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Removal of RDX From a Contaminated Groundwater by In Situ Bioremediation

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and Khankha Banerji

September 2000

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Final report

Approved for public release; distribution is unlimited

Prepared for U.S. Army Corps of Engineers
Washington, DC 20314-1000

Engineer Research and Development Center Cataloging-in-Publication Data

Removal of RDX from a contaminated groundwater by in situ bioremediation / by Scott A. Waisner ... [et al.]
; prepared for U.S. Army Corps of Engineers.

77 p. : ill. ; 28 cm. -- (ERDC/EL ; TR-00-14)

Includes bibliographic references.

1. Soil remediation. 2. Groundwater -- Purification. 3. In situ bioremediation. 4. Explosives, Military -- Biodegradation. I. Waisner, Scott A. II. United States. Army. Corps of Engineers. III. Engineer Research and Development Center (U.S.) IV. Environmental Laboratory (U.S.) V. Series: ERDC/EL TR ; 00-14.

TA7 E8 no.ERDC/EL TR-00-14

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Preface

This study was conducted by the U.S. Army Engineer Research and Development Center (ERDC) under funding from the Department of Army Environmental Quality Technology (EQT) program.

The study was conducted and the report was prepared by Mr. Scott A. Waisner, Dr. Herbert L. Fredrickson, and Mr. Lance D. Hansen, Environmental Laboratory (EL), ERDC, and Dr. Khankha Banerji, University of Missouri - Columbia. Significant contributions were made in design and implementation of the study by Dr. Mark E. Zappi and Mr. Glynn Myrick of ERDC and AScI, respectively.

The study was conducted under the supervision of Mr. Norman R. Francingues, Chief, Environmental Engineering Division, and Dr. John W. Keeley, Acting Director, EL.

At the time of publication of this report, Director of ERDC was Dr. James R. Houston, and Commander was COL James S. Weller, EN.

This report should be cited as follows:

Waisner, S. A., Fredrickson, H. L., Hansen, L. D., and Banerji, K. (2000). "Removal of RDX from a contaminated groundwater by in situ bioremediation," ERDC/EL TR-00-14, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

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Conversion Factors, Non-SI to SI Units of Measurement

Non-SI units of measurement used in this report can be converted to SI units as follows:

Multiply	By	To Obtain
acres	4.046873	square meters
cubic yards	0.7645549	cubic meters
feet	0.3048	meters
gallons (U.S. liquid)	0.003785412	cubic meters
miles (U.S. statute)	1.609347	kilometers
tons (2,000 lb)	907.1847	kilograms

1 Introduction

Scope of the Problem

Currently the U.S. Department of Defense (DoD)¹ has a total of 21,425 contaminated sites on 1,769 installations contaminated by energetic compounds, solvents, and heavy metals. Remedial response has been completed on only 9,640 of those sites. Through the end of fiscal year 1994, funds spent on studies, interim actions, design and cleanup totaled \$7 billion. The remaining cost to complete remedial response on all sites is currently estimated at \$26.5 billion (DoD 1995).

The production and processing of military explosives has led to the contamination of soil and water by energetic compounds at approximately thirty-five Army Ammunition Plants and Depots in the United States. In a report prepared for the Executive Director of the Strategic Environmental Research and Development Program (SERDP 1993), it was estimated that 706,000 cubic yards of soil and 10 billion gallons of groundwater have been contaminated by energetic compounds at these sites. Due to the fact that most sites were in the preliminary assessment and remedial investigation stages at the time of this report, these estimates are rough and most likely low. In the same report, an estimate for the volume of groundwater contaminated by energetic compounds at Cornhusker Army Ammunition Plant (CAAP) near Grand Island, NE, alone was 12 billion gallons (Cerar 1993). Contaminants typically found at these sites include trinitrotoluene (TNT), dinitrotoluene (DNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). The use of in situ biological degradation of these contaminants would provide significant cost savings over currently accepted treatment methods by eliminating the need for excavation of contaminated materials and the construction of treatment facilities.

Many researchers have established that these explosives can be mineralized through biological processes, but successful application of these techniques to in situ treatment of contaminated soils and waters has yet to be proven in the field. The purpose of the study reported herein was to determine the suitability of in situ biological degradation of explosives for the remediation of the groundwater and aquifer material at CAAP. Due to the extent to which RDX has

¹ For convenience, symbols and abbreviations are listed in the Notation (Appendix B).

migrated through the groundwater aquifer at this site, it is the major contaminant of concern and the focus of this study.

Significance of RDX Contamination

RDX poses two possible threats to the environment. Like all energetic compounds, it poses a detonation threat when its concentrations are high enough; fortunately at CAAP, as at most sites, this situation is rare. The second threat is to our health. Exposure to RDX, by inhalation of dust particles and fumes or ingestion, has been shown to have adverse effects on human health. The major toxicological effects of exposure to RDX are nausea, irritability, convulsions, unconsciousness, and amnesia. Due to these effects shown in humans, the U.S. Environmental Protection Agency (USEPA) has established drinking water health advisories (HA) for exposure to RDX (McLellan, Hartley, and Brower 1992).

The Longer-term HA (greater than 10 days) has been established as 100 µg/L for a child and 400 µg/L for an adult. These health advisory levels were based on the NOAEL (no observed adverse effect level) on the central nervous system of monkeys following ingestion of 10 mg/kg/day of RDX for 90 days. In the absence of adequate animal data, the Longer-term HA for a 10-kg child is used as a conservative estimate of the One-day and Ten-day HA. The Lifetime HA has been established as 2 µg/L for adults. This was established using the NOAEL of the increased incidence of suppurative inflammation in the prostate of male rats receiving 1.5 mg/kg/day of RDX in a 24-month continuous feeding study (McLellan, Hartley, and Brower 1992).

The health advisory levels for children were calculated based on an assumed body weight of 10 kg, an assumed water consumption of 1 L/day, and an uncertainty factor of 100. Adult health advisory levels were calculated based on a 70-kg adult, an assumed water consumption of 2 L/day, and an uncertainty factor of 100. Based upon limited evidence of carcinogenicity in animals and insufficient data in humans, RDX is classified as Group C, a possible human carcinogen. This cancer risk assessment was used to support selection of the uncertainty factors for the recommended Lifetime HA (McLellan, Hartley, and Brower 1992). Due to the risk to human health, the USEPA has established a target treatment level for remediation of sites contaminated with RDX at the Lifetime HA level of 2 µg/L.

Objectives and Scope of Study

The purpose of the study reported herein was to determine the feasibility of using in situ bioremediation techniques to remove RDX contamination in the groundwater and aquifer material at and near CAAP in Nebraska. To meet these objectives it was necessary to determine (1) if biological degradation and possibly mineralization of RDX could be achieved and (2) what conditions are necessary for this process to take place. Answers to these questions were sought

by examining the fate of ^{14}C -labeled RDX in biological shake flask experiments under several conditions. Radiolabeled RDX was added to shake flasks with soil and water from the contaminated aquifer. The use of contaminated material from the aquifer provided conditions similar to those found in the aquifer and a source of organisms native to the aquifer for this study.

While there are many variables that can influence the growth of microorganisms, an experimental matrix to investigate all of these variables would be far too large for the limited resources of this study. Hence, the focus of this study was limited to variables that could be reasonably controlled in the in situ environment at CAAP. These variables were the carbon and/or energy sources available to the microorganisms and the redox conditions necessary for the biological degradation of explosives. This study also investigated the use of microorganisms not native to CAAP that have been shown in other studies to degrade RDX. Determination of the fate and use of RDX by microorganisms was achieved by tracking ^{14}C from the radiolabeled RDX.

RDX degradation kinetics were not addressed in this study. However, first-order kinetics were used to describe the rate of $^{14}\text{CO}_2$ production from RDX mineralization in this study. Further studies using static soil columns have been proposed. The kinetics of RDX degradation are necessary for the application of biological degradation to the field. Three pieces of information can be obtained from the column studies: (1) the maximum degradation rate of RDX, (2) residence time in the system necessary to reach the maximum degradation rate, and (3) the concentration of RDX versus residence time in the system. The first two pieces of information will be obtained by operating the columns in a continuous feed mode until a steady state concentration of RDX is achieved in the column effluent. The third piece of information will be obtained by recycling column effluent until RDX concentration reaches a minimum.

Site History and Description

CAAP is located in central Nebraska, in Hall County on the west edge of the town of Grand Island. It was constructed in 1942 on approximately 12,000 acres and consisted of five major load lines for production of conventional munitions for World War II. For a short period of time after the war, the plant was placed on standby and used for the manufacture of ammonium nitrate fertilizer. The plant was reactivated during the Korean War for the production of artillery shells and rockets. The plant was again placed on standby between the Korean and Vietnam Wars. Munitions were manufactured for the Vietnam War from 1965 until 1973. The plant has remained on standby since the end of the Vietnam War. The plant manufactured TNT during all three of the wars, but RDX was manufactured only during the Korean War (Spalding and Fulton 1988).

The Initial Assessment Study completed in FY80 identified sixty-five contaminated sites. These sites included sumps, cesspools, and leaching pits used in the manufacture of explosives, and also disposal pits, old landfills, and open burning areas. In 1982, a groundwater plume of explosives contamination

was identified both on and off the site (DoD 1995). The cigar-shaped groundwater plume is estimated to be 5.5 miles long by 1 mile wide and to contain 12 billion gallons of water (Cerar 1993). Off base contamination affecting more than 500 private drinking water wells in Hall County and Grand Island forced the U.S. Army to provide bottled water for these residences. In FY86, the municipal water distribution system was extended to these residences, and in FY92, a study recommended extending the system to 60 additional residences (DoD 1995).

The site was placed on the National Priority List in 1987 and as of the end of FY94 has received \$31 million dollars in funding. Approximately 400,000 tons of contaminated soil have been removed and incinerated which has resulted in removal of nearly 95 percent of the sources of contamination. As of the end of FY94, Interim Remedial Actions have been completed at 59 of the 65 identified sites.

TNT and RDX are the major contaminants in the groundwater aquifer with maximum concentrations of 800 and 80 ppb, respectively. Although TNT is present in the groundwater at higher concentration, these concentrations are localized near the source of the contamination. RDX presents a much larger problem due to its high mobility in the groundwater aquifer. RDX is being transported by advection through the aquifer at a rate approximating the calculated Darcian velocity of the groundwater which is 0.5 m/day (Spalding and Fulton 1988).

The primary aquifer in the area of contamination is approximately 18 m deep with a relatively shallow water table ranging from 2 to 6 m below the surface. The aquifer is laterally unconfined and consists of gravel, sand, and silt deposited by streams from the west and northwest. The aquifer also contains lateral lenses of silt and sand which are sporadically spaced and discontinuous. The primary aquifer is bounded on the bottom by a silt and clay layer. This layer lies atop the Ogallala Group which is a significant source of water for many cities of the plains region of the United States (Spalding and Fulton 1988).

2 Literature Review of the Environmental Fate of RDX

The fate and transport of RDX in the environment can be influenced by many factors including sorption onto sediments, photolysis by sunlight, hydrolysis, and biologically mediated degradation. While the focus of this study is on the biological degradation of RDX, it is essential to understand other factors that can influence its degradation and fate in the environment. A search and review of available literature on this subject was conducted for this purpose.

Abiotic Fates

The solubility of RDX in water has been reported to be from 42 to 60 ppm (Banerjee, Yalkowsky, and Valvani 1980; Spangord et al. 1980a; Syracuse Research Corporation 1978), and its diffusion coefficient in water was estimated to be 7.15×10^{-6} cm²/sec (Rosenblatt et al. 1991). The solubility of RDX is high enough to allow significant quantities of RDX to be transported in water. However, the low diffusion coefficient of RDX in water indicates that the dispersion of RDX in waters will be highly dependent on advective processes. The highest concentrations of RDX found in the groundwater at CAAP were at or below 100 ppb. The half-life for volatilization of RDX from water is estimated to be 9×10^6 days (Spangord et al. 1980a). Because this rate is so low, volatilization is not a significant fate of RDX in water. A table of published physical and chemical properties of RDX is given in Appendix A. Although significant hydrolysis of RDX has been shown to occur, it is unlikely that hydrolysis will be a significant factor in the fate of RDX under the conditions normally found in the environment (Sikka et al. 1980; Hoffsommer and Rosen 1973). Calculated half-lives for alkaline hydrolysis of RDX at pH 8 or higher range from approximately one to several years (Spangord et al. 1980a).

Numerous studies have been conducted to determine the interaction of explosives with soils. Distribution coefficients ranged from 4.2 mL/g in high organic content soil to 0.8 mL/g for a sandy loam (Syracuse Research Corporation 1978). Sorption onto these soils appeared to be rapid and did not increase with extended contact times. Leggett (1985) found that sorption of RDX on bentonite drilling muds followed linear isotherms and was reversible in short-term experiments. Estimates of linear distribution coefficients with these drilling muds ranged from 4.92 to 6.75 mL/g. In another experiment RDX was shown to be completely released from a column of contaminated Kolin soil (a

mixture of fine silt and sand) by the continuous application of 20 pore volumes of 0.005-M $\text{Ca}(\text{NO}_3)_2$. From this Kolin soil 90% of the RDX was released in the first 8 pore volumes applied. Best fit for Freundlich parameters, K_d and $1/n$, were 0.8203 mL/g and 0.820, respectively, for RDX in Bentonite-sand (Selim and Iskandar 1994).

Ainsworth et al. (1993) conducted batch and columns sorption/desorption studies to determine the effects of different soil properties on the partitioning of RDX between soil and water. They found that RDX sorption on their soils was low, rapid, and totally reversible in the short term. RDX sorption was described well by a linear model for all soil types used in the experiment. A model for predicting the linear sorption coefficient, K_d , was proposed which was dependent on a soils cation exchange capacity, pH, clay content, organic carbon content, and extractable iron. Because the aquifer material at CAAP is a silty sand containing very little organic matter or clay, the linear distribution coefficient for RDX in this aquifer is expected to be less than 1.0.

A study of long-term sorption and desorption showed that RDX adsorption was rapid with only a small amount of adsorption occurring after one day (Brannon et al. 1992). However, the amount of extractable RDX from soil decreased with time. It was postulated that long-term sorption and reduction of the extractable fraction of RDX with time was due to diffusion of RDX into internal pore spaces of the particles. This report also showed that all soil types could act as a long-term sink for RDX and provide a slow release of this compound. This type of sorption behavior is the typical cause of the extended times required for pump-and-treat technology.

Pennington et al. (1995) determined that sorption kinetics would most likely be the rate-limiting factor for in situ remediation of explosives-contaminated soils when the contamination levels were low. In highly contaminated soils, the availability of explosives is controlled by the aqueous solubility of the explosives. It was also found that the addition of nonionic surfactants might increase the rate of desorption of explosives from soils.

Under normal environmental conditions, photodegradation of RDX occurs rapidly. The half-life of RDX when exposed to direct sunlight was found to be approximately 11 hours (Sikka et al. 1980). The major transformation product of RDX was found to be the mononitroso analog. In acidic aqueous solutions, transformation of RDX is caused by the cleavage of the N-N when exposed to a strong light source with a wavelength greater than 280 nm. Under neutral and slightly basic conditions transformation of RDX occurs due to cleavage of the N-O bond when it is exposed to wavelengths greater than 220 nm (Kubose and Hoffsommer 1977). Photolysis of RDX and the rate of photolysis do not appear to be dependent on the aqueous concentration of oxygen (Spanggord et al. 1980a). Other transformation products identified from the photolysis of RDX include nitrate, nitrite, formaldehyde, and nitrogen (Sikka et al. 1980). Burton and Turley (1995) concluded that photolysis of an RDX solution reduced its toxicity to daphnia and that it may reduce the toxicity to other aquatic organisms.

Biotic Fates

Evidence of microbial degradation was shown in experiments where contaminated river water was combined with 1% sediment from the same contaminated stream. Significant degradation of RDX occurred after a 20-day lag period. Little or no loss of RDX occurred in the river water alone or with an amendment of yeast extract. Approximately 80% of the RDX added was transformed within two weeks after degradation started. In radiolabeled studies, 80% of the [^{14}C]RDX added was evolved as $^{14}\text{CO}_2$ when 1% river sediment was added to the flasks. Evolution of $^{14}\text{CO}_2$ was preceded by a 10-day lag phase. It is believed that the river sediment provides a large seed of microorganisms capable of degrading RDX and nutrients for the growth of these microorganisms (Sikka et al. 1980)

Results from anaerobic studies suggested that degradation of RDX was a co-metabolic process. Results indicated that a source of organic carbon and RDX had to be present at the same time to achieve RDX degradation. These results suggest that the importance of the organic carbon added was as a co-metabolite and not just as a carbon nutrient to rapidly increase biomass. In flasks initially containing 10 ppm RDX and 50 ppm yeast extract, the RDX was completely transformed in three days. RDX has been found resistant to biodegradation under aerobic conditions (Spanggord et al. 1980b).

RDX in nutrient broth cultures disappeared in approximately four days when inoculated with anaerobic-activated sewage sludge. No transformation of RDX in nutrient broth was observed when inoculated with aerobic-activated sewage sludge and incubated aerobically. A pathway was proposed for anaerobic biological degradation of RDX. This pathway suggests that the one or more nitro groups are reduced to the point where destabilization of the triazine ring occurs, and the ring is fragmented by hydrolytic cleavage. Fragments of the ring are further reduced ultimately resulting in a mixture of hydrazines and methanol. Degradation intermediates identified were the mono-, di-, and trinitroso analogs of RDX, formaldehyde, methanol, hydrazine, and 1,1- and 1,2-dimethylhydrazine. It was suggested that an aerobic treatment may be used to mineralize these degradation intermediates (McCormick, Cornell, and Kaplan 1981, 1984; Walker and Kaplan 1992; Kaplan 1992).

Soil column studies were conducted to determine the feasibility of using land application for the treatment of pink water wastes. Simulated pink water was continuously applied to soil columns inoculated with microorganisms from aerobic-activated sludge and anaerobic sludge digest from municipal wastewater treatment plants and garden soil. Pink water was either applied fast (100 mL per day) or slow (40 mL per day) and with or without a 2000-g/L carbon supplement (glucose). From recovery patterns it was determined that carbon supplementation and longer retention times in the columns increased the level of biological transformation. It was also determined that land application was not a viable alternative for waste streams from explosives production facilities. Land application would likely result in the leaching of explosives and their transformation products to the groundwater (Greene, Kaplan, and Kaplan 1985).

Funk et al. (1993) conducted studies to determine the optimization of biological remediation of soils heavily contaminated with munition compounds. Although this study concentrated on the anaerobic degradation of TNT, it was found that RDX was removed to below the detection limit in approximately 24 days. Conditions in this experiment included the addition of a carbon supplement in the form of a potato-processing by-product, a pH of 7 held constant with a potassium phosphate buffer, and a temperature of 30 °C. A neutral or slightly acidic pH of 6.5 was found to be beneficial in preventing polymerization of azoxy compounds from TNT.

Three microorganisms isolated from nitramine explosive-contaminated soil were reported to be capable of mineralizing RDX under O₂ depleted conditions in pure cultures (Kitts, Cunningham, and Unkefer 1994). The microorganisms from the family *Enterobacteriaceae* were identified as *Morganell morganii*, *Providencia rettgeri*, and *Citrobacter freundii*. Recovery data from radiolabeled RDX experiments showed that *M. morganii*, *P. rettgeri*, and *C. freundii* converted 5, 8, and 9% of the radioactivity to ¹⁴CO₂, respectively. However, only *M. morganii* transformed HMX in the presence of RDX. This is the first reported instance of mineralization of RDX by a single organism, and it should be noted that all mineralization could be accounted for by radiochemical impurities and experimental error.

Rapid biodegradation of RDX in wastewater to CO₂ has been reported through a combination of aerobic and anoxic conditions (Ogden et al. 1994). This was achieved by growing bacteria isolated from horse manure first under anoxic conditions to achieve an anoxic consortium, then under aerobic conditions to rapidly increase the cell density. The culture was then grown under anoxic conditions for the remainder of the experiment. RDX at its aqueous solubility limit and any nitroso intermediates formed were removed from the solution in a total of 60 hours. A carbon supplement was added to the waste waters in the form of yeast extract. In experiments with radiolabeled RDX, 35% of the radioactivity injected was recovered as ¹⁴CO₂ in 150 hours.

A bacterium, *Stenotrophomonas maltophilia* PB1, capable of using RDX as a sole source of nitrogen has been isolated (Binks, Nicklin, and Bruce 1995). The bacterium was isolated from a mixed culture of microorganisms capable of metabolizing RDX. This culture was obtained from soil enrichments under aerobic and nitrogen-limiting conditions using soils heavily contaminated with RDX and HMX. The results implied that the bacterium utilized three of the six possible moles of nitrogen found in RDX. The bacterium was unable to utilize HMX as a sole source of nitrogen. Microbial growth and RDX degradation only occurred in the presence of sugars. This implies that the bacterium was only capable of using RDX as a nitrogen source when strong reducing conditions were present.

Composting has been investigated as a means of biologically treating explosives-contaminated soils. A field demonstration of composting at Louisiana Army Ammunition Plant (LAAP) showed that composting resulted in the biotransformation of munitions products. Although analysis of leachates detected from soils and materials treated by composting detected TNT, RDX,

HMX, and amino-dinitrotoluenes, toxicity tests of the leachate and materials showed that total toxicity was greatly reduced. Results showed that transformation of munitions compounds proceeded much faster under thermophilic conditions. Cost analysis of results from this study indicated that treatment of soil at LAAP with the amendment conditions tested used would cost twice as much as incineration on a unit soil mass basis (Keehan and Sisk 1996, Williams and Myler 1990, Griest et al. 1990).

A study was conducted at Umatilla Army Depot Activity to optimize throughput of munitions-contaminated soils. Results from this study indicated that amendment composition is an important parameter in achieving maximum reduction of RDX and HMX and minimizing unit processing costs. The study also showed that both static pile and mechanically agitated in-vessel composting approaches are effective in transforming explosives. The optimized unit cost determined from these studies was reduced to approximately half the cost of on-site incineration (Keehan and Sisk 1996, Williams and Myler 1990).

Studies conducted on soils contaminated with 49,000 mg/kg of RDX and 4,600 mg/kg of HMX showed that composting of the soils resulted in the transformation of 61% of the RDX and 49.4% of the HMX (Hitner et al. 1996). However, no analysis for transformation products or investigation of toxicity reduction of soils and leachate was performed in this study. The treatment of this soil by composting also resulted in a 242% increase in total treated waste due to the addition of amendments and bulking agents.

Immobilization and transformation of RDX in the rhizosphere of soils is likely to be mediated by plants. It has been demonstrated that RDX is adsorbed by the roots of plants and then transported to other parts of the plant. RDX-derived residues have been found to be metabolized and accumulated in all parts of the plant. The relative order of plant tissue concentration of RDX-derived residues was found to be seed > leaves ≥ stem > root > pod (Cataldo, Harvey, and Fellows 1990).

3 Experimental Design

In order to develop an experimental matrix that would not be too large for the practical limitations of this study, the number of growth variables investigated had to be limited. Several growth-influencing factors were ruled out immediately. The control of temperature at a site covering over five square miles was definitely not feasible, so only the maintenance of a constant temperature around the shake flasks was sought. While pH has been shown in several studies to be influential on the fate of explosives, it was felt that the control of pH would be impractical on a site of this size. The list of possible amendments to the water had to be kept short and could not include chemicals whose injection into the groundwater would possibly raise objections from regulators or the public.

Because the aquifer material was very low in total organic carbon, it was felt that the addition of a substrate for biological growth and energy would be of the largest benefit to the degradation of RDX. Four growth substrates were decided on for this study: acetate, ethanol, potato starch, and corn starch. Previous studies at ERDC have also shown the addition of a nonionic surfactant to be beneficial to the degradation of explosive compounds in soil matrices (Pennington et al. 1995, Zappi et al. 1993). Therefore, the addition of Tween 80 with a growth substrate was decided on as a condition for this study. Although a study by the Idaho Agricultural Experiment Station (Funk et al. 1993) showed the addition of both phosphate and ammonium to be beneficial to the initial-phase of munitions degradation, it was decided that the addition of ammonium to the groundwater could meet with resistance. Therefore, only phosphate was added to the flasks.

