in only the flasks amended with Na_3-citrate suggested that these systems were either limited in carbon, as RDX is known to be used as a source of nitrogen (Binks et al. 1995; Boopathy et al.

1998; Boopathy et al. 1998; Coleman et al. 1998; Ronen et al. 1998), or that the oxidation of citrate rapidly decreased the redox potential, providing conditions favorable for the reduction of RDX. Furthermore, sodium ions are well known to defloculate and destroy soil structure (Sherard et al. 1976; Schaller and Sutton 1978), which could result in a release of additional carbon sources. This action was evident immediately upon addition of the Na_3-citrate solution to slurry flasks.

The presence of additional carbon in the system would stimulate the heterotrophic bacterial population, resulting in the rapid consumption of oxygen. The exogenous carbon source provided (citrate), acting as electron donor, would also increase the level of reducing power produced by the bacterial cells. This in turn can be used for the reduction of RDX, either by biologically mediated mechanisms such as oxygen-insensitive type 1 nitroreductase (NRI) (Kitts et al. 2000), or by the shuttling of electrons from electron transport chains to abiotic electron sinks such as humic acids (Lovely et al. 1996; Bradley et al. 1998; Coates et al. 1998). These reduced compounds could in turn transfer electrons to RDX indirectly through iron, an abiotic remediation technology currently being investigated (Hundal et al. 1997; Singh et al. 1998; Singh et al. 1999).

In contrast, results of the anaerobic microcosm experiment point to different mechanisms operating under strictly anaerobic conditions. The presence of Na_3-citrate reduced the level of RDX mineralization by approximately 40% as compared to the unamended microcosms. This was in direct contrast to the results observed in the aerobically incubated flask experiments, where the addition of Na_3-citrate stimulated the degradation of RDX while unamended slurries did not attain a 50% reduction in RDX concentration after 300 days.

The exact nature of these differences was not immediately evident as the two approaches examine different aspects of the biodegradation of RDX. The aerobic flasks examined the initial biotransformation of RDX, resulting in the production of the reduced compounds MNX, DNX, and TNX. The subsequent intermediates produced by the reduced compounds are not known to date, and therefore it was not possible to determine whether complete mineralization was occurring in this open system. In contrast, the closed system of the anaerobic microcosms provided the opportunity to monitor mineralization of RDX, but because all intermediates produced during biotransformation are not known and were radioactive, they were not analyzed.

Despite these experimental differences, several conclusions can be drawn with respect to the mechanisms involved in the aerobic vs. anaerobic systems. In the case of the aerobic slurry flasks, the presence of reduced intermediates suggested a nitroreductase activity. Purified type I nitroreductase was able to catalyze the aerobic reduction of RDX using NADPH (Kitts et al. 2000). However, the RDX degrading microorganism from which it was isolated (*Morganella morganii* (Kitts et al. 1994)) was only able to degrade RDX in pure culture under oxygen limited conditions. This lead the authors to speculate that a high level of reductant (NAD(P)H) would be required to reduce RDX under aerobic conditions (Kitts et al. 2000). In the case of the flask experiments, reduction of oxygen concentrations within the slurry due to stimulation of heterotrophs by the presence of additional carbon would permit the accumulation of sufficient reductant for RDX reduction to occur. Other studies have observed an increase in the level of RDX mineralization when anaerobic degradation is followed by an aerobic polishing step (Ronen et al. 1998; Shen et al. 1998). Increases of up to 7% mineralization have been observed (Shen et al. 1998), possibly due to the further degradation of anaerobically recalcitrant RDX reduction intermediates. In the case of the anaerobic microcosms described

here, the higher levels of RDX mineralization observed in the microcosms containing an initial oxygen headspace can be explained by this polishing step.

