UNIVERSITY OF CALIFORNIA

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Biological Transformation and Mineralization of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX)

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Civil Engineering

by

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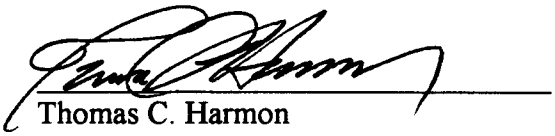
The thesis of Jennifer Hui-yun Chiou is approved



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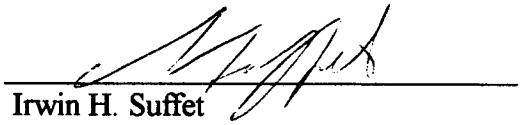

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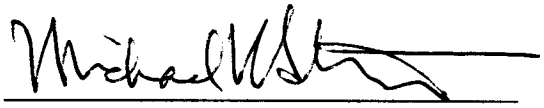

Michael K. Stenstrom, Committee Chair

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ABSTRACT OF THE THESIS

Biological Transformation and Mineralization
of
Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX)

by

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Master of Science in Civil and Environmental Engineering

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Professor Michael K. Stenstrom, Chair

Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) is a common high explosive chemical. Large amounts of RDX need to be disposed every year in the United States. RDX has toxic effects on humans, especially on the central nervous system. It is also a possible carcinogen. Therefore it is essential to find a safe and efficient way to dispose RDX. Anoxic denitrification is found to be an effective method to biodegrade RDX

RDX biotransformation is a co-metabolic process. Ethanol is a good carbon co-substrate that supports RDX transformation process. Ethanol concentration is one of the factors that affect RDX transformation efficiency. In a continuous-flow packed-bed reactor, higher ethanol concentrations yield higher RDX transformation efficiencies. An

ethanol concentration of 0.1 % is the minimum ethanol concentration required to achieve a satisfactory RDX transformation efficiency (60%).

In an anoxic denitrifying batch experiment with ^{14}C-RDX added to trace the biotransformation metabolites, 38.02% of RDX can be mineralized to carbon dioxide under well buffered conditions. The degree of Mineralization increases with increasing amount of nitrate available as the electron acceptor. The nitroso-derivatives of RDX does not accumulate in this system. The majority of RDX metabolites are very water soluble, polar, and volatile.

Chapter I

Introduction

Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) is one of the important high explosives used by the U.S. and European munitions industries. Mixtures of RDX and other explosives such as TNT and HMX are the major components in nuclear and conventional weapons as detonators, primers and boosters. During World War II, RDX production in the U.S. and Germany averaged 15,200,000 and 7,100,000 Kg per month, respectively (Urbanski, 1967).

The recent dismantling activity is producing large amounts of explosives that need to be disposed. Currently the U.S. Department of Energy (DOE) reports that 45,000 kg of high explosives waste are disposed every year at the Pantex Plant in Texas. Because of the Intermediate Range Nuclear Forces Treaty and the Strategic Arms Reduction Treaties (Wilkie, 1994), another 50,000 kg of high explosives will need to be treated every year. In addition, wastewaters produced during production, packing and washing of explosives from munitions plants are often contaminated by RDX. Wastewaters contaminated by RDX are highly problematic and needed to be treated properly before disposal.

In the past, RDX-contaminating wastewater was disposed in lagoons, which often resulted in the contamination of groundwater via the leaching of hazardous explosives through soil. Currently high explosives are being destroyed by open burring and incineration. However, the combustion of RDX leads to various unwanted toxic products

such as hydrocyanic acid (HCN); therefore, this process needs to be phased out as soon as possible. A more effective and safer way to dispose of RDX needs to be developed.

Presently, activated carbon adsorption is one of the most widely used methods to remove RDX from contaminated wastewater. However the disposal of the explosive-laden activated carbon is a largely unresolved problem. Alkaline hydrolysis of the laden-carbon is a potential solution because alkaline hydrolysis can decompose RDX to small molecules such as formate, nitrite, acetate and ammonia. The high pH wastewater produced by alkaline hydrolysis is another potential problem, which needs to be neutralized before being discharged.

An alternate process, investigated in this research, is the biological transformation and mineralization of RDX. Biological treatment is often less expensive when compared to physio-chemical treatment, and is a promising method for in-situ bioremediation. Research on biological treatment of RDX has been conducted over a period of almost twenty years, and the results have shown that RDX can be effectively transformed under anaerobic and anoxic conditions. However, none of the previous research has shown a significant amount of total mineralization of RDX (conversion of RDX to carbon dioxide and inorganic nitrogen). It is suspected that some of the biologically transformed RDX forms metabolic intermediates such as nitroso derivatives, dimethylhydrazine and hydrazine. Some of these intermediates are mutagenic and carcinogenic. Therefore the goal of this research is to identify some of the metabolic intermediates and the proper conditions to completely mineralize RDX.

In this research, ethanol is the organic co-substrate to support the growth and activity of microorganism (Wilkie, 1994). Nitrate is the electron acceptor to provide an anoxic denitrifying condition (Hesselman, 1993). ^{14}C-RDX is used to monitor the production of carbon dioxide and metabolic intermediates transformed from RDX.

Chapter II

Literature Review

2.1. Physical Properties of RDX

RDX, which stands for Royal Demolition eXplosive, is one of the most commonly used military explosives. Its synonyms include cyclonite, hexagon and cyclotrimethylenetrinitramine. RDX is a colorless polycrystalline, heterocyclic triazine. The structure is shown in Figure 1. RDX has stability similar to TNT but higher explosive power.

There are two common processes to synthesize RDX. One is a direct nitrolysis process and the other is an indirect nitration process using hexamine to react with ammonium nitrate/nitric acid. Both techniques also produce HMX as an impurity at 10% (w/w) or less (Yinon, 1990). HMX stands for High Melting eXplosives because it has higher density and higher melting point than RDX. The presence of HMX in RDX is usually not objectionable, since it has explosive properties that are at least as desirable as RDX. The structure of HMX is also shown in Figure 1.

RDX is not very soluble in water. The solubility of RDX in water at 20°C about 40 mg/L. The solubility of RDX is greater in polar organic solvents such as acetone and acetonitrile. Table 1 lists the physical and chemical properties of RDX.

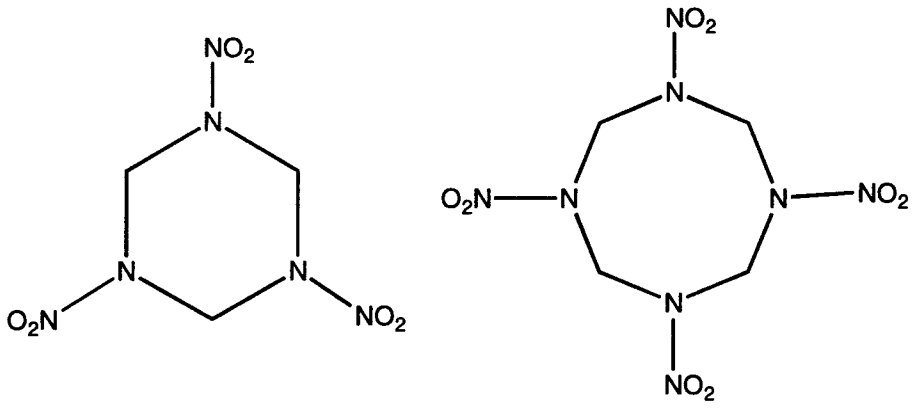


Figure 1. Chemical structures of RDX and HMX

Table 1. Physical and chemical properties of RDX

(Yinon, 1990; Rosenblatt *et al.*, 1991)

Property	
CAS Reg. No.	121-82-4
Molecular Weight	222.26
Empirical Formula	$C_3H_6N_6O_6$
Crystal form	white orthothombic crystals
Melting Point	204°C
Vapor Pressure (25°C)	4.03×10^{-9}
Heat of Fusion	38.26 cal/g
Ignition temperature	229°C
Aqueous solubility	in g/100g solvent
Water	0.006 (25°C)
Chloroform	0.008 (20°C)
Toluene	0.02 (20°C)
Benzene	0.05 (20°C)
Ether	0.055 (20°C)
Ethanol	0.11 (20°C)
Ethyl acetate	1.5 (20°C)
Acetonitrile	5.5 (25°C)
Acetone	8.2 (25°C)

2.2. Toxicity and Regulation of RDX

The major toxic effect of RDX to humans is damage to the central nervous system (CNS). Exposure to workers in munitions plants via inhalation of dust containing RDX has caused nausea, irritability, convulsion, unconsciousness and amnesia (Etnier, 1989). Ingestion of RDX causes similar CNS effects (Woody *et al.*, 1986). When in contact with human skin, RDX acts primarily as an irritant (Etnier, 1989). There is only little chance that RDX is adsorbed by skin due to its lipophobic nature (Rosenblatt, 1980).

RDX was not found to be carcinogenic in rats (Levin *et al.*, 1983). However, there was a significant increase in incidents of hepatocellular carcinomas and adenomas in female mice receiving 100 mg/kg/day for two years (Mclellan, 1988). RDX was therefore classified as Group C: possible human carcinogen.

The U.S. Environmental Protection Agency (US EPA) determined the lifetime health advisory (HA) of RDX. The concentration in drinking water at which no adverse health effects would be expected to occur following lifetime ingestion is 2 μg/L. The Chemical Effects Information Task Group also proposed a water quality criterion (WQC). A criterion of 105 μg/L was proposed for ingestion of drinking water alone, and 103 μg/ L was proposed for ingestion of drinking water and aquatic foodstuffs (Etnier, 1989). The US EPA's lifetime HA is more conservative than the WQC because the US EPA uses an additional uncertainty factor for the group C chemicals while WQC does not have this policy. In addition US EPA uses a 20 % relative source contribution in the absence of site specific information when calculating lifetime HA (Etnier, 1990).

RDX at its solubility limit in water causes no significant toxicity to the *Ceriodaphnia dubia, Hydra littoralis, and Paratanytarsus parthenogeneticus* freshwater invertebrates (Turley *et al.*, 1991). However a maximum water concentration of 0.3 mg/L has been suggested to protect aquatic life (Sullivan, 1979).

2.3. Environmental Fate

RDX has a vapor pressure of 1.0×10^{-6} mm Hg. It may exist in both vapor phase and particulate phase in the atmosphere. This very low vapor pressure suggests that RDX volatilization is quite low (Lyman *et al.*, 1982).

When RDX is in the atmosphere, it can be decomposed by reacting with the photochemical generated hydroxyl radical and the half-life for this reaction in the vapor phase is 1.5 hours (Atkinson, 1987). In addition, RDX absorbs ultraviolet light between 240 and 350 nm so RDX in the atmosphere is expected to undergo photolysis (Etnier, 1986).

RDX is stable in an aqueous solution with pH close to natural water. This suggests that hydrolysis of RDX is not occurring in natural water. The major process that degrades RDX in natural water is photolysis. Experiments showed that RDX in water exposed to ultraviolet light decomposed rapidly with a half-life of 3.7 minutes (Burrows *et al.*, 1984). RDX in water decomposed by natural sunlight in 9 to 13 hours (Sikka, 1980). Formaldehyde and nitrosoamines were identified as the by-products of photolysis.

Although nitrosoamines are suspected to be mutagenic and carcinogenic, they do not accumulate because they are also photoreactive (Sikka, 1980).

When RDX is in contact with soil, the value of the soil sorption coefficient (Koc) of RDX ranges from 63.1 ml/g (Spanggord, 1983) to 270 ml/g (Layton, 1987). This indicates a medium to high mobility of RDX in soil which suggests that RDX disposed in lagoon or surface water can be expected to leach into groundwater. A later study showed that the adsorption rate constant of RDX in soil is slow and the rate depends on the clay content, pH, and cation exchange capacity (Ainsworth *et al.*, 1993).

Plants growing in RDX contaminated water bioaccumulate RDX. Bush beans grown in 10 mg/L RDX solution accumulated 97 mg/L of RDX in aerial tissue in 7 days of exposure. The bioconcentration observed during this relatively short-term exposure indicates that the plants which grow around RDX contaminated sites may have accumulate a large amount of RDX (Harvey *et al.*, 1991). The accumulation of RDX in any edible plants suggests an undefined and perhaps large human intake of RDX via the food-chain.

2.4. Treatment of RDX

2.4.1. Physiochemical Processes

An important chemical treatment method to remove RDX from contaminated water is activated carbon adsorption. Wujcik *et al.* (1992) demonstrated that a continuous flow granular activated carbon (GAC) column is able to remove RDX from contaminated groundwater. They also found that two columns in series produce greater removal efficiency than a single column. However the disposal of the RDX-laden carbon is another problem. Thermal regeneration is one possible method to recover laden activated carbon. Waer *et al.* (1992) stated that the capacity of GAC loaded with naturally occurring organic material and methylene blue could be returned to that of virgin carbon at a temperature of 850°C in 15 min. However this process is energy intensive and thermal regeneration with explosive-laden carbon is very risky.

In view of the problem of the activated carbon adsorption, another group of scientists evaluated alkaline hydrolysis of bulk RDX in aqueous solutions. This process is a simple and fast way to convert RDX to smaller, less harmful compounds such as acetate, format, and nitrite which can be treated in conventional wastewater treatment systems (Spontarelli *et al.*, 1993). Spontarelli *et al.* (1993) also found that hydrolysis rate can be increased by reducing particle size, stirring, and adding organic solvents to the alkaline solution. One mole of RDX can be completely hydrolyzed by three moles of sodium hydroxide.

Heilmann (1994, 1995) evaluated activated carbon adsorption combined with alkaline hydrolysis carbon regeneration. The RDX-contaminated wastewater is first passed through granular activated carbon columns to concentrate RDX waste on carbon and to reduce treatment volume. Next the laden carbon is treated with alkaline hydrolysis and the regenerated carbon is then able to treat another charge of RDX contaminated water. Through this process, it is possible to use alkaline hydrolysis to indirectly destroy RDX contained in wastewater or groundwater. Directly using alkaline hydrolysis on RDX-contaminated wastewater is probably not commercially feasible due to the large quantity of base and acid that would be required to raise the pH of wastewater and then neutralize it after hydrolysis reactions are completed.

Heilmann also evaluated the kinetics of RDX-laden carbon regeneration. The experiment was performed in a water bath at different temperatures. Heilmann derived a pseudo first-order rate equation for use in the presence of excessive hydroxide concentration. Using the Arrhenius-equation he concluded that the reaction rate constant is temperature dependent and is much more rapid at a higher temperature. An increase in temperature of 10°C in the range of 50°C to 80°C produces an increase in reaction rate from 2.6 to 3.3 fold. At 80°C and pH above 11, hydrolysis is completed in a few minutes.

Alkaline hydrolysis followed by activated carbon adsorption appears to be a fast and easy way to decompose RDX chemically; however, there are still some potential problems with the process. The products of RDX alkaline hydrolysis, i.e., nitride ion, format ion, acetate ion and ammonia are not the most desirable end products. The hydrolysates need to

be further decomposed to nitrogen gas, carbon dioxide and water. In addition, the solution which contains high pH and salinity must be neutralized before entering a normal wastewater treatment system. This process can not be applied for in-situ bioremediation because of the base and acid requirements.

2.4.2. Biological Processes

Biological transformation and mineralization is an alternative process to remove RDX from contaminated water. The process is achieved through a co-metabolic reaction. A significant amount of work has been carried out on the biological degradation of RDX in the past fifteen years. Scientists have tried to degrade RDX under aerobic, anaerobic, and anoxic conditions.

2.4.2.1. Aerobic Processes

Several experiments showed that no RDX can be degraded under aerobic conditions (McCormick, 1981; Haas, 1990; Ro and Stenstrom, 1991). The transformation mechanism of microorganisms is probably inhibited when oxygen is present. However, Thiboutot *et al.* (1994) isolated two strains, designated "A" and "C" that were both able to mineralize 34% of RDX in 2 days in liquid culture under an aerobic condition. The strains used glucose as a co-substrate and RDX as nitrogen source. This

result has not been confirmed by others and biotransformation metabolic pathways and intermediates are unknown.

A white rot fungus *Phanerochate chrysosporium* can transform 66.6% of ^{14}C-RDX to $^{14}CO_2$ in 30 days under aerobic conditions (Fernando and Aust, 1991). However, the growth of *P. chrysosporium* is inhibited by the presence of TNT (Spiker *et al.*, 1992). The initial concentration of RDX was 0.028 mg/L, which is much lower than the concentration normally found in RDX contaminated wastewater and soil. Also no one has been able to sustain the growth of *P. chrysosporium* in a continuous culture, such as those need for wastewater treatment plants. Therefore *Phanerochate chrysosporium* might not be a good choice for biotrasformation of RDX at explosives contaminated sites.

2.4.2.2. Anaerobic Processes

McCormick and his group reported in 1981 that RDX can be biodegraded under anaerobic conditions. The structure of RDX undergoes a successive reduction of the nitro groups. The cleavage reaction occurs when microorganisms attack and destabilize the ring structure. The degradation products include hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), hydrazine, 1,1-dimethylhydrazine, 1,2-dimethylhydrazine, formaldehyde and methanol. Figure 2 shows the metabolic by-products and pathways of RDX biodegradation. Base on the metabolites that were detected, they proposed a detailed RDX biotransformation pathways as shown in Figure 3.

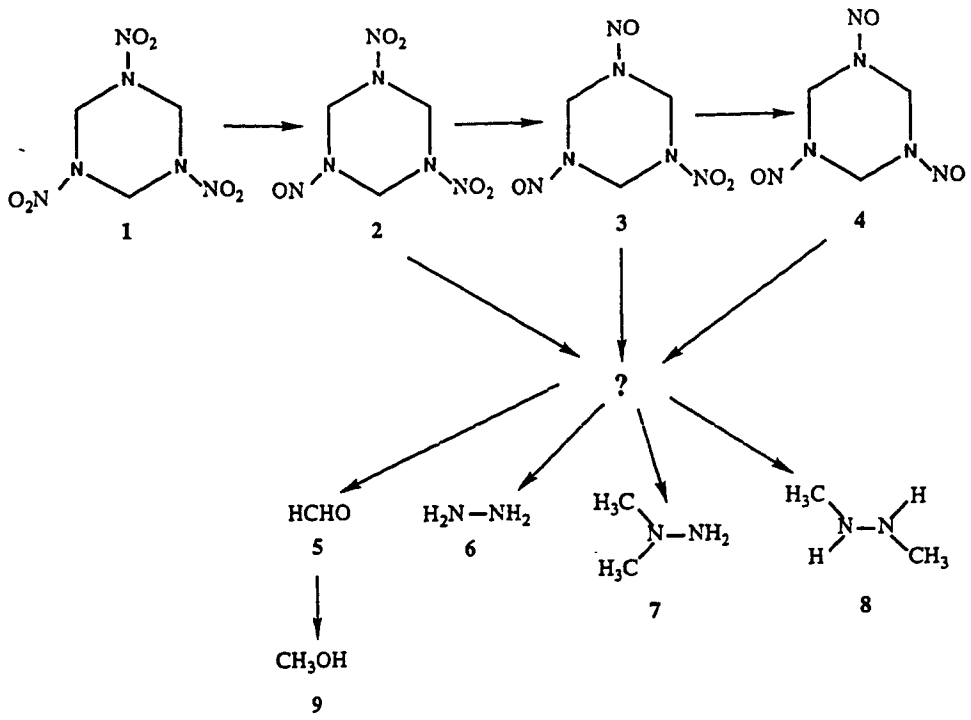


Figure 2. The biodegradation by-products of RDX under an anaerobic condition identified by McCormick *et al.*, 1981; 1, RDX; 2, MNX; 3, DNX; 4, TNX; 5, formaldehyde; 6, hydrazine; 7, 1,1-dimethylhydrazine; 8, 1,2-dimethylhydrazine; 9, methanol

In the anaerobic batch system of McCormick *et al.* (1981), nitroso derivatives of RDX were accumulated in the first three days. The maximum amount of MNX reached half of the initial RDX concentration on the second day. After the third day all of the nitroso derivatives of RDX decreased and were mostly removed by eighteen days. The amount of formaldehyde reached a maximum on the first day and decreased thereafter. Methanol was detected but without kinetic data. Methanol can be oxidized to CO_2 readily in aerobic conditions. The biodegradability of hydrazine and dimethylhydrazine are still unknown (McCormick *et al.*, 1981).