All of the literature found suggest that RDX degradation occurs only under anoxic or anaerobic conditions, but both aerobic and anaerobic conditions were investigated in this study for confirmation of this information. The use of an anaerobic period of growth followed by an aerobic period of growth was also investigated.

4 Materials and Methods

Materials

Soil and water used in this experiment were obtained from CAAP. Soil samples were obtained by drilling to a depth of 40 ft with a continuous flight auger. The auger was then withdrawn and the soil pulled from the bottom 10 ft of the auger flights. This procedure was repeated several times until several 5-gal buckets of soil could be obtained. Two 55-gal barrels of water were withdrawn from a monitoring well which had previously shown the highest levels of RDX contamination and was located approximately 0.8 mile west of the drill site. Buckets of soil were kept in ice chests until they could be transferred to coolers along with the barrels of water.

Soil from the aquifer is characterized as a silty sand with gravel-sized particles. The soil has a pH of 7.3, a TOC (total organic carbon) content of 362 mg/kg, and a CEC (cation exchange capacity) of 0.370 meq/100 g. Due to the presence of gravel-sized particles, the soil was passed through a #10 sieve and manually homogenized prior to use and analysis. The particle-size distribution, after being passed through a #10 sieve, of soils from two CAAP monitoring wells is shown in Figure 1. Analysis of the soil by fluorescence direct-count screening showed the soil to contain approximately 440,000 cells/g of dry soil. Results of HPLC analysis for explosives content of the soil and water are shown in Table 1.

Shake-Flask Methods

Experiments were performed in 250-mL shake flasks from Ace Glass as shown in Figure 2. Five replicates were used for each condition unless noted otherwise. Flasks were checked for radioactivity, cleaned, and autoclaved prior to use. Soil and water from the aquifer were added to the flasks as a source of organisms and background chemicals from the aquifer. Approximately 20 g (dry weight) of soil and 80 mL of water obtained from the aquifer at CAAP were added to each flask, and 5 mL of an autoclaved additive solution was then added to each flask. Additive solutions contained phosphate, carbon for growth/energy, and in some cases a nonionic surfactant (Tween 80). The initial concentration of these additives in each flask is given in Table 2. Sterility was maintained in abiotic control flasks by the addition of 0.3 g of mercuric chloride to each flask. In the first experiments, 1 mL of a 10-ppm RDX solution and

1 mL of a uniformly ring-labeled [^{14}C]RDX solution were added to each flask. In the second experiments, an additional 1 mL of 10-ppm RDX solution was added to each flask. This resulted in the contaminant content in each flask as shown in Table 2. The [^{14}C]RDX solution added to all flasks except those in the first aerobic experiment contained 9.8 ppb RDX with a radiochemical purity greater than 99%. The [^{14}C]RDX solution added to flasks in the first aerobic experiment contained 6.3 ppb RDX with a radiochemical purity of 93% and 33.7 ppm acetone. Flasks were assembled, and 2.5 mL of 1 N KOH was added to the center well of each flask to capture CO_2 evolved in the flask. Flasks were then sealed. Assembly of all flasks and additions to them were performed inside sterile hoods.

After assembly, aerobic flasks were placed on incubated orbital-shaker tables operating at 100 rpm. The shaker tables were inside incubated chambers which shielded the flasks from light and kept the flasks at a constant temperature of 25 ± 2 °C. Flasks were removed from the incubator every two to three days and placed under a sterile hood. Flasks were opened and allowed to breath for 5 to 10 minutes. During this time, KOH in the well was exchanged with fresh KOH, and at selected times the pH and ORP of the flasks were measured and recorded. Flasks were then resealed and returned to the incubator.

In the anaerobic experiments, the flasks were placed on orbital shaker tables inside a Coy[®] glove bag which is kept in a temperature controlled room. The Coy[®] bag was purged and filled with a 96% nitrogen/4% hydrogen gas mixture. Any oxygen in the bag was reacted with hydrogen by a catalyst which maintained an oxygen level of 0% by volume inside the glove bag. KOH exchanges occurred through two 6-in. needles placed through the septum which seals the top of the KOH well. Stoppers were removed from the needles and two syringes, one empty and one filled with 2.5 mL of KOH, were placed on the needles. KOH was withdrawn by the empty syringe, and then injected by the filled syringe. The syringes were removed and stoppers replaced in the needles. Because the tips of needles remained in the KOH whenever the needles were open, there was minimal or no exchange of gases between the atmospheres inside and outside the flasks. This method allowed the organisms to create an isolated anaerobic environment inside the flasks. To prevent increases in gas pressure inside the flasks from forcing KOH out of the needles, it was necessary to add helium-grade balloons to the side ports of the flasks. This allowed the pressure to remain constant and prevent expansion of gases inside the flask.

After completion of an experimental run, KOH was removed from the flask wells and the pH and ORP of the flasks were measured. Approximately 12.5 g of wet soil and 10 mL of water were then removed from each flask for further analysis. KOH was again added to the flask wells, and the flasks were resealed. Then 1 mL of concentrated phosphoric acid was injected through the septum of the side port in order to convert any carbonates in the remaining slurry to CO_2 . Flasks were returned to shaker tables and left overnight before KOH was removed for analysis.

Soil and water removed from flasks were placed in 25-mL tubes and centrifuged to separate solids and liquid. The supernatant was then removed and

analyzed for radioactivity. A modified Bligh-Dyer extraction method (Ward et al. 1994, Bligh and Dyer 1959) was then used to extract lipids and any other extractable material from the soil.

Following extraction of the soil, three samples of approximately 0.5 g of wet soil were then prepared and oxidized in a Packard Model 307 Sample Oxidizer. This equipment fully oxidizes the sample and captures the CO₂ and tritium in separate vials of scintillation cocktail for radioactive analysis. The remainder of the extracted soil in the centrifuge tube was used for water soil water content estimation. Radioactivity of all samples was determined by liquid scintillation counting on a Packard 2500TR Liquid Scintillation Analyzer.

Biochemical Oxygen Demand Method

The biochemical oxygen demands (BOD) produced by each of the five additives (acetate, ethyl alcohol, Tween 80, potato starch, and corn starch) used in the shake-flask experiments were investigated. Experiments on each condition were performed in duplicate. BOD was determined with BI-1000 Electrolytic Respirometers by Bioscience, Inc. Two respirometer units were used, each equipped with temperature-controlled water baths, and capable of controlling eight electrolysis cells each. A 1-L reactor vessel fitted to each electrolysis cell, as shown in Figure 3, was used in this experiment. Data were collected by BI-1000 Control Software, version 1.0, via one 8-bit data acquisition card in an 80386 model computer for each respirometer.

As oxygen was consumed and CO₂ was produced and captured by the potassium hydroxide trap, the pressure in the reactor vessel was decreased. This pressure decrease caused the electrolyte solution in the outer chamber of the electrolysis cell to lower and lose contact with the switch electrode. When contact with the switch electrode is broken a current is passed between the oxygen and hydrogen electrode which produces oxygen in the inner chamber and hydrogen in the outer chamber of the electrolysis cell. The hydrogen is vented to the atmosphere, and the oxygen is vented into the reactor vessel raising the pressure. The increase in reactor vessel pressure caused the electrolyte to reestablish contact with the switch electrode and stop the current. The software measures the length of time that current is produced. Knowing the current and length of time, the software then calculates the mass of oxygen produced. The cumulative oxygen produced versus time is recorded in a separate file for each cell.

Reactor vessels were prepared for each of the five organic additives and a control with only dilution water. The vessels were cleaned with a caustic soap, thoroughly rinsed with DI water, and then closed. Inorganic nutrients, as shown in Table 3, were added to each reactor vessel after being filled with approximately 500 mL of deionized water. Then 500 mg of the appropriate additive was added to each vessel and total volume of the solution increased to 1 L. Reactor vessels were then closed, placed in the water baths, and stirred for 15 min. Water bath temperatures were maintained at $20 \pm 0.1^\circ\text{C}$.

After reactor vessel temperatures had equilibrated, 10 mL of a seed solution was added to each vessel. The seed solution was produced by mixing one POLYSEED[®] capsule in 500 mL of deionized water for 2 hr. Polyseed[®], by Polybac Corporation, is a broad spectrum of bacteria developed as a uniform seed population for the BOD₅ test on both municipal and industrial wastes. After addition of the seed the electrolytic cells were added to the reactor vessels, and a leak test was performed on the vessels. After each vessel passed the leak test, data acquisition was begun on that vessel.

Data from the BI-1000 software were imported into a spreadsheet and fitted to a first-order reaction curve (Equation 1) by the method of least squares. Only the first 120 hours of data were used to find the solution. This was to avoid BOD which may have been due to nitrification. This was a concern due to the addition of ammonia by the inorganic nutrient solutions, and the lack of availability of a nitrification inhibitor. Equation 1 is a typical first-order reaction curve with exception of the addition of a time constant to compensate for the lag phase due to acclimation by the microorganisms. The solution was found using the Microsoft Excel solver function with ultimate BOD, reaction rate constant, and lag phase time as unknowns subject to the following constraints:

$$\begin{aligned} L &\geq 0 \\ 0 &\leq k \leq 1 \\ \ell &\geq 0 \end{aligned}$$

$$BOD = L(1 - e^{-k(t-\ell)}) \quad (1)$$

where

$$BOD = \text{biochemical oxygen demand exerted at time } t \left(\frac{\text{mass} \cdot O_2}{\text{volume of sample}} \right)$$

$$L = \text{ultimate BOD} \left(\frac{\text{mass} \cdot O_2}{\text{volume of sample}} \right)$$

$$K = \text{reaction rate constant (time}^{-1}\text{)}$$

$$\ell = \text{total time of lag phase}$$

$$\text{if } t - \ell < 0 \Rightarrow t - \ell = 0$$

Analytical Methods

Modified Bligh-Dyer (1959) total lipid extraction method

This method, Figure 4, involved estimation of water content of the soil and the addition of dichloromethane and methanol to form a MeOH-DCM-H₂O volumetric ratio of 2:1:0.8. Tubes were then sealed, thoroughly mixed with a vortex mixer, sonicated for 10 minutes, and allowed to sit at room temperature overnight. DCM and water were then added to each tube to change the volumetric ratio of MeOH-DCM-H₂O to 1:1:0.9. Tubes were again mixed with a vortex mixer and centrifuged to separate the extraction mixture. Centrifuge tubes were carefully removed from the centrifuge, and a glass pipette was used to reach through the aqueous methanol top phase and remove the DCM phase from the bottom corner of the centrifuge tube. The DCM phase was rinsed through baked sodium sulfate into another tube with clean DCM to remove any water from the lipid sample. A known fraction of the DCM containing the lipids was then analyzed for radioactivity. The aqueous methanol phase of the extraction was also removed from the centrifuge tube and analyzed for radioactivity.

Preparation of water samples for HPLC analysis of explosives

Water samples were preconcentrated and prepared for analysis by solid phase extraction (SPE) with Waters Porapak[®] R_{DX} Sep-Pak[®] Vac Cartridges. These cartridges consist of a divinylbenzene/vinylpyrrolidone copolymer, packed in high-purity polyethylene syringe barrels. As water samples are drawn through the SPE cartridges, nitroaromatic and nitramine compounds present in the water are adsorbed onto the copolymer material of the SPE cartridges. The adsorbed compounds are then eluted from the columns with acetonitrile which is collected for analysis.

Porapak R_{DX} cartridges were conditioned to activate the packing material. Cartridges were placed on a vacuum manifold and a Sep-Pak vacuum adapter was placed in the open end of the syringe barrel of each cartridge. A 60-mL Sep-Pak reservoir was connected to each vacuum adapter. Each reservoir was filled with 15 mL of acetonitrile. The vacuum was pulsed in order to start flow, and the acetonitrile was allowed to drip through the cartridge under gravity alone. Just before each reservoir ran dry, 30 mL of distilled/deionized water was added to each of the reservoirs. The water was then pulled through the reservoirs at a flow rate no greater than 10 mL/min. Just before each reservoir was drained of water, the water sample was added to the reservoir and pulled through the cartridge at a flow rate which did not exceed 10 mL/min. After each cartridge was empty, full vacuum was applied to the manifold to remove residual water from the cartridge. Reservoirs were not allowed to run dry from the beginning of the cartridge conditioning process until the entire sample was loaded. If a conditioned cartridge is exposed to oxygen prior to the completion of sample loading it will not perform properly.

After cartridges were cleaned and loaded, the vacuum manifold was drained and a rack of clean, solvent-rinsed test tubes was added to the reservoir as sample collection vessels. Reservoirs and vacuum adapters were removed and 5 mL of acetonitrile was added to each cartridge. The acetonitrile was allowed to drip through the cartridges at a flow rate less than or equal to 1 mL/min. After the cartridges were empty, vacuum was applied to remove the remaining acetonitrile from the cartridges. Test tubes of the sample eluent were then removed and sealed with a Teflon-lined cap. Test tubes were then refrigerated in the dark until analyzed.

HPLC analytical methods

Water samples were analyzed for RDX on a Waters liquid chromatography unit. No standards were available for RDX degradation products at the time of analysis. Equipment used included a Hewlett Packard ODS Hypersil (C18) column, 100 × 4.6 mm with a 5- μ m particle size, and a Waters 996 Photodiode Array UV Detector. Signal from the detector was analyzed using Millennium™ 2010 version 2.15 chromatography software by Waters. Combination of this detector and software allowed for traditional analysis of chromatograms and peak identity and purity confirmation by spectral analysis.

A 25- μ L sample volume was injected into a mobile phase containing 26% of a 92:8 methanol:butanol mixture and 74% water buffered with 20 mM ammonium nitrate. Data were collected for 17 minutes from 210 to 600 nm with a 2.5-nm resolution. A chromatogram for analysis was produced by a derived channel of the maximum absorbance from 250 to 350 nm. Peak purity and confirmation were performed by analyzing the peak spectrum from 210 to 450 nm. Duplicate injections were performed on each sample.

Kinetic analysis of $^{14}\text{CO}_2$ vs time data

To estimate the rate of RDX mineralization, it was assumed that the mineralization of RDX was an irreversible process following first-order kinetics with respect to the concentration of RDX in solution (Equation 2). It was also assumed that RDX mineralization will be complete provided no substrate required for cellular growth becomes limiting and no mineralization occurred during the acclimation phase of the microorganisms to conditions in the flask. Given these assumptions Equation 2 can be rearranged and integrated (Equation 4). In order to calculate the rate of mineralization of RDX, Equation 4 must be rewritten in terms of the $^{14}\text{CO}_2$ data collected in this study. Assuming the fraction of the original concentration of RDX in solution is equal to the fraction of the radioactivity remaining in solution, and knowing that the radioactivity in solution is equal to one minus the radioactivity recovered as CO_2 , Equation 4 can be rewritten as Equation 5.

Equation 5 was fit to the $^{14}\text{CO}_2$ recovery data by the method of least squares with reaction rate constant and lag-phase time as best fit parameters. $^{14}\text{CO}_2$ recovery data for many of the shake flasks exhibited decreasing rates of mineralization long before mineralization was complete. It was assumed that

this limitation was caused by a limit of growth substrate needed by the microorganisms for growth in the flasks, therefore these data were ignored when fitting the results.

$$\frac{dRDX_t}{dt} = -k \cdot RDX_t \quad (2)$$

where

RDX_t = concentration of RDX in solution at time t

t = time elapsed since start of experiment

k = reaction rate constant

$$\int_l^t \frac{dRDX_t}{RDX_t} = -k \int_l^t dt \quad (3)$$

where

RDX_t = concentration of RDX in solution at time t

l = lag phase time required for microorganisms to acclimate

$$\frac{RDX_t}{RDX_i} = e^{-k(t-l)} \quad (4)$$

where

RDX_i = initial concentration RDX

$$c_t = 1 - e^{-k(t-l)} \quad (5)$$

where

c_t = fraction of radioactivity recovered as CO_2

5 Results

Results from the shake-flask experiments include both radioactive tracer analysis and HPLC analysis of the final shake-flask contents. The radioactive tracer analysis shows the fate and distribution of carbon from RDX in the slurry system. These data were analyzed for (1) mass balance recovery of radiolabeled RDX put into the system and (2) the distribution of carbon from the RDX between the soil, aqueous phase, biological matter, and the carbonate species. Time-dependent data of radioactive CO₂ evolution are also presented and analyzed in an attempt to determine the rate of RDX mineralization. HPLC analysis of the final aqueous concentration of RDX in slurry is also presented to determine the extent of RDX transformation under each condition. The results of BOD tests on the carbon and surfactant additives used in these experiments are presented. These data sets were analyzed to estimate the ultimate BOD produced per unit mass of additive and the rate of oxygen uptake by a generic population of microorganisms utilizing each additive. From this information total BOD added to the flasks in each condition was calculated and used as a basis to explain the differences in degradation and mineralization achieved with different additives.

Bar graphs showing ¹⁴C recovery are divided into carbonates, nonpolar Bligh-Dyer extract, polar Bligh-Dyer extract, supernatant, and soil. The sum of these five areas of recovery gives a mass balance for ¹⁴C in the flasks. The carbonates included total recovery of ¹⁴C in the CO₂ traps during the experiment and after acidification of the flask contents. Because mineralized ¹⁴C can remain in solution as any of the carbonate species, and the distribution of these species is determined by the solution pH, the remaining flask contents were acidified to convert all carbonate species in solution to gaseous CO₂, after removal of soil slurry for the Bligh-Dyer extraction. No attempt was made to trap volatile organic compounds, which could in some cases account for a low mass balance. The nonpolar extract included ¹⁴C recovered in the DCM phase from the Bligh-Dyer extraction. ¹⁴C recovered in the nonpolar extract represents the fraction of ¹⁴C assimilated into the microbial cells and the extractable [¹⁴C]RDX sorbed to the cell walls and the soil. The polar extract included ¹⁴C recovered in the MeOH/water phase of the Bligh-Dyer extraction. ¹⁴C recovered in the polar extract represents the extractable fraction of ¹⁴C-containing compounds which were sorbed to the soil or cell walls of organisms. The supernatant included ¹⁴C in the water recovered after centrifuging the soil slurry withdrawn from flasks for the Bligh-Dyer extraction. ¹⁴C recovered in the supernatant represents soluble ¹⁴C-containing compounds excluding the carbonate species. The levels of radioactivity detected in the supernatant were corrected for carbonates by

subtracting the levels of radioactivity obtained during the acidification step of the flask contents. The soil included ^{14}C recovered from trapped CO_2 which was produced by the oxidation of previously extracted soil samples. ^{14}C recovered from the soil samples represents the unextractable fraction of ^{14}C -containing compounds which were sorbed to the soil.

Graphs of $^{14}\text{CO}_2$ recovery versus time are shown for each experiment. Points on these graphs represent the cumulative amount of $^{14}\text{CO}_2$ recovered in the CO_2 traps at a particular time. Values on these graphs do not reflect radiolabeled carbonate species which remained in solution, because the levels of [^{14}C]carbonate species in solution versus time could not be determined. This accounts for the differences between the values of the final points on these graphs with the values shown for carbonate species on the bar graphs. Although there is not enough data in this experiment to suggest what the factors are controlling the rate of RDX mineralization nor the order of the reaction, the $^{14}\text{CO}_2$ recovery versus time data were fit to a first-order reaction equation (Equation 5) for a basis of comparing the rate of $^{14}\text{CO}_2$ production under different conditions.

Graphs of ORP and pH versus time were also included for aerobic experiment 2, and the anaerobic-aerobic experiment. These graphs were produced to address the concern that the aerobic flasks may be cycling between aerobic and anaerobic conditions.

Bar graphs showing the final RDX concentrations in the aqueous phase of the slurries are presented. The detection limit for RDX by the HPLC method used was approximately $60\ \mu\text{g/L}$, but with the small amount of preconcentration achieved by use of the SPE cartridges in the sample preparation method, the effective detection level was lowered to $45\ \mu\text{g/L}$. Although levels below this were occasionally detected, the accuracy of these measurements is doubtful.

The results of BOD produced by additives used in the experiments are presented as graphs of BOD versus time over approximately a six-day time period. The ultimate BOD produced per unit mass and the first-order reaction rate constants of these additives were determined. The estimated levels of ultimate BOD added to the flasks by these additives in each experimental condition are also presented.

Biochemical Oxygen Demand of Additives

The BOD produced by additives used in the shake-flask experiments was determined in electrolytic respirometers. This information was sought to help explain the different levels of RDX transformation and mineralization achieved in the shake-flask experiments by the addition of different additives. Results from the analysis of the BOD data are shown in Figure 5 through Figure 12 and Table 4. Although the experiment was performed with duplicates for each condition, it should be noted that the data for only one of the duplicate cells were used for ethyl alcohol, potato starch, and corn starch. Data for the discarded

cells were determined to be faulty due to a loss of the airtight seal somewhere in the reactor vessel or the electrolytic cell. The loss of the seal resulted in a near infinite BOD exertion rate for these cells.

Because the rate at which BOD is exerted is estimated to be dependent on the BOD remaining in the solution, a first-order equation (Equation 1) was fit to the data obtained from the respirometer studies. The fit of this first-order equation to the data is shown in Figure 5 through Figure 10.

The first-order reaction rate constants obtained for the five additives tested are presented in Table 4 along with the estimated ultimate BOD exerted per unit mass of additive. The results show that acetate and ethyl alcohol are oxidized approximately twice as fast as the starches and surfactant (Tween 80). These results correlate to molecule complexity as was expected. The results also showed that ethyl alcohol caused a much higher BOD to be exerted on a unit mass basis than the other additives. Acetate, corn starch, and Tween 80 showed approximately the same BOD exerted per unit mass. It was interesting to note that the starch from potatoes exerted approximately twice as much BOD per unit mass as the starch from corn.

Using the ultimate BOD produced per unit mass of additive estimated from the data, the ultimate BOD added in each condition of the shake-flask study was calculated. These calculated results are presented in Figure 11 and Figure 12.

Aerobic Shake Flasks

Experiment 1

In the initial experiment, six conditions were investigated. These conditions included the addition of acetate, acetate with Tween 80 (nonionic surfactant), ethanol, potato starch, corn starch, and a no-carbon-added condition. The source of microorganisms for the study was the water and soil from CAAP which was added to each flask. Sterile control flasks were also maintained for each condition investigated except for the addition of corn starch. The two conditions, addition of potato starch and addition of corn starch, were considered similar enough to exclude a sterile control for the addition of corn starch. Sterility in the control flasks was obtained by double autoclaving CAAP soil and water before addition to the flasks. Flasks were kept in incubated shakers with a constant temperature of 28 ± 1 °C. Uniformly ^{14}C ring-labeled RDX was added to each flask from a stock solution. In this experiment a five-year-old stock [^{14}C]RDX solution was used due to unavailability of newer material. The stock solution contained 7% radioactive impurities as determined by liquid chromatography and autoradiography. This impurity level is shown on the graphs corresponding to results from these experiments. No correction was made in the calculations because the identity of non-RDX ^{14}C containing compounds in the stock solution and how they reacted in the experiments could not be determined.

The first thing noted from the ^{14}C recovery data was that the sterile controls did not remain sterile. The recovery of $^{14}\text{CO}_2$ versus time (Figure 15) showed $^{14}\text{CO}_2$ evolution in the sterile flasks lagging behind that found in the nonsterilized flasks early in the experiment, but for most conditions the levels of $^{14}\text{CO}_2$ evolved were the same in sterile control and nonsterilized flasks by the end of the three-week experiment. From this evidence it is assumed that the entire population of microorganisms was not killed by the double autoclaving procedure. Because the KOH exchanges in this experiment were performed outside of a sterile hood, another possibility is that the flasks were contaminated by organisms in the lab air.