Comparison of the results of Na_3-citrate amendment on the degradation of RDX under aerobic and anaerobic conditions presented potentially conflicting results. In the aerobic flask 1 (50 mg RDX), RDX and its reduction intermediates were not detectable after 35 to 44 days. However, the maximum amount of label recovered as $^{14}CO_2$ after 94 days in the anaerobic microcosms was 44%. There are several hypotheses that may explain this difference. The first is that, in the aerobic flasks, RDX was completely mineralized to CO_2, and that there was inhibition of RDX mineralization by Na_3-citrate under anaerobic conditions. The second is that the RDX and it reduced intermediates were further degraded under aerobic conditions, producing compounds recalcitrant to complete mineralization. Mass balance of the microcosms revealed that the majority of the ^{14}C label added as RDX was either mineralized or remained in the aqueous phase. This result agrees with other observations that RDX and its degradation intermediates are not strongly bound to soil particles (Pennington et al. 1999; Sheremata et al. 2001).

A third possible explanation would be that different microbial communities were responsible for the degradation of RDX under aerobic and anaerobic conditions. This hypothesis was supported by the results of the unamended slurries under both aerobic and anaerobic conditions. In the aerobic slurries, less than 50% of the RDX was transformed after 301 days (i.e. in flask 1 (50mg), the concentration did not drop below saturation at 50 mg/L), whereas in the anaerobic microcosms, 87% to 92% of the RDX was mineralized after 94 days. Under these conditions, it was evident that different mechanisms were involved, and most likely related to differences in the stimulated bacterial populations.

DGGE results of the aerobic slurries clearly demonstrated the stimulation of at least two OTUs in the presence of RDX (A and B bands) and the inhibition of a third OTU (D band). Band C was present in all samples, suggesting it was stimulated by the added citrate and/or the physico-chemical changes caused by the Na_3-citrate. Examination of the day 1 sample revealed that the OTUs stimulated by the presence of RDX were below detection levels of approximately 1% (Muyzer et al. 1993; Stephen et al. 1999) but quickly increased in numbers over a 2 week period.

The 16S rDNA sequences revealed several interesting relationships. Band A-3 was tentatively identified as a *Stenotrophomonas* sp. by 16S rDNA comparison. A previously isolated RDX degrader (Binks et al. 1995), which functions under aerobic conditions, has been identified as a *Stenotrophomonas maltophilia* strain (strain PB1). This microorganism isolated from a previously contaminated soil, was able to use RDX as a sole source of nitrogen and required the addition of carbon sources (glucose, succinate, and glycerol) for aerobic degradation of RDX to occur. It was postulated that the reason for this was that a large amount of reducing power was required, possibly in the form of NADH or NADPH.

Sequence data for band A-5 suggested its relationship to members of the beta Proteobacterial Alcaligenaceae group, notably *Alcaligenes, Achromobacter* and *Bordetella* spp. Members of this group have been previously isolated with the ability to degrade several environmentally important compounds, including 2,4-dichlorobenzoate (*Achromobacter* and *Alcaligenes*) and the herbicide atrazine (*Alcaligenes*) (Singh et al. 1999) which is structurally related to RDX by its S-triazine backbone. Comparison of bands B-1 and B-5 to Blast and FASTA databases returned similarities of 100% to several *Sphingomonas* sp. which are

also known to play important roles in the degradation of numerous environmental pollutants (Singh et al. 1999).

In conclusion, it was apparent that in unamended flasks, the degradation of RDX under aerobic and anaerobic conditions was accomplished by two different, unknown mechanisms. The results for the Na_3-citrate amended slurries are less conclusive, but point to a similar conclusion. The molecular biological analysis of the Na_3-citrate amended aerobic slurry flasks suggested that several microorganisms, one of which had been previously identified as an aerobic RDX degrader, were stimulated by the presence of RDX under aerobic slurry conditions. This fact, combined with the rapid disappearance of RDX under Na_3-citrate amended aerobic conditions suggested that a consortium of bacteria was responsible for the degradation of RDX.

Chapter 7. General Discussion

In recent years, the links between environmental pollution and human health problems have increased. This in turn has driven the study and development of remediation technologies to clean up contaminated environments. In the search for efficient and cost effective strategies, bioremediation has become an attractive option. In studying pollutant biodegradation, one of the first considerations is always, 'can the pollutant in question be broken down?' The exact biochemical pathways and the genetic mechanisms regulating this activity can be examined in great detail *ex situ*, once the microorganism(s) demonstrating this activity have been isolated. Application of this *ex situ* information to *in situ* technologies raises several questions, including, ' will this microorganism perform these functions *in situ*?', ' is this microorganism an important component of the *in situ* activity?', and ' what are the overall effects of this pollutant on the entire bacterial community?'