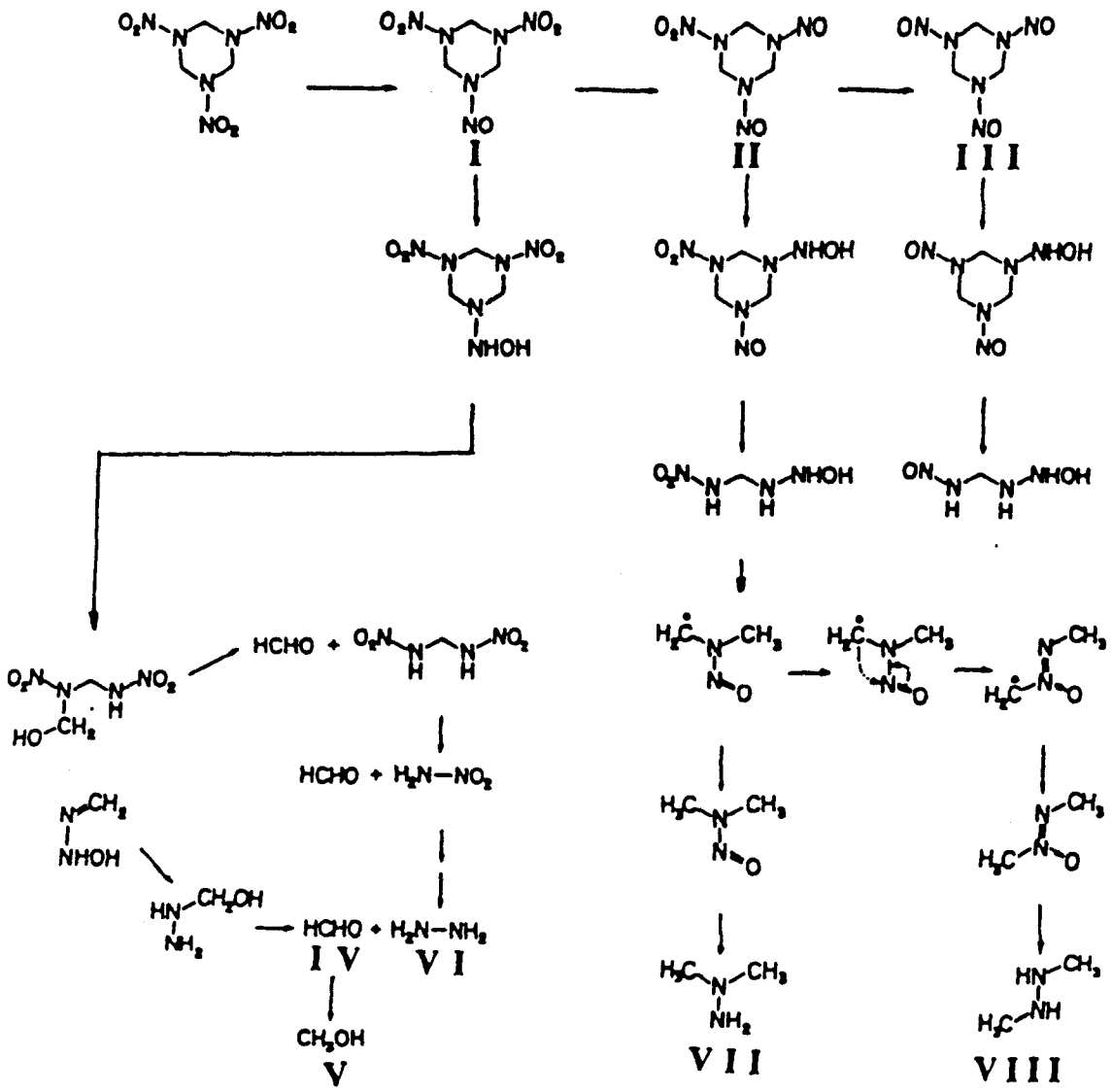


Figure 3. The proposed biotransformation pathways of RDX under anaerobic conditions by McCormick *et al.*, 1981.

Under anaerobic conditions RDX transformation requires a certain amount of organic carbon. In the absence of an yeast extract no RDX was transformed in the Holston River water. This suggests that transformation of RDX by microorganism is a co-metabolic process (Spanggord *et al.*, 1980). McCormick *et al.* (1984) stated that transformation efficiency increases when the culture is supplied with a nutrient broth high in organic substrate. They concluded that the activity of biotransformation is directly proportional to the concentration of available carbon in the medium.

To solve the problem of metabolic intermediates accumulation from RDX transformation in anaerobic conditions, Roberts *et al.* (1996) proposed an aerobic polishing stage after treatment of munitions-contaminated soils. RDX and TNT are transformed in an anaerobic stage and the following aerobic stage reduces the metabolites, as indicated by a total organic carbon (TOC) reduction from 400 mg/L to 30 mg/L in 7 days. A high concentration of carbon substrate in the reactors increases the transformation rate of nitroaromatic compounds in the anaerobic stage but also increases the residual carbon to be destroyed in the aerobic stage. 0.25 % (w/w) of glucose was the best concentration for most rapid treatment by both stages.

Hesselman *et al.* (1992 a,b) applied an "indirect-off line-bioregeneration" system to treat the water contaminated with low concentration of RDX to reduce the reaction volume. The contaminated water is first concentrated by running through a series of activated carbon columns. The RDX adsorbed on the activated carbon is then desorbed

by a polar organic solvent. The organic solvent containing RDX is biologically treated to transform RDX to more degradable compounds.

In order to find the solvent which works best for both desorption of RDX from activated carbon and as the co-substrate for RDX biotransformation, Hesselman evaluated several different organic substrates under fermentative, sulfate and nitrate reducing conditions. The experiments were performed with batch reactors. He found that under fermentative conditions, peptones and proteins are good substrates for RDX transformation while in sulfate-reducing conditions, peptones and amino acids support RDX transformation. Under nitrate reducing conditions, successful co-substrates include substrates that are good for sulfate-reducing conditions, as well as ethanol, propanol, sodium acetate and sodium propionate. Hesselman detected a temporary accumulation of RDX metabolites under fermentative and sulfate reducing conditions. Denitrifying cultures using nitrate as electron acceptor and ethanol or acetic acid as co-substrate appear to be best for RDX biotransformation. Hesselman also found that desorption of RDX from activated carbon increases with increasing temperature.

Using the cultures developed by Hesselman, Wilkie (1994) compared the transformation efficiency using seven different organic co-substrates which include ethanol, acetic acid, propionic acid, formic acid, ethyl acetate, acetone and methanol in an anoxic system. She concluded that ethanol and acetic acid yielded the highest degradation efficiency and the two co-substrates also support the greatest cell growth. In

addition, ethanol is an effective RDX desorption solvent and it can be used bi-functionally in the "indirect-off-line-bioregeneration" system.

Wilkie continued her research to evaluate the effects of ethanol concentration on RDX biotransformation. She began experiments at 0.1% (1 ml EtOH/L) ethanol concentration and found that RDX transformation efficiency increased with increasing ethanol concentration (Figure 4). At 2.0 % ethanol concentration, 60% of RDX transformation was observed after the system reached steady state in two weeks. When ethanol concentration reached 5%, RDX removal efficiency dropped to be below 10%. The removal efficiency recovered in ten days after ethanol concentration was decreased to 1 % (Figure 5). This result suggests that 5% ethanol concentration might be inhibitory, but the brief exposure did not cause permanent damage. Wilkie concluded that ethanol at 2.0 % concentration was a reasonable value for the continuous-flow system experiments (Wilkie, 1994).

In addition to the co-substrate experiments, Wilkie also performed experiments to determine the effects of retention time and temperature on degradation efficiency. She found that at 2.0% ethanol concentration, the degradation efficiency increased from 55% to 80 % after raising the biological reactor temperature from 27°C to 35°C. She also discovered that an increase in retention time in bioreactors from 3 hours to 6 hours resulted in a 60 % increase in RDX degradation.

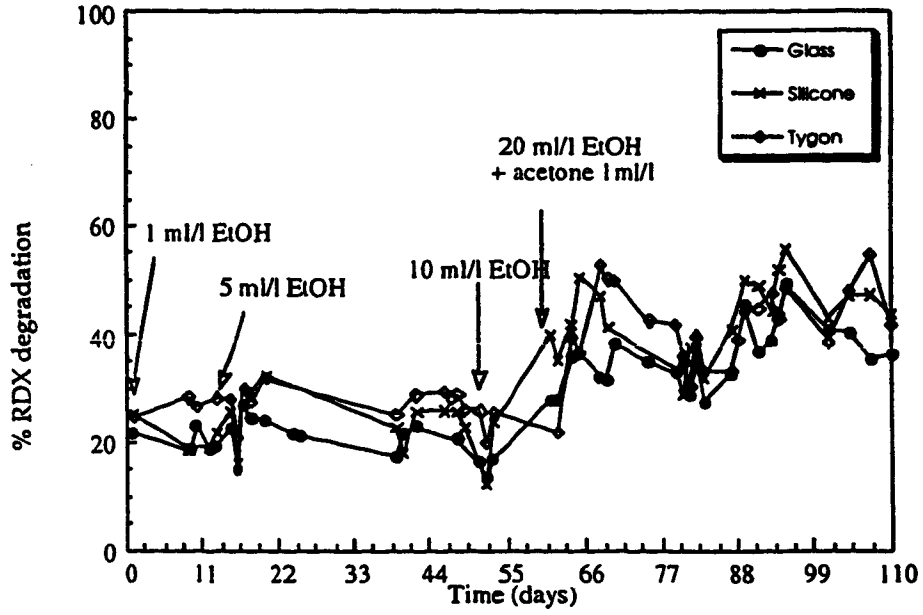


Figure 4. The effects of increasing ethanol concentration on RDX transformation efficiency in continuous flow reactors (adopted from Wilkie, 1994)

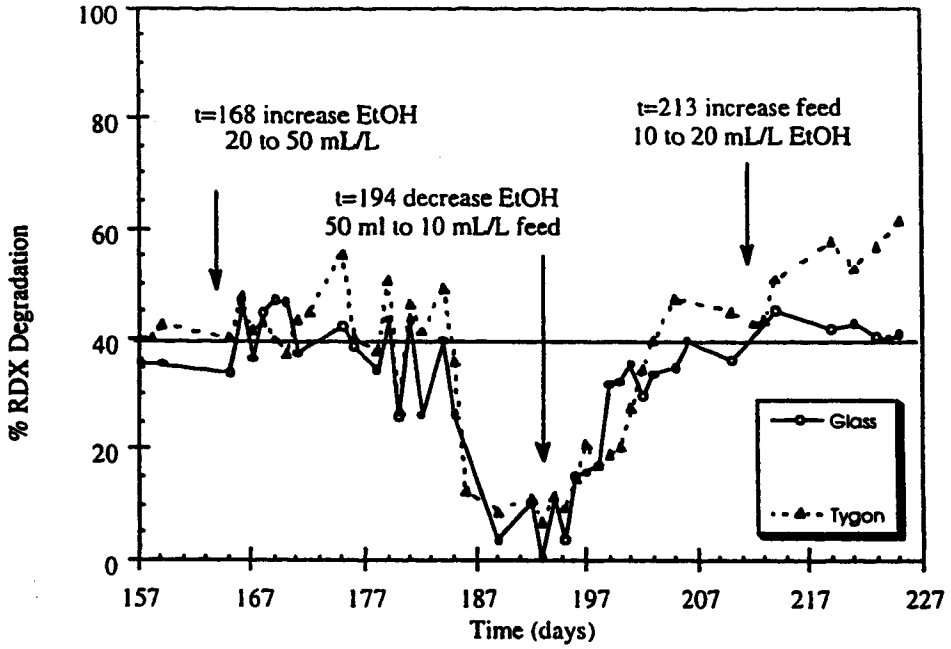


Figure 5. Inhibitory effect on microorganism at 5% ethanol concentration (adopted from Wilkie, 1994)

Kitts *et al.*(1994) at the Los Alamos National Laboratory isolated three RDX degrading species. Two of the isolates, *Morganella morganii* and *Providencia rettgeri* completely transformed RDX and its nitroso-derivatives in 45 days. The third isolate, *Citrobacter freundii* partially transformed RDX and generated high concentration of RDX nitroso-derivatives. All three isolates mineralized less than 10 % of the carbon ^{14}C from labeled RDX under oxygen depleted culture conditions.

Alatriste-Mondragon (1996) identified RDX degrading species in an anaerobic system. Using a variety of biochemical tests, volatile fatty profiles and mole percentages of guanine-cytosine (G+C), the isolate was identified as *Clostridium putrificum.* The optimum temperature and pH for the growth and RDX transformation are 40°C and 5.8 respectively. The isolate co-metabolizes RDX using glucose as carbon source and peptone as nitrogen source.

With radioisotope experiments Alatriste-Mondragon measured the production of CO_2 and other metabolites. He found that less than 3% of the ^{14}C-RDX was mineralized to $^{14}CO_2$. Radio isotope experiments also showed that there were at least six metabolites produced from RDX biotransformation; three were more polar compounds and three were less polar compounds. Comparing with standards, two of the less polar metabolites were identified to be the mono and tri-nitroso derivatives of RDX and the third less polar metabolite was proposed to be the di-nitroso derivative of RDX. A small percentage (<1 %) of the radio labeled carbon was detected in bio-mass.

2.5. Proposed Research Processes

There were three major goals in this research project. The first was to determine the minimum ethanol requirement for satisfactory (at least 60%) RDX transformation in continuous flow reactors using nitrate as the electron acceptor. The second goal was to identify the metabolites from RDX biotransformation in the same anoxic denitrifying reactors. The third goal was to increase the amount of CO_2 produced from RDX by biotransformation.

Wilkie (1994) determined the maximum tolerable ethanol concentration by microorganisms in order to minimize the dilution factor for the "indirect-off-line-bioregeneration" system. The desorption of RDX from granular activated carbon is successful only with ethanol concentration above 50 %. Process economics are more favorable at higher ethanol concentration. However follow-on processes have to remove excess ethanol from the anoxic column effluent to minimize the organic material being discharged to the environment. In addition, if biotransformation of RDX-contaminated wastewaters is desirable, process economics are more favorable at the lowest ethanol concentration. This results because ethanol must be added as a co-substrate; it is not available from activated carbon regeneration. Therefore the goal was to determine the minimum concentration of ethanol that is required to maintain the biotransformation efficiency above 60 %. Ethanol concentration was gradually decreased from 2.0 %. When transformation efficiency decreased below 50 %, an increase in retention time or a

decrease in initial RDX concentration was evaluated as an effort to recover RDX transformation efficiency.

The second goal of the project was to detect the metabolites of RDX using ^{14}C-RDX. Previous research and preliminary ^{14}C-RDX experiments showed that more than 80% of the radioactivity from ^{14}C-RDX remained in the liquid phase and less than 10% of the labeled RDX converted to CO_2. McCormick (1981) stated that mono-. di- and tri-nitroso derivatives, hydrazine, dimethylhydrazine, formaldehyde and methanol were the metabolites of RDX biotransformation in an anaerobic system. This project was to determined if the metabolites found in an anaerobic system also existed in an anoxic denitrifying system, and if the metabolites of RDX accumulated.

The third goal of this project was to increase the degree of RDX mineralization in anoxic denitrifying conditions. Various conditions for improving CO_2 production was evaluated. It was proposed that more RDX and its metabolites can be further transformed if there was sufficient nitrate as the electron acceptor. However increasing nitrate in batch reactors was difficult because of pH change, nutrient depletion and the potential accumulation of inhibitory intermediates. Acid titration and increased buffer concentration were used to control pH in the batch reactors. Diluting the batch system with fresh medium was also proposed to decrease metabolites accumulation and to solve the nutrient shortage problem.

Chapter III

Materials and Methods

3.1. Materials

3.1.1. Chemicals

RDX and [2,4,6-^{14}C]-RDX were supplied by the Lawrence Livermore National Laboratory. High Performance Liquid Chromatography (HPLC) analysis showed that on average raw RDX contains 10% of HMX. The purity of ^{14}C-RDX is greater than 99% and the specific activity is 7.75 mCi/mmol, based upon the data sheet from the supplier (Chemsyn Science Lab., Lenexa, KS). The mono-nitroso derivative of RDX (MNX) and the tri-nitroso derivative of RDX (TNX) were provided by the Naval Surface Warfare Center (Indian Head, MD, USA).

All chemicals used for nutrients and solvents were obtained from Fisher Scientific Co. Absolute grade, pure ethanol was purchased from Quantum Chemical Co. (Tuscola, IL). Sterile tuberculin syringes and needles were purchased from Owens and Minor Co. The sterile 0.2 mm ACRODISC filters were also obtained from Fisher Scientific Co. The C_8 cartridges for Solid Phase Extraction (SPE) were purchased from Varian (Harbor City, CA). The Ecolite$^{(+)}$ scintillation cocktail for ^{14}C-RDX analysis was purchased from ICN (Costa Mesa, CA). β-phenylethylamine as one of the constituent of CO_2 absorber was purchased from Sigma (St. Louis, MO).

3.1.2. Denitrifying Cultures

Anaerobic digested sludge was collected by Wilkie (1994) from the Hyperion wastewater treatment plant in El. Segundo, CA, as well as from several RDX contaminated sites near Amarillo, TX. All the sludge was diluted with oxygen free phosphate buffer, filtered and incubated in a minimal medium with oxygen free ethanol, potassium nitrate and phosphate buffer for a period of four years. All constituents of the feedings are listed in Tables 2 and 3.

3.1.3. Packed Bed Biological Reactor(Bioreactor)

The biological transformation of RDX was performed in 200 mm long Plexiglas columns packed with either glass beads (diam.~3mm, Fisher Scientific Co.) or cut Tygon tubing, size 13 (diam.~2mm, Cole-Parmer). The internal diameter of each column was 25 mm and an empty bed volume of each column was 98.17 mL. The retention time of RDX in bioreactor was three hours when flow rate was 0.3 ml/min and six hours when flow rate was 0.15 ml/min. Two glass bead columns were set-up in series to increase degradation efficiency.