Flasks with the addition of acetate with Tween 80 showed much more $^{14}\text{CO}_2$ evolution than the other flasks. These same flasks also showed greater than 75% (below the detection limit) of the RDX in the flask to have been transformed, while the other conditions produced approximately 50% transformation of RDX (Figure 13). Later tests on the additives also showed that the ultimate BOD added to the flasks with Tween 80 was higher than others by a factor of approximately twenty-three (Figure 11).

Curves were fit to the data of $^{14}\text{CO}_2$ recovery versus time (Figure 16), but no meaningful information could be seen in these kinetic rates. In all flasks except those with the addition of acetate with Tween 80, a high initial rate of $^{14}\text{CO}_2$ recovery was followed by a short period where $^{14}\text{CO}_2$ recovery almost stops. The initial high rate of $^{14}\text{CO}_2$ recovery is probably a result of radioactive impurities which were easily mineralized by the microorganisms. In these same conditions the rate of $^{14}\text{CO}_2$ recovery began to increase again at approximately ten days which most likely corresponded to the mineralization of RDX. The flasks with the addition of acetate with Tween 80 did appear to follow the first-order reaction equation for the mineralization of RDX as described in Equation 5. The first-order reaction rate constant was 0.021 which was close to the values seen in the second aerobic experiment.

The mass balance of ^{14}C recovered from the flasks (Figure 14) showed that the average of each condition was $100\% \pm 8\%$. In all conditions except the addition of acetate and Tween 80, the recovery of ^{14}C in the form of carbonates was approximately 7%. It is assumed that 7% of the ^{14}C recovered as carbonate in all conditions is most likely the result of the mineralization of radioactive impurities from the stock [^{14}C]RDX used in this experiment. Therefore, in all of the conditions except those flasks with the addition of acetate and Tween 80, mineralization of these impurities would account for almost all of the ^{14}C recovered as carbonates. The remainder of ^{14}C recovered as carbonates for most conditions is most likely attributable to experimental error. The only condition that indicated mineralization of RDX is therefore the addition of acetate and Tween 80.

The most significant fraction of the ^{14}C remained in the aqueous phase either as RDX or as intermediates of degradation. Approximately 3% of the ^{14}C was found in the nonpolar fraction of the Bligh-Dyer extraction, and 1 to 2% was found in the polar extract. Very little of the ^{14}C was unextractable from the soil.

Experiment 2

Results from the initial aerobic experiment raised questions about the effects of Tween 80 on the mineralization of RDX. It was not known if the positive effects on RDX mineralization were due to Tween 80's properties as a surfactant or if Tween 80 simply acted as an additional source of carbon or energy for the microorganisms. To determine if the addition of acetate at a higher concentration would produce results similar to those of the addition of Tween 80, another aerobic experiment was conducted. In this experiment the total mass of additives, including the Tween 80, in each condition were equal. The additions included acetate with Tween 80 at the same levels as the initial experiment, Tween 80 alone, and acetate alone. In contrast to the first experiment, the ultimate BOD added to the flasks in each of these conditions was approximately the same (Figure 12).

Sterile control flasks were kept for the high acetate and acetate Tween 80 conditions. Sterility was maintained through the addition of 0.3 g (1.5% of the estimated dry weight of soil) of mercuric chloride. Addition of the mercuric chloride did appear to maintain sterility as indicated by the lack of RDX transformation (Figure 13) in the control flasks. Sterile control flasks were not kept for the Tween 80 only condition. It was felt that the Tween 80 only and acetate with Tween 80 conditions were similar enough to exclude this sterile control. A fresh stock of uniformly ^{14}C ring-labeled RDX was used in this experiment. This radiolabeled compound was synthesized by NEN Research Products and was verified to have a radiochemical purity greater than 99% as determined by liquid chromatography and autoradiography.

Bioaugmentation was also investigated in the second aerobic experiment. Three microorganisms were isolated which were shown to mineralize RDX when they are together. The microorganisms were isolated from Hastings Army Ammunition Plant (AAP) soil in work performed at ERDC. The three microorganisms were unofficially identified as *Alcaligenes denitrificans*, *Pseudomonas fluorescens*, and *Pseudomonas nitroreducens*.¹ CAAP soil and water was double autoclaved and added to these flasks followed by an inoculum of each cultured organism.

The mass balance of ^{14}C recovered from the flasks (Figure 17) ranged from a low of 76.6% to a high of 106.3% by condition. The average total recovery of ^{14}C for all conditions was 87.6% for this experiment. The lower levels of recovery were found in conditions with the highest levels of mineralization. The most likely cause of the low recovery is the loss of $^{14}\text{CO}_2$ from the flask head-space during the exchanges of KOH solutions in the CO_2 traps, but could also be caused by the production of other ^{14}C containing gases which were not trapped. The levels of total ^{14}C -recovery above 100% would have to be attributed to experimental error.

¹ Personal Communication, December 1995, Wayne Evans, ASi Corporation, Vicksburg, MS.

The ^{14}C recovery data (Figure 17) from the second aerobic experiment showed that the Hastings Triplet of organisms mineralized larger amounts of RDX than the organisms native to CAAP soil for all conditions. However, it is believed that these bioaugmented flasks started with a larger mass of viable organisms than the flasks with the native CAAP organisms. Unfortunately, levels of biological mass, total or viable, were not measured in these experiments. Although the level of mineralization was higher in the flasks with Tween 80, the high acetate condition was the only aerobic condition to produce transformation of RDX to below the detection limit (Figure 13) with both the native CAAP and Hastings organisms. As in the first experiment, the most significant fraction of the ^{14}C remained in the aqueous phase. Very little of the ^{14}C was unextractable from the soil. Small amounts were recovered in the polar and nonpolar fraction of the Bligh-Dyer extraction.

Results of fitting the first-order equation of RDX mineralization (Equation 2) through the $^{14}\text{CO}_2$ recovery data are shown in Table 5 and Figure 20. Even though the Hastings Triplet flasks mineralized higher levels of RDX, the first-order rates of $^{14}\text{CO}_2$ production from the Hastings Triplet flasks and the Native flasks appear to be approximately equal for the same conditions. These rates indicate that the ability to mineralize RDX was approximately the same for the organisms isolated from Hastings AAP soil and the organisms native to the CAAP aquifer. The first-order reaction rates also indicate that RDX mineralization proceeded approximately three times faster in flasks with Tween 80 as part of the carbon addition to the flasks. Mineralization may have been slowed in the flasks with the addition of acetate only by the higher pH values, above 9, observed in these flasks (Figure 19).

There were concerns after the first aerobic experiment that the conditions in the flasks may have been cycling between aerobic and anaerobic conditions. To assure that conditions had remained aerobic during the experiment, ORP and pH were monitored throughout the second experiment. ORP and pH versus time are presented in Figure 19. Although the ORP levels in the experiments did not drop to levels that would be considered strictly anaerobic, they were in the border region between aerobic and anoxic activity (Wareham, Hall, and Mavinic 1991; Peddie, Mavinic, and Jenkins 1990; Jenkins and Mavinic 1989; Koch and Oldham 1985; Brannon et al. 1978). The ORP in flasks inoculated with the Hastings organisms did drop to levels which would be considered as anoxic. Even though analysis for nitrogen-containing species was not performed on the flask contents, the levels of E_h observed are a strong indicator of denitrification activity in the flasks. The Hastings flasks did show higher levels of mineralization, although the flasks with CAAP native organisms also showed significant mineralization of RDX. The ORP data collected in this experiment strongly indicate that the oxygen levels in the flasks were very low, and that both aerobic and anaerobic respiration may have been occurring in the flasks.

Anaerobic Shake Flasks

Experiment 1

The same six conditions were investigated in the initial anaerobic experiment as in the initial aerobic experiment. These conditions included the addition of acetate, acetate with Tween 80 (nonionic surfactant), ethanol, potato starch, corn starch, and a no-carbon-added condition. The source of microorganisms for the study came from the water and soil obtained from CAAP which was added to each flask. Sterile controls were also maintained for each condition except for the addition of corn starch. A sterile control was not kept for this condition because it was assumed to be similar enough to the sterile control for the potato-starch-added condition. Sterility in the control flasks was maintained by the addition of 0.3 g (1.5% of the estimated dry weight of soil) of mercurous chloride to each flask. Mercurous chloride was used instead of mercuric chloride in this experiment by accident. The mercurous chloride did appear to prevent the formation of $^{14}\text{CO}_2$ in the flasks as shown in Figure 22, but it did not prevent transformation of RDX (Figure 21). [^{14}C]RDX used in this experiment was the same as that used in the second aerobic experiment and had a radio-chemical purity greater than 99%. A constant temperature of 24 ± 1 °C was maintained throughout the experiment.

Flasks with the addition of acetate with Tween 80 showed the highest level of RDX mineralization at the end of the six-week experiment (Figure 22). This condition showed 30% of ^{14}C recovered as carbonates while other biologically active conditions showed approximately 10% mineralization. It was noted that the potato starch showed slightly higher levels of mineralization than the other conditions with the exception of acetate with Tween 80. Transformation of RDX was significant in all conditions (Figure 21). All conditions except the no-carbon addition reduced the aqueous concentration of RDX to below the detection limit of 45 $\mu\text{g/L}$. RDX was detected slightly below the detection limit, 40 $\mu\text{g/L}$, in the flasks with only acetate addition. The most significant fraction of the ^{14}C remained soluble in the aqueous phase. Small fractions of ^{14}C were recovered from the soil and the Bligh-Dyer extraction.

Analysis of the $^{14}\text{CO}_2$ data as a first-order reaction (Equation 5) showed the condition of acetate with Tween 80 addition to have the highest rate of RDX mineralization. A fit of a curve based on a first-order reaction through these data (Figure 24) showed a reaction rate constant of $2 \times 10^{-3} \text{ day}^{-1}$ for the acetate with Tween 80 condition. This rate was double that of the other conditions (Table 8), but the rate of $^{14}\text{CO}_2$ production appeared to still be increasing in all conditions.

Because flasks were not opened for KOH exchanges during the anaerobic experiments, only the final pH and ORP were measured for each flask (Table 6). The final pH of the flasks ranged from 9 to 10. The pH of biologically active flasks was above 9.5 while the pH of the sterile flasks was 9.5 or below. The E_h of sterile controls was approximately +450 mV which indicates that the reduction of oxidized-nitrogen-containing species had not occurred and the possible presence of residual oxygen in the flasks. Dissolved oxygen levels in

the flasks were not measured. In contrast to the sterile flasks, the E_h of biologically active flasks ranged from -297 to -476 mV. ORP values observed in the biologically active flasks indicate that chemical species containing oxidized forms of nitrogen had been reduced and that methanogenesis may be occurring (Brannon et al. 1978). Methanogenesis in the biologically active flasks could account for the recovery of $^{14}\text{CO}_2$.

Experiment 2

The results from the initial anaerobic experiment showed higher levels of mineralization in the condition of acetate with Tween 80 addition. These results raised the same questions as those in the first aerobic experiment. Were the higher levels of mineralization in this condition the result of Tween 80's properties as a surfactant, an additional carbon source, or both? To answer this question similar carbon addition conditions were selected for this experiment as in the second aerobic experiment. However potato starch was used instead of acetate due to the better results observed with this substrate in the initial anaerobic experiment. In this experiment the total mass of additives, including the Tween 80, in each condition were equal. The following conditions were used: potato starch, Tween 80, and potato starch with Tween 80. It should be noted that the ultimate BOD added in the potato starch condition was twice the ultimate BOD added in the other conditions. Sterile control flasks were kept for the potato starch with Tween 80 and the high potato starch conditions. Due to tight standard deviations of the results in sterile flasks in previous experiments, only three replicates were kept for sterile controls in this experiment. No sterile controls were kept for the Tween 80 added condition because it was felt that this condition was sufficiently similar to the potato starch with Tween 80 condition. Sterility was maintained in the controls by the addition of 0.3 g of mercuric chloride.

Bioaugmentation was also investigated in this experiment. A microorganism was obtained from the American Type Culture Collection which was isolated by a group of scientists at Los Alamos National Laboratory (Kitts, Cunningham, and Unkefer 1994). The bacteria *Morganella morganii* was shown to degrade and mineralize RDX under O_2 depleted conditions. Flasks inoculated with *M. morganii* were maintained for each of the conditions listed above. CAAP soil and water added to the flask were double autoclaved prior to being added to the flasks.

Flasks with the addition of high levels of potato starch showed the most significant evolution of $^{14}\text{CO}_2$. Both the native CAAP organisms and the *M. morganii* converted approximately 23% of the ^{14}C in the flasks to carbonates in the six-week experiment with the high potato starch condition (Figure 25). Under the other conditions of carbon addition, the *M. morganii* mineralized approximately 14% of the ^{14}C RDX, but the native organisms mineralized far less under the same conditions. All biologically active conditions resulted in the transformation of RDX to below the detection level of 45 $\mu\text{g/L}$ except the addition of Tween 80 only with native CAAP organisms (Figure 21). The addition of Tween 80 with native CAAP organisms was also the biologically

active condition with the highest ORP at the end of the experiment (Table 7). Small fractions of ^{14}C were recovered in the Bligh-Dyer extraction and on the soil. As in other experiments the majority of the ^{14}C remained in the aqueous phase either as RDX or intermediates of degradation.

Analysis of the $^{14}\text{CO}_2$ recovery with time data showed the rate of $^{14}\text{CO}_2$ recovery to be slowing down after three weeks in most of the biologically active flasks (Figure 26). This is an indication that something in the system required for evolution of $^{14}\text{CO}_2$ was becoming limiting. Estimates of the reaction rate constants based on the first-order reaction equation (Equation 5) are given in Table 8. The first-order reaction equation showed good correlation to the data. Based on first-order reaction rate constants, the native organisms mineralized RDX much faster with the addition of potato starch alone than the other conditions. The rate of mineralization was approximately the same for all conditions for flasks inoculated with *M. morganii* (Figure 27).

ORP and pH were checked at the conclusion of the six-week experiment (Table 7). Values of E_h ranged from +134 mV down to -339 mV in the biologically active flasks. The lower ORP values corresponded to the conditions which produced the most $^{14}\text{CO}_2$. The E_h remained high for the sterile control flasks and was approximately greater than +600 mV indicating that oxygen remained in the flasks. The E_h value was also much higher in the flasks with native CAAP organisms with the addition of Tween 80 only as an amendment. This condition also showed much less transformation of RDX than other biologically active conditions. The pH values for all flasks were around 9.5 except for the sterile flasks with the addition of a high level of potato starch which had a pH of 7.3.

Anaerobic - Aerobic Shake Flasks

After five weeks of anaerobic incubation in the second anaerobic experiment, several of the flasks from the experiment were removed from the glove bag and exposed to aerobic conditions for two more weeks. Two flasks were taken from each of the conditions with the CAAP native organisms, and one flask was taken from each of the sterile control conditions. Care was taken when selecting the flasks to ensure that the average of the $^{14}\text{CO}_2$ evolution data for the remaining flasks was equal to the average of all flasks at the time these flasks were removed. Because only one sterile flask was removed for each sterile control, no standard deviation could be given for these flasks in the $^{14}\text{CO}_2$ versus time graph (Figure 30). Although only two flasks for biologically active conditions were used, the standard deviation of each condition remained very low except for the potato starch with Tween 80 condition.

The rate of RDX mineralization was estimated based on a best fit of the first-order reaction equation (Equation 5) to the $^{14}\text{CO}_2$ evolution with time data (Figure 30). The estimated order and reaction constants are given in Table 9, and graphs of the curves fit to the data are given in Figure 32. The first-order reaction equation showed good correlation to the data. The data were analyzed

as a whole, anaerobic and aerobic together. Reaction constants from the analysis of data for the potato starch with Tween 80 and Tween 80 conditions were dominated by $^{14}\text{CO}_2$ evolution from the aerobic phase of this experiment and showed much higher reaction rate constants. During the aerobic phase the potato starch with Tween 80 showed a very high reaction rate coefficient, 0.046 day^{-1} . This was the highest first-order reaction rate coefficient demonstrated throughout the study.

During the five-week anaerobic phase of this experiment only the high potato starch condition showed significant levels of $^{14}\text{CO}_2$ evolution (Figure 30). The $^{14}\text{CO}_2$ recovered versus time data also showed that this was the only condition that indicated a limiting condition occurring during the anaerobic phase. The limiting condition could be caused by a reduction in the level of RDX intermediates in solution or a reduction of carbon substrate, but most likely was caused by a low pH condition (5.4) which prevents methanogens from functioning (Tchobanoglous and Burton 1991). During the aerobic phase the rate of RDX mineralization for this condition continued with little increase until the end of the two-week aerobic phase. This resulted in 30% (Figure 29) of the [^{14}C]RDX being mineralized in 7 weeks and an overall first-order rate constant of 0.007 day^{-1} . This condition also showed the lowest ORP at the end of the anaerobic phase, E_h of -200 (Figure 31), and the greatest RDX transformation at the end of the experiment (Figure 28).

The Tween 80 and potato starch with Tween 80 conditions showed very little $^{14}\text{CO}_2$ evolution during the anaerobic phase but demonstrated significant increases in the $^{14}\text{CO}_2$ evolution during the aerobic phase (Figure 30). Potato starch with Tween 80 showed the largest amount of RDX mineralization, 50% of the total ^{14}C in the flasks (Figure 29), in the seven-week experiment. Greater than 95% of this mineralization occurred during the two-week aerobic phase (Figure 30). The extremely high rate of RDX mineralization during the aerobic phase suggests that intermediates of degradation were formed under anaerobic conditions which were easily converted to carbonates under aerobic conditions. This possibility was suggested by McCormick et al. (1981).

Small amounts of ^{14}C were recovered in the Bligh-Dyer extraction, and a small fraction remained bound to the soil. As in all other experiments a large fraction of the ^{14}C was found to remain in the aqueous phase either as RDX or products of degradation (Figure 29).

ORP levels of the high potato starch and potato starch with Tween 80 conditions both dropped to an E_h below -100 mV during the five-week anaerobic portion of this experiment (Figure 31). At this ORP level denitrification is usually complete and sulfate reduction is occurring or has completed in natural systems. The ORP level of the Tween 80 condition only dropped to an E_h of 150 mV during the anaerobic portion of this experiment. This is in the lower ORP range at which nitrate reduction is usually occurring and suggests that the nitro groups of all the RDX may not be reduced yet. Less than 50% of the RDX in this condition was transformed by the end of the experiment.

6 Discussion

Mineralization of RDX was demonstrated in both the aerobic and anaerobic experiments. However, ORP levels in the second aerobic experiment indicated that conditions in the flasks were anoxic during the aerobic experiments. Biological degradation of RDX under anaerobic conditions has been demonstrated in many other studies (Sikka et al. 1980; Spanggard et al. 1980b; McCormick, Cornell, and Kaplan 1981; Walker and Kaplan 1992; Kaplan 1992; Funk et al. 1993; Kitts, Cunningham, and Unkefer 1994; Ogden et al. 1994; Binks, Nicklin, and Bruce 1995). Mineralization of RDX, however, did proceed several times faster in the aerobic experiments than in the anaerobic experiments. Mineralization of RDX in the anaerobic flasks was probably the end result of methanogenesis which is a very slow process. In the aerobic flasks, mineralization most likely resulted from a combination anoxic respiration (denitrification) and aerobic respiration. Both aerobic and anoxic respiration proceed several times faster than methanogenesis.

The initial steps of RDX degradation were most likely the same in both the aerobic and anaerobic experiments. Oxygen in the flask was depleted rapidly through aerobic respiration as microorganisms oxidized available carbon sources. Following the removal of oxygen from the flasks, one or more of the nitro groups on the triazine ring were reduced until the ring became unstable. The ring was then broken by hydrolytic cleavage as suggested by McCormick, Cornell, and Kaplan (1981).

Because the reduction of nitrate only takes place in the presence of suitable microorganisms, reduction of the nitro groups of the RDX molecule was most likely mediated by microorganisms capable of denitrification. Flasks biologically augmented with the microorganisms from Hastings AAP showed higher levels of RDX mineralization. These microorganisms were unofficially identified as *Alcaligenes denitrificans*, *Pseudomonas fluorescens*, and *Pseudomonas nitroreducens*, all of which are capable of nitrate reduction. During reduction of the nitro groups on the RDX molecule and any other forms of oxidized nitrogen in the system, the E_h exhibited by the slurry should be between +200 and +400 mV (Brannon et al. 1978; Koch and Oldham 1985; Peddie, Mavinic, and Jenkins 1990). ORP levels may fall below this level prior to reduction of all nitro groups if a carbon source for the denitrifying organisms becomes limiting. Measured ORP levels in the second aerobic experiment stayed in this ORP range during most of the experiment. Only flasks that exhibited an E_h near or below +200 reduced RDX concentration to below the detection limit.

While most flasks in the anaerobic experiments transformed most of the RDX, flasks in the aerobic experiments with low levels of BOD added exhibited little transformation of RDX. The small amount of carbon added to the flasks in the aerobic experiment was most likely consumed quickly during the periods of aerobic respiration. Because the organisms mediating the denitrification process require a source of carbon for cell synthesis, they were able to reduce only small amounts of the nitro groups. In the anaerobic experiments only the oxygen in the flask at the beginning of the experiment was available to microorganisms for respiration. Therefore, the amount of carbon which could be reduced by aerobic respiration was limited, which resulted in a much larger fraction of the carbon added being available for use by the denitrifying organisms. The only biologically active condition from the anaerobic experiments which had a large concentration of RDX remaining was CAAP organisms and the addition of Tween 80. This would indicate that Tween 80 may not be a good source of carbon for denitrifying organisms.

In the aerobic experiments, fresh air was allowed to circulate into the flasks every two or three days. As oxygen transfer into the slurry occurred, the enzyme system needed for denitrification would be suppressed by the presence of oxygen. Oxygen transferred into the slurry was most likely consumed rapidly by aerobic respiration utilizing the added carbon sources and RDX degradation products formed by cleavage of the triazine ring. This process would result in the mineralization of these RDX degradation products and return the slurry to anoxic conditions. This cycle not only allowed the degradation products from RDX to be rapidly mineralized, but also provided periods of rapid growth of the facultative microorganisms

Because alkalinity is produced during denitrification, conditions where denitrification is dominant in the flask should also be accompanied by an increase in the pH of the slurry. In contrast, aerobic respiration is accompanied by the production of CO₂ which tends to make aerobic cultures become slightly acidic. The high pH (9.5), high level of RDX transformation, and low level of RDX mineralization of the high acetate condition in the second aerobic experiment is a result of a biological culture dominated by denitrifying organisms. The lowest pH reading in the second aerobic experiment corresponds to the highest levels of mineralization. These pH levels, approximately 7.8, indicate significant levels of both anoxic and aerobic activity which resulted in higher levels of mineralization.

In the anaerobic experiments, mineralization of RDX would have required several steps mediated by microorganisms: denitrification, hydrolysis and fermentation, and methanogenesis. After denitrification of the flask contents is complete, the ORP of the flask should fall below an E_h of +200 mV. The final E_h of all anaerobic conditions was at least 150 mV below this level except for the flasks with Tween 80 addition and native CAAP organisms (Table 6 and Table 7). The final E_h of the Tween 80 with CAAP organisms was +134 mV, and this was the only biologically active condition not to show RDX concentrations to have been reduced below or near the detection limit.

Following the denitrification process in the flasks, identified by a sharp drop of the ORP below +200 mV, hydrolysis and fermentation of the complex organic compounds by anaerobic organisms should become dominant. Degradation products from these processes are then converted to methane and CO₂ by methanogens. The production of ¹⁴CO₂ under anaerobic conditions is an indication that the RDX was mineralized by the process of methanogenesis. Gas production due to methanogenesis usually occurs in an E_h range from -250 to -310 mV (Koch and Oldham 1985). The E_h in most biologically active conditions was in or below this range. It is believed that readings significantly outside this range may be the result of slow electrode response and lack of experience taking ORP readings.