In attempting to answer some of these questions, several community level approaches have been adopted from clinical research, including the Biolog system and PCR-DGGE. The main goal of this work was to examine the effect of contamination on soil microbial communities using a polyphasic

biochemical/molecular biological approach. The first stage dealt with the effect of petroleum hydrocarbon pollution on bacterial communities using the Biolog system and PCR-DGGE. The results of this stage (i.e. the applicability of both Biolog and PCR-DGGE for community analysis) were then used for a more rigorous examination of RDX contamination and its effect on soil bacterial communities. The goal of this second stage was to visualize changes in the microbial community caused by RDX contamination, identify the microorganisms stimulated by RDX contamination, and examine several factors which affect the biodegradation of RDX in soil.

The results of the initial work dealing with petroleum hydrocarbon contaminated sites in Northern Canada determined that the Biolog system is easily adaptable to various experimental systems (e.g. incubation at lower temperatures). Analysis of the Biolog CLPP patterns also supported the conclusions of the PCR-DGGE analysis that the samples demonstrated more similarity based on their geographic origin, than the level of petroleum hydrocarbon contamination.

Despite the agreement of the Biolog analysis with the PCR-DGGE data, the Biolog system has several important shortcomings with respect to its suitability

for *in situ* analysis. Firstly, the Biolog system is culture based, which may exclude environmentally important K-strategists from analysis. In oligotrophic environments such as the Canadian North, slow growing K-strategists may be much more important than fast growing r-strategists in the overall biochemical activity of the soil. Several studies have demonstrated the selective nature of the Biolog system, which favors fast growing heterotrophs such as pseudomonads, and not the numerically dominant members of the microbial community (Garland 1997; Konopka et al. 1998). Secondly, the CLPP patterns generated are not simply a combination of the individual member bacterial species substrate utilization patterns (Verschuere et al. 1997; Smalla et al. 1998). Instead, it appears that the patterns generated can be strongly influenced by one or more of the microorganisms present. A third factor is the continuing debate which encompasses the nature of the information contained within the CLPP and the most appropriate method for its analysis (Howard 1997; Garland and Mills 1999; Garland and Mills 1999; Howard 1999; Insam and Hitzl 1999). Where the Biolog systems appears to provide the most pertinent information is in dynamic eutrophic environments where fast growing heterotrophs are responsible for the main biochemical activities and represent the majority of microorganisms.

The development of PCR-DGGE for molecular biological analysis of environmental systems has provided a very powerful technique to circumvent many of the shortcomings of the Biolog system. This includes simultaneous examination of multiple samples, visualization of OTUs which are present at 1% or more of the total bacterial population (i.e. many OTUs at high resolution) (Muyzer et al. 1993; Stephen et al. 1999), and the ability to assign a phylogenetic relationship to a band of interest.

The ability to assign phylogenetic relationships to bands of interest, i.e. OTUs which appear to be stimulated or inhibited by the imposed physico-chemical conditions, provides a tremendous amount of information relating to the community structure. In the course of the work presented here, several OTUs, identified as being prominent in their environments, were sequenced and the phylogenetic relationships determined. In the case of the petroleum hydrocarbon contaminated samples, several OTUs were found to be related to the Actinomycetales, many members of which possessed petroleum hydrocarbon degradative activities. In the RDX slurries, one of the bands displayed significant similarity to *Stenotrophomonas maltophilia*, a microorganism previously isolated for its ability to aerobically degrade RDX. Of the other bands sequenced, two

were 100% identical to *Sphingomonas* while the final band displayed similarity to the Alcaligenaceae group, members of which are known to degrade several herbicides, including atrazine which is structurally similar to RDX. Although phylogenetic relatedness does not guarantee a specific biochemical activity (Dojka et al. 1998; Hugenholtz et al. 1998), the presence of related microorganisms would suggest that the potential for such an activity is high.