Table 2. Basal Medium Composition

Component	Concentration (mg/L)
K_2HPO_4	5000
$NaH_2PO_4 \cdot H_2O$	2875
KNO_3	1600
NH_4Cl	200
$MgCl \cdot H_2O$	100
$CaCl_2 \cdot 2H_2O$	40
Na_2SO_3	19.7

Table 3. Trace Mineral Composition

Component	Concentration (mg/L)
$FeCl_3$	3.9
$MnCl_2$	0.95
$ZnCl_2$	0.66
$CoCl_2 \cdot 6H_2O$	0.58
$CuCl_2 \cdot 2H_2O$	0.30
$Na_2Mo_4 \cdot 2H_2O$	0.46
$Na_2B_4O_7 \cdot 10H_2O$	0.24

3.1.4. Continuous-flow System Set-up

The continuous-flow experiments were conducted under anoxic conditions which were achieved by degassing the feeding solution with nitrogen gas during preparation, and providing a constant supply of nitrate in the feeding solution to serve as the electron acceptor. Glass columns were operated at room temperature while the Tygon column was operated at 37°C using a circulating hot water bath. The feeding constituents are listed in Table 2 and 3. Feeding solution was continuously pumped through the bioreactors in an up flow direction using a Masterflex Model 7524-10 pump drive equipped with a multiple cartridge pump head. Tygon tubing was used to deliver the solution. Feeding solution was changed every other day and the tubing was cleaned with acid solution to prevent growth of microorganisms in feed container and tubing. The influent and effluent samples were collected and analyzed with HPLC to obtain the percentage of RDX transformed in bioreactor (Figure 6).

3.1.5. Batch Reactors Set-up

Batch experiments were conducted in 24 ml or 64 ml glass bottles with butyl-rubber stoppers. The medium for the batch experiments contained exactly the same type and concentration of nutrients as used for the continuous flow bioreactor. The inoculum for batch reactors was collected from the continuous flow system. RDX solution was prepared in acetone at 2g/L and diluted to the desired concentration in batch reactors. The glass bottles were flushed with nitrogen gas throughout the preparation to maintain

oxygen free conditions. At the end of the preparations, the batch reactors were stored in a 35°C incubator, which was the optimum temperature for cell growth. Samples for RDX removal and nitrate consumption analysis were obtained by inserting a needle-tipped tuberculin syringe through the rubber stopper to withdraw a proper amount of aliquots.

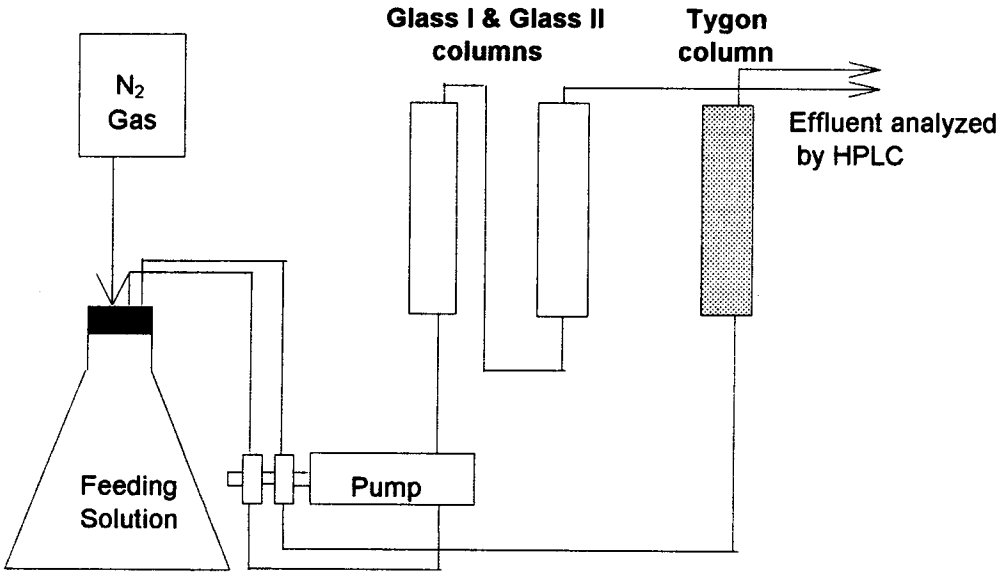


Figure 6. Continuous-flow packed bed bioreactor set-up

3.2. Analytical Methods

3.2.1. Solid Phase Extraction (SPE)

In earlier research with RDX (Wilkie, 1994), sterile 0.2 mm ACRCODISC-13 mm filters were used to filter all the samples before HPLC analysis. Lau and Lee (1995) developed a process which used Solid Phase Extraction (SPE) to further clean biological samples to increase the life span of HPLC columns. The process also concentrates the samples to achieve a lower detection limit. The C_8 cartridges were rinsed with methanol and deionized water before sample loading . After loading the samples, the cartridges were allowed to dry for twenty minutes using a vacuum. Acetonitrile was used to desorb the RDX collected by C_8 cartridges. Recovery was evaluated by the comparing directly injected RDX standards and the RDX samples eluted from SPE. 90% RDX recovery was achieved when using 500 mg C_8 cartridges.

3.2.2. High Performance Liquid Chromatography (HPLC)

HPLC analysis was performed with a Hewlett Packard 1050 Series Liquid Chromatography with a variable wavelength detector. An autosampler and a Hewlett Packard 3396 Series II integrator (Avondale, PA) were also used. The main analytical column was a 10μm, Adsorsphere, C_{18} reversed phase column (250 mm x 4.6 mm) from Alltech (Deerfield, IL). A 5 mm guard column was used to protect the main analytical column. The mobile phase consisted of 40% water, 30% methanol and 30% acetonitrile

at a flow rate of 1 ml/min. The sample injection volume was 20 µl and the detection wavelength was 236 nm. A calibration standard was prepared with 0, 2, 10, 20, and 40 mg/L in water and acetonitrile. The HPLC detection limit of RDX was 0.1 mg/L.

3.2.3. Ion Chromatography

Nitrate (NO_3^-) was measured using Dionex Ion Chromatography with suppressed conductivity detection and an Ion Pac AS9-SC column (Dionex, Sunnyvale, CA). The mobile phase consisted 0.75 mM $NaHCO_3$ and 2mM Na_2CO_3 with a flow rate of 2 ml/min. Samples were filtered through 0.2 µm Acrodisc before injection. The injected sample loop was 50 µl. Peaks were detected at approximately 1.8 minutes. A calibration standard curve using standards at 1, 2, 3, 4, 5 and 10 mg/L of KNO_3. was developed.

3.3. Effects of Ethanol Concentration Experiments

A series of experiments were conducted to investigate the effect of ethanol concentration on the transformation efficiency of RDX in continuous flow bioreactors. Throughout the analysis, all of the feeding components were kept constant except ethanol concentration. When Wilkie (1994) determined the maximum tolerable ethanol concentration for feeding the continuous flow bioreactor, the initial ethanol concentration was 0.1%. After obtaining steady state, ethanol concentration was increased step-by-step until a consistent decrease in degradation deficiency was observed. In a similar fashion, the minimum ethanol concentration requirement was determined by decreasing

ethanol concentration from 2.0 % and observing the decline of RDX removal efficiency. When RDX removal efficiency fell below 50 %, an increase in retention time of RDX from 3 hours to 6 hours in bioreactors and a decrease in initial RDX concentration from 10 mg/L to 1 mg/L were evaluated to recover the transformation efficiency.

The effect of ethanol concentration experiments were also conducted in a batch system to determine the minimum ethanol concentration required to achieve 90 % of RDX transformation in seven days. Ethanol concentration of 0.5%, 1.0%, 1.5%, and 2.0 % were evaluated.

3.4. Kinetic Study of RDX Transformation and Effects of Nitrate Concentration

The kinetic study of RDX transformation was conducted with batch reactors. The initial RDX concentration was 10 mg/L. Aliquots (0.5 ml) were withdrawn every 24 hours from batch reactors to monitor the remaining RDX concentration. The amount of nitrate was also measured by Ion Chromatography. Before HPLC and IC analysis, the samples were deproteinized by Trichloroacetic Acid Solution (TCA), centrifuged for 5 minutes and filtered by 2 μm Acrodisc to minimize the contamination on HPLC and IC analytical columns.

To observe the effect of increased electron acceptor, a specific amount of potassium nitrate was added to batch reactors each day. The amount of nitrate added was based on preliminary experiments which determined the amount of nitrate that was

consumed by batch cultures. The rate of RDX transformation was again monitored and compared to the batch cultures without additional nitrate.

3.5. pH Control Experiments

In order to maintain the normal biological functions of cells in batch reactors, the pH has to be controlled properly. Three methods to control pH in batch reactors were evaluated: the first method was to increase the buffer capacity by increasing the phosphate buffer concentration from 50 mmol to 250 mmol and to 500 mmol. The second was to use Tris-base buffer instead of phosphate buffer. The pKa of Tris-base is 8.2 so Tris buffer has better buffer capacity from pH 7 to 9 than phosphate buffer. Before using Tris buffer, the pH of Tris-base was pre-adjusted to pH 7 and pH 8 with 1 M HCl. The third method was to titrate batch cultures with acid. The acid used for titration was nitric acid which also contributed nitrate to the culture. The pH value in batch reactors was monitored with pH papers.

3.6. Analysis of By-Products

3.6.1. CO_2 production and distribution of metabolites

^{14}C-RDX was used to investigate the mineralization of RDX by measuring $^{14}CO_2$ production in batch reactors. 0.05 μCi of ^{14}C-RDX was added to a batch reactor with

total culture aliquot volume of 3.2 ml, which included 2 ml of medium, 0.7 ml of 40 mg/L unlabeled RDX and 0.5 ml of inoculum. In some of the experiments, the volumes of all the constituents were scaled up two times proportionally. In either case, the final medium concentration was the same as that in the continuous flow reactor. To measure the initial radioactivity of RDX, 0.1 ml of the aliquot was withdrawn from batch reactors and transferred to scintillation vials containing 10 ml of Ecolite$^{(+)}$ scintillation cocktail. Samples were assayed with a Beckman liquid scintillation spectrometer (Model LS 1800, Beckman, Irvine, CA) for radioactivity. The batch reactors were incubated at 35°C for varying periods of time before $^{14}CO_2$ analysis.

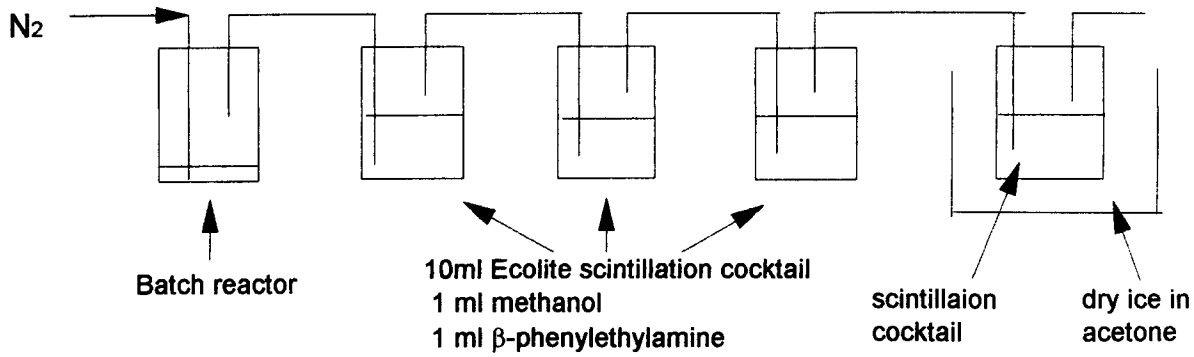
To measure $^{14}CO_2$ production 0.1 ml of 3.4 % H_3PO_4 per 3.2 ml of liquid culture to batch reactors was added to transform all the CO_2 in aqueous solution to gaseous state. Batch cultures were then purged by nitrogen gas with a minimum flow rate for five minutes. CO_2 was trapped by three scintillation vials in series with CO_2 absorbers which included 10 ml of Ecolite$^{(+)}$ scintillation cocktail, 1 ml of methanol and 1 ml of β-phenylethylamine. A cold trap containing acetone and dry ice was attached at the end of the series to trap any volatile compounds that were not absorbed in CO_2 traps (Figure 7).

Two other CO_2 trapping systems were also evaluated. The first one used a microcentrifuge tube containing the same CO_2 absorber as above in batch system. The second one contained 500g/L of KOH as CO_2 absorber. The tubes were placed inside the batch reactors at the beginning of an experiment (Figure 7). At the end of an experiment 0.1 ml of the liquid absorber was analyzed for radioactivity. The cultures were purged

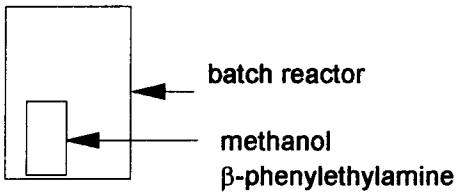
with nitrogen gas and trapped by regular CO_2 traps to capture any CO_2 that was not trapped in the absorber inside the batch reactors.

After gas purging, the liquid cultures were transferred to plastic test tubes for centrifugation. The final volume of the batch sample was measured during transfer. After the samples were centrifuged at 7,500 rpm for 8 minutes to separate cells from supernatant, 0.1 ml of the supernatant was withdrawn to measure the ^{14}C-RDX radioactivity in the liquid phase. The cells were washed twice with 1ml of 1M NaCl solution. 0.1 ml of the wash aliquot was transferred to a vial with 10 ml Ecolite$^{(+)}$ scintillation cocktail to be assayed for radioactivity. The pellets at the bottom of the tube were re-dissolved in 0.1 ml of 1M NaCl. The whole 0.1 ml of the mixture was then assayed for radioactivity.

a. The Ragular gas purging system



b. Methanol and β-phenylethylamine trap



c. KOH trap

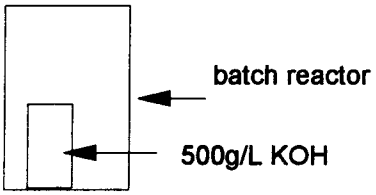


Figure 7. The CO_2 trapping systems. a. Three vials in series, purged with nitrogen gas at the end of an experiment. b. A microcentrifuge tube containing 1:1 methanol and β-phenylethylamine is inserted in the batch reactor to trap CO_2. c. A microcentrifuge tube containing 500 g/L of KOH is inserted in the batch reactor to trap CO_2.

3.6.2. The Measurement of Water-Soluble Metabolites of RDX

3.6.2.1. Standard curve of RDX nitroso-derivatives.

Different solvent (water, methanol and acetonitrile) percentages and flow rate were evaluated to obtain the solvent composition that best separated RDX and the three nitroso-derivatives. After the best solvent composition was determined, 0, 2, 10, 20 and 40 mg/L of TNX, MNX and RDX were injected to HPLC to obtain the standard curve (Appendix I).

3.6.2.2. Liquid-Liquid Extraction and Measurement for nitroso-derivatives of RDX

To analyze the metabolites in the liquid portion of a batch reactor, liquid samples were first concentrated by liquid-liquid extraction. The entire aliquot was first centrifuged at 10,000 rpm for 10 minutes to separate cells and supernatant. The liquid culture was pre-acidified to pH=1 to observe most peaks on the HPLC chromatogram. The aliquots were transferred to glass culture tubes (25 x150 mm). Next, the same volume of ethyl acetate was added to the aliquot. The glass culture tubes were vortexed for 15 seconds and left to settle for 10 minutes to ensure a good separation between organic and liquid layers. The organic layer was then collected and pooled in an another glass culture tube. The extraction processes were repeated three times. The pooled organic portion was then evaporated with a gentle stream of nitrogen gas to dryness and the metabolites were re-dissolved in 0.3 ml of acetonitrile which does not give a solvent

peak on HPLC chromatogram. The kinetics of RDX transformation and nitroso-derivative production were established over a five day period.

3.6.2.3. Measurement of Water-Soluble Metabolites with ^{14}C-RDX

^{14}C-RDX was added to batch cultures to increase the sensitivity and specificity of RDX nitroso-derivatives analysis as well as the analysis of all the other water-soluble metabolites. The same liquid-liquid extraction method for unlabeled RDX was applied to the labeled RDX analysis. The final extract was in 100 µl of ethyl acetate. For the labeled RDX metabolites analysis, an HPLC radiochromatographic system consisting a Varian Vista model 5500 (Varian, Walnut Creek, CA), a Rheodyne injector (Rheodyne Inc., Cotati, CA) with a 20 µl sampling loop and a reverse-phase column (Adsorbosphere C18; 10 µm; 250mm x 4.6 mm; Alltech Associates Inc., Deerfield, IL). Fractions (0.5 ml) were collected using a microfractionator model FC-80K (Gilson, Middleton, WI). 20 µl of the extract was injected into HPLC for fractionation. 300 µl of the sample from each fraction were transferred to vials containing 10 ml of Ecolite$^{(+)}$ scintillation cocktail for radioactivity assayed by LSS. The results of the counting were used to construct radiochromatograms. The whole aliquot in each fraction was also counted in order to perform a mass balance. A solvent composition of 90% water and 10% methanol with flow rate at 1.5 ml/min was used to separate the peaks of RDX and its nitroso-derivatives. The relative retention time between the peaks on HPLC-LSS profile were calculated and

compared to the relative retention time of the standard RDX, MNX and TNX on the HPLC-UV chromatogram.

3.6.3. Formaldehyde analysis

The method to detect formaldehyde in batch reactors was adopted from Kuwata *et al.* (1979) with minor modifications. Formaldehyde was to be derivatized by 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone. The derivatives were extracted by solid phase extraction with C_8 bond phase cartridges and then desorbed by acetonitrile. The sample was analyzed by HPLC with a reverse phase C_{18} column. The mobile phase consisted of 40% acetonitrile and 60% water with a flow rate at 1.5 ml/min. The detection wavelength was 254 nm. The detection limit for formaldehyde was 0.1 mg/L.

3.6.4. Methanol Analysis

The aliquots from batch reactors were analyzed for methanol. The aliquot was first deproteinized by TCA, centrifuged and filtered. TCA was also used as an internal standard during gas chromatography analysis. The methanol analysis was performed on a Varian Vista model 6000 gas chromatography (Varian Instrument Group, Palo Alto, CA) equipped with a flame ionization detector and a reporting integrator. The bond stationary phase was a Supelcowax 10 (30m x 0.25 mm id) from Supelco (Bellefonte, PA). The

sample was delivered with split injection at a split ratio of 15:1. The injector and detector temperature were maintained at 250 °C and 290 °C, respectively. The initial oven column temperature was 60 °C for 12 minutes then elevated rapidly (30°C/min to a final temperature of 180°C to remove water and contaminants from the column). The temperature was maintained for 5 minutes prior to cooling for the analysis of the next sample.

Chapter IV

Results and Discussion

4.1. The Effects of Ethanol Concentration on RDX Transformation Efficiency

Before starting the experiments to determine the minimum ethanol concentration required to transform RDX, the continuous-flow packed bed reactors were maintained with 2.0 % ethanol in the feeding solution for two years. The transformation of RDX reached almost 100% during this time of incubation, although fluctuation in RDX transformation efficiency was observed constantly (Figures 8, 9). The influents and effluents from glass and Tygon columns were collected and analyzed once a week.