7 Conclusions

Results from this study indicate that RDX can be mineralized biologically through either anaerobic processes or by a combination of anoxic and aerobic respiration. There was no conclusive evidence of a strictly aerobic pathway for the degradation of RDX. Mineralization of RDX does occur through a strictly anaerobic pathway but it is much slower, and there is the possibility of the formation of hydrazines as suggested by McCormick, Cornell, and Kaplan (1981). Emphasis should be placed on the anoxic-aerobic pathway due to a much higher rate of mineralization.

Under anoxic conditions the reduction of all NO_x groups in solution, including the nitro groups of the RDX molecule, should be indicated by a significant and rapid drop of the ORP below an E_h of approximately 200 mV. Hydrolytic cleavage of the triazine ring can occur during the denitrification step, but it is unclear from these results if hydrolytic cleavage of all triazine rings will occur with the completion of denitrification. An aerobic phase following hydrolytic cleavage of the triazine ring will result in the mineralization of RDX intermediates. In the anaerobic-aerobic experiment, mineralization of these intermediates was shown to proceed at a rapid rate.

A sufficient source of carbon which can be easily used by denitrifying organisms for cell synthesis is important to the reduction of the NO_x groups in RDX. Results from the first aerobic experiment show that insufficient levels of carbon available for cell synthesis limited the transformation of RDX. There was evidence in the anaerobic studies that the nonionic surfactant Tween 80 is not a good source of carbon for the denitrification step.

The final pH of most flasks in this study was above 9. Because the optimal pH range for denitrification is between 7 and 8, control of the pH should provide an increase in the rate of denitrification and transformation of RDX.

Results from experiments with bioaugmentation did not show an increased rate of RDX mineralization. Experiments conducted under anoxic conditions with denitrifying microorganisms isolated from Hastings AAP did show that the acclimation phase can be shortened by bioaugmentation. Most contaminated sites will probably contain microorganisms capable of reducing the nitro groups associated with RDX under the right conditions, but contaminated sites showing very small populations of viable microorganisms may benefit significantly from bioaugmentation.

8 Future Research

Further research should be devoted to the optimization of the anoxic-aerobic process. Particular attention should be focused on the ORP levels and reduction of NO_x groups during the anoxic phase. Reduction of these NO_x groups should be verified by monitoring the concentrations of nitrate, nitrite, and the nitroso analogs of RDX. Emphasis should also be placed on identification of possible recalcitrant organic degradation products from this optimized process. The benefits of using a buffer to hold the pH in the optimum range for denitrification (between 7 and 8) during the anoxic phase of the experiments should also be investigated.

Biological experiments are currently being conducted at ERDC using static-soil columns. Studies will include both continuous flow to determine the maximum rate for RDX transformation, and recirculation to determine maximum removal of RDX and its intermediates from contaminated waters.

References

- Ainsworth, C. C., Harvey, S. D., Szecsody, J. E., Simmons, M. A., Cullinan, V. I., Resch, C. T., and Mong, G. H. (1993). "Relationship between the leachability characteristics of unique energetic compounds and soil properties," Project Order No. 91PP1800, U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.
- Banerjee, S., Yalkowsky, S. H., and Valvani, S. (1980). "Water solubility and octanol/water partition coefficients of organics. Limitations of the solubility-partition coefficient correlation," *Environmental Science and Technology* 14(10), 1227-1229.
- Binks, P. R., Nicklin, S., and Bruce, N. C. (1995). "Degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Stenotrophomonas maltophilia* PB1," *Applied and Environmental Microbiology* 61(4), 1318-1322.
- Bioscience, Inc. (1991). *BI-1000 Electrolytic respirometer owner's manual*. Bioscience, Inc., Bethlehem, PA.
- Bligh, E. G., and Dyer, W. J. (1959). "A rapid method of total lipid extraction and purification," *Canadian Journal of Biochemistry and Physiology* 37(8), 911-917.
- Brannon, J. M., Adrian, D. D., Pennington, J. C., and Myers, T. E. (1992). "Slow release of PCB, TNT, and RDX from soils and sediments," Technical Report EL-92-38, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.
- Brannon, J. M., Gunnison, D., Butler, P. L., and Smith, I., Jr. (1978). "Mechanisms that regulate the intensity of oxidation-reduction in anaerobic sediments and natural water systems," Technical Report Y-78-11, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.
- Burton, D. T., and Turley, S. D. (1995). "Reduction of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) toxicity to the Cladoceran *Ceriodaphnia dubia* following photolysis in sunlight," *Bulletin of Environmental Contamination and Toxicology* 55, 89-95.

- Cataldo, D. A., Harvey, S. D., and Fellows, R. J. (1990). "An evaluation of the environmental fate and behavior of munitions material (TNT, RDX) in soil and plant systems - environmental fate and behavior of RDX," Project Order No. 88PP8853, U.S. Army Biomedical Research and Development Laboratory, Fort Detrick, Frederick, MD.
- Cerar, R. (1993). Interview by Labat-Anderson Inc. personnel on March 16. See SERDP (1993).
- DoD. (1995). "Defense environmental restoration program - annual report to Congress for fiscal year 1994," U.S. Department of Defense, Washington, DC.
- Funk, S. B., Roberts, D. J., Crawford, D. L., and Crawford, R. L. (1993). "Initial-phase optimization for bioremediation of munition compound-contaminated soils," *Applied and Environmental Microbiology* 59(7), 2171-2177.
- Glover, D. J., and Hoffsomer, J. C. (1973). *Bulletin of Environmental Contamination and Toxicology* 10, 302.
- Greene, B., Kaplan, D. L., and Kaplan, A. M. (1985). "Degradation of pink water compounds in soil - TNT, RDX, HMX," Technical Report NATICK/TR-85/046, U.S. Army Natick Research and Development Center, Natick, MA.
- Griest, W. H., Stewart, A. J., Tyndall, R. L., Ho, C.-h., and Tan, E. (1990). "Characterization of explosives processing waste decomposition due to composting: final, phase I report," ORNL/TM-11573, Oak Ridge National Laboratory, Oak Ridge, TN.
- Hitner, J. M., Merk, S. A., Quintana, R. L., and Carpenter, P. (1996). "Investigation of composting as a means of treating soils contaminated with low levels of nitramine explosives," NAWCWPNS TP 8255, Naval Air Warfare Center Weapons Division, China Lake, CA.
- Hoffsommer, J. C., and Rosen, J. M. (1973). "Hydrolysis of explosives in sea water," *Bulletin of Environmental Contamination and Toxicology* 10(2), 78-79.
- Jenkins, C. J., and Mavinic, D. S. (1989). "Anoxic-aerobic digestion of waste activated sludge: part II - supernatant characteristics, ORP monitoring results and overall rating system," *Environmental Technology Letters* 10, 371-384.
- Kaplan, D. L. (1992). "Biological degradation of explosives and chemical agents," *Current Opinion in Biotechnology* 3, 253-260.

- Keehan, K. R., and Sisk, W. E. (1996). "The development of composting for the remediation of explosives-contaminated soils," *Biotechnology in industrial waste treatment and bioremediation*. R. F. Hickey and G. Smith, eds. CRC Press, Boca Raton.
- Kitts, C. L., Cunningham, D. P., and Unkefer, P. J. (1994). "Isolation of three hexahydro-1,3,5-trinitro-1,3,5-triazine degrading species of the family *Enterobacteriaceae* from nitramine explosive-contaminated soil," *Applied and Environmental Microbiology* 60(12), 4608-4711.
- Koch, F. A., and Oldham, W. K. (1985). "Oxidation-reduction potential - a tool for monitoring, control and optimization of biological nutrient removal systems," *Water Science and Technology* 17(Paris), 259-281.
- Kohler, J., and Meyer, R., eds. (1993). *Explosives*, 4th ed., VCH Publishers, New York.
- Kubose, D. A., and Hoffsommer, J. C. (1977). "Photolysis of RDX in aqueous solution. initial studies," NSWC/WOL/TR 77-20, Naval Surface Weapons Center.
- Leggett, D. C. (1985). "Sorption of military explosive contaminants on bentonite drilling muds," Report 85-18, U.S. Army Corps of Engineers Cold Regions Research and Engineering Laboratory, Hanover, NH.
- Lindner, V. (1980). "Explosives and propellants," *Kirk-Othmer Encyclopedia of Chemical Technology*. M. Grayson and D. Eckroth, eds. 3rd ed., vol. 9, Wiley, New York.
- McCormick, N. G., Cornell, J. H., and Kaplan, A. M. (1981). "Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine," *Applied and Environmental Microbiology* 42(5), 817-823.
- _____. (1984). "The anaerobic biotransformation of RDX, HMX, and their acetylated derivatives," Technical Report NATICK/TR-85/007, U.S. Army Natick Research and Development Center, Natick, MA.
- McGrath, C. J. (1995). "Review of formulations for processes affecting the subsurface transport of explosives," Technical Report IRRP-95-2, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.
- McLellan, W. L., Hartley, W. R., and Brower, M. E. (1992). "Hexahydro-1,3,5-trinitro-1,3,5-triazine," *Drinking water health advisory: munitions (USEPA Office of Drinking Water Health Advisories)*, W. C. Roberts and W. R. Hartley, ed. Lewis Publishers, Boca Raton.
- Ogden, K. L., Byrnes, C. M., Hanners, J. L., and Unkefer, P. J. (1994). "Combination of aerobic and anoxic conditions to increase the rate of RDX biodegradation." University of Arizona, Tucson, AZ. (unpublished).

- Peddie, C. C., Mavinic, D. S., and Jenkins, C. J. (1990). "Use of ORP for monitoring and control of aerobic sludge digestion," *Journal of Environmental Engineering* 116(3), 461-471.
- Pennington, J. C., Myers, T. E., Davis, W. M., Olin, T. J., McDonald, T. A., Hayes, C. A., and Townsend, D. M. (1995). "Impacts of sorption on in-situ bioremediation of explosives-contaminated soils," Technical Report IRRP-95-1, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.
- Rosenblatt, D. H. (1986). "Contaminated soil cleanup objectives for Cornhusker Army Ammunition Plant," Technical Report 8603, U.S. Army Medical Bioengineering Research Development Laboratory, Fort Detrick, Frederick, MD.
- Rosenblatt, D. H., Burrows, E. P., Mitchell, W. R., and Parmer, D. L. (1991). "Organic explosives and related compounds," *The handbook of environmental chemistry*, O. Hutzinger, ed., vol. 9, part G, Springer-Verlag, Berlin.
- Selim, H. M., and Iskandar, I. K. (1994). "Sorption-desorption and transport of TNT and RDX in soils," Report 94-7, U.S. Army Corps of Engineers Cold Regions Research and Engineering Laboratory, Hanover, NH.
- SERDP. (1993). *An approach to estimation of volumes of contaminated soil and groundwater for selected army installations*. Labat-Anderson Inc., (May). Prepared for the Executive Director, Strategic Environmental Research and Development Program.
- Sikka, H. C., Banerjee, S., Pack, E. J., and Appleton, H. T. (1980). "Environmental fate of RDX and TNT," Technical Report 81-538, U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.
- Spalding, R. F., and Fulton, J. W. (1988). "Groundwater munition residues and nitrate near Grand Island, Nebraska, USA," *Journal of Contaminant Hydrology* 2, 139-153.
- Spangford, R. J., Mill, T., Chou, T. -W., Mabey, W. R., Smith, J. H., and Lee, S. (1980a). "Environmental fate studies on certain munition wastewater constituents, final report, phase I - literature review," SRI Project No. LSU-7934, SRI International, Menlo Park, CA.
- _____. (1980b). "Environmental fate studies on certain munition wastewater constituents, final report, phase II - laboratory studies," SRI Project No. LSU-7934, SRI International, Menlo Park, CA.
- Syracuse Research Corporation. (1978). "Environmental fate of RDX and TNT." Progress reports from June 1977 to May 1978 for U.S. Army Medical Research and Development Command Contract DAMD-17-77-C-7026. Referenced in *see* Spangford et al. 1980a.

- Tchobanoglous, G., and Burton, F. L. (1991). *Wastewater engineering: treatment, disposal, and reuse/Metcalf & Eddy, Inc.*, 3d ed. McGraw-Hill, Inc., New York.
- Walker, J. E., and Kaplan, D. L. (1992). "Biological degradation of explosives and chemical agents," *Biodegradation* 3, 369-385.
- Ward, D. M., Panke, S., Kloppel, K., Christ, R., and Fredrickson, H. (1994). "Complex polar lipids of a hot spring *Cyanobacterial* mat and its cultivated inhabitants," *Applied and Environmental Microbiology* (SEP).
- Wareham, D. G., Hall, K. J., and Mavinic, D. S. (1991). "Preliminary investigation into using oxidation-reduction potential (ORP) for real-time control of aerobic-anaerobic sludge digesters," *Proceedings of 1991 Special Conference in Reno, Nevada*, by ASCE, New York, 271-276.
- Williams, R. T., and Myler, C. A. (1990). "Bioremediation using composting," *Biocycle* (NOV).
- Zappi, M., Gunnison, D., Pennington, J., Teeter, C., Coyle, C., and Rope, C. (1993). "Evaluation of bioslurry systems for treating explosives contaminated soils from the Hastings East Industrial Park." U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS. (unpublished).