In the case of the RDX slurries, other more traditional biochemically based methods were employed. Mineralization assays provide insight into the ability of the microorganisms to completely breakdown the pollutant in question to CO_2 and H_2O, whereas chemical analysis of the slurries revealed the presence of several important degradation intermediates. Culturing of the slurry microbes did not result in the isolation of RDX degrading bacteria, but suggested that this activity was due to a community effort.

In conclusion, the polyphasic approaches used during the course of this work provided useful information as to the *in situ* conditions which influenced bacterial populations within contaminated sites. The petroleum hydrocarbon contaminated samples appeared to be more influenced by the geographical positioning of the sites rather than contamination levels. In the case of RDX

contamination in clean soils, the indigenous bacterial population was able to effectively remove RDX without significant changes to the microbial community structure. In the slurry systems, the mechanism of RDX degradation was dependent on the physico-chemical conditions (i.e. aerobic versus anaerobic, presence of Na_3-citrate). In both the column and slurry systems, the ability to degrade RDX does not appear to be the sole activity of one bacterial species, but rather a community effort.

These experiments demonstrate the utility and power of a polyphasic approach in describing and understanding *in situ* activities of soil bacterial communities under contaminated conditions. The application of polyphasic monitoring programs in contaminated environments will provide a more comprehensive account of the condition of impacted bacterial communities, including the effect on biodiversity, biochemical functioning and the degradative mechanisms involved. This in turn will provide environmental regulators with more detailed and valuable information on the effectiveness of bioremediation technologies, so that more informed regulatory decisions can be made and implemented.

Chapter 8. Appendix

8.1. Soil DNA Extraction and Purification

Extraction and purification of total soil DNA was performed as described by Berthelet et al. 1996.

The soil sample (0.2-0.5 g) was placed in a 2 mL screw cap tube containing 2.5 g of sterile 0.1 mm zircona/silica beads (Biospec Products, Inc., Bartlesville, OK) and 500 µL of sterile sodium phosphate buffer (100 mM, pH 8.0) was added. To this, 250 µL of lysis buffer was added (100 mM NaCl, 500 mM Tris-HCl (pH 8.0), 10% Sodium dodecylsulfate (SDS)) and the sample homogenized using a Mini Beadbeater-8 (Biospec Products, Inc., Bartlesville, OK) for 5 minutes at 4°C at 3200 rpm.

8.2. RDX Analysis

Detailed protocol as supplied in Sheremata et al. 2001.

RDX concentrations were determined by reversed-phase high-pressure liquid chromatography (HPLC) with a photodiode array (PDA) detector. The Waters (Waters Associates, Milford, MA) HPLC system consisted of a model 600

pump, 717 Plus autosampler, and a 996 photodiode array (PDA) detector (λ=254 nm). A Supelcosil LC-CN column (25 cm x 4.6 mm, particle size 5 µm) was coupled with a Temperature Control Module held at 35 °C. The methanol/ water gradient was at a flow rate of 1.5 mL/min. The initial solvent composition was 30% methanol and 70% water that was held for 8 minutes. Following this, a linear gradient was run from 30 to 65% methanol over 12 minutes. The solvent ratio was changed to the initial conditions over 5 minutes and held for another 5 minutes. The system was outfitted with Millenium data acquisition software.

Liquid chromatography/mass spectrometry (LC/MS) was used to verify the presence of RDX, MNX, DNX, and TNX. Analyte ionization was achieved in a negative electrospray ionization ES (-) mode. This system consisted of a Micromass Platform II benchtop single quadrupole mass detector fronted by a Hewlett-Packard 1100 series HPLC system equipped with a photodiode array detector. Samples (50 µL) from the microcosms were injected into a Supelcosil LC-CN column (25 cm x 4.6 mm; 5 µm particle size) at 35 °C. The instrument conditions used are reported elsewhere (9). Confirmation of the identity of targeted metabolites (MNX and TNX) was accomplished by comparison with

reference compounds simulated according to the methods of Brockman et al. (1949).

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