The ethanol concentration was first decreased from 2.0 % to 0.2 %. Three weeks was allowed for the transformation process to reach a steady state. The flow rate was 0.3 ml/min, Tygon column temperature was 35°C and the initial RDX concentration was 10 mg/L. The results of Tygon column showed a drop in RDX transformation efficiency from almost 100% to around 80 % following the decrease of ethanol concentration (Figure 10). After four months, the ethanol concentration was decreased from 0.2 % to 0.1 %. RDX transformation efficiency decreased to a range between 60 % to 70 %. When ethanol concentration was decreased again to 0.04%, almost zero transformation efficiency was observed. This fact demonstrated that 0.1% ethanol concentration is the minimum ethanol concentration required to achieve at least 60% of

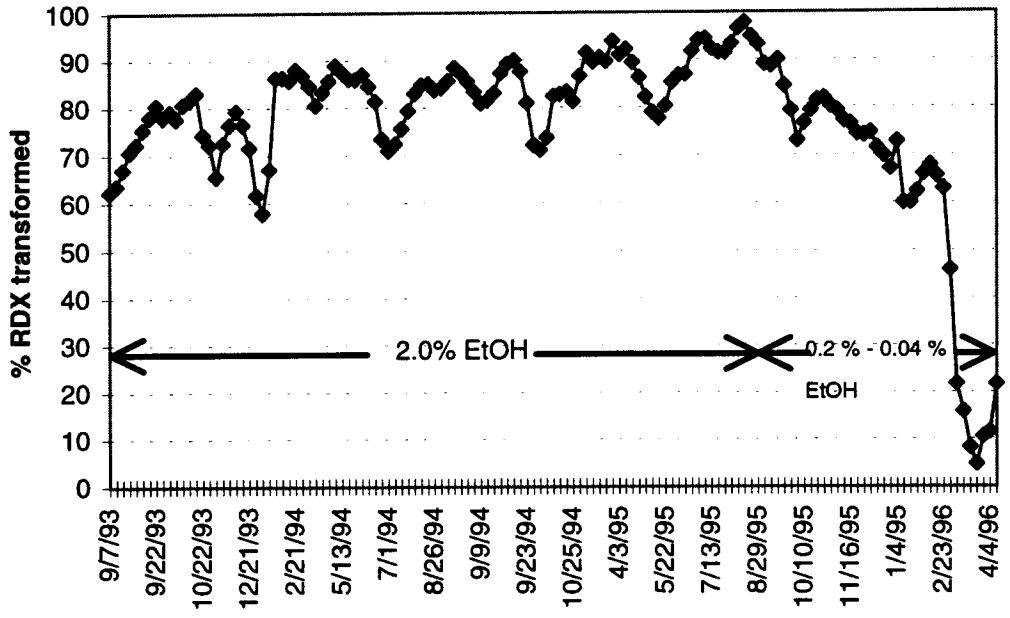


Figure 8. Transformation efficiency of RDX in Tygon column continious-flow packed bed reactor from 9/7/93 to 4/9/96

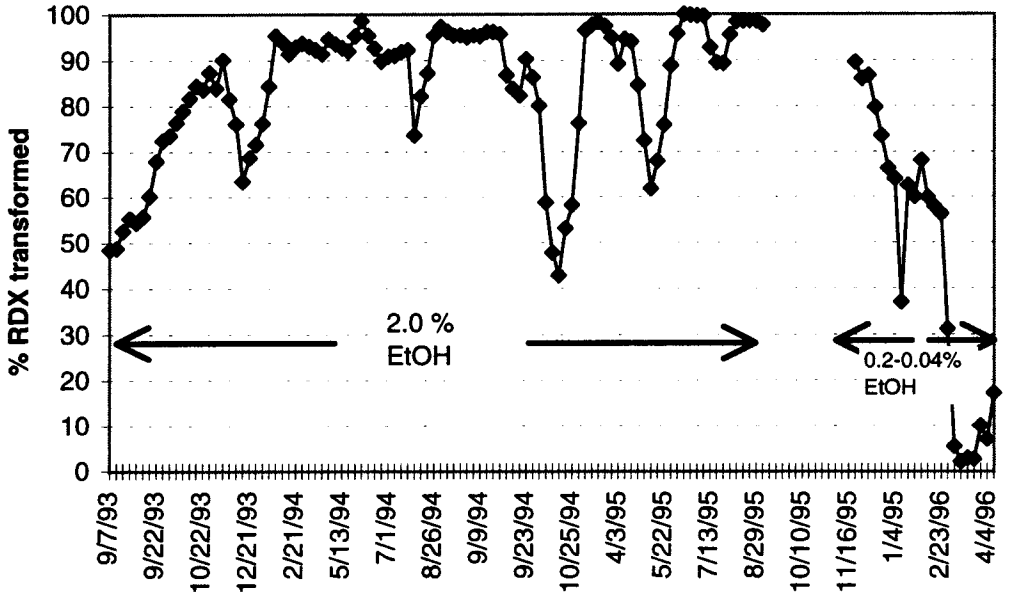


Figure 9. Transformation efficiency of RDX in two glass columns-in-series continious-flow packed bed reactor from 9/7/93 to 4/9/96

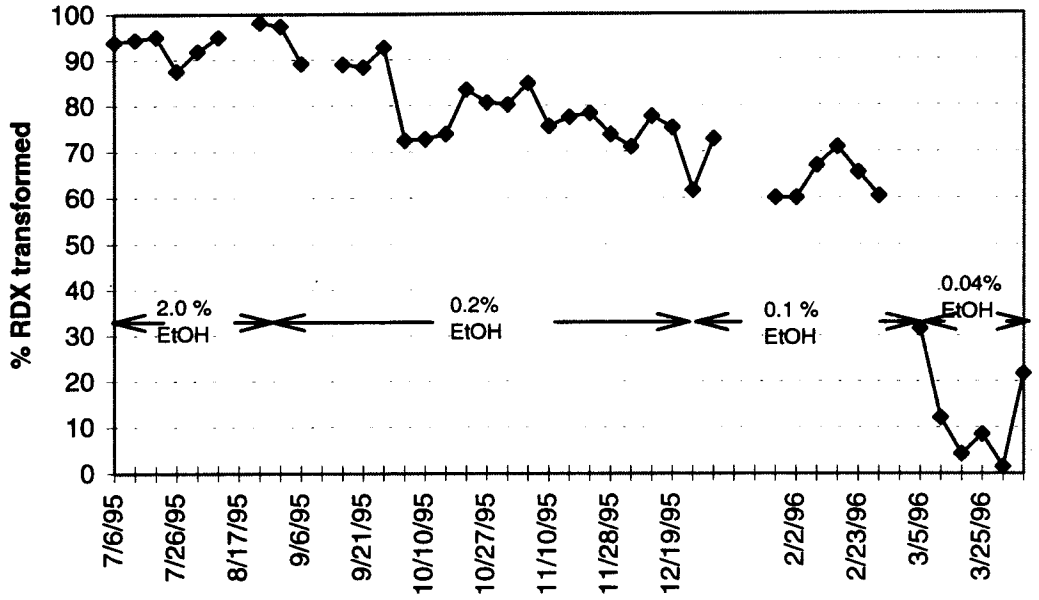


Figure 10. The effects of decreasing ethanol concentration on RDX transformation efficiency in Tygon bioreactor

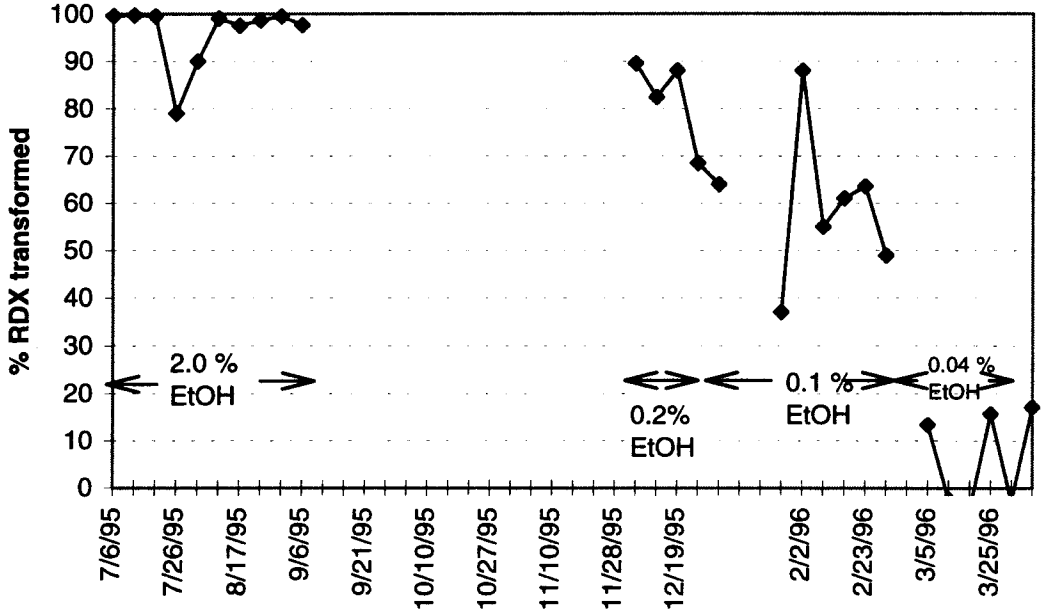


Figure 11. The effects of decreasing ethanol concentration on RDX transformation effeciency in two glass columns-in-series bioreactor.

RDX transformation in a continuous-flow packed bed reactor under the present conditions. The comparison between glass and Tygon columns showed that an elevation in temperature did not help to increase RDX transformation efficiency (Figures 10,11).

For the actual wastewater treatment process, 0.1 % (100 ml/L) ethanol in wastewater treatment system is considered a high value. This ethanol concentration can cause an undesirable microorganism growth. To achieve a higher RDX transformation efficiency with low ethanol concentration, two solutions were proposed: (1) decrease the flow rate through the continuous-flow reactor to increase the retention time of RDX in reactors and (2) decrease the initial RDX concentration.

The flow rate was decreased from 0.3 ml/min to 0.15 ml/min which increased the retention time in reactors from three hours to six hours. The ethanol concentration was maintained at 0.1 %. After six weeks of incubation, the removal efficiency was between 60 % to 70 %, which was about the same as the experiments without an increased in retention time (Figure 12). The results showed that an increase in retention time was not enough to compensate the decrease in ethanol concentration; an increase in retention time was only effective in increasing RDX transformation efficiency when ethanol concentration was 2.0% as stated by Wilkie (1994), but not when ethanol concentration was as low as 0.1%.

The second alternative was to decrease RDX concentration in feeding solution from 10 mg/L to 1 mg/L. Ethanol concentration was 0.04% and the flow rate was 0.15

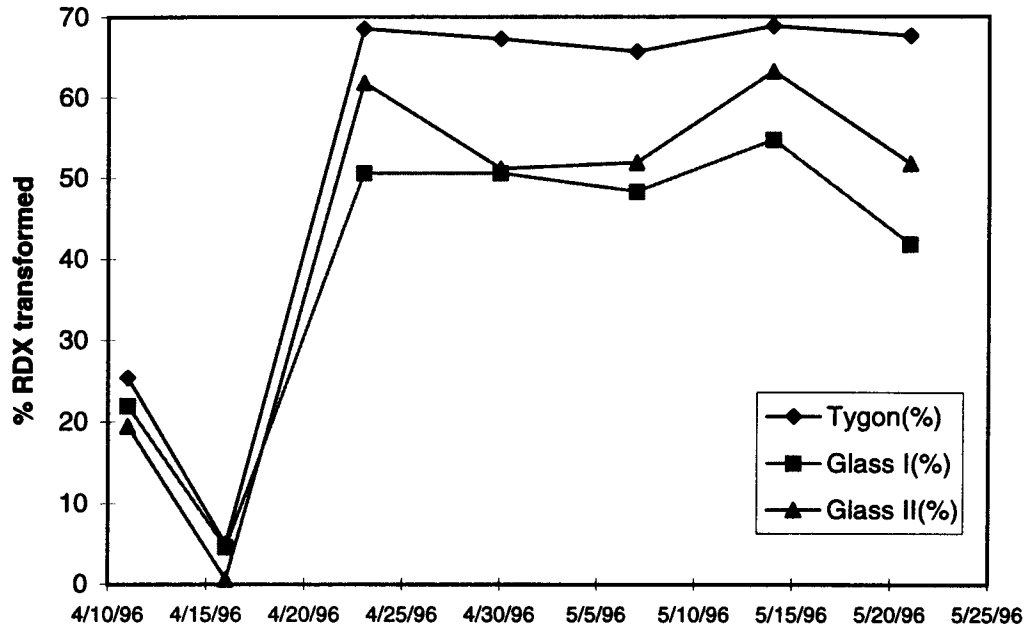


Figure 12. RDX transformation efficiency after the retention time of RDX in bioreactor was increased from 3 hours to 6 hours. Ethanol concentration was 0.1 %

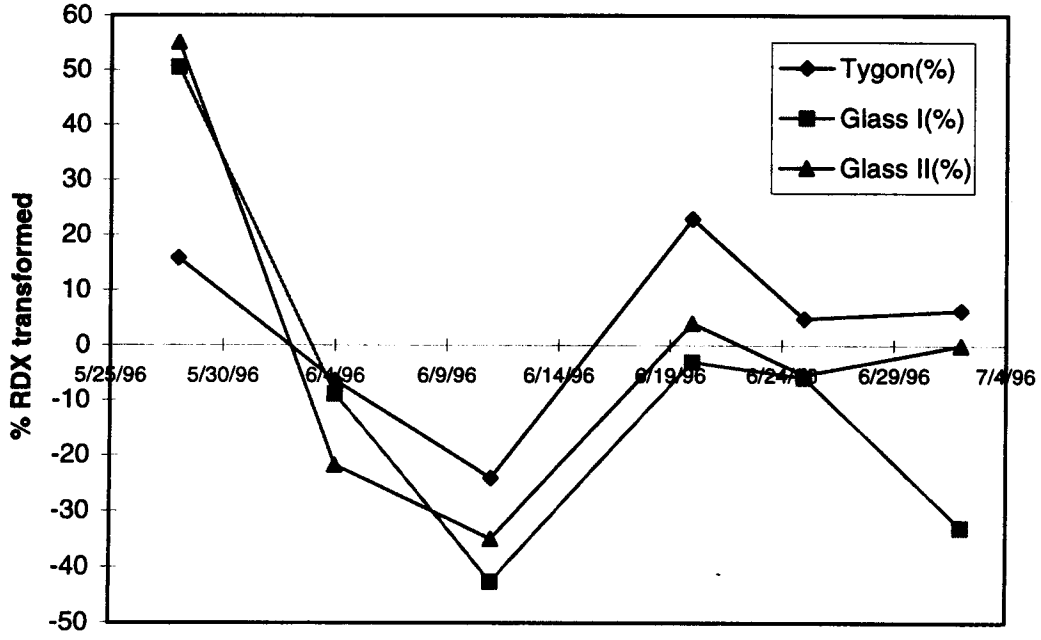


Figure 13. RDX transformation efficiency after the retention time of RDX in bioreactor was increased from 3 hours to 6 hours and the initial RDX concentration was decreased from 10 mg/L to 1 mg/L. Ethanol concentration was 0.04%

ml/min. The results of this experiment showed that over six weeks of incubation, the transformation efficiency stayed close to 0 % (Figure 13). The decrease in initial RDX concentration along with an increase in retention time did not yield good RDX transformation efficiency. Other methods have to be investigated to decrease the ethanol concentration in feeding solution.

The results of the effect of ethanol concentration in batch reactors are shown in Table 4. After seven days, the RDX transformation efficiency was relatively constant for ethanol concentration above 0.5 %, however 0.2% ethanol concentration resulted in significantly lower transformation efficiency. Therefore 0.5 % ethanol is the sufficient ethanol concentration for batch experiments.

Table 4. Effects of ethanol concentration on RDX transformation efficiency in batch reactors

Ethanol conc.	KNO_3 conc.	RDX transformed in seven days
0.20%	1.6 g/L	83.00%
0.50%	1.6 g/L	93.40%
1.00%	1.6 g/L	95.00%
1.50%	1.6 g/L	91.60%
2.00%	1.6 g/L	91.00%

4.2. RDX Transformation Kinetic Study and the Effects of Nitrate Concentration

A RDX transformation kinetic study was conducted in batch reactors. The initial concentration of RDX was 10 mg/L and the RDX and nitrate remaining in the reactors were monitored daily. Figure 14 shows that the initial amount of nitrate (1.6 g/L KNO_3) was depleted during the first three days; however the RDX continued to decrease after nitrate was depleted. This suggests that the culture can transfer RDX even after the primary electron acceptor is depleted. The continued transformation of RDX after the depletion of the terminal electron acceptor suggests that the enzyme responsible for RDX transformation is in abundance, or the storage products are available to temporarily continue the reaction. Comparing Figure 14 and Figure 15 shows that RDX transformation is more rapid in the presence of excess nitrate.

Figure 15 shows a decline in RDX transformation rate around 70 hours. The reason that decreased RDX transformation rate after the second potassium nitrate addition was probably due to increasing pH or to the accumulation of inhibitory metabolites or end products.

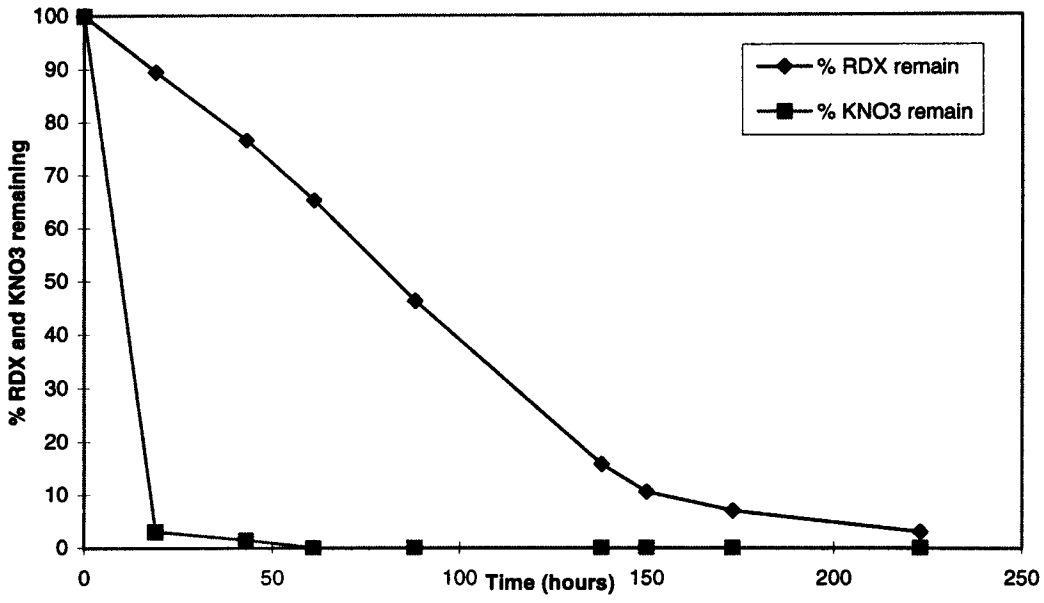


Figure 14. Kinetic study of RDX transformation and nitrate removal in batch reactors. Initial RDX concentration was 10.4 mg/L; initial potassium nitrate concentration was 2.0 g/L and initial ethanol concentration was 0.5 %.

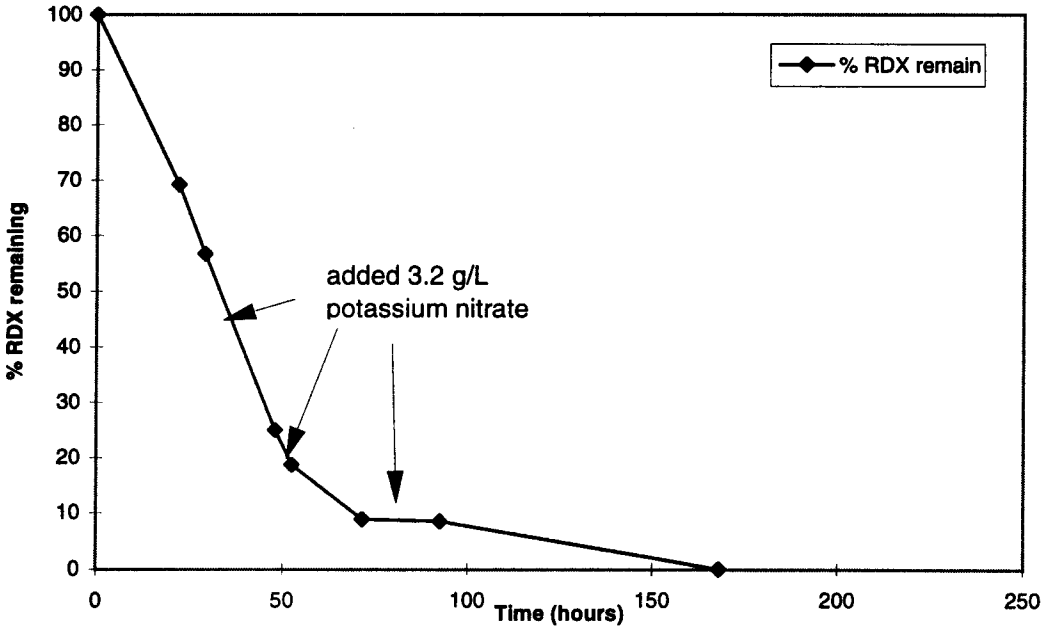


Figure 15. Kinetic study of RDX transformation with fresh nitrate added to batch reactors every 24 hours. Initial RDX was 12.0 mg/L, initial potassium nitrate conc. was 2.0 g/L and initial ethanol conc. was 0.5 %.

4.3. pH Control Experiments

In order to understand the reasons for the decreased RDX transformation rate in batch reactors, the pH in batch reactors was monitored. Figure 16 shows that in a batch reactor without additional nitrate, pH in the reactor increased form 7 to 8 in 24 hours. In a batch reactor with nitrate added to maintain electron acceptor, pH increased from 7 to 8.5 in 24 hours and to 9 in 48 hours. One reason that increased pH in batch reactors was the denitrification reaction which transformed NO_3^-, an acid, to nitrogen gas. The other reasons may include the alkalinity produced from potassium nitrate after nitrate was depleted and from RDX biotransformation metabolites.

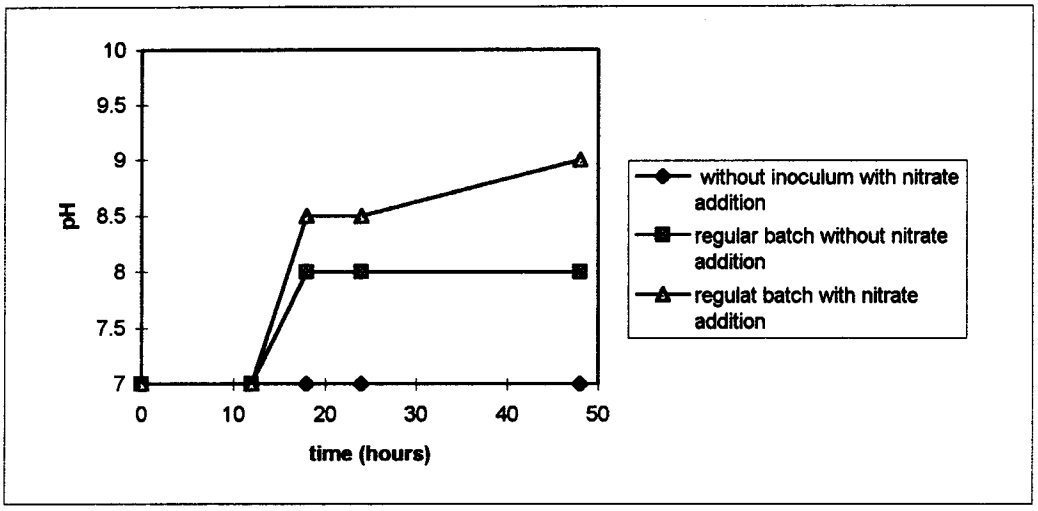


Figure 16. The pH change in batch cultures with or without nitrate addition

The pH increase in the batch reactors can be very unfavorable to microorganisms' growth and function. In order avoid pH increase in the batch reactors, three methods were tried: 1) Increasing the initial phosphate buffer concentration to increase buffer capacity; 2)Using Tris-base instead of phosphate buffer; and 3)Applying acid titration to neutralize the alkalinity in a batch culture. The effectiveness of above pH control methods were evaluated by the maximum amount of nitrate that was consumed by microorganism. As long as nitrate was being consumed, it was assumed that the culture was not inhibited by high pH.

The experimental results showed that when the initial buffer concentration was increased five times (250 mmol), two times more nitrate could be added to the reactor before inhibiting microorganisms' activity; however an increase in initial buffer concentration to ten times as much (500 mmol) did not increase nitrate consumption. The excessive potassium and sodium from phosphate buffer might have inhibited the growth of microorganism.

Acid titration also appeared successful. However the amount of acid required to neutralize batch systems was difficult to control because the experiments were on a small scale (6.4 ml total batch volume). In addition, there was not a full understanding of alkalinity producing mechanism by batch cultures. The amount of acid added was first calculated to balance the hydroxide ion that was produced during the denitrification process and the amount of free potassium ion that was produced when nitrate ion was consumed. The pH of batch cultures was monitored constantly to observe the pH change

and to adjust the acid to be added to batch reactors to maintain a constant pH value between 7 and 8.

To effectively control the pH in a batch reactor, a combination of increasing initial phosphate concentration and applying acid titration was evaluated. Experimental results showed that this process was successful. With the proper amount of acid added to the batch culture and enough buffer capacity in the system, three times as much of potassium nitrate can be consumed by a batch culture.

The experiments with Tris-base showed that it was than phosphate buffer because it buffered the system better around pH=8. Phosphate buffer was initially selected for the batch experiments because it was also used in the continuous flow reactors. The continuous flow reactors did not exhibit pH problems.

Another attempt to solve the problem of pH increase was to add fresh medium to batch reactors. This method not only reduces the increase in pH but also dilutes the accumulation of metabolites that might be harmful to microorganism. However the dilution experiments were not successful with either 5 times dilution or 0.5 times dilution. The rate of nitrate consumption decreased may be caused by the dilution of a key enzyme or a nutrient. A continuous flow reactor might not have this problem because fresh medium was constantly supplied and metabolites was constantly removed.

4.4. Optimization of $^{14}CO_2$ Production

At the beginning of CO_2 production experiments, the conditions in the batch reactors were adjusted to match with the conditions in continuous flow system. The batch system was supplied with the same potassium nitrate and initial RDX concentrations and similar cell density as in the continuous flow reactor. 2.0 % ethanol was provided which was the threshold ethanol concentration for continuous flow system. At this initial stage of experiments, less than 10% of the radioactivity from ^{14}C-RDX was transferred to $^{14}CO_2$, more than 80% of the radioactivity stayed in liquid phase after four days. The duration of experiments was not a factor.

The second attempt was to increase the amount of nitrate in batch reactor to fully oxidize ethanol as the co-substrate. The stoichiometry is the following:

$$5C_2H_5OH + 12\ NO_3^- + 12H^+ \rightarrow 10CO_2 + 6N_2 + 21H_2O \quad \text{.....(1)}$$

Based on this stoichiometry, the next experiment used 9.9 g/L of potassium to fully oxidize the 0.2 % ethanol. However this attempt was not successful in increasing CO_2 production. The most probable reasons were the low ethanol concentration (0.2%) or the excessive initial potassium nitrate concentration.

In order to avoid high initial nitrate concentration, less amounts of potassium nitrate were added to reactors in three to five days interval. This was successful as shown in Table 5. The percentage of $^{14}CO_2$ production increased as the amount of

potassium nitrate added to reactors increased. Radioactivity in liquid phase also decreased significantly with increasing nitrate addition.

Table 5. Effects of potassium nitrate concentration on RDX mineralization

Days	EtOH conc.	KNO_3 conc. (g/L)	$^{14}CO_2$ produced	label in liquid	label in cells	total label recovery
5	2%	1.6	9.03%	79.97%	0.09%	89.51%
7	2%	3.2	10.70%	73.42%	0.05%	83.38%
12	2%	3.2+4.8*	12.20%	68.81%	0.44%	81.45%
16	2%	3.2+4.8+4.8**	17.80%	53.42%	0.21%	71.75%

* fresh potassium nitrate was added to batch culture on the fifth day
** fresh potassium nitrate was added to batch culture on the fifth and the tenth day

A kinetic study of nitrate utilization showed that a batch culture was able to deplete 3.2 g/L of potassium nitrate in 24 hours. In the next experiment 3.2g/L potassium nitrate was added to each batch reactor daily. Ethanol concentration was also decreased from 2% to 0.5%, which was the sufficient ethanol concentration to transform more than 90% of RDX in seven days. The results again showed an increasing trend of $^{14}CO_2$ production with increasing amount of nitrate added to the reactor (Table 6).

Figure 17 shows the data from Table 6. The horizontal axis in Figure 17 is the ratio of electron acceptor to ethanol masses. When the ratio equals 1, there is just enough of nitrate to fully oxidize the ethanol. Figure 17 shows that CO_2 production increased with increasing nitrate to ethanol ratio A batch culture with nitrate to ethanol ratio of 0.75 transformed 38.02% of the radioactivity from ^{14}C-RDX to $^{14}CO_2$, which was the maximum amount of RDX mineralization at this point.

Table 6. The complete data of RDX mineralization experiments and the distribution of metabolites

Days	EtOH Conc.	KNO_3 Conc. (g/L)	KNO_3/EtOH	label in liquid	label in cell mass	$^{14}CO_2$ produced	total recovery	normalized CO_2 production
7	2%	1.6	0.02	65.20%	N/A	4.40%	69.30%	6.35%
2	2%	1.6	0.02	71.20%	N/A	6.80%	78%	8.72%
5	0.20%	9.9	1.09	88.70%	N/A	6%	94.70%	6.34%
5	2%	1.6	0.02	88.40%	0.08%	10.10%	99.30%	10.17%
5	2%	1.6	0.02	78.60%	0.06%	9.40%	88.00%	10.68%
5	2%	1.6	0.02	73.50%	0.12%	7.60%	81.22%	9.36%
7	2%	3.2	0.04	73.44%	0.05%	10.70%	83.38%	12.83%
12	2%	3.2+4.8	0.09	68.81%	0.44%	12.20%	81.45%	14.98%
16	2%	3.2+4.8x2	0.11	52.42%	0.21%	17.80%	71.45%	24.91%
7	0.50%	2.0+3.2	0.23	59.82%	0.29%	26.35%	86.51%	30.46%
7	0.50%	2.0+3.2X2	0.37	64.80%	1.50%	21.20%	87.51%	24.23%
7	0.50%	2.0+3.2X2	0.37	76%	0.09%	34.17%	110.26%	30.99%
7	0.50%	2.0+3.2X2	0.37	58.75%	0.14%	26.17%	85.06%	30.77%
7	0.50%	2.0+3.2X2	0.37	71%	N/A	21%	92.36%	23.02%
7	0.50%	2.0+3.4X5	0.75	54.20%	0.90%	35.20%	92.50%	38.05%
16	1.10%	2.0+3.4X8	0.54	59.50%	1.20%	29%	89.60%	32.25%

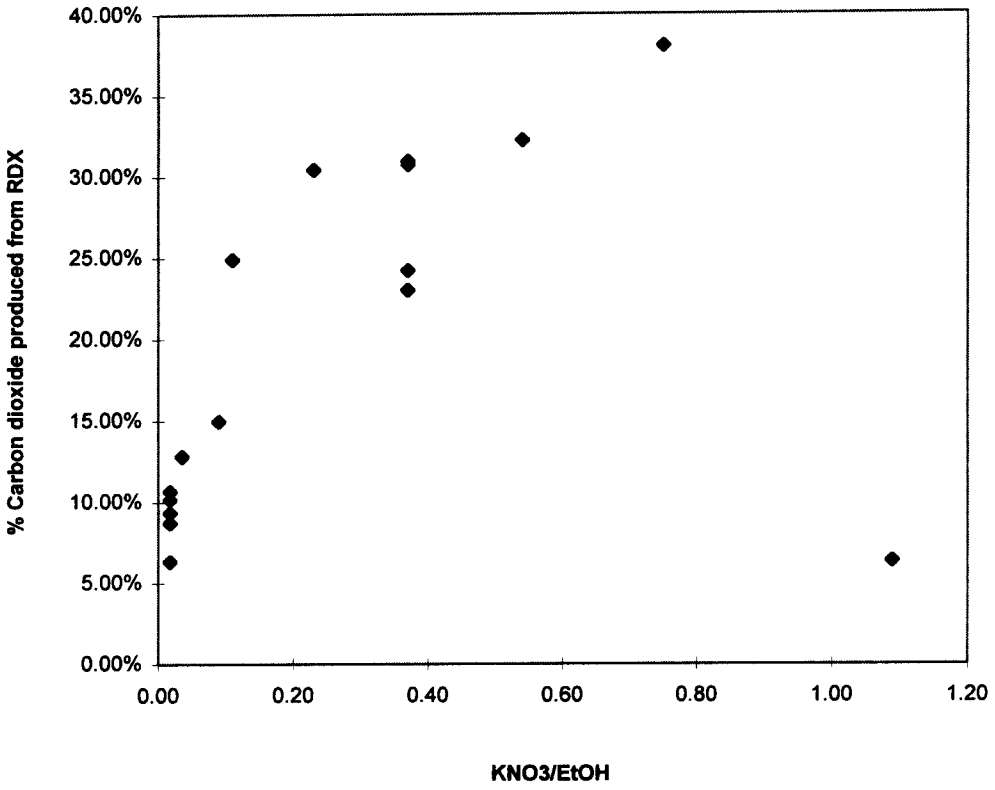


Figure 17. The relation between RDX mineralization and nitrate/ethanol redox ratio. The % CO_2 produciton is the normalized values. The point at right bottom corner represents insufficient ethanol concentration

Maximum amount of mineralization was achieved in a batch culture with properly controlled pH. The CO_2 mineralized from RDX might increase if more nitrate were added to the reactor; however there is an upper limit of the nitrate that can be consumed by the batch culture. Other unknown reasons may inhibit nitrate utilization and RDX mineralization.

The alternative CO_2 trapping methods did not increase CO_2 recovery and the set up was inconvenient to work with. Therefore the alternative methods was abandoned and CO_2 in all the experiments was collected by using the regular nitrogen purging method (Figure 7, a).

4.5. Kinetics of Nitroso Derivatives of RDX

Nitroso derivatives of RDX are the biotransformation metabolites with the most concern because they are carcinogenic and mutagenic. The kinetics of RDX nitroso derivatives production was observed over a period of five days in batch system. A recovery test showed that ethyl acetate was able to extract above 98% of RDX and MNX and above 80% of TNX from the liquid phase. Due to ethyl acetate interference with the HPLC, the extract was exchanged into acetonitrile before HPLC analysis.

RDX, MNX and TNX were separated in the HPLC using mobile phase 40% methanol and 60% water, with a flow rate of 1.0 ml/min. This composition gave the best separation and resolution of RDX, MNX and TNX. A sample HPLC chromatogram is shown in Figure 18. The detection limit of MNX and TNX on HPLC was 0.25μg/L and with a 25 fold concentration by liquid-liquid extraction, the minimum detectable concentration of MNX and TNX was 10 μg/L.

To study the kinetics of transformation, RDX and its metabolites were observed for five days. Figure 19 shows that RDX decreased to 2% of its initial concentration after five days; MNX reached a maximum on the first day and decreased to almost 0% on the fifth day. DNX and TNX were never observed in the five-day kinetic study. The results show that MNX was produced from RDX transformation and reached a maximum amount on the first day. It was quickly transformed to other compounds and did not accumulate. DNX and TNX were either not produced or transformed quickly to other compounds that they were never detected.

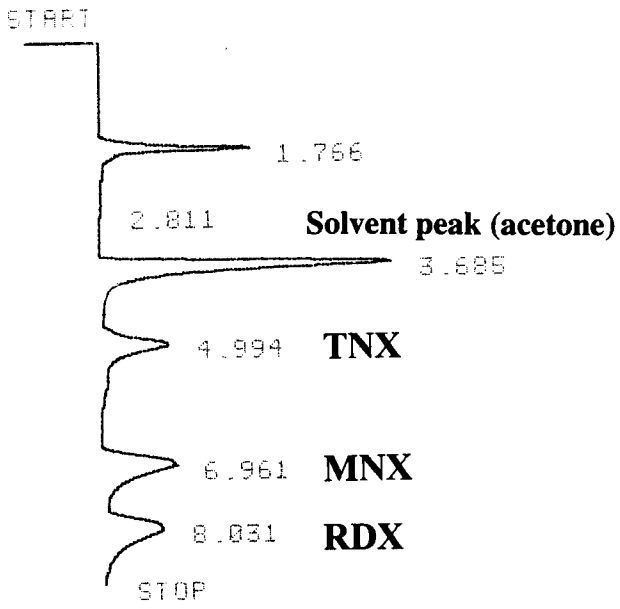


Figure 18. The sample HPLC-UV chromatogram of standard RDX, MNX and TNX. The mobile phase was 40% methanol and 60% water with a flow rate of 1.0 ml/min.

Table 7. Five days kinetic data of RDX nitroso-derivatives production

Days	RDX (mg/L)	MNX (mg/L)	TNX (mg/L)
0	11.36	0	0
1	5.25	0.3	0
2	3.31	0.05	0
3	2.15	0.02	0
5	0.24	0.07	0

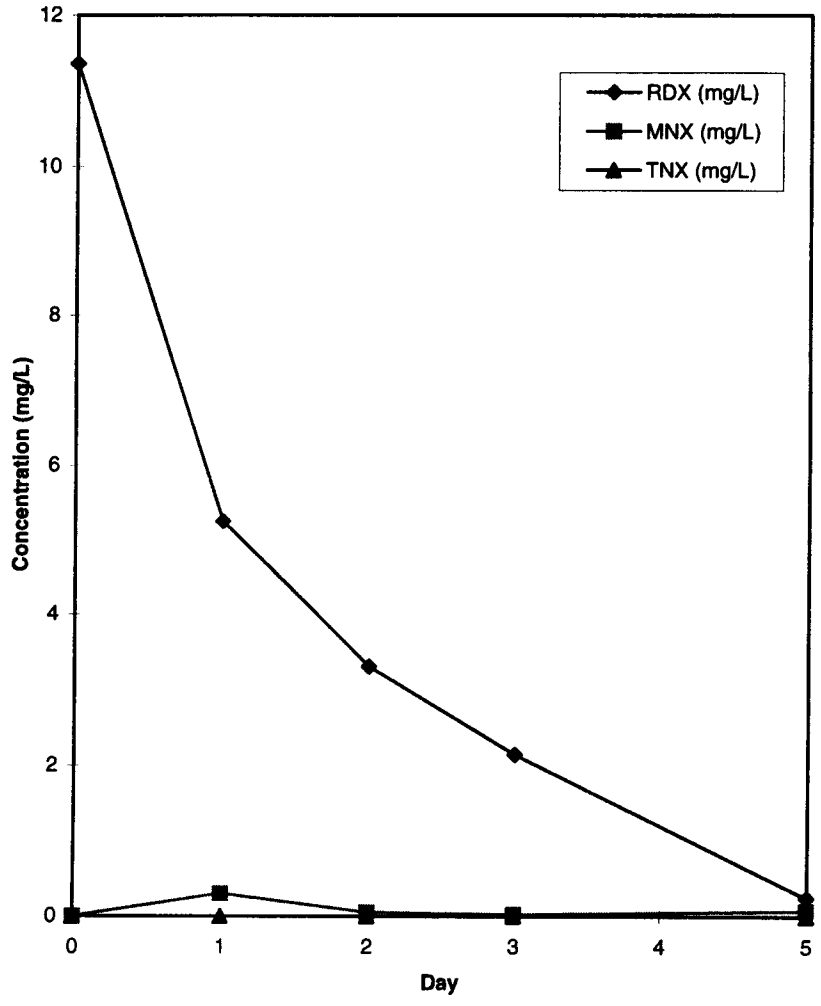


Figure 19. Kinetics of RDX and its nitroso-derivatives under anoxic denitrifying conditions

In addition to the nitroso derivatives of RDX, the HPLC chromatogram also shows unknown peaks at earlier retention times (2 to 3 minutes). These peaks were also observed in the controls without RDX. For this reason they were assumed to be metabolites from ethanol degradation. However, the peaks could also hide RDX metabolites and other solvent compositions will have to be examined in order to further examine them. Alternatively, a normal phase HPLC method could be used instead of the reverse phase method.

The effluents of the continuous flow bioreactor were also analyzed for RDX nitroso derivatives. Samples were collected at an arbitrary time and were prepared the same way as the samples form batch cultures. The results of the continuous flow bioreactor matched the results of the batch reactor. With 10 mg/L influent RDX concentration, 0.03 mg/L to 0.07 mg/L of MNX was observed. One sample showed a peak that could have been DNX. The peak area was smaller than that of MNX. TNX was never observed.

4.6. Water Soluble Metabolites of ^{14}C-RDX

The water soluble metabolites of RDX were analyzed using ^{14}C-RDX. The stock ^{14}C-RDX solution was first analyzed for its purity by capturing all fractions from an HPLC analysis. Two impurities in ^{14}C-RDX stock solution were found. Figure 20 shows one impurity between fractions 5 to 7. The other impurity appeared between fractions 35 to 40. A preliminary ^{14}C-RDX metabolites study showed that the metabolites produced from RDX (Figure. 21) had exactly the same retention time as that of ^{14}C-RDX impurities. In order to eliminate the uncertainty between metabolites and impurities, stock ^{14}C-RDX was first purified.

^{14}C-RDX was purified by HPLC fractionation. Stock ^{14}C-RDX solution was injected into HPLC, and the fractions which contained pure ^{14}C-RDX were collected and pooled in one test tube. The pure ^{14}C-RDX in methanol and water as the mobile phase for HPLC fractionation was then extracted by ethyl acetate, evaporated to dryness and re-dissolved in acetone. The purity of the purified ^{14}C-RDX was checked again by HPLC-LSS. Figure 22 shows a single RDX peak, which confirms the purification process. The recovery of the pure ^{14}C-RDX from the stock solution was 90%.

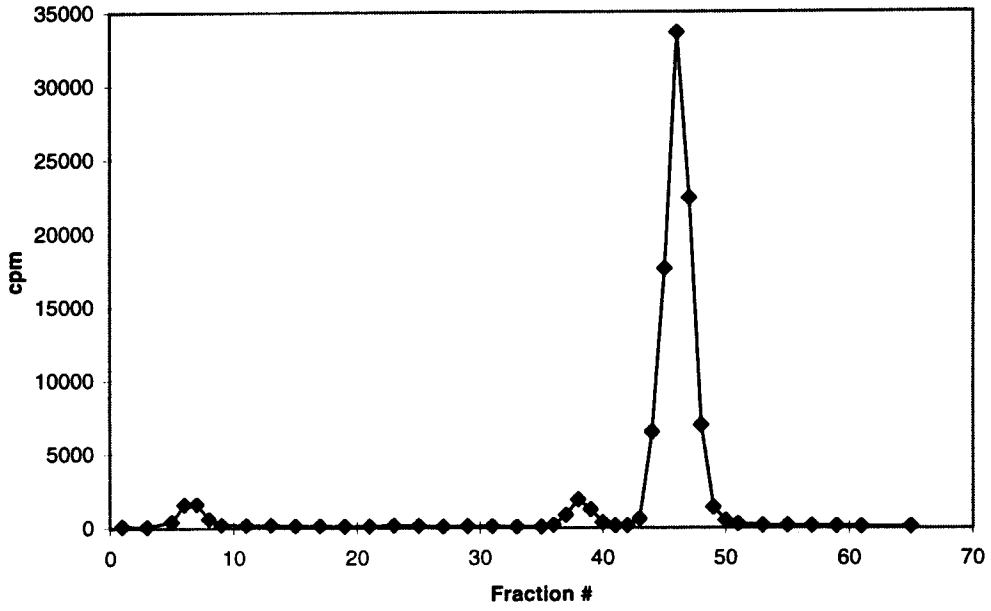


Figure 20. The radiochromatogram of ^{14}C-RDX stock solution. There were at least two impurities that contributed to about 9 % of the total radioactivity

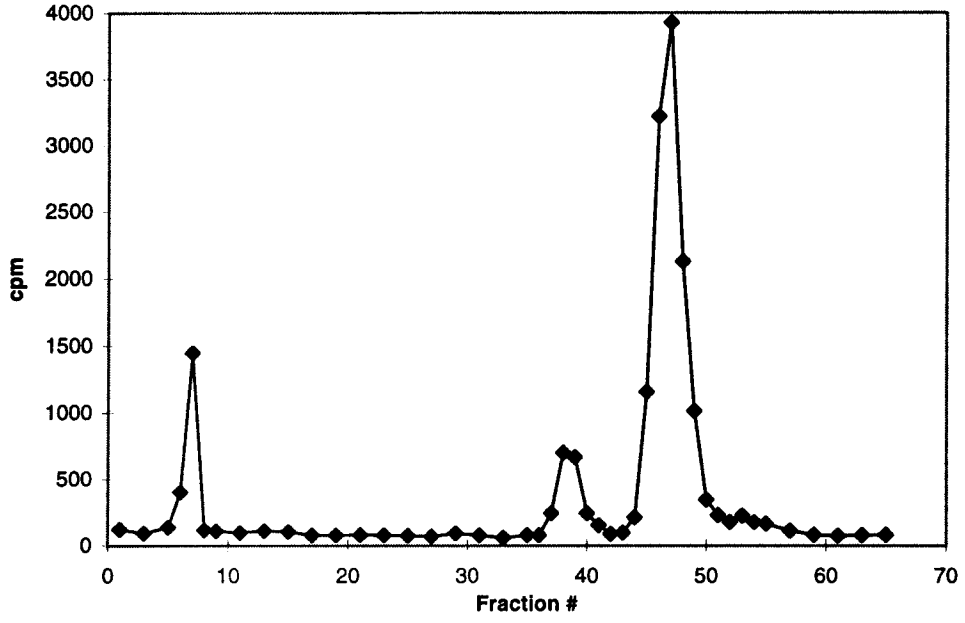


Figure 21. The radiochromatogram of RDX and its metaboites after two days of incubation. The metabolites of RDX overlaped with the impurities of RDX

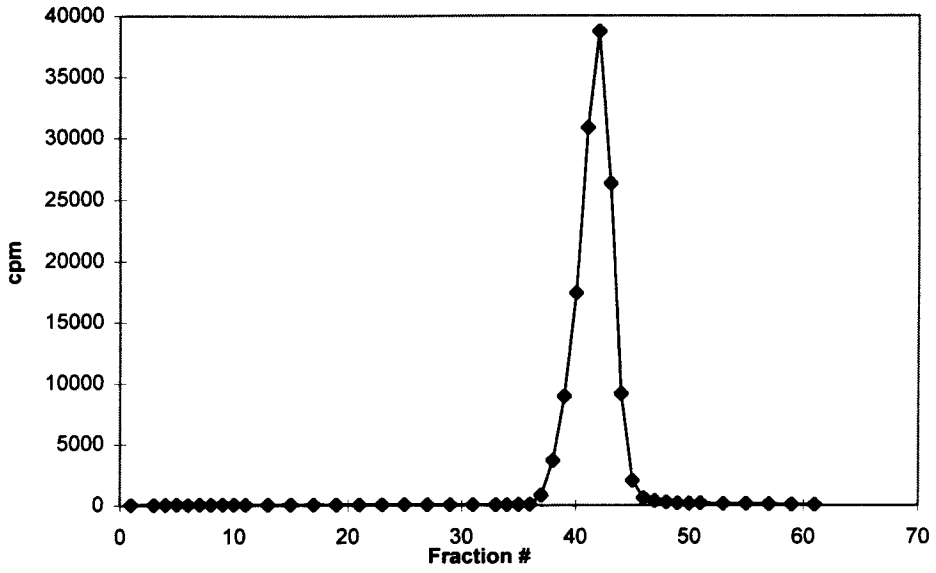


Figure 22. The radiochromatogram of the purified RDX stock solution. the purification was performed by HPLC fractionation.

A five day ^{14}C-RDX transformation and metabolites distribution kinetic study was conducted in batch reactors with purified ^{14}C-RDX. Batch samples were analyzed on the first day, third day and fifth day.

Figure 23 is a flow chart of the experiments conducted for ^{14}C-RDX metabolites distribution analysis. The production of $^{14}CO_2$ was measured first. The distribution of the radioactivity in supernatant and in cells was also assayed. The difference between the initial radioactivity and the sum of the radioactivity in CO_2, cell and supernatant was the loss. The loss could be the volatile metabolites from RDX which escaped during CO_2 purging. An cold trap was used to trap the volatile compounds but less than 0.1 % of radioactivity was recovered.

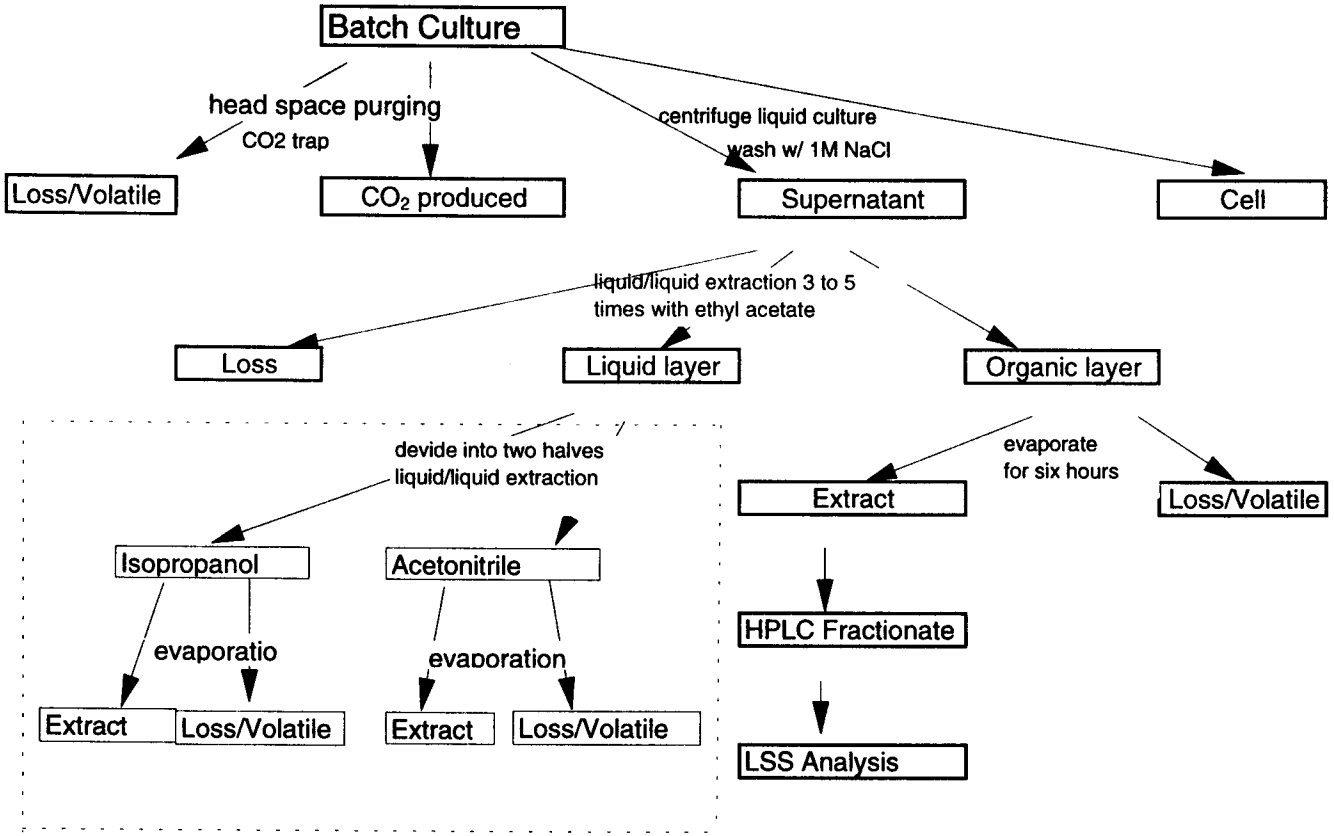


Figure 23. Flow chart of the experiments conducted for the analysis of ^{14}C- RDX biotransformation metabolite

The five day kinetic study of ^{14}C-RDX biotransformation metabolites showed that the amount of radioactivity in supernatant was always above 70%. It indicated that the majority of RDX biotransformation metabolites were water soluble. After acidification and centrifugation, the supernatant was extracted with ethyl acetate three to five times or until the percentage of radioactivity that can be recovered in one extraction was below 3 %. The experiments on days one, three and five all showed that approximately 20% of the radioactivity could not be recovered in ethyl acetate which indicates that approximately 20% of RDX metabolites are very water soluble.

The metabolites in liquid phase was extracted by acetonitrile and isopropanol. The liquid phase was saturated with sodium chloride to avoid acetonitrile and isopropanol dissolution. The results show that 70% of the radioactivity in the liquid phase can be extracted by acetonitrile and 78% of the activity can be extracted by isopropanol. It suggests that the metabolites in the liquid phase are very water soluble. Acetonitrile and isopropanol extracts were then evaporated under a soft stream of nitrogen gas. After six hours of evaporation over 90% of the radioactivity was lost. The result indicates that the RDX metabolites in liquid phase were not only very water soluble but also volatile. Other methods such as solid phase extraction needed to be used to concentrate the metabolites.

The ethyl acetate layers from the liquid-liquid extraction were pooled together and evaporated under a slow stream of nitrogen gas for 5 to 6 hours until 100 µl of the solvent remained. During evaporation a significant amount of the radioactivity from ^{14}C-RDX

was lost again. The amount lost during evaporation increased over five days, indicating that the amount of volatile metabolites produced from RDX increased with time.

The ethyl acetate extract was fractionated by HPLC. Sixty-five fractions of the effluent from the HPLC column were collected and the radioactivity in each fraction was assayed by scintillation counter. Approximately three fractions were collected per minute. The radiochromatogram of RDX and its metabolites on day one, day three and day five are shown in Figures 24 , 25 and 26, respectively.

The first day analysis shows that most of the radioactivity that was extractable by ethyl acetate was from the untransformed RDX (Figure 24) . Two major metabolites were produced. One of the metabolites appeared at fractions 36 to 42. By comparing to the unlabeled RDX nitroso derivative standards using relative retention time (Table 8), the peak is most likely the mono-nitroso derivative of RDX. The other peak that appeared at fractions 6 to 9 was a more polar compound. There could be more than one compound in that peak because C_{18} reverse phase HPLC column is only effective in separating non-polar compounds. Polar compounds as appearing early on the chromatogram may not separate well.

The radiochromatogram on day three shows two more peaks (Figure 25). The peak which appeared at fractions 26 to 28 might be the di-nitroso derivative of RDX because its relative retention time was between the mono-nitroso derivative and the tri-nitroso derivative of RDX. Another more polar compound at fractions 11 to 14 was also observed.

On the radiochromatogram from day five (Figure 26), the peak corresponding to the di-nitroso derivative was not present. The peak corresponding to mono-nitroso derivative also decreased. The tri-nitroso derivative of RDX was never observed. The results of ^{14}C-RDX nitroso derivative analysis match the results from the unlabeled RDX experiments as indicated by the HPLC analysis along.

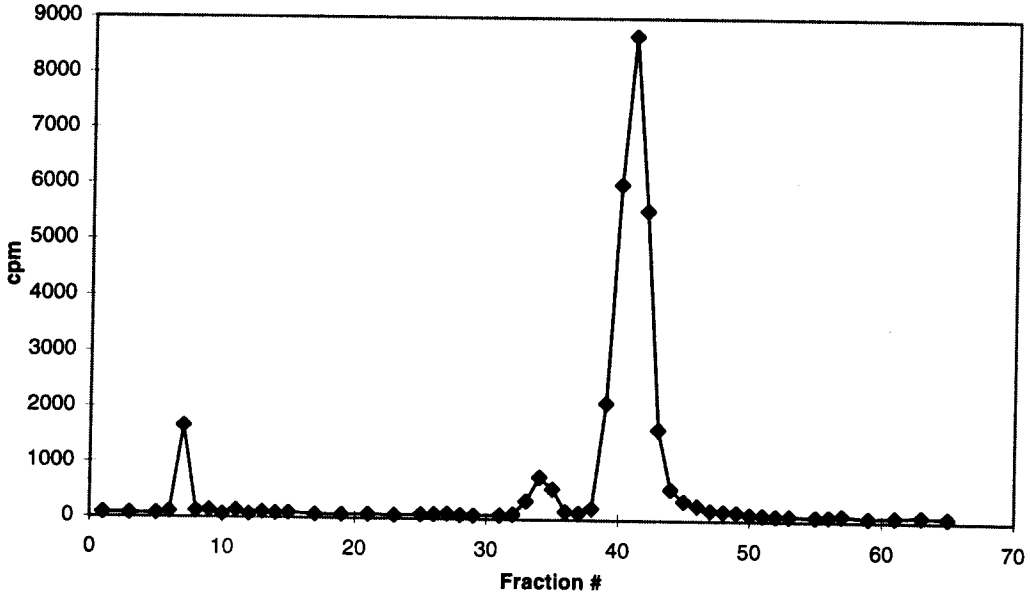


Figure 24. The radiochromatogram of ^{14}C-RDX biotransfomation after one day of incubation under an anoxic denitrifying condition.

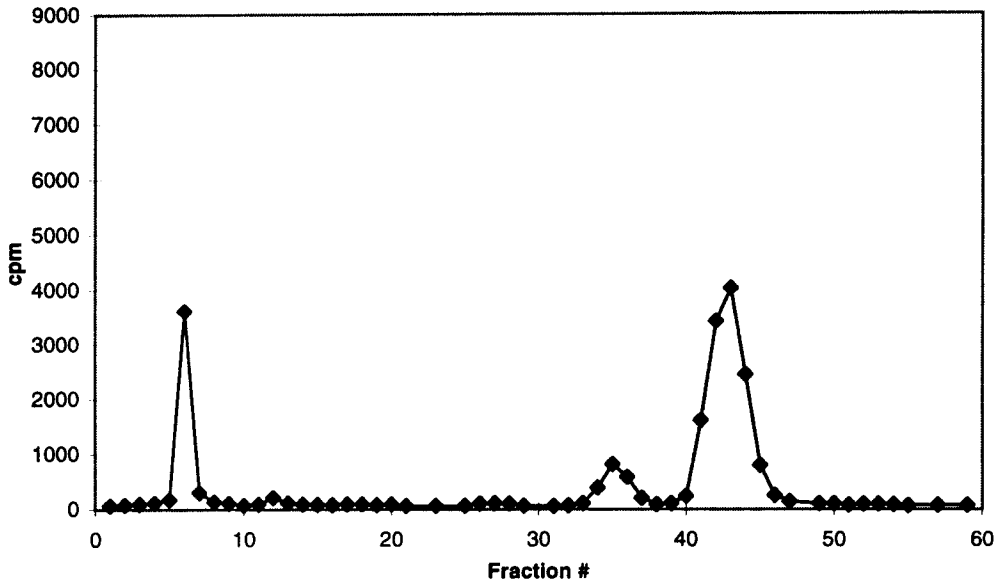


Figure 25. The radiochromatogram of ^{14}C-RDX biotransfomation after three days of incubation under an anoxic denitrifying condition.

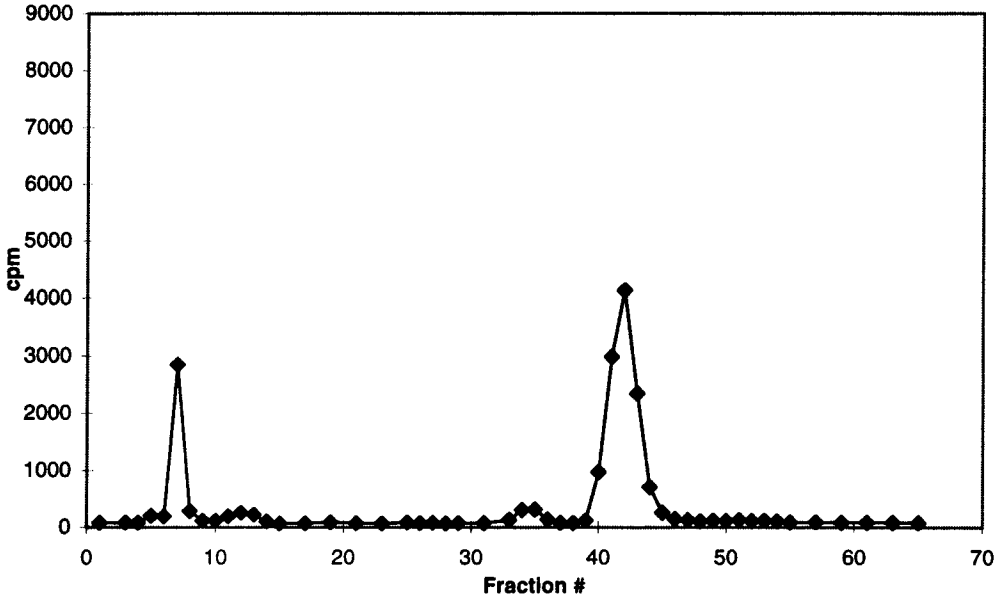


Figure 26. The radiochromatogram of ^{14}C-RDX biotransfomation after five days of incubation under an anoxic denitrifying condition.

Table 8. Relative retention times of the metabolites produced by the mix culture in batch reactors under the anoxic transformation of RDX

mobile phase= 90/10 water/methanol
Flow rate=1.5 ml/min

metabolite HPLC LSS	RRT*	standard HPLC-UV	RRT
fraction 6	0.140		
fraction 12.5	0.291		
		TNX	0.459
fraction 27	0.628		
fraction 35	0.812	MNX	0.817
fraction 43 (RDX)	1	RDX	1

* RRT was calculated with the fractions that contain the most radioactivity. The fraction numbers were the average of at least three replicates.

mobile phase= 60/40 water/methanol
Flow rate=1.0 ml/min

Standard HPLC-UV	RRT
TNX	0.621
MNX	0.867
RDX	1

Table 9. The percentage distribution of radioactivity from ^{14}C-RDX biotansformation under anoxic denitrifying conditions.

Days	0	1	3	5
RDX	100	45.89	19.2	17.22
CO_2	0	5.5	6.26	17.15
cell	0	0.66	0.77	1.25
Fraction 3-9	0	3.12	5.82	5.28
Fraction 11-14	0	0	0.3	0.75
Fraction 26-28	0	0	0.18	0
Fraction 33-42	0	3.35	3.1	0.89
very water soluble metabolites	0	**20.15**	**20.74**	**19.93**
Loss/ volatile	0	**21.31**	**43.62**	**37.51**

Table 9 is the distribution of metabolites from ^{14}C-RDX over a period of five days. RDX decreased rapidly during the first three days but then decreased. This may be due to unfavorable conditions, such as the elevated pH, however, the rate of CO_2 production was not affected, and continue to increase. This suggests that the microbial process for CO_2 production might be different than the RDX transformation process.

Figure 27 shows the time series of the metabolites that were extractable by ethyl acetate. It showed that there was no accumulation of RDX nitroso-derivative (fractions 20 to 40). Nitroso-derivatives are known to be mutagenic and carcinogenic and the absence of such compounds is a desired result. Comparing to anaerobic processes in which nitroso derivatives of RDX usually accumulate, this anoxic process appears to be a better choice than an anaerobic process.

The metabolites that appeared early on radiochromatogram (fractions 3 to 14) which contributed to approximately 5 % of the total radioactivity need to be identified. Further research should be conducted to identify these compounds with LC-MS after good separation of the compounds is obtained.

Subtracting the percentage of metabolites extractable by ethyl acetate, most of the metabolites from RDX appeared to be water soluble and volatile (Figure 28). According to McCormick *et al.* (1981) the low molecular weight, polar and neutral compounds were most likely to be methanol and formaldehyde. The other two possibility were dimethylnitrosamine and 1,2-dimethyldiazine-1-oxide, according to the proposed pathway (Figure 3). If the end products that contribute to the majority of radioactivity in the volatile and very water soluble portion of the metabolites are methanol and formaldehyde, it suggests that RDX can be further mineralized because both compounds can be easily oxidized to CO_2 under aerobic conditions or methanogenic anaerobic conditions. However if some of the other intermediates along the biotransformation pathway were produced and accumulated, the toxicity and biodegradability of such compounds still needs to be investigated.

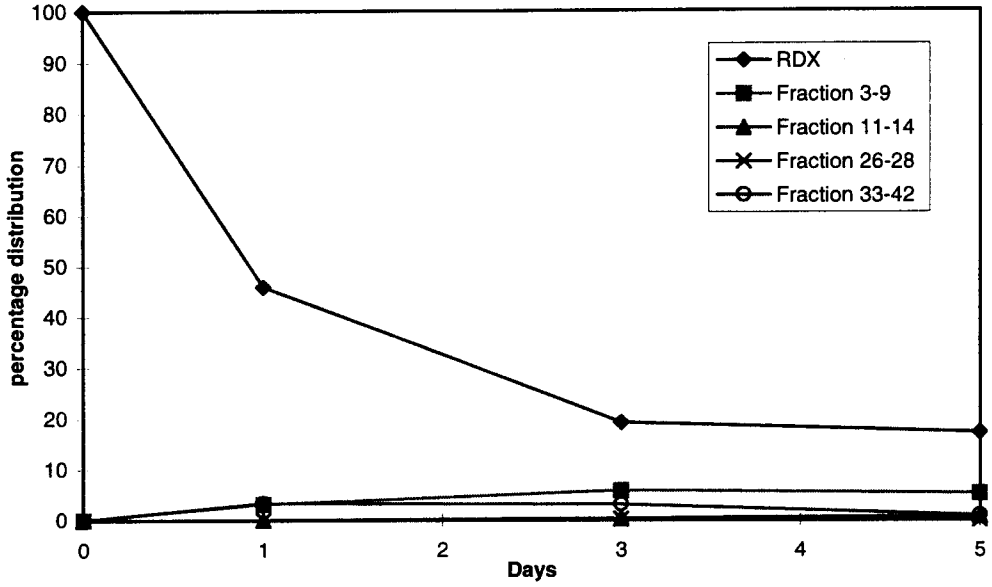


Figure 27. Time series of ^{14}C-RDX biotransformation metabolites which are extractable by ethyl acetate

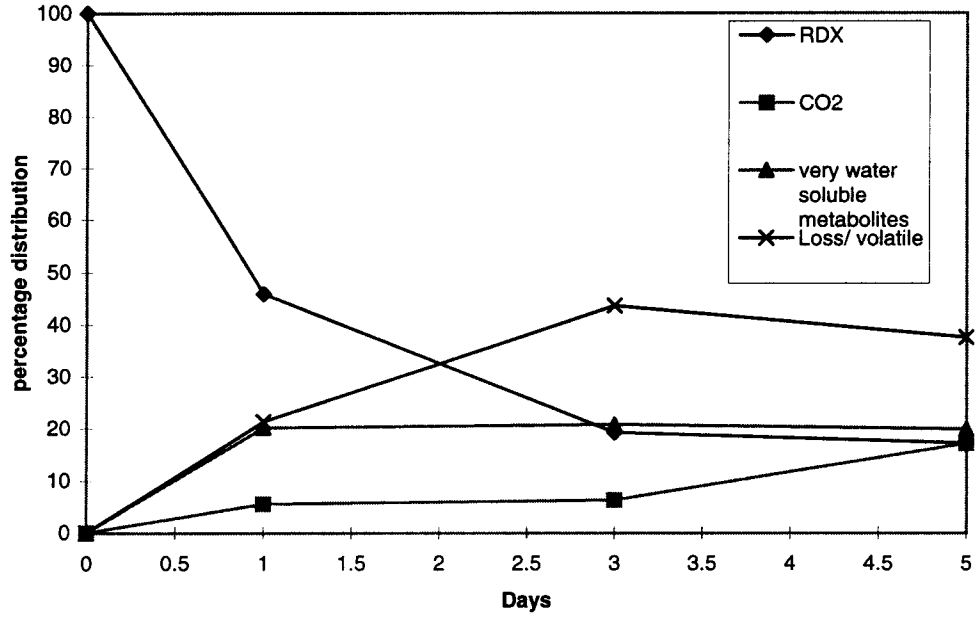


Figure 28. Time series of ^{14}C-RDX biotransfomation metabolites which are polar, volatile and with small molecular weights

4.7. Detection of Hydrazine and 1,1-dimethylhydrazine

The detection of hydrazine as one of the metabolites of RDX biotransformation was conducted at both UCLA and LLNL. The detection of hydrazine at UCLA was performed by GC-MS (Ruth, 1995). One liter of the effluent from continuous flow bioreactor was first filtered by a microfiber filter system and then concentrated by liquid-liquid extraction with dichloromethane for 6 hours in a pyrex accelerated one-step extractor-concentrator. The GC- MS analysis showed that there was no hydrazine-like compounds.

The analysis of hydrazine and 1,1-dimethylhydrazine at Lawrence and Livermore National Laboratory was by HPLC equipped with a PDA detector. Hydrazine and dimethylhydrazine was first derivatized by salicylaldehyde due to the lack of hydrazine and dimethylhydrazine UV absorbance. The detection limit was 50 ppb. The analysis showed also negative results on the presence of both compounds.

4.8. Detection of Formaldehyde

The analysis of formaldehyde using Kuwata's method showed negative results on the presence of formaldehyde in the batch culture. The reasons of no detection may be either formaldehyde was transformed to other compounds quickly after it was produced or because of the high volatility of formaldehyde. Large amount of formaldehyde lost during liquid culture transfer for the preparation of formaldehyde analysis. A better

formaldehyde collecting device such as a formaldehyde trap needs to be used to confirm the absence of formaldehyde in batch cultures.

4.9. Detection of Methanol

Figure 29 is a typical GC chromatogram of standard methanol and ethanol. The detection of methanol produced by batch cultures was performed by direct GC injection of liquid culture samples after deproteination and centrifugation. This analysis also showed negative results. Similar to formaldehyde, methanol is also very volatile. In order to increase the concentration of methanol to a sufficient level to be detected by GC, concentrating methods such as vacuum distillation or super critical liquid extraction needs to be applied for methanol analysis. A more direct way to capture methanol, as well as formaldehyde is to use a purging trap prior to GC analysis.

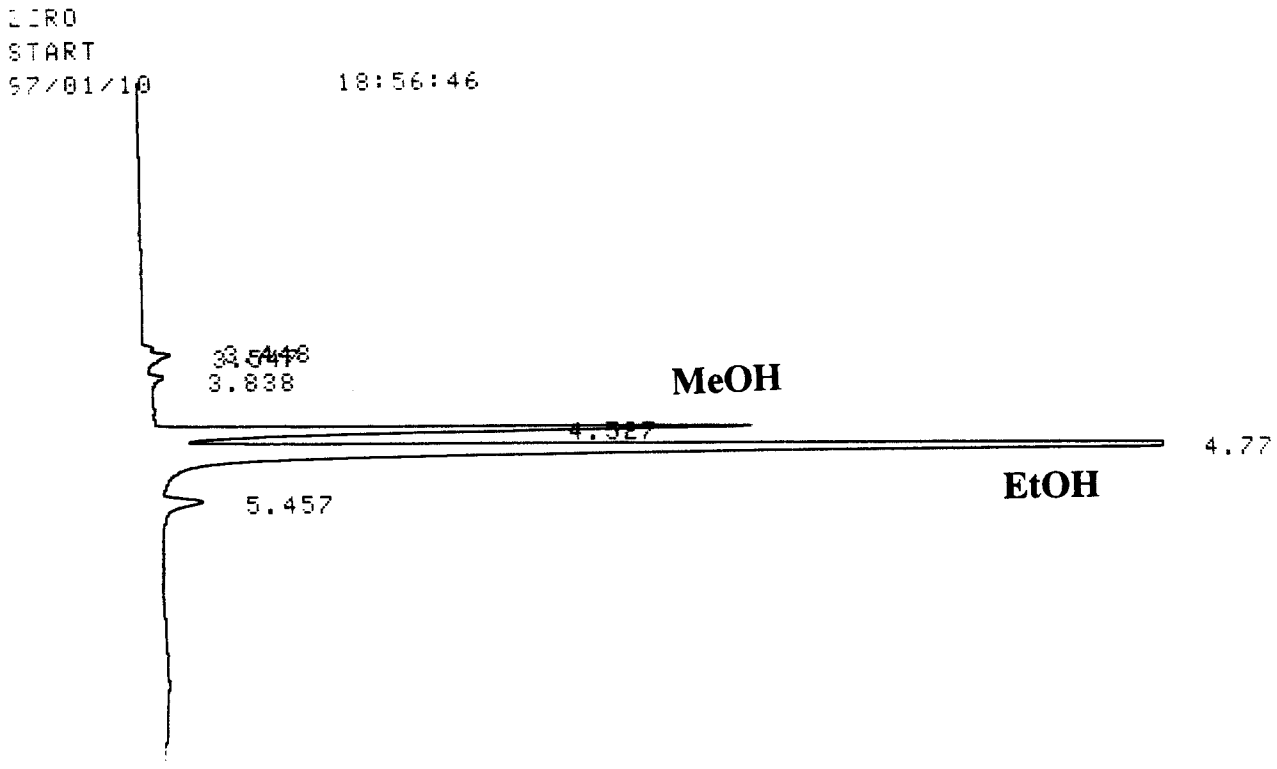


Figure 29. Sample GC chromatogram for methanol and ethanol analysis

Chapter V

Conclusions

RDX, a toxic explosive chemical, can be efficiently transformed in a continuos-flow packed bed bioreactor under anoxic denitrifying conditions. Significant amount of RDX is mineralized in a similar batch system. The followings are the conclusions of RDX biological transformation and mineralization experiments based on fourteen months of bench scale study.

- 90% to 100% of RDX can be transformed in a continuos-flow packed bed bioreactor in three hours with 2.0 % of ethanol concentration in feeding solution. The reactor temperature is 35°C.

- RDX transformation efficiency decreases with decreasing ethanol concentration. 0.1 % ethanol concentration in feeding solution of a continuous-flow packed-bed reactor is the minimum ethanol concentration required to transform 60% of RDX.

- Ethanol concentration of 0.04 % resulted in less than 10% of RDX transformation efficiency. An increase in retention time of RDX in continuous-flow packed bed reactor did not increase transformation efficiency, although at 2.0 % ethanol

concentration, an increase in retention time from three hours to six hours restored transformation efficiency.

- 90% of RDX can be transformed in 7 days in a batch reactor at 0.5 % initial ethanol concentration.

- RDX transformation continued after the terminal electron acceptor, nitrate, was depleted, but at a slower rate compared to the rate in the presence of nitrate. It is unknown how long the culture can continue the transformation.

- Mineralization of RDX increases with increasing nitrate supply as the terminal electron acceptor. 38% of mineralization can be achieved with 0.75 nitrate and ethanol redox ratio (19 g/L potassium nitrate and 0.5% ethanol).

- The pH value in batch reactors increases when reaction proceeds resulting in inhibition of microbial activity. To control the pH value in a batch system, increasing the initial buffer concentration or titrating the system with nitric acid to neutralize the pH increase were the feasible solutions.

- Under anoxic denitrifying conditions, no nitroso derivatives of RDX accumulated in a batch reactor as oppose to under anaerobic conditions, significant amount of mono-nitroso and di-nitroso derivatives of RDX accumulated.

- The majority of RDX biotransformation metabolites under anoxic conditions were very water soluble, volatile and of low molecular weights. The characteristics and biodegradability of those metabolites were not yet identified.

- The preliminary analysis of methanol, formaldehyde, hydrazine and 1,1-dimethylhydrazine showed negative results on the presence of all these compounds. Because they are all very volatile, a better trapping system needed to be developed to confirm the absence of these compounds.

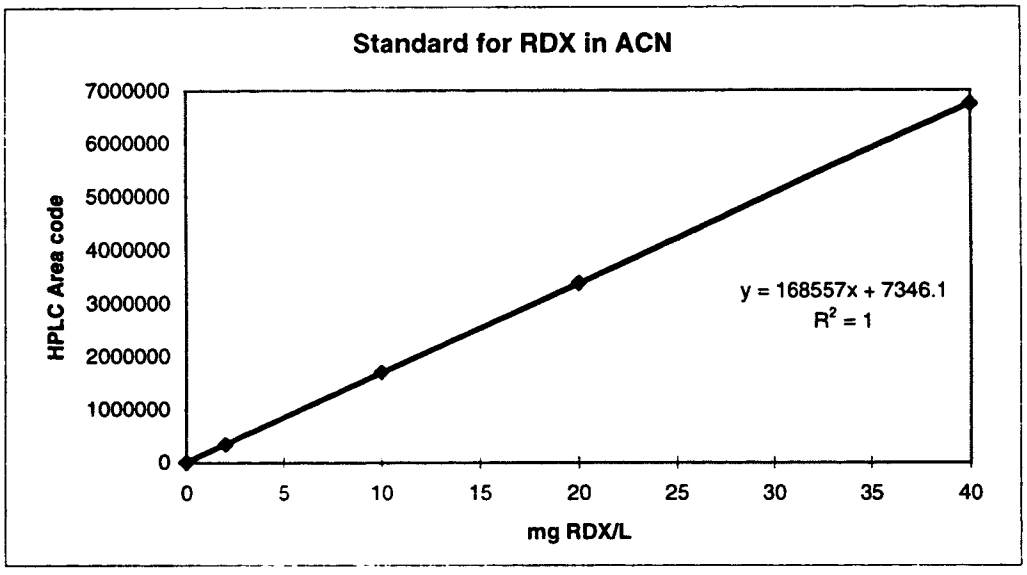
Appendix I. Sample Calibration Curves

HPLC Standard Calibration for RDX in ACN

2/23/97 for column received on 10/4/96

Solvents: 40% methanol, 60% water
Flow rate: 1.0 ml/min
Injection V: 20 µL
Standard solution: 40 mg/L RDX

		Area Codes					
injection V	mg RDX/L	X1	X2	X3	**Average**	standard dev	% cv
0 mL	**0**	0	0	0	**0**	0	0
1 mL	**2**	336418	337126	346137	**339893.667**	5418.461436	1.5941637
5 mL	**10**	1704695	1680478	1724936	**1703369.67**	22258.61232	1.30673997
10 mL	**20**	3378629	3389584	3390344	**3386185.67**	6555.288577	0.19358917
20 mL	**40**	6738387	6743280	6748554	**6743407**	5084.689666	0.07540238

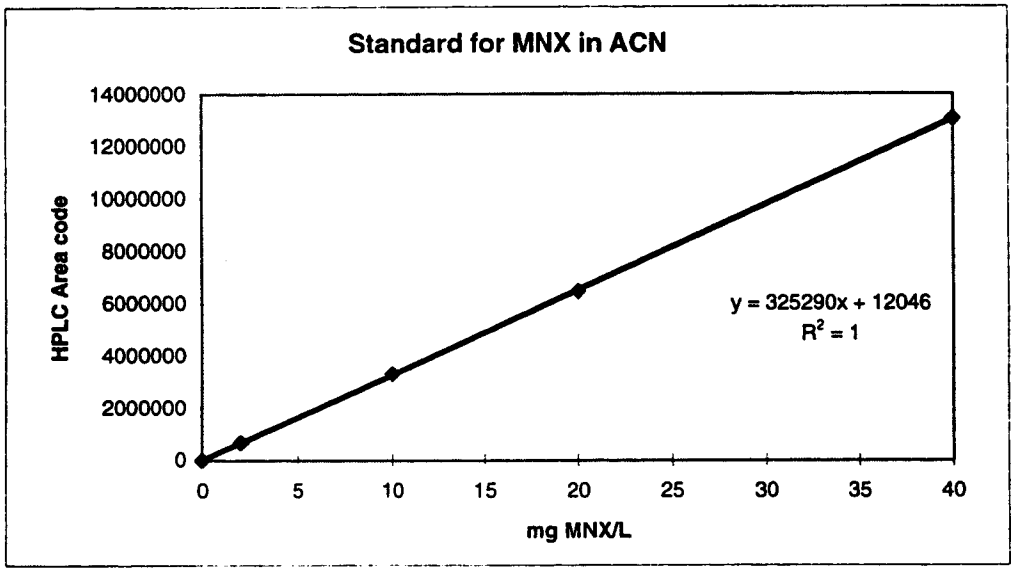


HPLC Standard Calibration for MNX in ACN

2/23/97 for column received on 10/4/96

Solvents: 40% methanol, 60% water
Flow rate: 1.0 ml/min
Injection V: 20 µL
Standard solution: 40 mg/L MNX

		Area Codes					
injection V	mg MNX/L	X1	X2	X3	**Average**	standard dev	% cv
0 mL	**0**	0	0	0	**0**	0	0
1 mL	**2**	667477	690218	651972	**669889**	19236.747	2.87163202
5 mL	**10**	3271229	3313262	3338094	**3307528.333**	33799.2346	1.02188798
10 mL	**20**	6528506	6535635	6328698	**6464279.667**	117471.26	1.81723666
20 mL	**40**	13061792	13049200	13007320	**13039437.33**	28518.0985	0.21870651

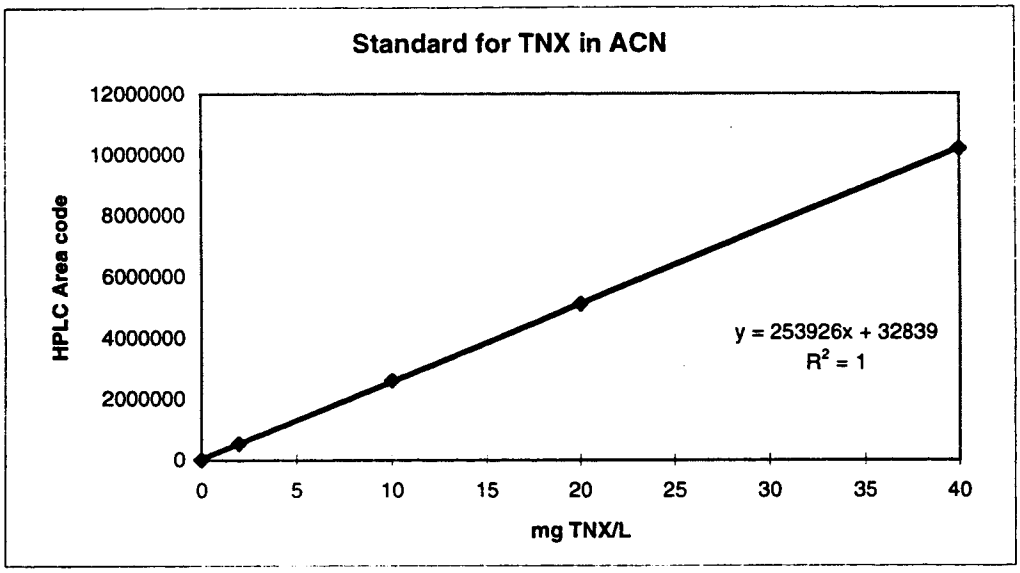


HPLC Standard Calibration for TNX in ACN

2/23/97 for column received on 10/4/96

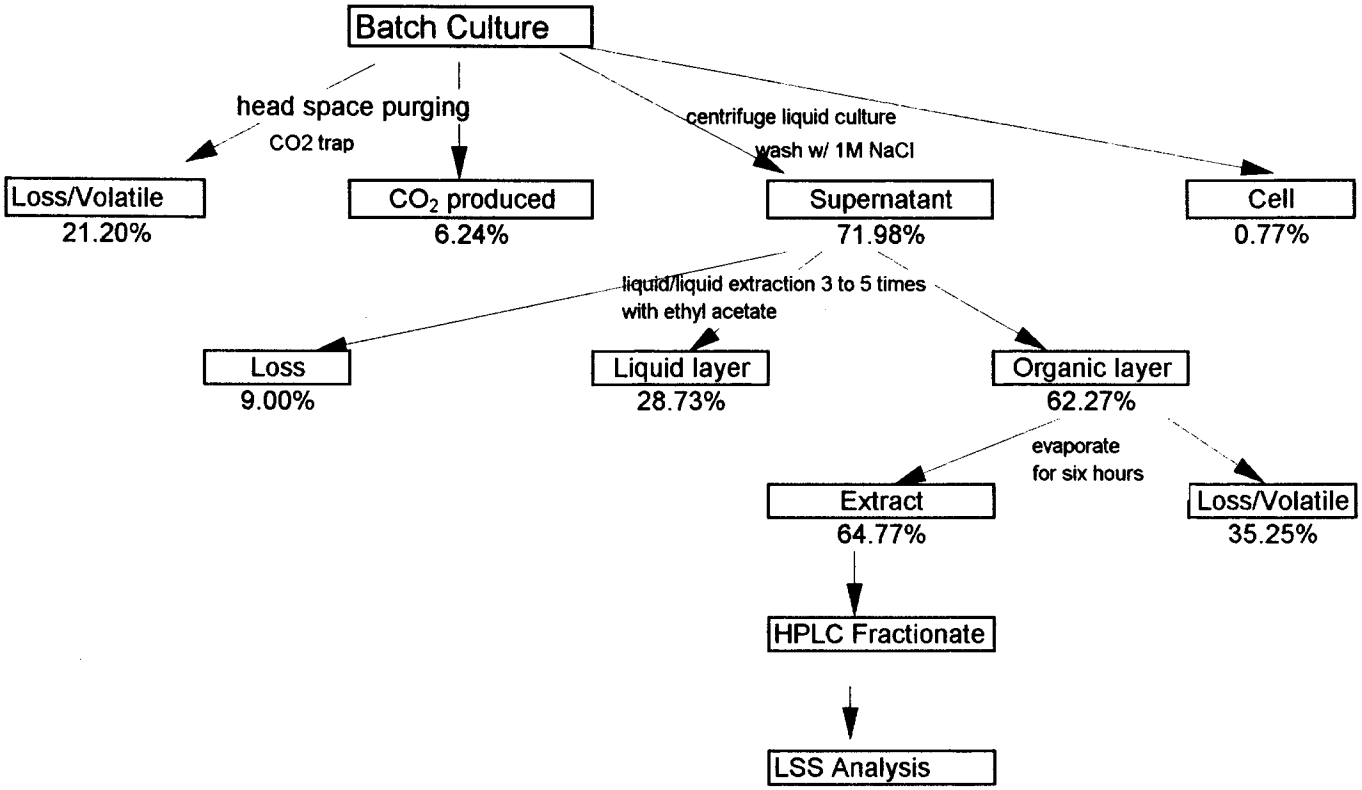
Solvents: 40% methanol, 60% water
Flow rate: 1.0 ml/min
Injection V: 20 µL
Standard solution: 40 mg/L TNX

		Area Codes					
injection V	mg TNX/L	X1	X2	X3	**Average**	standard dev	% cv
0 mL	0	0	0	0	**0**	0	0
1 mL	2	561758	548309	519856	**543307.6667**	21394.02726	3.93773704
5 mL	10	2630837	2596037	2613094	**2613322.667**	17401.12687	0.66586216
10 mL	20	5122115	5132867	5075667	**5110216.333**	30399.73193	0.59488151
20 µL	40	10202072	10182096	10155824	**10179997.33**	23195.31585	0.22785189

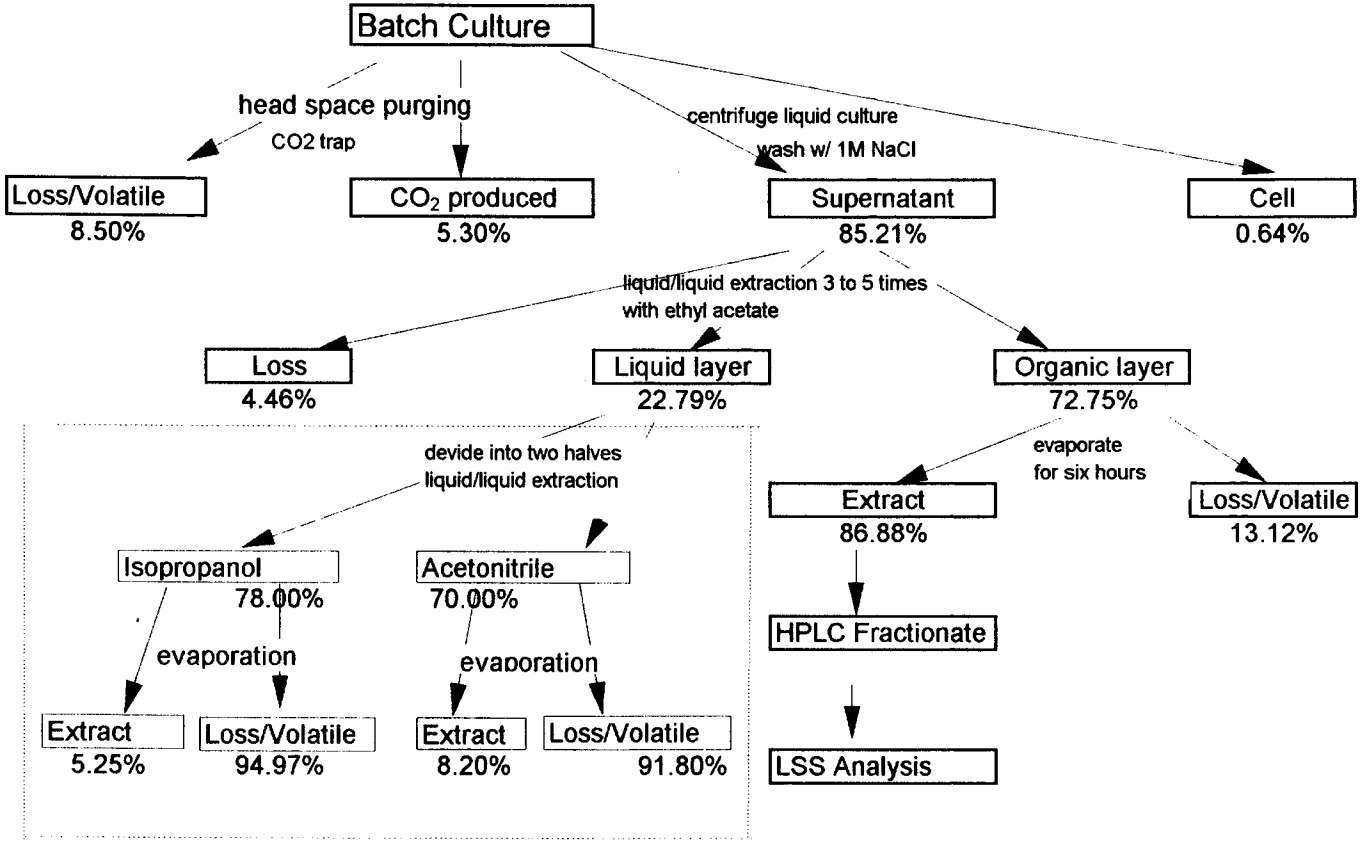


Appendix II. Distribution of Radioactivity

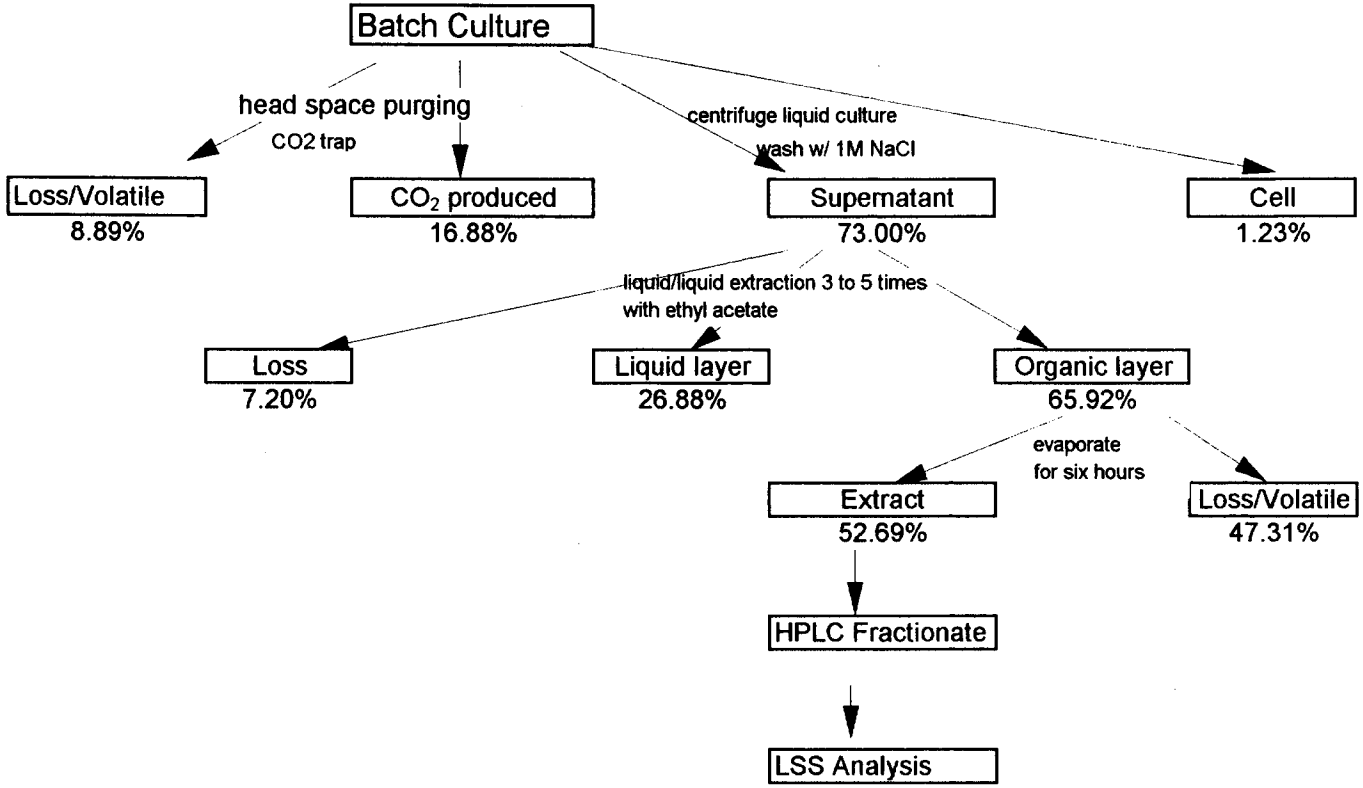
The Distribution of Radioactivity on Each Step on **Day Three** in an Anoxic Denitrifying Batch Reactor



The Distribution of Radioactivity on Each Step on **Day One** in an Anoxic Denitrifying Batch Reactor



The Distribution of Radioactivity on Each Step on **Day Five** in an Anoxic Denitrifying Batch Reactor



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