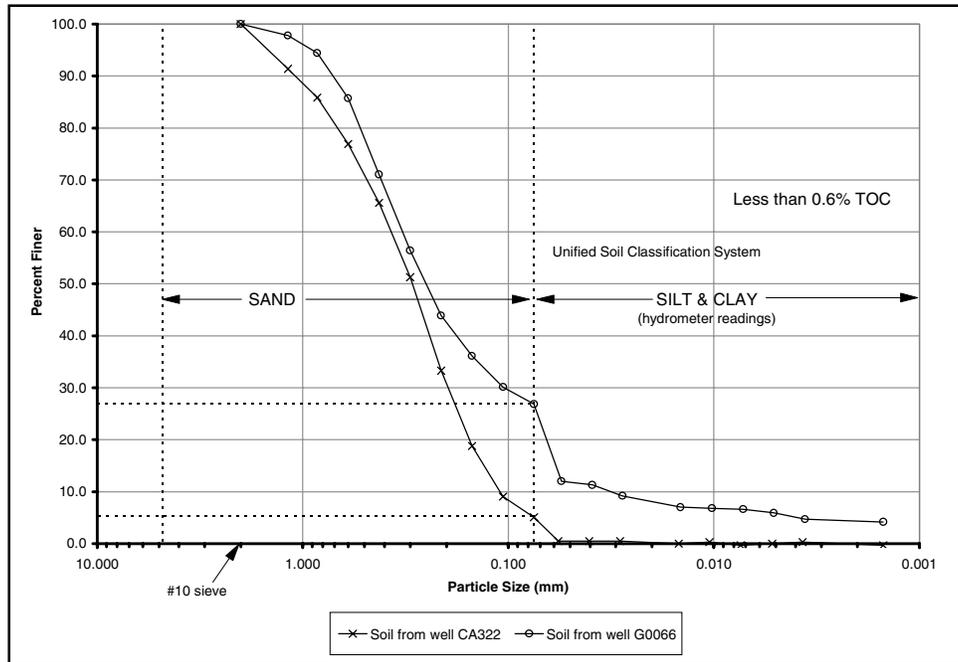


Figure 1. Soil particle size distribution

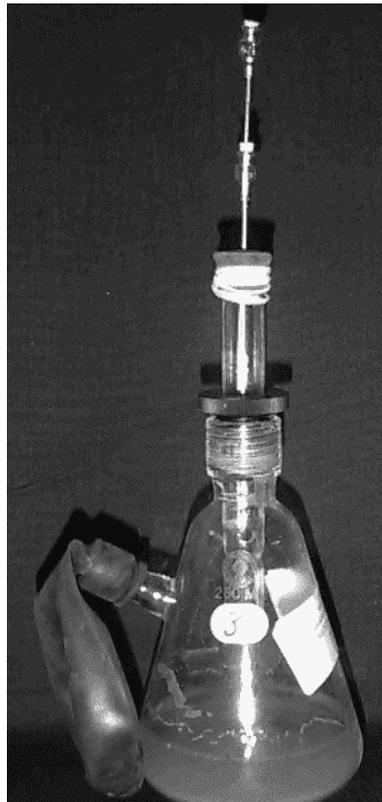


Figure 2. Anaerobic shake-flask assembly

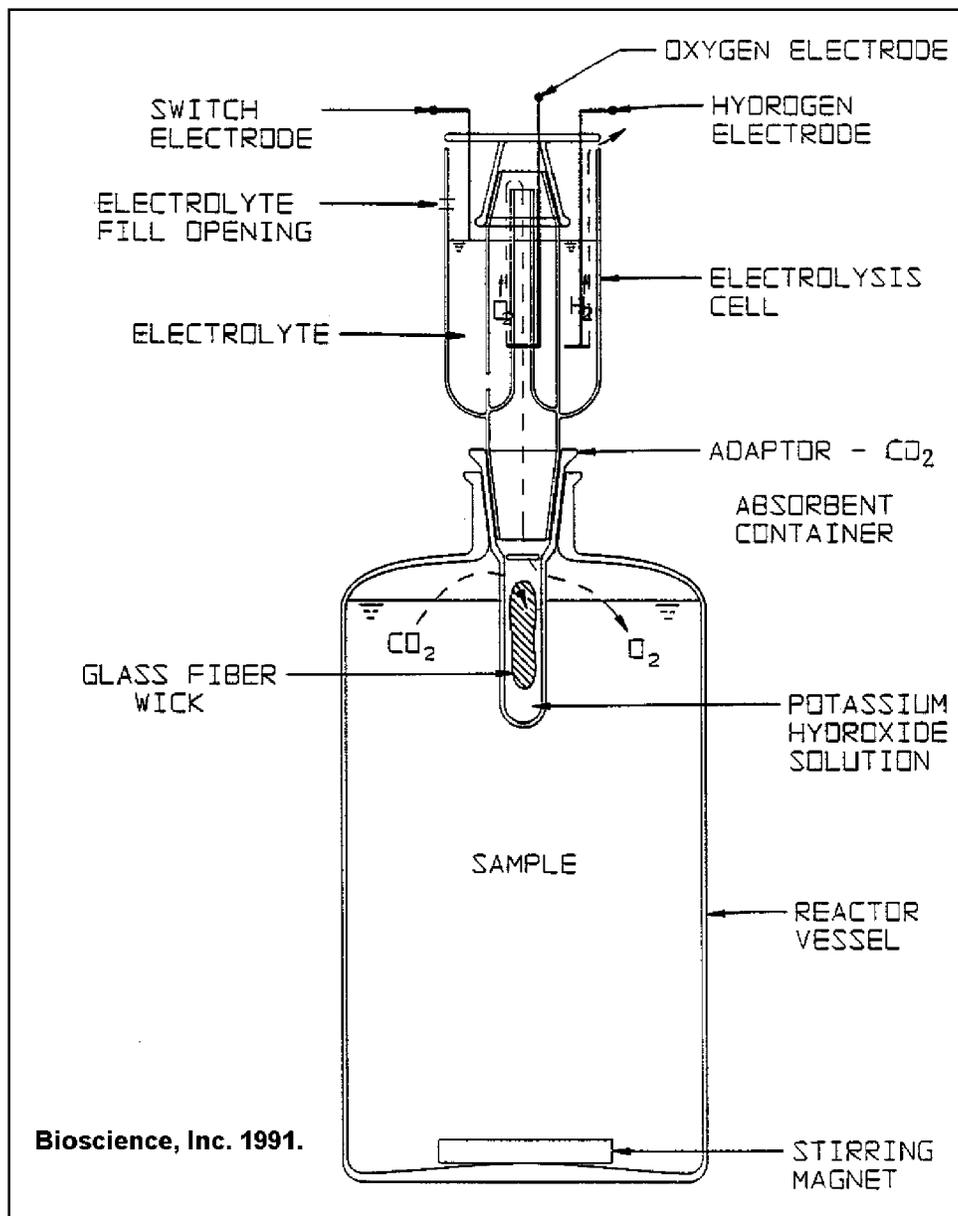


Figure 3. Reactor vessel with electrolysis cell

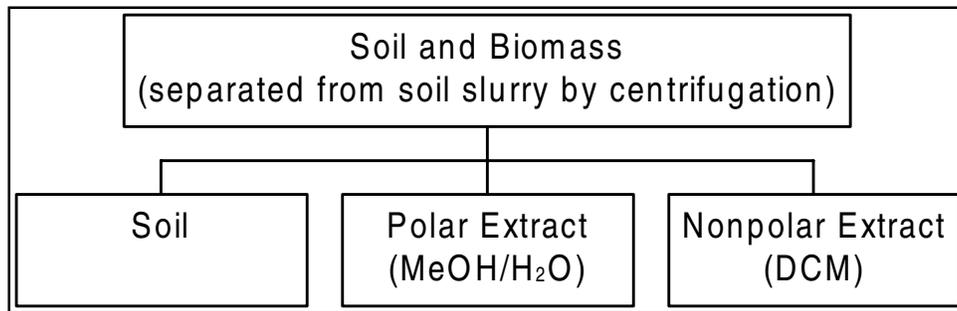


Figure 4. Bligh-Dyer (1959) method of separation

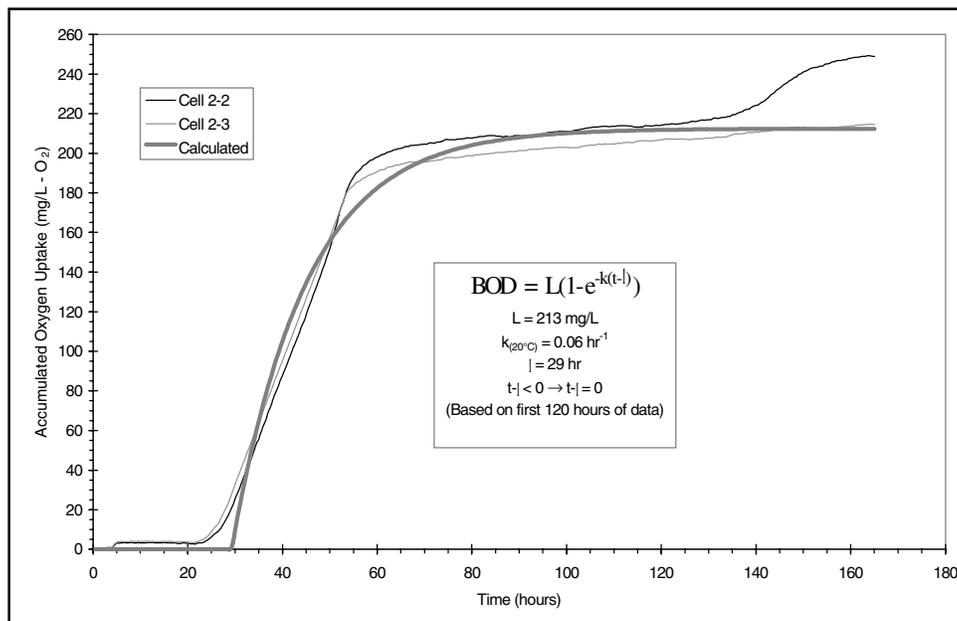


Figure 5. Acetate (500 mg/L) - BOD vs time

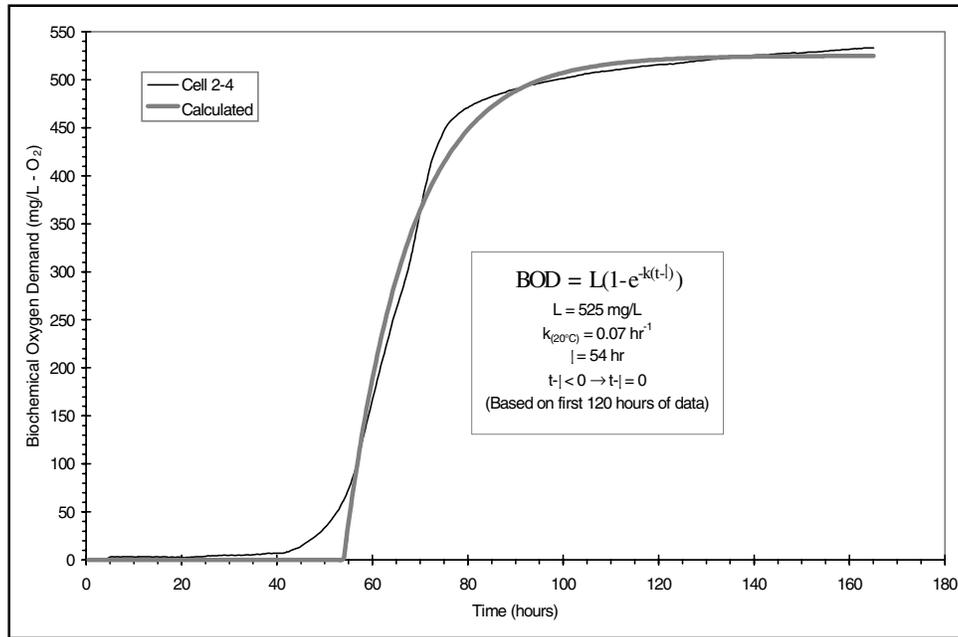


Figure 6. Ethyl alcohol (500 mg/L) - BOD vs time

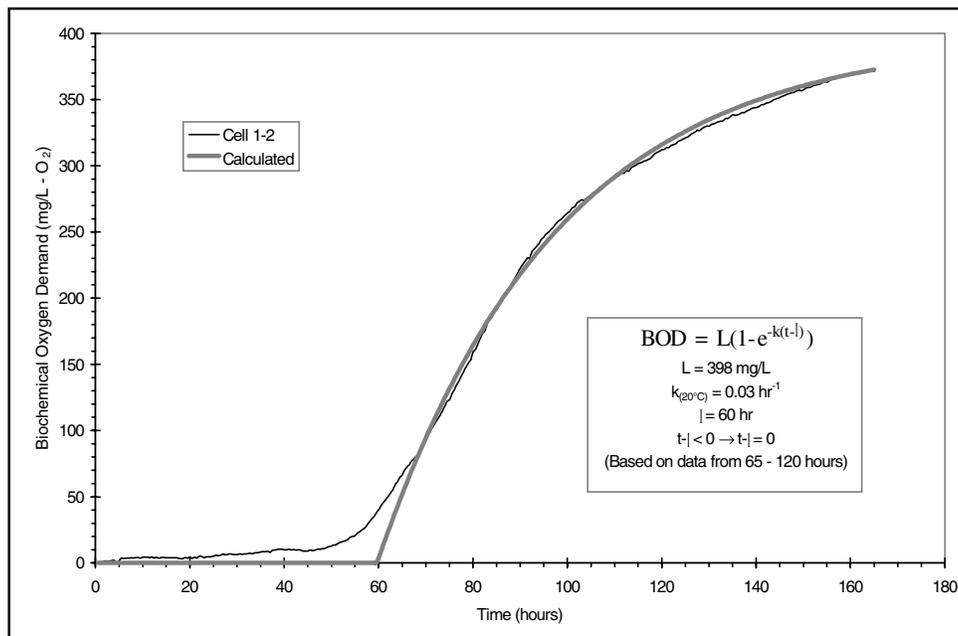


Figure 7. Potato starch (500 mg/L) - BOD vs time

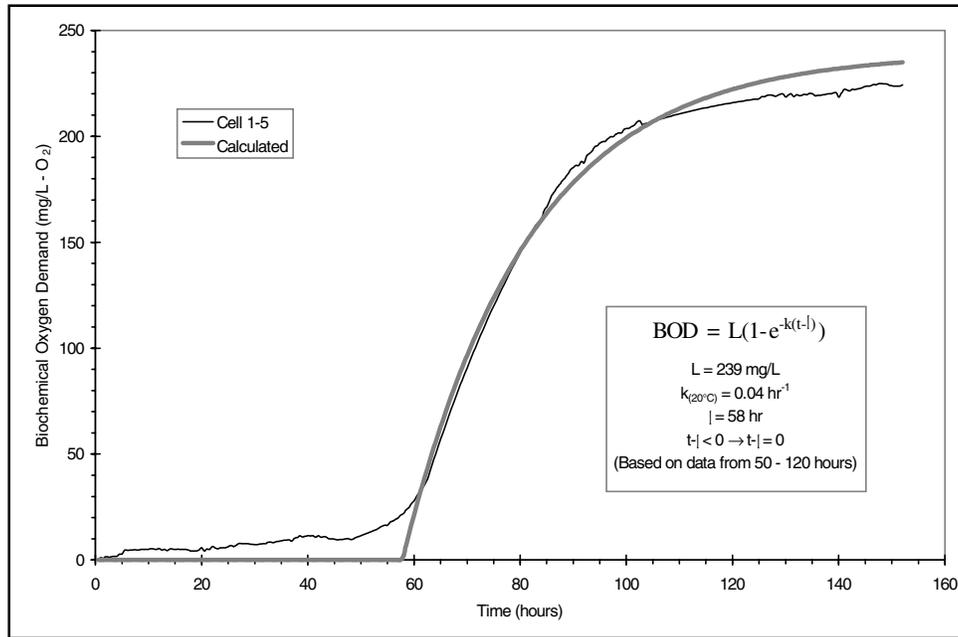


Figure 8. Corn starch (500 mg/L) - BOD vs time

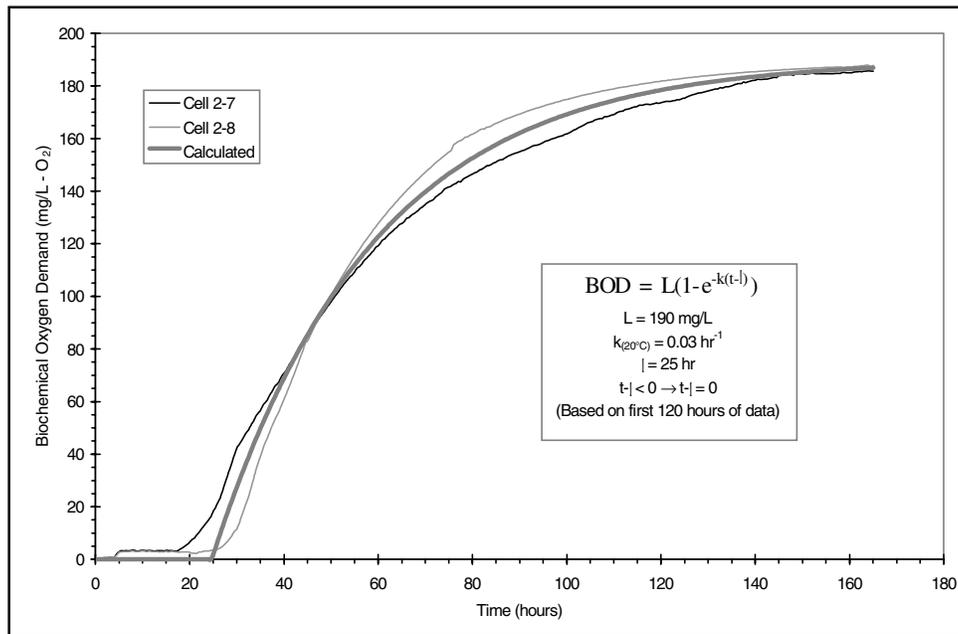


Figure 9. Tween 80 (500 mg/L) - BOD vs time

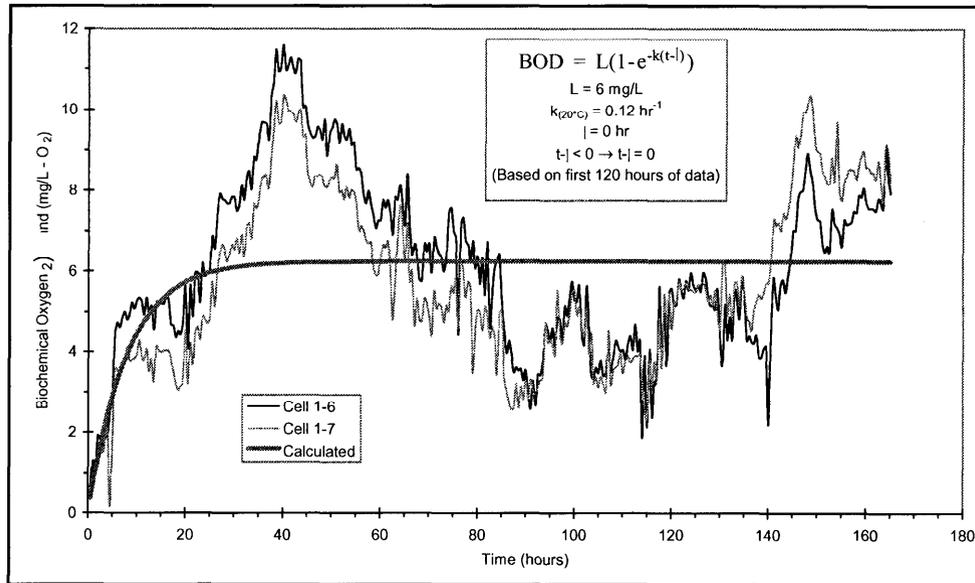


Figure 10. Seeded blank - BOD vs time

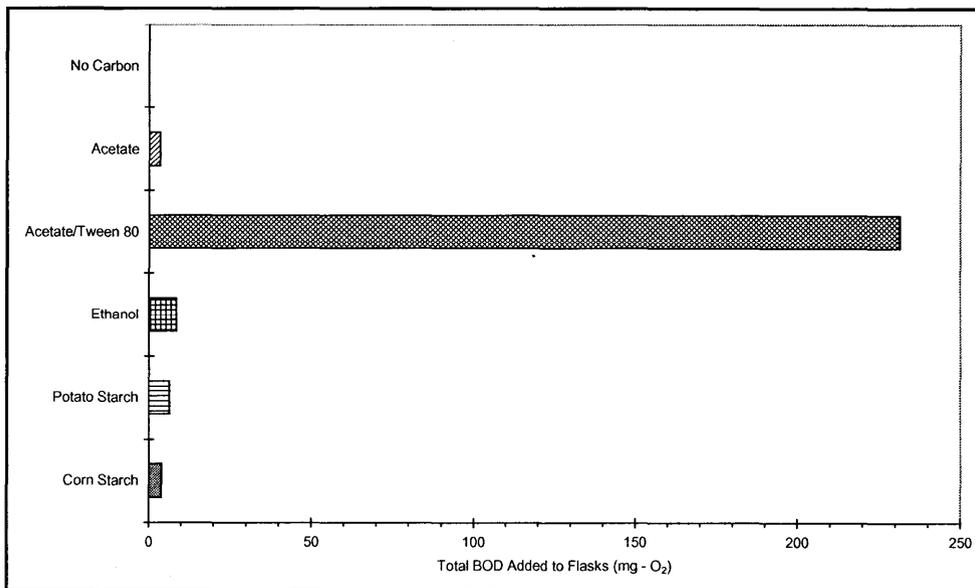


Figure 11. Estimated BOD added by condition (experiment 1)

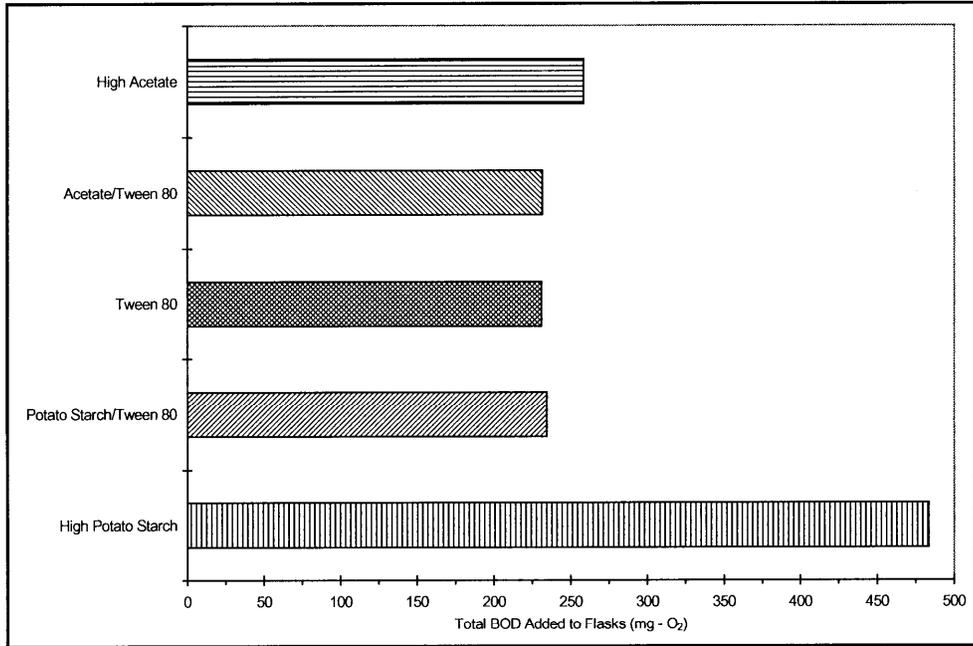


Figure 12. Estimated BOD added by condition (experiment 2)

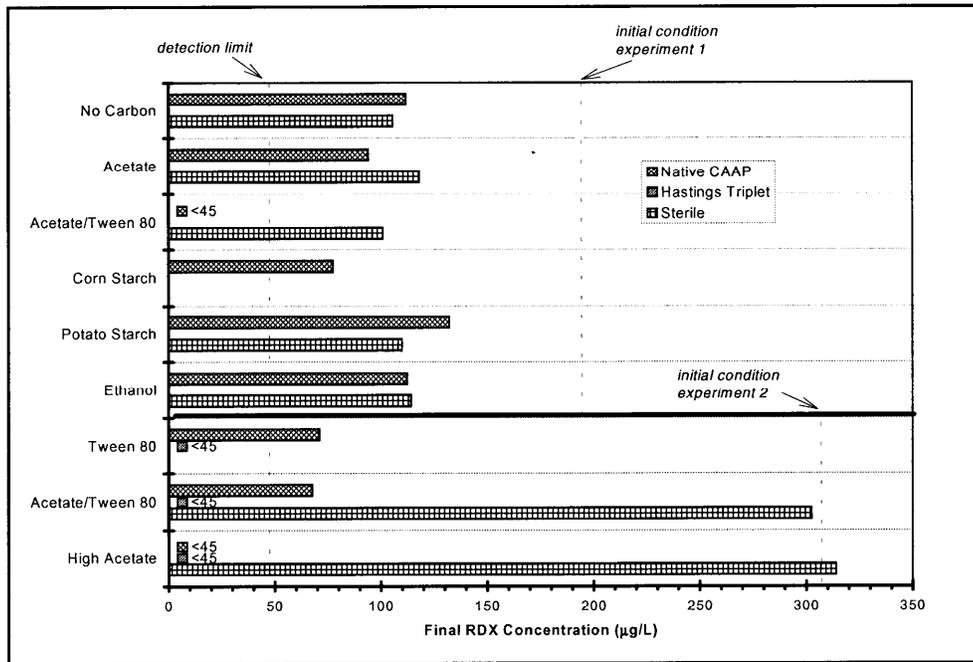


Figure 13. Final RDX concentrations, aerobic experiments

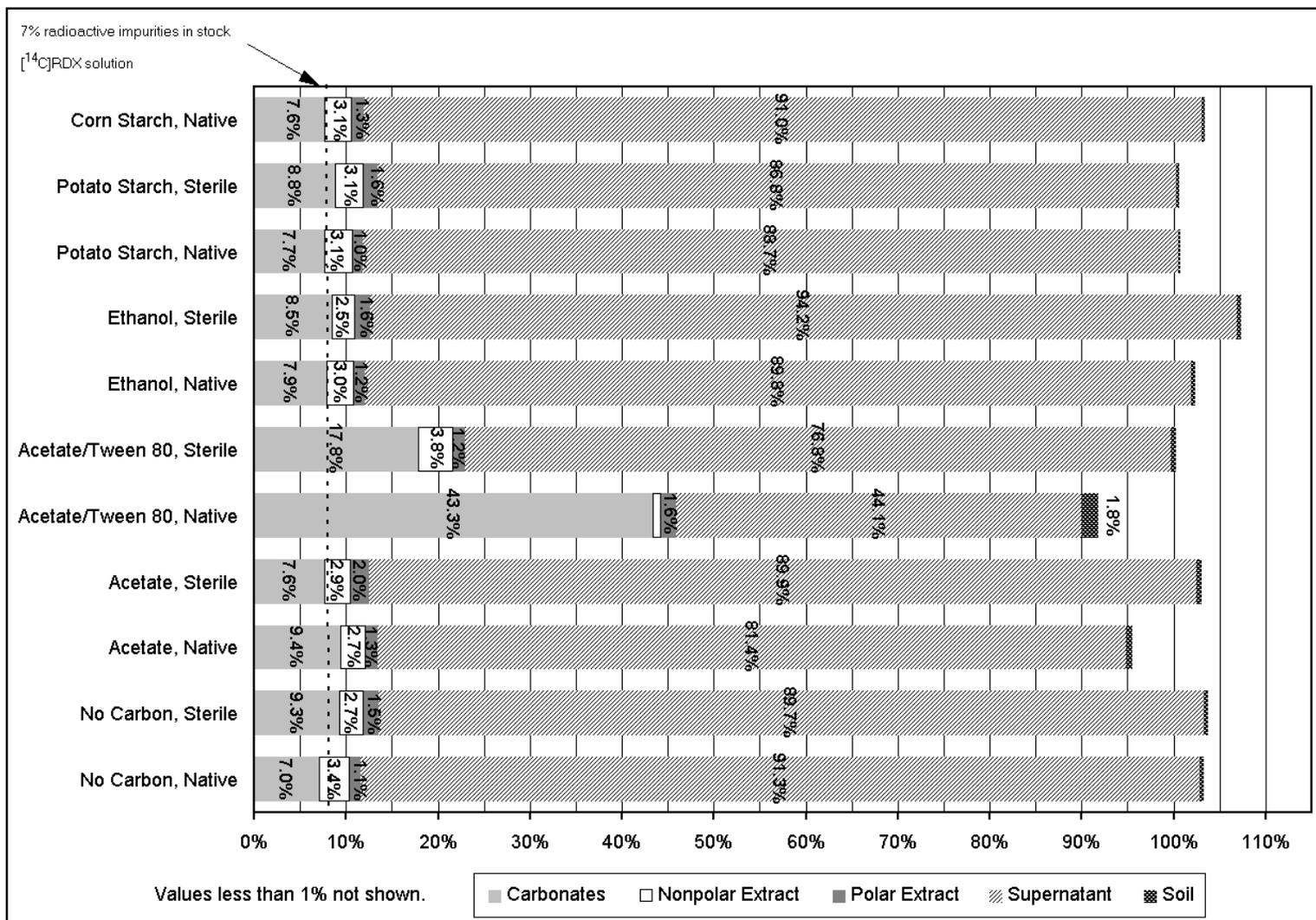


Figure 14. ^{14}C mass balance in aerobic experiment 1

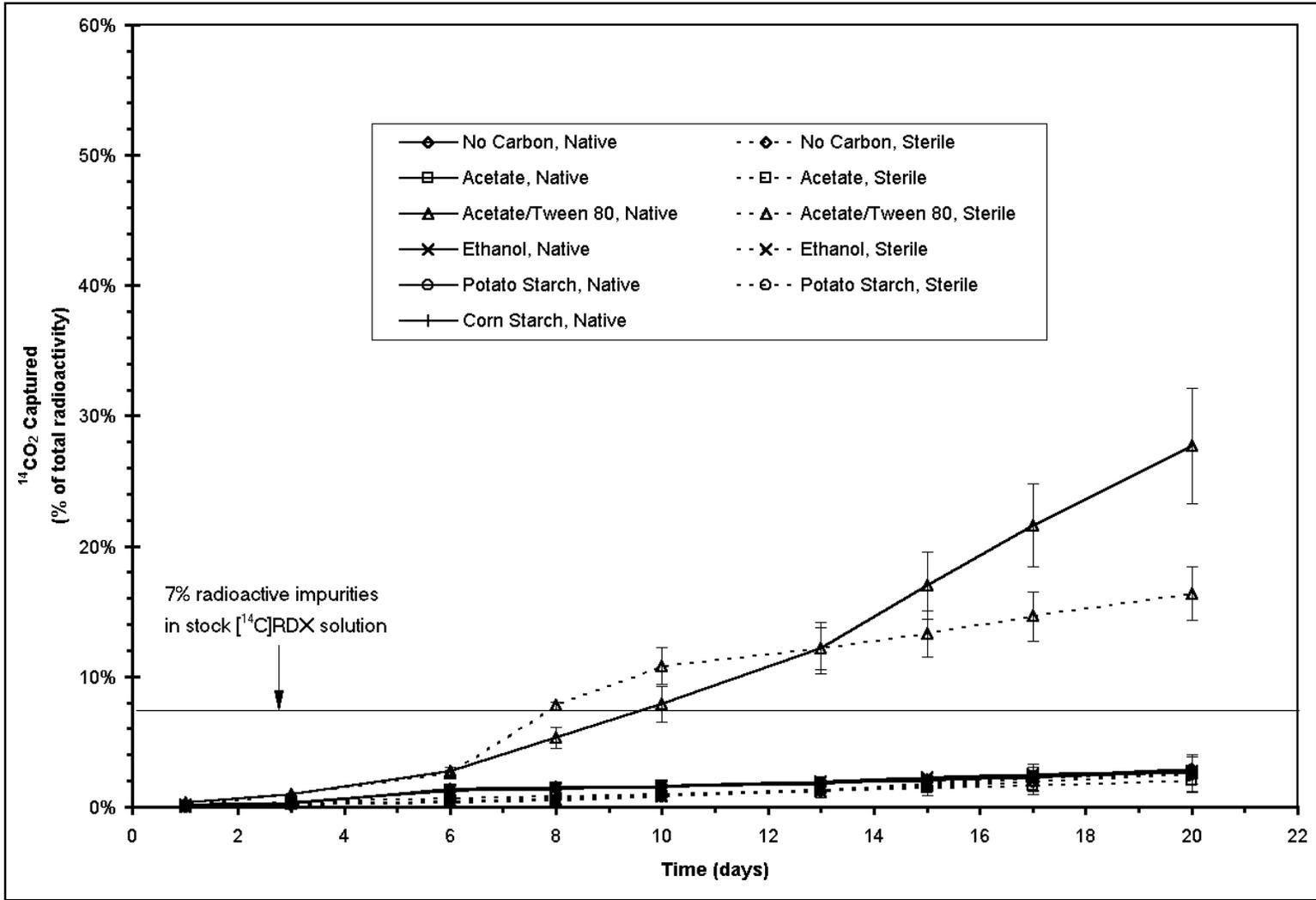


Figure 15. $^{14}\text{CO}_2$ vs time in aerobic experiment 1

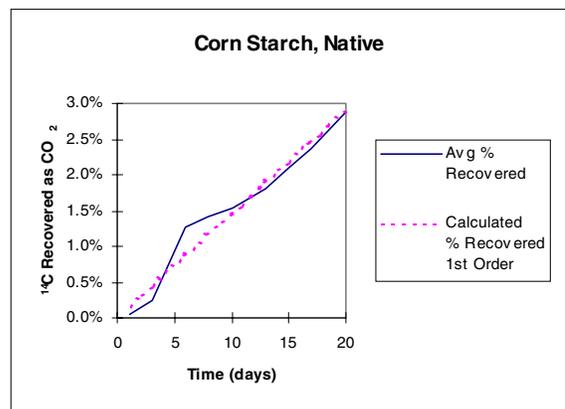
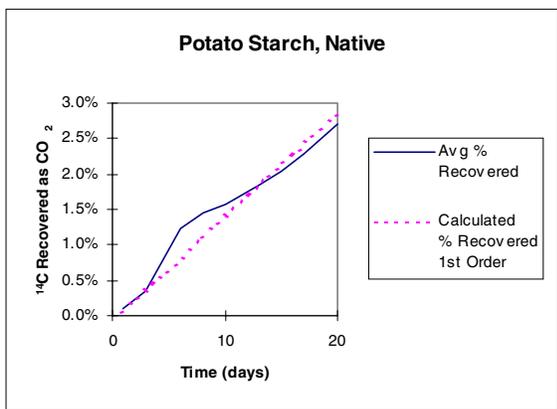
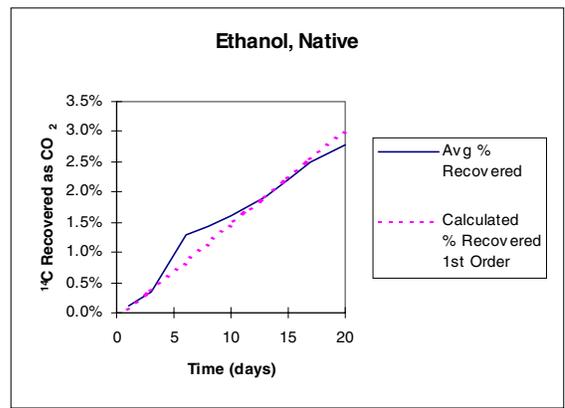
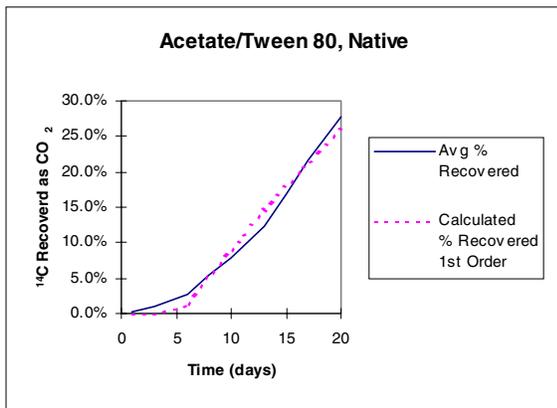
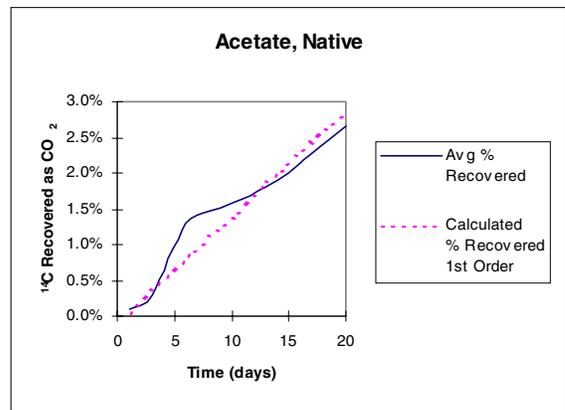
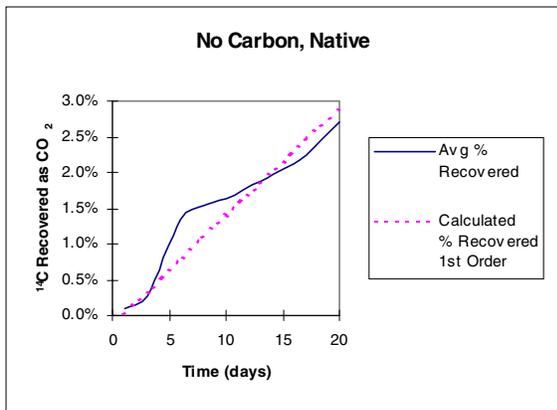


Figure 16. First-order reaction fit to $^{14}\text{CO}_2$ recovery data, aerobic experiment 1

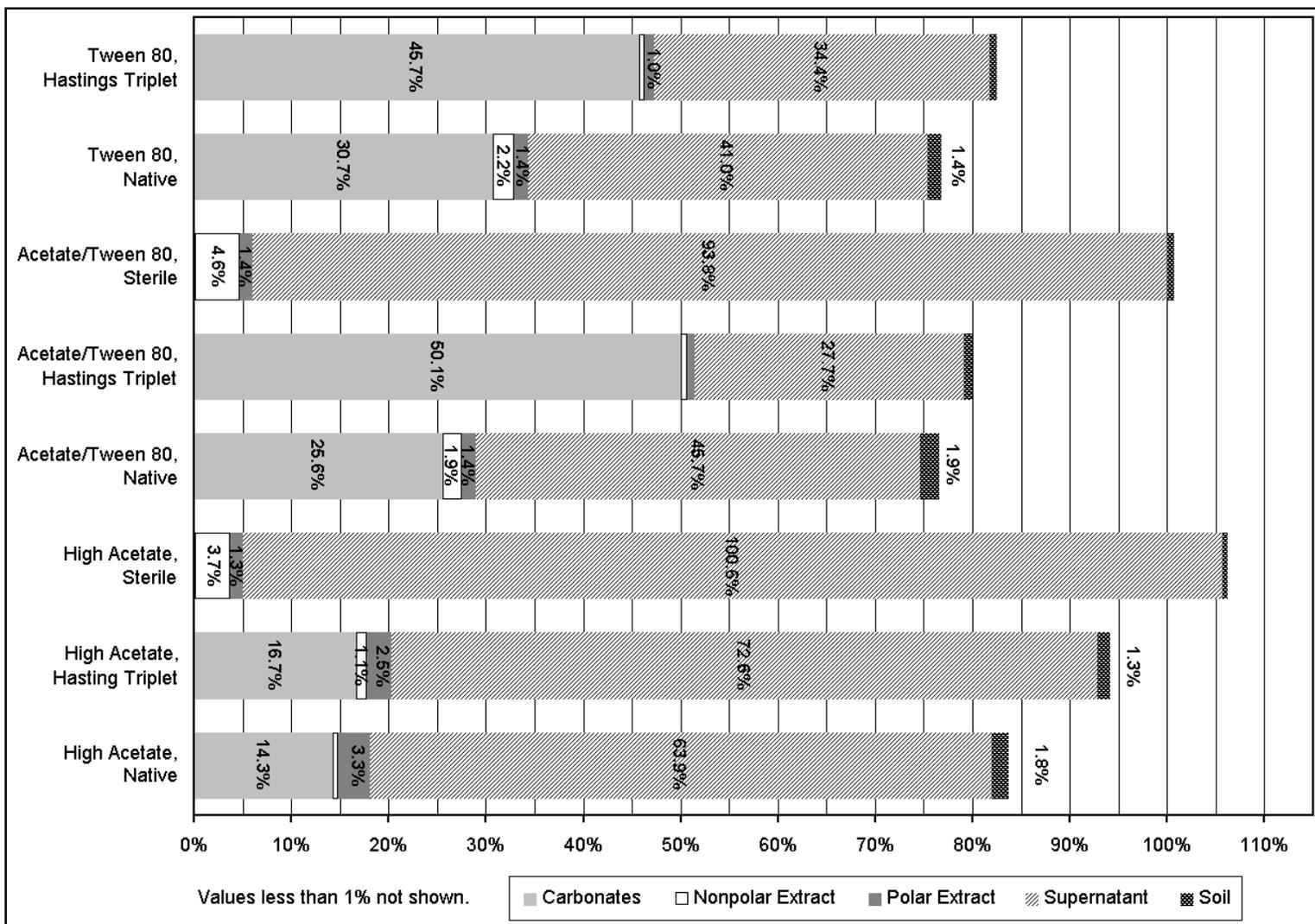


Figure 17. ¹⁴C mass balance in aerobic experiment 2

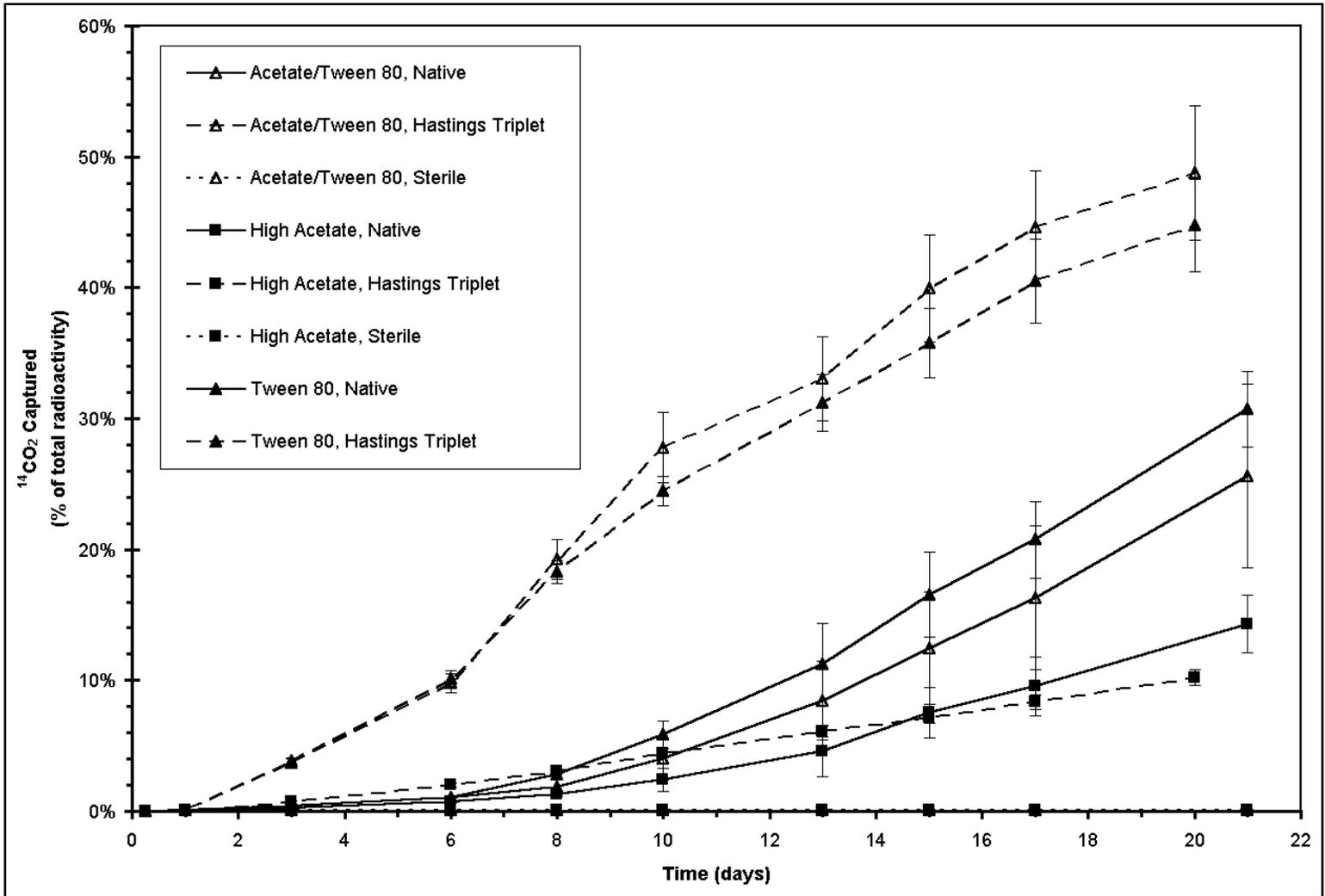


Figure 18. $^{14}\text{CO}_2$ vs time in aerobic experiment 2

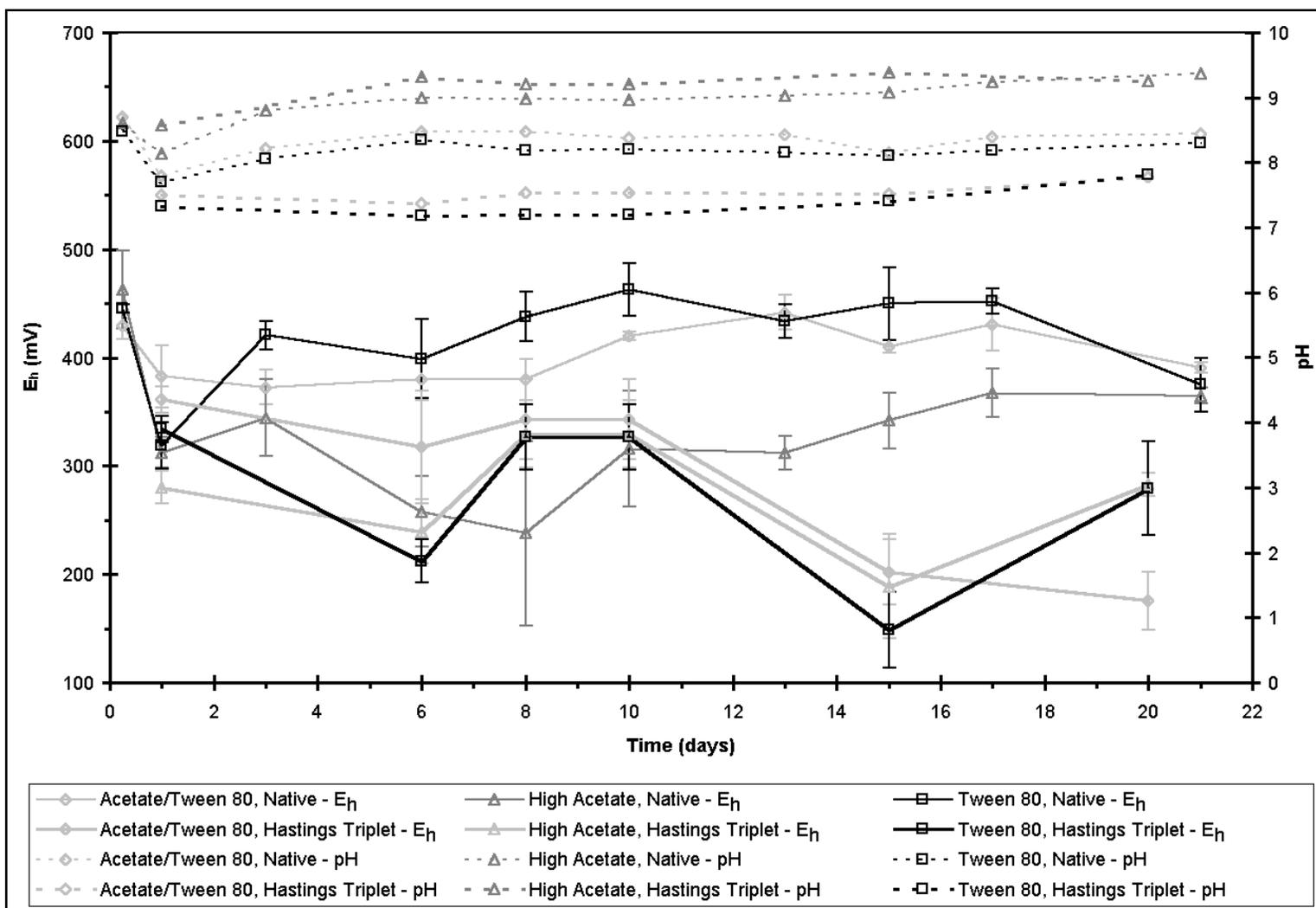


Figure 19. ORP and pH vs time for flasks with native organisms in aerobic experiment 2

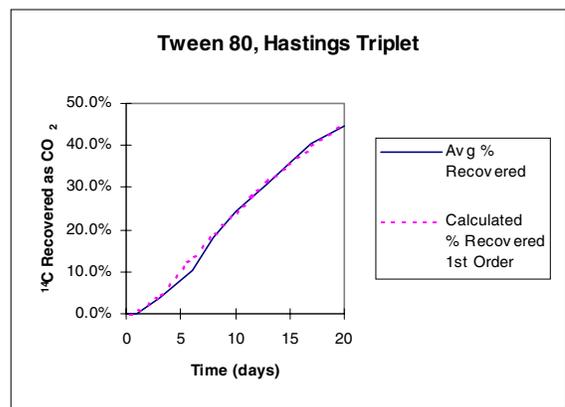
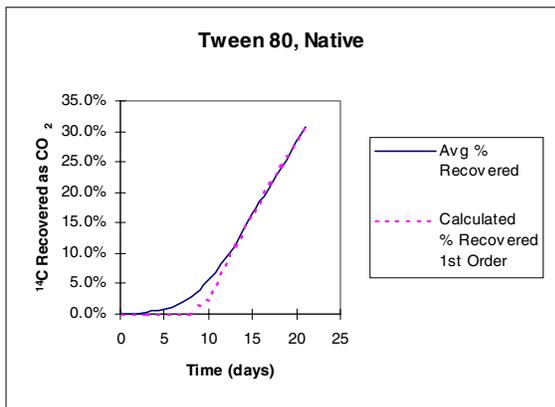
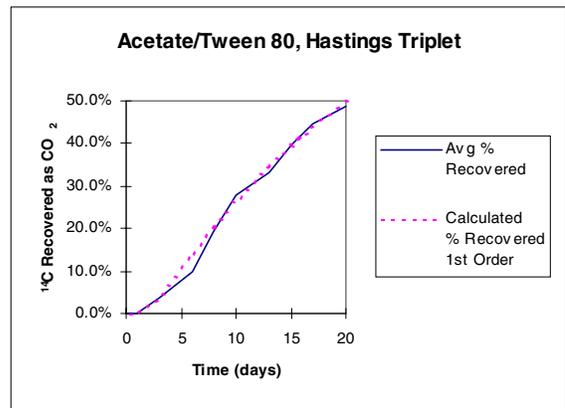
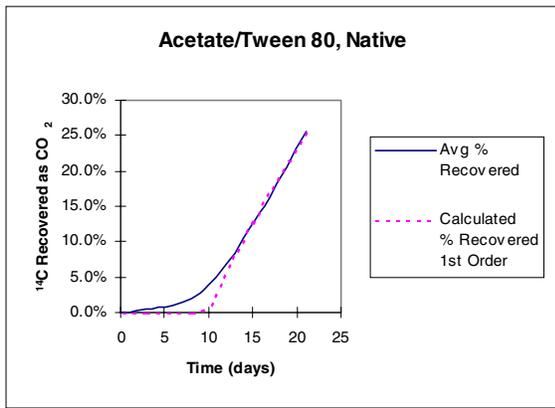
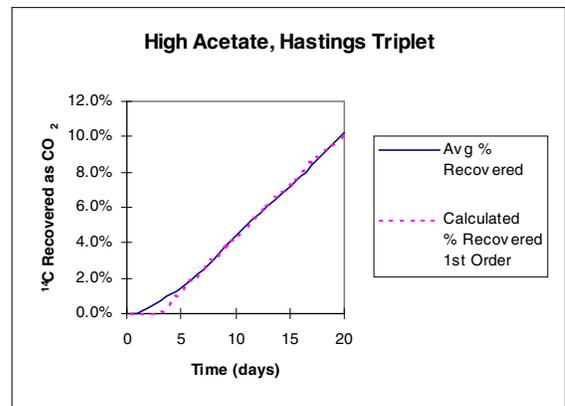
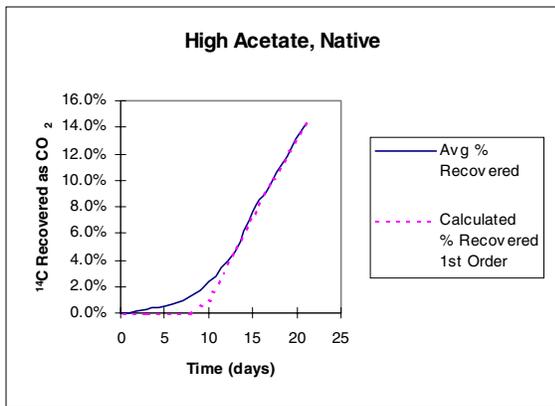


Figure 20. First-order reaction fit to $^{14}\text{CO}_2$ recovery data, aerobic experiment 2

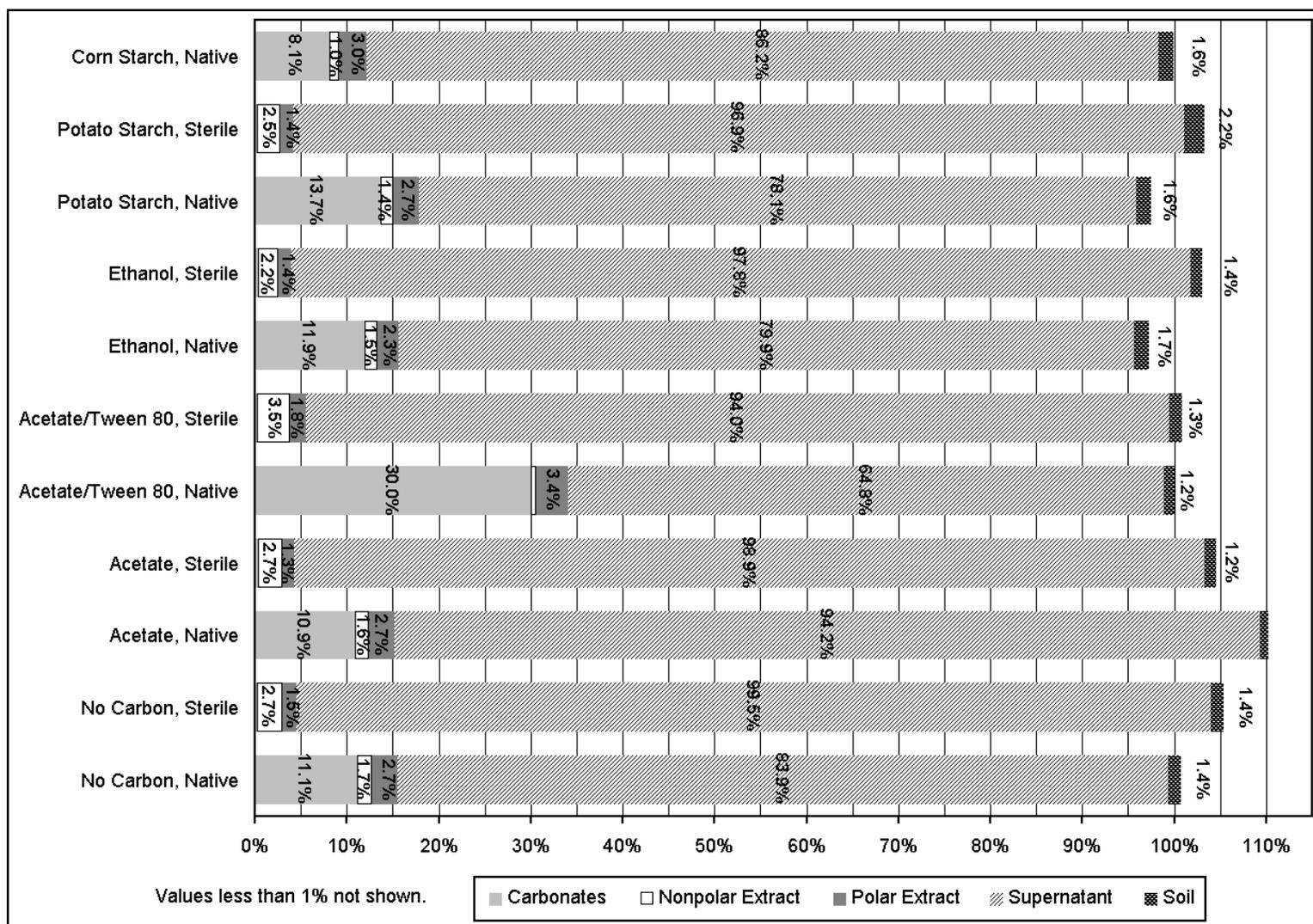


Figure 22. ¹⁴C mass balance in anaerobic experiment 1

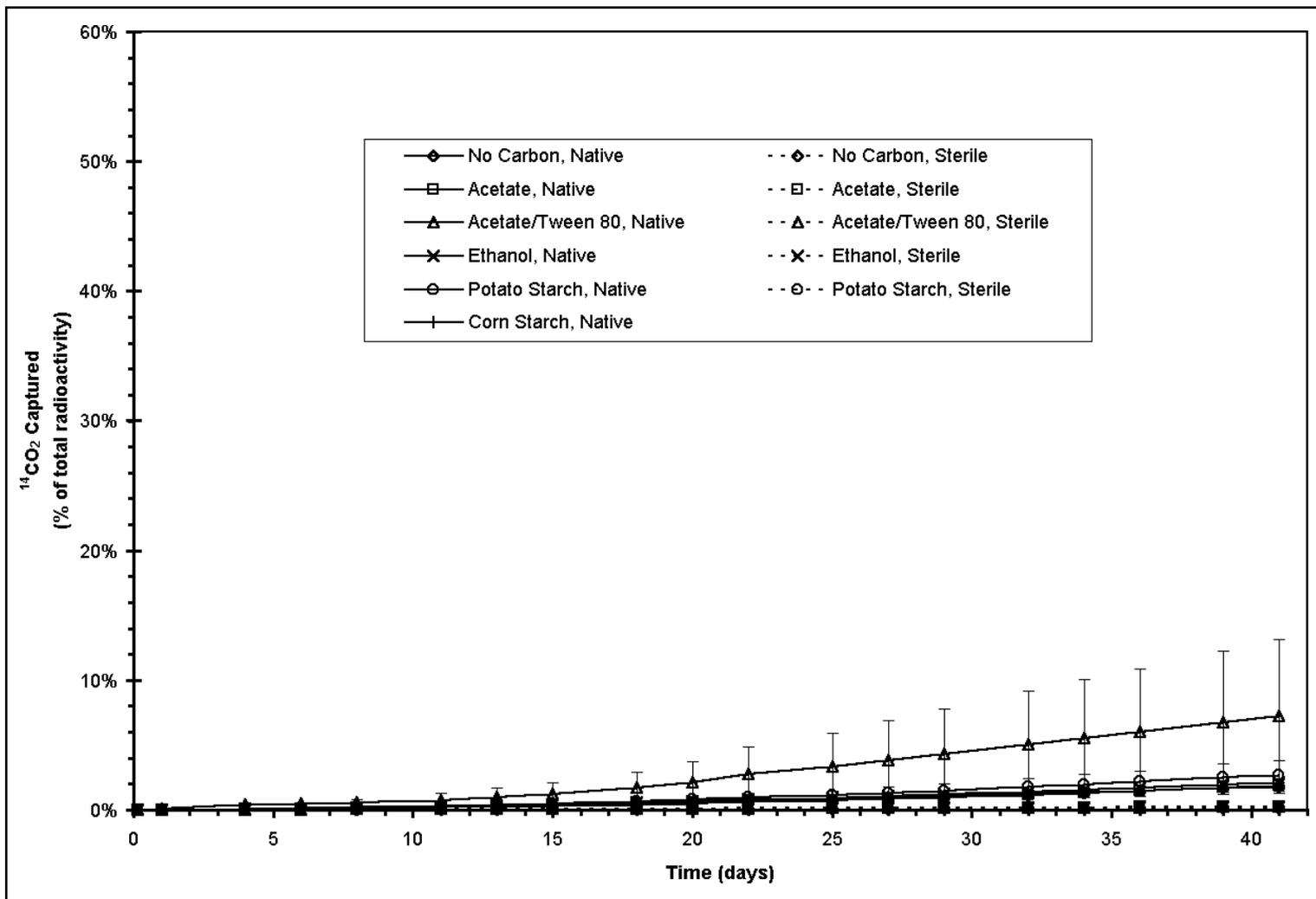


Figure 23. $^{14}\text{CO}_2$ vs time in anaerobic experiment 1

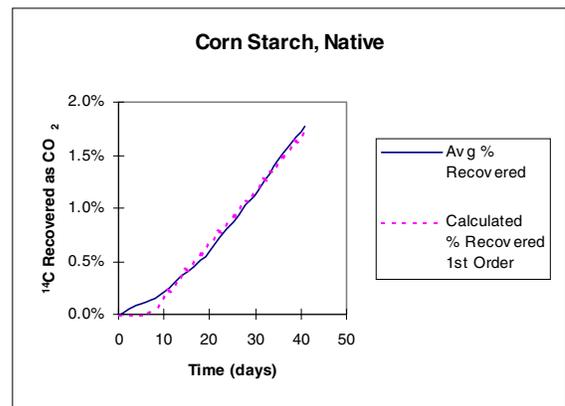
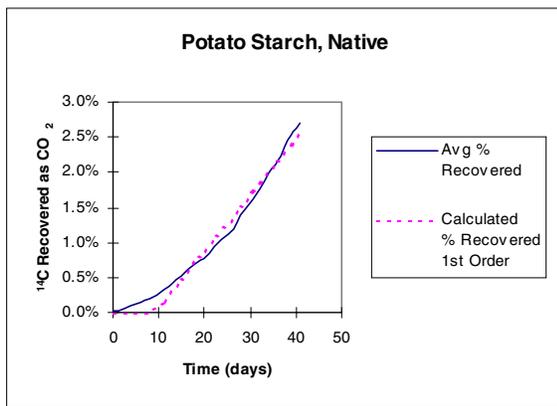
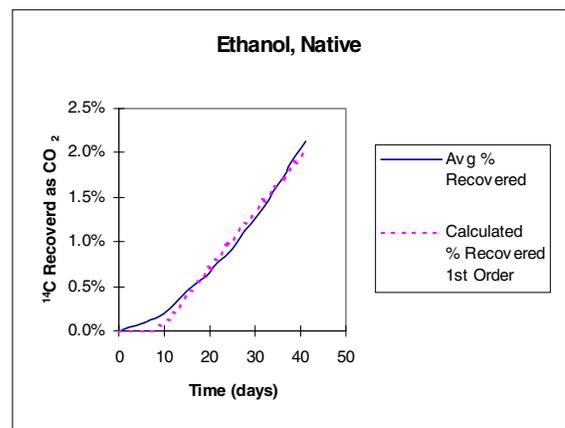
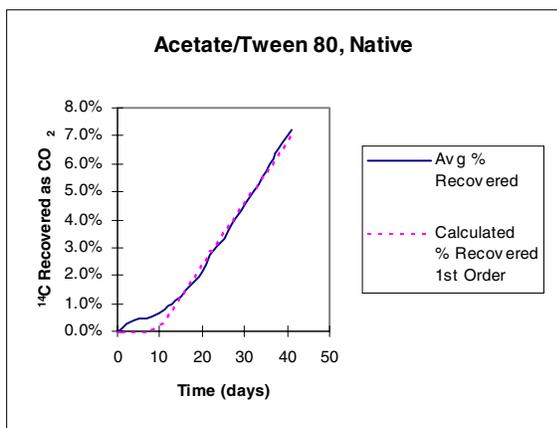
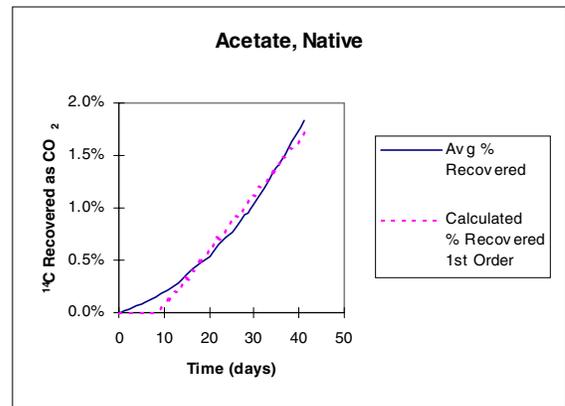
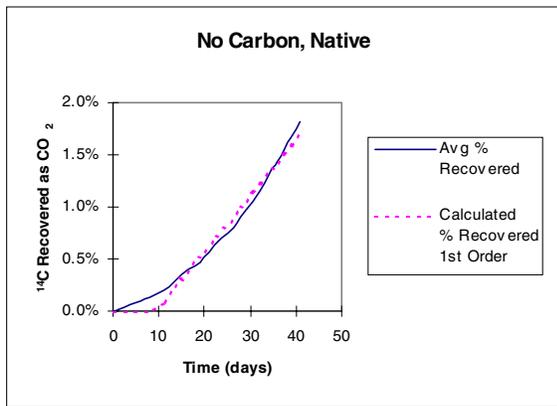


Figure 24. First-order reaction fit to $^{14}\text{CO}_2$ data, anaerobic experiment 1

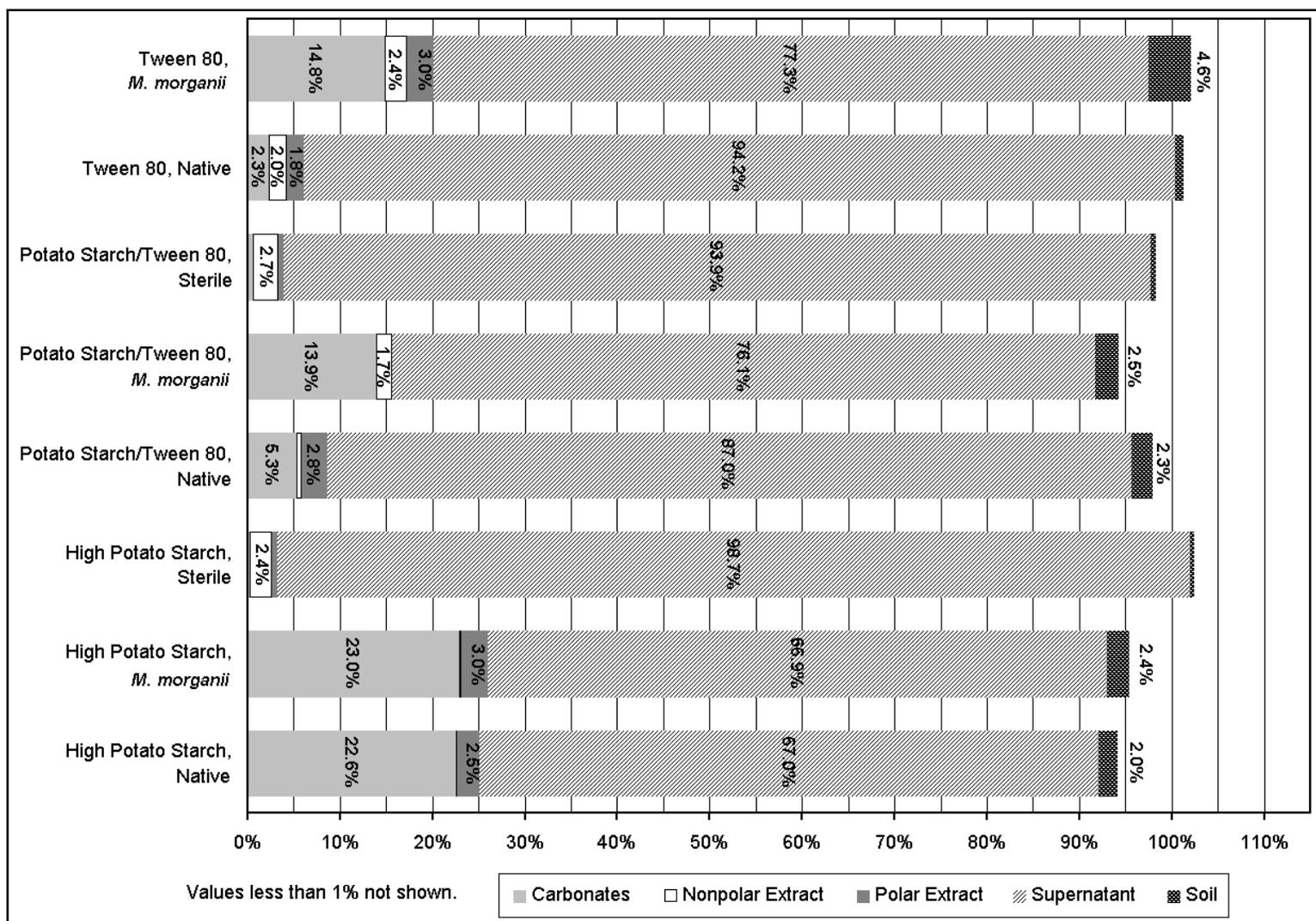


Figure 25. ¹⁴C mass balance in anaerobic experiment 2

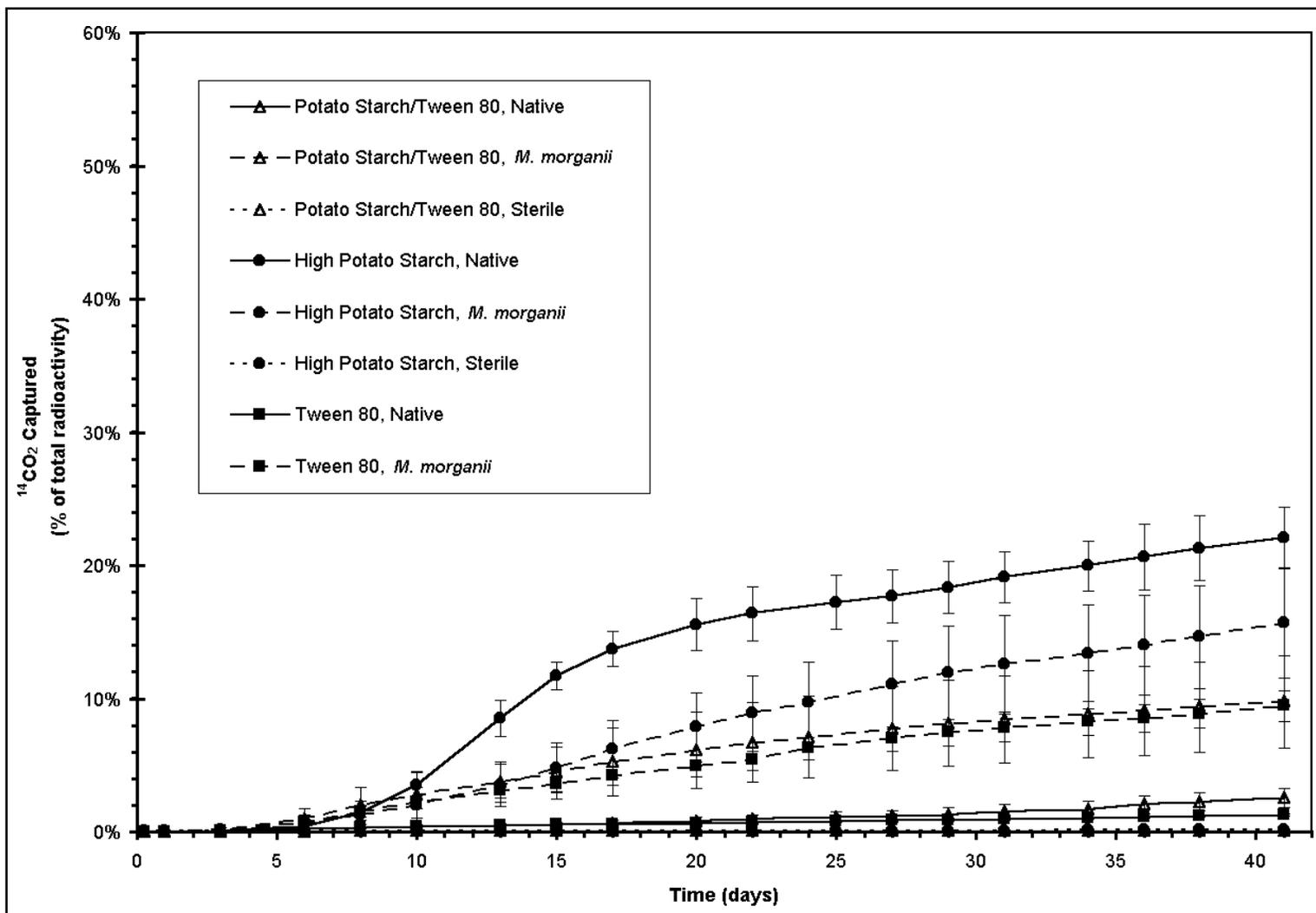


Figure 26. $^{14}\text{CO}_2$ vs time in anaerobic experiment 2

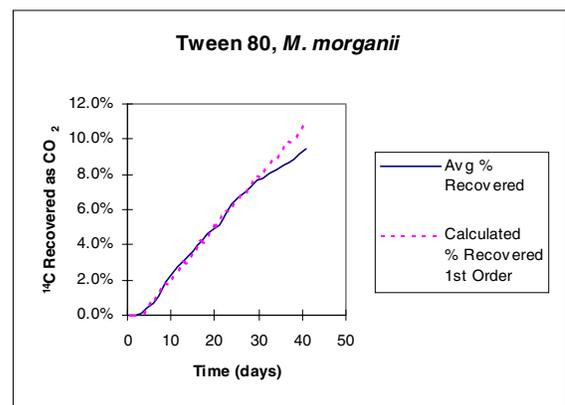
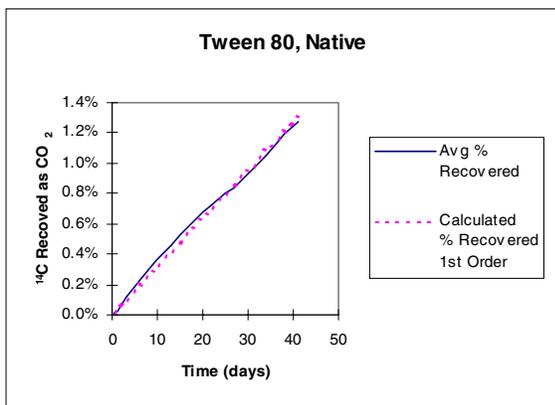
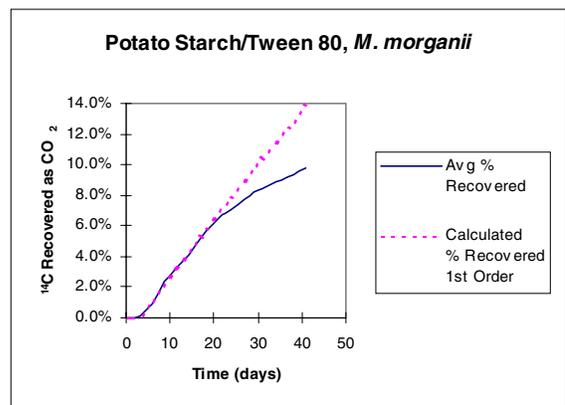
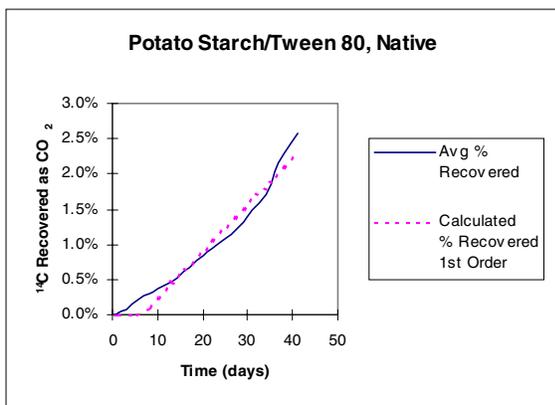
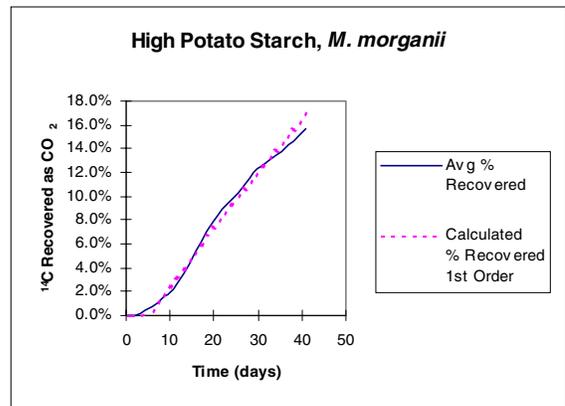
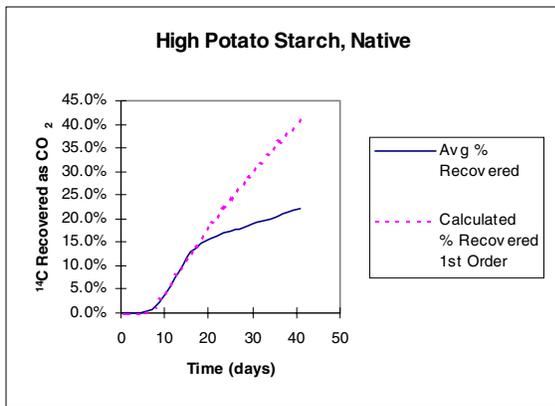


Figure 27. First-order reaction fit to $^{14}\text{CO}_2$ recovery data, anaerobic experiment 2

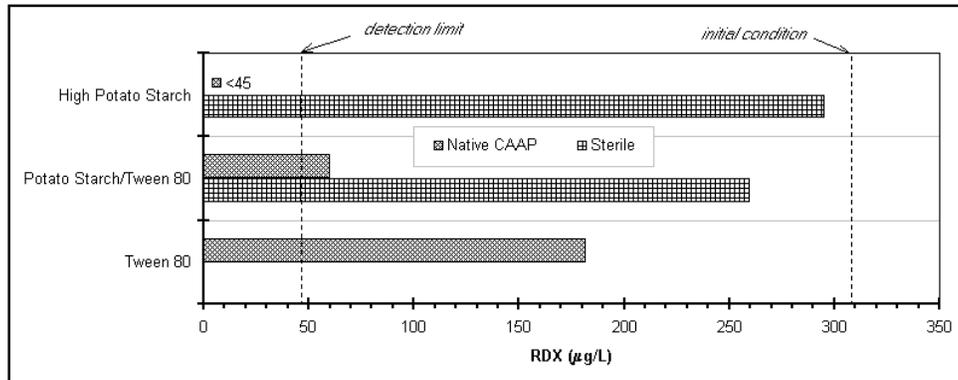


Figure 28. Final RDX concentrations, anaerobic/aerobic experiment

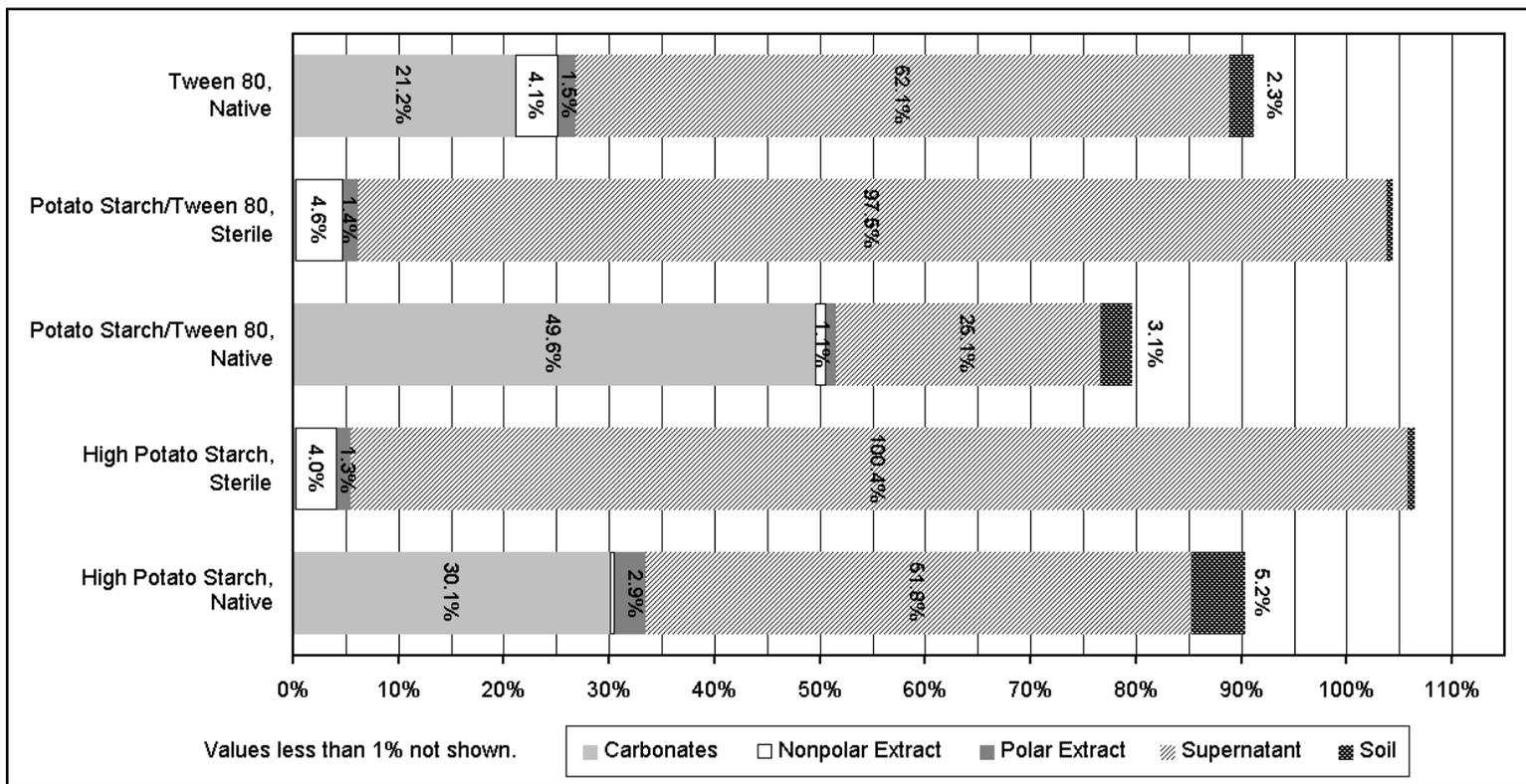


Figure 29. ¹⁴C mass balance in anaerobic/aerobic experiment

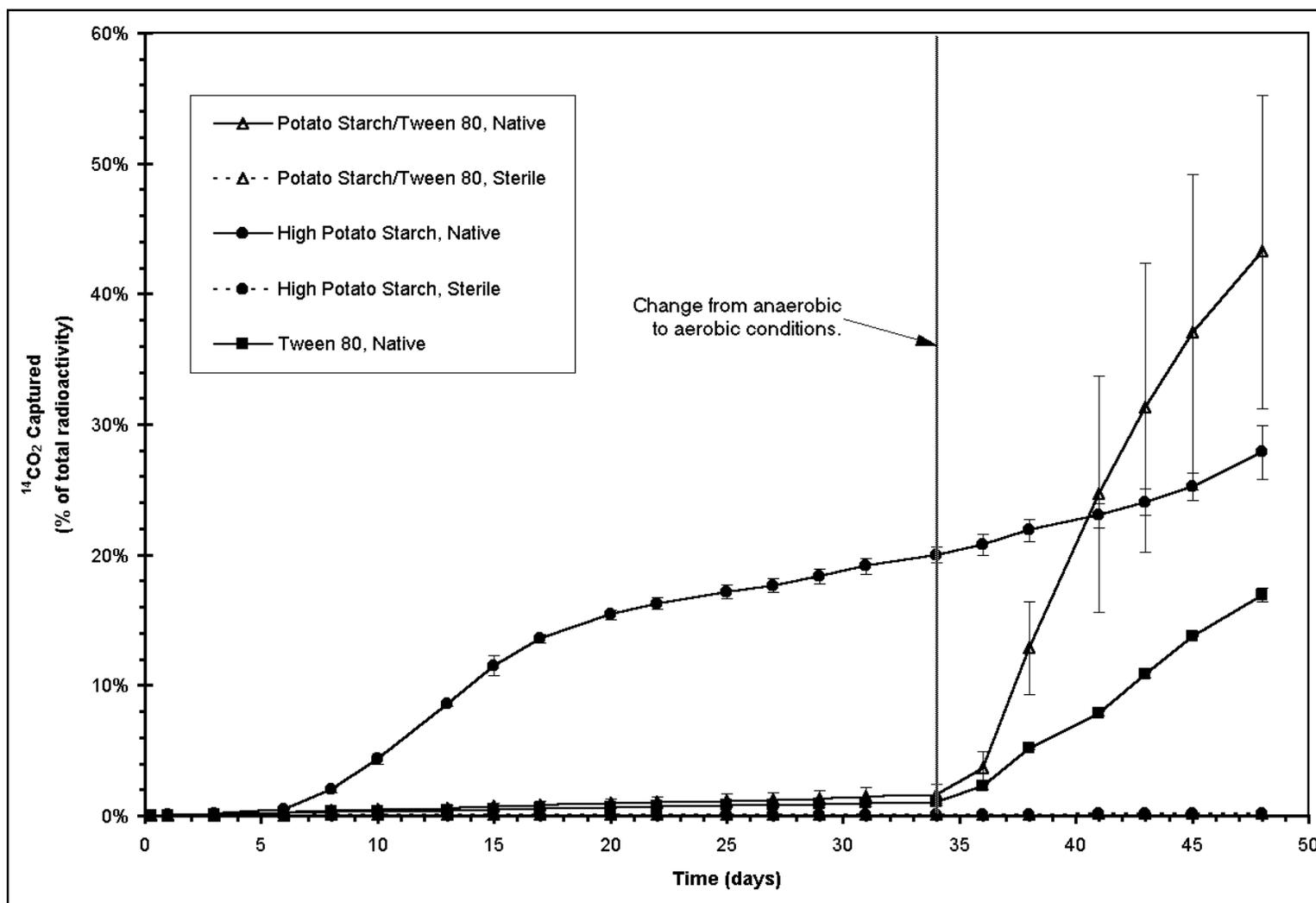


Figure 30. $^{14}\text{CO}_2$ vs time in anaerobic/aerobic experiment

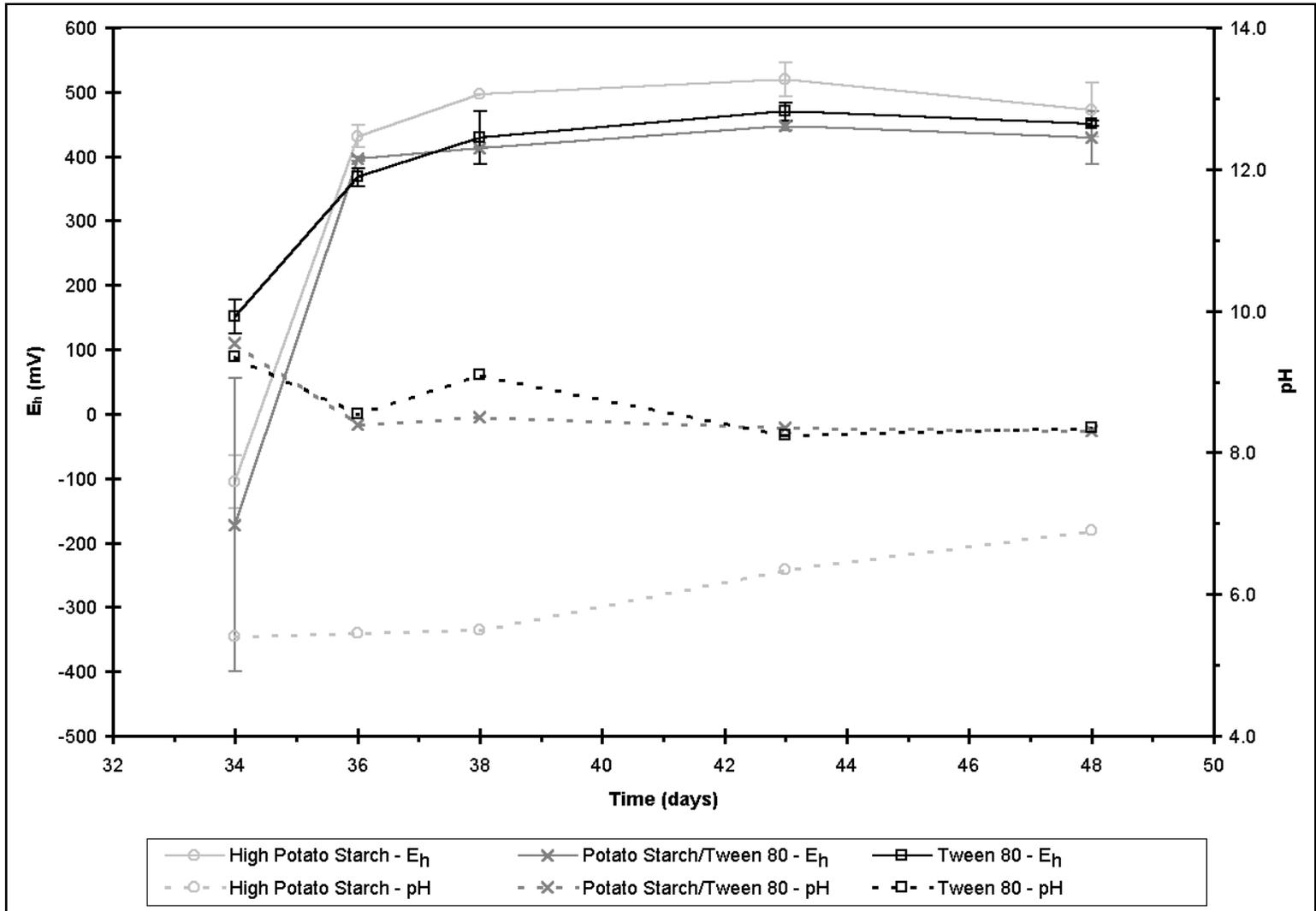


Figure 31. E_h and pH vs time for anaerobic/aerobic experiment

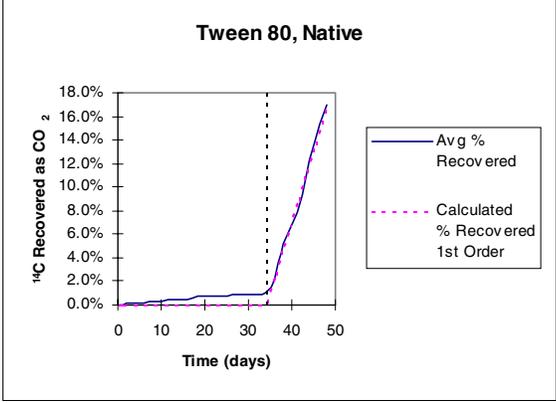
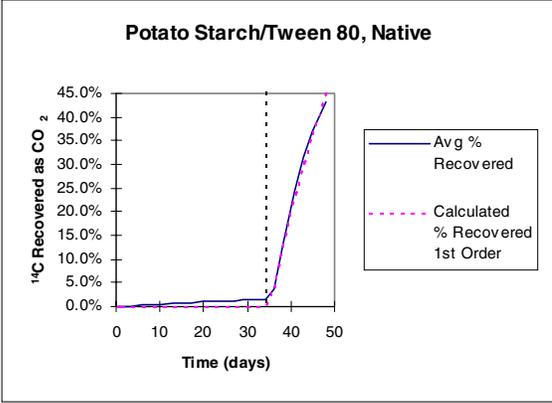
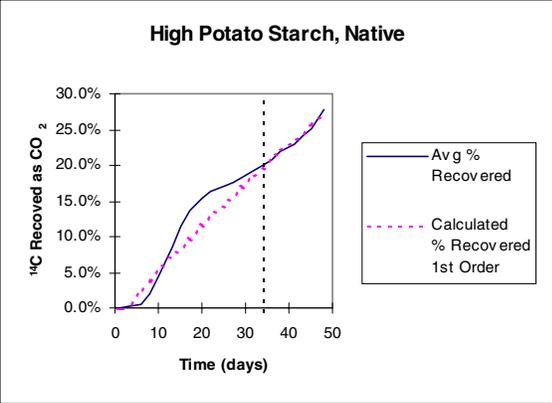


Figure 32. First-order reaction fit to $^{14}\text{CO}_2$ recovery data, anaerobic/aerobic experiment

Table 1
HPLC Analysis of CAAP Water and Soil Used in Shake Flasks

Analyte	Water (µg/L)	Soil (µg/L)
HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine)	4.53	<20
RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine)	16.40	<20
TNB (1,3,5-trinitrobenzene)	93.60	<20
DNB (1,3-dinitrobenzene)	<0.20	<20
TETRYL	<0.50	<20
TNT (2,4,6-trinitrotoluene)	330.00	<20
4A-DNT (4-amino-2,6-dinitrotoluene)	51.40	<20
2A-DNT (2-amino-4,6-trinitrotoluene)	54.90	<20
2 6-DNT (2,6-dinitrotoluene)	<0.20	<20
2,4-DNT (2,4-dinitrotoluene)	0.56	<20

Table 2
Initial Shake-Flask Contents

All flasks included:

RDX total (experiment 1).....	16.82 µg	157 µg/kg (slurry)
RDX total (experiment 2).....	26.82 µg	251 µg/kg (slurry)
¹⁴ C-RDX.....	5.51 µg	51 µg/kg (slurry)
RDX (experiment 1)	10.00 µg	93 µg/kg (slurry)
RDX (experiment 2)	20.00 µg	187 µg/kg (slurry)
RDX in situ.....	1.31 µg	12 µg/kg (slurry)
HMX in situ	0.34 µg	3 µg/kg (slurry)
TNT in situ	24.75 µg	230 µg/kg (slurry)
TNB in situ	7.02 µg	66 µg/kg (slurry)
4A-DNT in situ.....	3.86 µg	36 µg/kg (slurry)
2A-DNT in situ.....	4.12 µg	38 µg/kg (slurry)
KH ₂ PO ₄	0.50 mg	4.67 mg/kg (slurry)

In situ indicates that the source of the compound is from the native CAAP soil and water added.

Acetate:

sodium acetate.....	8 mg	75 mg/kg (slurry)
---------------------	------	-------------------

High Acetate:

sodium acetate.....	608 mg	5682 mg/kg (slurry)
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Acetate/Tween 80:

Tween 80	600 mg	5607 mg/kg (slurry)
sodium acetate.....	8 mg	75 mg/kg (slurry)

Potato Starch:

potato starch	8 mg	75 mg/kg (slurry)
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High Potato Starch:

potato starch	608 mg	5682 mg/kg (slurry)
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Potato Starch/Tween 80:

potato starch	8 mg	75 mg/kg (slurry)
Tween 80	600 mg	5607 mg/kg (slurry)

Corn Starch:

corn starch	8 mg	75 mg/kg (slurry)
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Ethanol:

ethanol	8 mg	75 mg/kg (slurry)
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Tween 80:

Tween 80	608 mg	5682 mg/kg (slurry)
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Table 3 Inorganic Nutrient Additions to Reactor Vessels	
Addition	Amount, g
Phosphate buffer:	
KH ₂ PO ₄	4.3
K ₂ HPO ₄	10.9
Na ₂ HPO ₄	31.5
NH ₄ Cl	8.5
MgSO ₄	23.0
CaCl ₂	13.8
FeCl ₃	0.2

Table 4 Unit BOD and Rate Constants for Additives		
Additive	Unit BOD (mg O₂/mg additive)	First-order Reaction Rate Constant (Equation 1)
Acetate	0.426	0.06
Ethyl alcohol	1.050	0.07
Potato starch	0.796	0.03
Corn starch	0.478	0.04
Tween 80	0.380	0.03

Table 5 Estimated First-Order Reaction Rate Constants for RDX Mineralization Based on ¹⁴CO₂ Recovery Data, Aerobic Experiments		
Condition	Reaction Rate Constant	Correlation Coefficient
No Carbon, Native, Experiment 1	0.002	0.964
Acetate, Native, Experiment 1	0.002	0.972
Acetate/Tween 80, Native, Experiment 1	0.021	0.991
Ethanol, Native, Experiment 1	0.002	0.983
Potato Starch, Native, Experiment 1	0.001	0.978
Corn Starch, Native, Experiment 1	0.001	0.980
High Acetate, Native, Experiment 2	0.013	0.999
High Acetate, Hastings Triplet, Experiment	0.006	0.999
Acetate/Tween 80, Native, Experiment 2	0.026	0.997
Acetate/Tween 80, Hastings Triplet, Experiment	0.039	0.994
Tween 80, Native, Experiment 2	0.031	0.999
Tween 80, Hastings Triplet, Experiment 2	0.033	0.998

Table 6 Final pH and ORP Levels in Anaerobic Experiment 1		
Condition	pH ± σ	E _h ± σ (mV)
No Carbon - Native	10.0 ± 0.6	-297 ± 19
Acetate - Native	9.7 ± 0.0	-374 ± 34
Acetate w/Tween 80 - Native	9.8 ± 0.1	-444 ± 49
Ethanol - Native	9.8 ± 0.2	-354 ± 7
Potato Starch - Native	9.8 ± 0.2	-363 ± 7
Corn Starch- Native	10.0 ± 0.8	-476 ± 16
No Carbon - Sterile	9.3 ± 0.1	+454 ± 3
Acetate - Sterile	9.5 ± 0.2	+441 ± 12
Acetate w/Tween 80 -Sterile	9.3 ± 0.0	+442 ± 4
Ethanol - Sterile	9.1 ± 0.0	+446 ± 4
Potato Starch - Sterile	9.3 ± 0.2	+459 ± 5

Table 7
Final pH and ORP Levels in Anaerobic Experiment 2

Condition	pH ± σ	E _n ± σ (mV)
High Potato Starch - Native	5.8 ± 0.0	-80 ± 90
High Potato Starch - <i>M. Morganii</i>	9.5 ± 0.0	-339 ± 35
High Potato Starch – Sterile	7.3 ± 0.0	624 ± 2
Potato Starch w/Tween 80 - Native	9.4 ± 0.1	34 ± 18
Potato Starch w/Tween 80 - <i>M. Morganii</i>	9.4 ± 0.4	-194 ± 104
Potato Starch w/Tween 80 - Sterile	9.5 ± 0.0	604 ± 2
Tween 80 - Native	9.2 ± 0.7	134 ± 6
Tween 80 - <i>M. Morganii</i>	9.5 ± 0.1	-318 ± 27

Table 8
Estimated First-Order Reaction Rate Constants for RDX Mineralization Based on ¹⁴CO₂ Recovery Data, Anaerobic Experiments

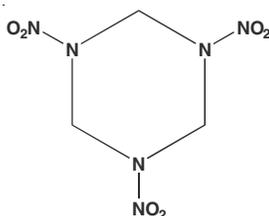
Condition	Reaction Rate Constant	Correlation Coefficient
No Carbon, Native, Experiment 1	0.001	0.992
Acetate, Native, Experiment 1	0.001	0.992
Acetate/Tween 80, Native, Experiment 1	0.002	0.996
Ethanol, Native, Experiment 1	0.001	0.994
Potato Starch, Native, Experiment 1	0.001	0.992
Corn Starch, Native, Experiment 1	0.001	0.996
High Potato Starch, Native, Experiment 2	0.016	0.998
High Potato Starch, <i>M. morganii</i> , Experiment 2	0.005	0.992
Potato Starch/Tween 80, Native, Experiment 2	0.001	0.984
Potato Starch/Tween 80, <i>M. morganii</i> , Experiment 2	0.004	0.999
Tween 80, Native, Experiment 2	0.000	0.998
Tween 80, <i>M. morganii</i> , Experiment 2	0.003	0.999

Table 9
Estimated First-Order Reaction Rate Constants for RDX
Mineralization Based on ¹⁴CO₂ Recovery Data, Anaerobic/Aerobic
Experiment

Condition	Reaction Rate Constant	Correlation Coefficient
High Potato Starch, Native	0.007	0.985
Potato Starch/Tween 80, Native	0.046	0.998
Tween 80, Native	0.014	0.996

Appendix A

Properties of RDX



Parameter	Value	Reference
Empirical formula	C ₃ H ₆ N ₆ O ₆	
Molecular weight	222.15	
Density (g/cm ³)	1.82 1.83	Kohler and Meyer 1993 Lindner 1980
Melting point (°C)	204 205	Kohler and Meyer 1993 Banerjee, Yalkowsky, and Valvani 1980
Solubility in water (mg/L)	43.2 at 23 ± 2°C 59.9 ± 1.2 at 25 ± 0.2°C 44.7 at 18°C 42 at 20°C	Leggett 1985 Banerjee, Yalkowsky, and Valvani 1980 Spanggord et al. 1980a Syracuse Research Corporation 1978
Diffusion coefficient in water (cm ² /s)	7.15 × 10 ⁻⁶ (estimated)	Rosenblatt et al. 1991
Log K _{ow}	0.88 at 23 ± 2°C 0.87	Leggett 1985 Banerjee, Yalkowsky, and Valvani 1980
Log K _{oc}	2.00	Rosenblatt 1986
Vapor pressure (torr)	4.03 × 10 ⁻⁹ at 25°C	Rosenblatt et al. 1991
Henry constant, K _H (calculated) (atm × m ³ /mol)	1.96 × 10 ⁻¹¹ at 25°C 2.6 × 10 ⁻¹¹ at 20°C	Rosenblatt et al. 1991 Spanggord et al. 1980a

Appendix B

Notation

AAP	Army Ammunition Plant
BOD	Biochemical oxygen demand
CAAP	Cornhusker Army Ammunition Plant
CEC	cation exchange capacity
DCM	dichloromethane
DNT	dinitrotoluene
DoD	U.S. Department of Defense
EPA	U.S. Environmental Protection Agency
HA	health advisory
HMX	her majesties explosive; octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	high performance liquid chromatography
LAAP	Louisiana Army Ammunition Plant
MeOH	methyl alcohol
NOAEL	no observed adverse effect level
ORP	oxidation-reduction potential
RDX	royal demolition explosive; hexahydro-1,3,5-trinitro-1,3,5-triazine
SERDP	Strategic Environmental Research and Development Program
SPE	solid phase extraction
TNT	trinitrotoluene
TOC	total organic carbon

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2000	3. REPORT TYPE AND DATES COVERED Final report	
4. TITLE AND SUBTITLE Removal of RDX From a Contaminated Groundwater by In Situ Bioremediation		5. FUNDING NUMBERS	
6. AUTHOR(S) Scott A. Waisner, Herbert L. Fredrickson, Lance D. Hansen, and Khankha Banerji			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) U.S. Army Engineer Research and Development Center Environmental Laboratory, 3909 Halls Ferry Road, Vicksburg, MS 39180-6199; University of Missouri - Columbia Columbia, MO 65211		8. PERFORMING ORGANIZATION REPORT NUMBER ERDC/EL TR-00-14	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Corps of Engineers Washington, DC 20314-1000		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution is unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) <p>Controlled experiments involving biodegradation of RDX were conducted with 14C-tagged RDX in aerobic, anaerobic, and anaerobic/aerobic slurries to identify the conditions maximizing RDX-mineralization. Indigenous microorganisms present in the CAAP groundwater and soil were evaluated along with a consortium of three microbes isolated from Hastings soil under aerobic conditions. A culture collection bacterium reported to be able to degrade RDX under anaerobic conditions was also evaluated. Several different carbon supplements were also investigated. Highest degree of mineralization (50%) was obtained under aerobic conditions when the contaminated groundwater was supplemented with large quantities of carbon source and Hastings triplet. Anaerobic/aerobic operation with native CAAP organisms and potato starch also resulted in rapid mineralization during the aerobic phase of the experiment. Under suitably chosen conditions, it was possible to transform RDX in solution to non-detect levels.</p>			
14. SUBJECT TERMS Biodegradation Biological degradation Explosives Explosives bioremediation		In situ RDX RDX bioremediation Treatability study	15. NUMBER OF PAGES 77
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT