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ANOXIC BIOLOGICAL TRANSFORMATION AND MINERALIZATION OF RDX

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ABSTRACT

RDX, a high explosive used in both conventional and nuclear weapons, was biodegraded in upflow, packed bed biocolumns using ethanol as a co-substrate and nitrate as a terminal electron acceptor. Three parallel reactors were operated with media composed of glass beads and rings made from silicone and Tygon tubing. Over 90% removal was obtained in the reactors, which operated with 3 hours or less hydraulic retention time. Ethanol concentrations were varied from 0.04 %(v/v) to 5%, with 2% being optimal. Mineralization was confirmed in batch experiments using ^{14}C labeled RDX, and up to 38% mineralization was observed. Mineralization increased with increasing nitrate concentration. Nitroso derivatives of RDX did not accumulate.

KEYWORDS

RDX, high explosives, biodegradation, mineralization, anoxic denitrification,

INTRODUCTION

Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) is one of the important high explosives used by the US and European munitions industries. Present dismantling activity is producing large amounts of explosives that require disposal. In addition, wastewaters generated during production, packing and washing of explosives from munitions plants are often contaminated with RDX. Wastewaters contaminated by RDX are highly problematic and need to be treated properly before disposal.

The major toxic effect of RDX to humans is damage to the central nervous system. RDX is also classified as Group C: possible human carcinogen. The US Environmental Protection Agency determined the lifetime health advisory of RDX at which no adverse health effects would be expected to occur following ingestion is 2 μg/L.

Presently, activated carbon adsorption is one of the most widely used methods to remove RDX from contaminated wastewater. Wujcik *et al.* (1992) demonstrated that a continuous flow granular activated carbon (GAC) column was able to remove RDX from contaminated groundwater. However, the disposal of the explosives-laden activated carbon is a largely unresolved problem. Alkaline hydrolysis of the laden carbon is a potential solution to this problem because it is able to convert RDX to smaller, less harmful compounds which can be treated in conventional wastewater treatment systems (Spontarelli *et al.*, 1993). Heilmann (1994, 1996) evaluated activated carbon adsorption combined with alkaline hydrolysis carbon regeneration. The RDX-contaminated wastewater is first passed through granular activated carbon columns to concentrate RDX waste onto the carbon and to reduce treatment volume. Next, the laden carbon is treated with alkaline hydrolysis and the regenerated carbon is then able to process another charge of RDX contaminated water.

An alternate process, investigated in this research, is the biological transformation and mineralization of RDX. Biological treatment is often less expensive when compared to physio-chemical treatment, and is a promising method for in-situ bioremediation. Anaerobic biodegradation has shown promising results on the transformation of RDX in contaminated wastewater. McCormick *et al.* (1981) reported that a digester inoculum metabolized 50 mg/L RDX in four days with peptone in medium at 37°C. Kitts *et al.* (1994) at the Los Alamos National Laboratory isolated three RDX degrading species. Two of the isolates, *Morganella morganii* and *Providencia rettgeri,*

completely transformed RDX and its nitroso-derivatives in 45 days. The third isolate, *Citrobacter freundii,* partially transformed RDX and generated high concentrations of RDX nitroso-derivatives. All three isolates mineralized less than 10 % of the carbon (^{14}C) from labeled RDX under oxygen depleted culture conditions.

Treating low concentrations of RDX with anaerobic biodegradation would contaminate the otherwise "clean" water with high amounts of organic co-substrate, bacteria and oxygen scavengers. Therefore, we propose a new treatment process which first involves adsorption of RDX onto activated carbon to concentrate RDX, followed by a novel carbon regeneration method, which desorbs the RDX using an organic solvent which also serves as the co-substrate in co-metabolic biodegradation of RDX. This regeneration process is described as an "Indirect-off-line-bioregeneration" (IOBR). The process is shown schematically in Figure 1. Such a combination is especially suitable to treat low RDX concentrations in large water volumes such as those found at contaminated sites and in wash down water.

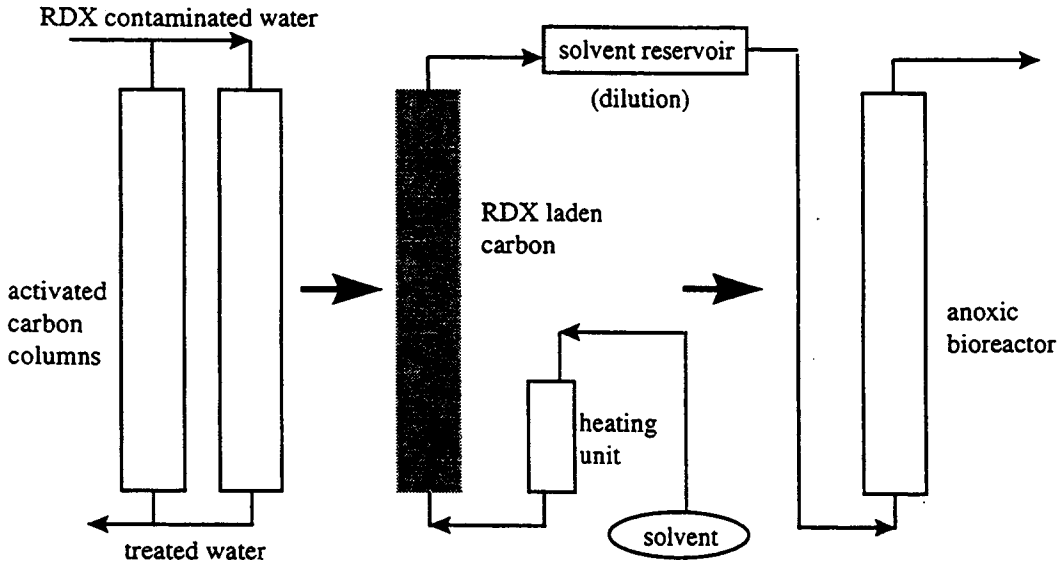

Figure 1. Proposed RDX treatment scheme with "indirect-off-line-bioregeneration"

MATERIAL AND METHODS

Denitrifying Cultures

Anaerobic digested sludge was collected from the Hyperion Wastewater Treatment Plant in El. Segundo, CA. In addition, a mixture of sludge and wastewater was collected from several RDX contaminated sites near Amarillo, TX. All the samples were diluted with oxygen free phosphate buffer, filtered, and incubated for three weeks in a medium consisting of ethanol, potassium nitrate and phosphate buffer.

Description of the Continuous-Flow Packed Bed Reactor (Bioreactor)

The biological transformation of RDX was performed in 200 mm long Plexiglas columns packed with three materials (glass beads, silicone and Tygon tubing). The internal diameter of each

column was 25 mm and the empty bed volume of each column was 98.17 mL. Columns were inoculated with mixed cultures. The feed flow was initiated two weeks after inoculation to allow culture growth and attachment to the packing material. The feed constituents are listed in Tables 1 and 2. Feed solution was continuously pumped through the bioreactors in an up flow direction using a Masterflex Model 7524-10 pump drive equipped with a multiple cartridge pump head. Tygon tubing was used to deliver the solution. Influent and effluent samples were collected and analyzed by HPLC to obtain the percentage of RDX transformed in bioreactor.

Table 1. Basal Medium Composition

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

Table 2. Trace Mineral Composition

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

CO_2 production and distribution of metabolites

^{14}C-RDX was used to investigate the mineralization of RDX by measuring $^{14}CO_2$ production in batch reactors. 0.05 µCi of ^{14}C-RDX was added to a batch reactor containing a total culture aliquot volume of 3.2 ml. The mineralization product, $^{14}CO_2$, produced in the head space of batch reactors was purged by a gentle stream of nitrogen gas and trapped by three scintillation vials in series. The three scintillation vials contained CO_2 absorber which included 10 ml of Ecolite(+)scintillation cocktail, 1 ml of methanol and 1 ml of β-phenylethylamine.

Liquid-Liquid Extraction and Measurement for Nitroso-derivatives of RDX

To analyze the metabolites in the liquid portion of a batch reactor, the sample was first concentrated by liquid-liquid extraction. The whole aliquot was first centrifuged at 10,000 rpm for 15 minutes to separate cells and liquid. The liquid culture was pre-acidified to pH 1 to create the most peaks on the HPLC chromatogram. The aliquots were transferred to 100 ml glass test tubes and equal volumes of ethyl acetate were added to each aliquot. Each test tube was vortexed for 15 seconds and settled for 10 minutes to obtain good separation between the organic and liquid layers. The organic layer was then collected and pooled into another glass culture tube (25 x150 mm). The extraction process was repeated three times. The pooled organic portion was then evaporated with a gentle stream of nitrogen gas to dryness and the metabolites were re-dissolved in 0.3 ml of acetonitrile. The extract was analyzed by HPLC.

High Performance Liquid Chromatography (HPLC)

HPLC analysis was performed with a Hewlett Packard 1050 Series Liquid Chromatography with a variable wavelength detector. An autosampler and a Hewlett Packard 3396 Series II integrator (Avondale, PA) were also used. The main analytical column was a 10mm, Adsorbosphere, C_{18} reversed phase column (250 mm x 4.6 mm) from Alltech (Deerfield, IL). A 5 mm guard column was used to protect the main analytical column. The mobile phase consisted of 40% water, 30% methanol and 30% acetonitrile at a flow rate of 1 ml/min. The sample injection volume was 20 ml and the detection wavelength was 236 nm. The HPLC detection limit of RDX was 0.1 mg/L. For the analysis of ^{14}C-RDX biotransformation metabolites, the solvent composition was modified to 90% water and 10 % ethanol to obtain the best separation of RDX and its nitroso derivatives. The HPLC analytical column was connected to a microfractionator model FC-80K (Gilson, Middleton, WI) to collect the effluent from the column. The radioactivity in each fraction was assayed with a liquid scintillation spectrometer LS 1800 (Beckman, Irvine, CA) to construct a radiochromatogram.

RESULTS

The saturation capacity for an initial RDX concentration of 40 mg/L on Filtrasorb-400 was found to be 417 mg/g GAC, which is the best activated carbon among three different carbon tested (Filtrasorb, Norita and Darco). Water is not a good solvent for the desorption of RDX on activated carbon due to the poor solubility of RDX in water. Polar organic solvents at elevated temperatures (~50 °C) markedly enhanced the desorption of RDX from activated carbon. For a mixture of ethanol and water, at least 50% (v/v) must be used to facilitate enhanced desorption.

The optimum ethanol concentration for RDX biodegradation was 2.0% (V/V). Over 90% of RDX can be removed in three hours in a continuous flow packed bed reactor. 5.0% ethanol in the feed solution inhibited the biological activity. Biodegradation efficiency decreased with decreasing ethanol concentration (Figure 2). At 0.04 % ethanol concentration, less than 10% of RDX could be transformed by the same bioreactor. An increase in RDX degradation from 55 % to 80 % occurred after raising the reactor temperature from 27°C to 35°C (Figure 3). An increase in retention time in the reactor from 3 hours to 6 hours produced an approximately 20 % increase in RDX biodegradation (Figure 4).

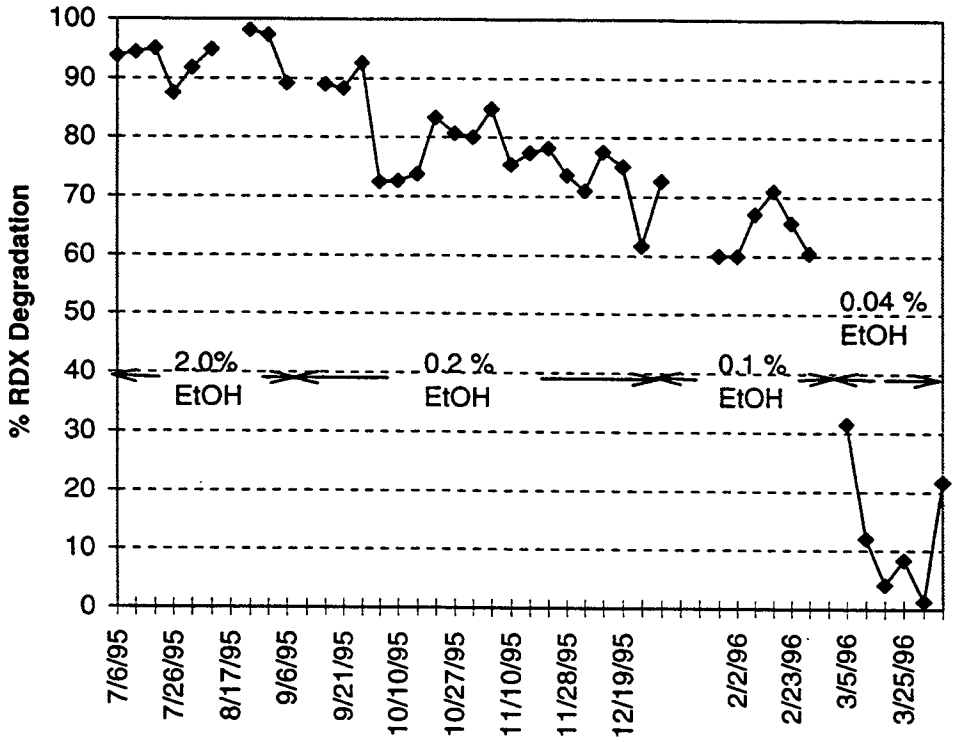

Figure 2. Effect of decreasing ethanol concentration on RDX biodegradation efficiency

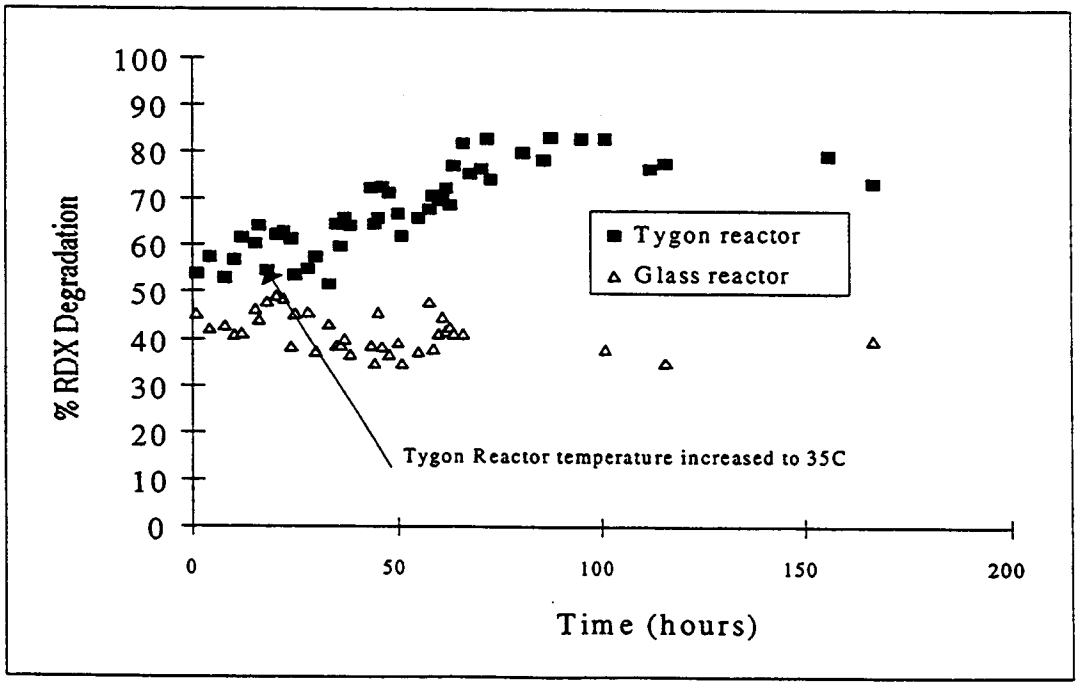

Figure 3. Effect of temperature on RDX biodegradation efficiency

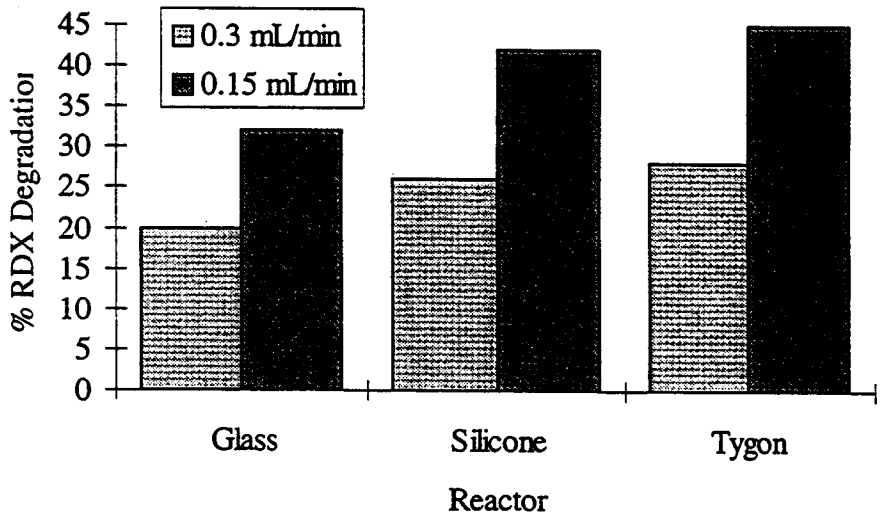

Figure 4. Effect of flow-rate (retention time) on RDX biodegradation efficiency

A kinetic study was performed by sampling at different lengths along the reactor. A salt tracer study was performed to determine the mixing characteristics (velocity and dispersion coefficient) of the reactor (Wilkie, 1994). A numeric model using a pseudo-first order reaction was used to describe the overall degradation of RDX in the reactors. The rate constant calculated for glass, silicone and Tygon reactors were 1.7, 4.2 and 4.2 hr^{-1}, respectively. The squared regression term (r^2) of observed versus predicted was 0.99 for the Tygon and glass reactors and 0.95 for the silicone reactor. A summary of all three transport equation parameters (D,V and k) are listed in Table 3.

Table 3. Transport Model Parameters (inlet flowrate of 0.3 ml/min)

Reactor	Glass	Silicone	Tygon
hydraulic retention time (hr)	2.57	2.93	3.00
dispersion coefficient D (cm^2/hr)	11.0	14.0	12.5
average pore water velocity V (cm/hr)	7.8	6.8	6.7
pseudo-first order rate constant k (hr^{-1})	1.7	4.2	4.2

The results of the batch experiments with ^{14}C-RDX showed that RDX mineralization was limited by the nitrate concentration. RDX mineralization increased with increasing nitrate to ethanol molar ratio (Figure 5). 38.1 % of the radioactivity on ^{14}C-RDX could be mineralized to $^{14}CO_2$ in seven days with a nitrate/ethanol ratio of 0.75 (1 = nitrate required to oxidize the ethanol to CO_2). Higher ratios were not possible due to the difficulties in maintaining neutral culture pH. A carefully buffered system was needed to avoid increasing pH in the batch cultures. The nitroso derivatives of RDX did not accumulate, although they could be briefly observed in kinetic experiments. A preliminary study showed that the same batch culture could mineralize 10 % of ^{14}C-HMX in eleven days.

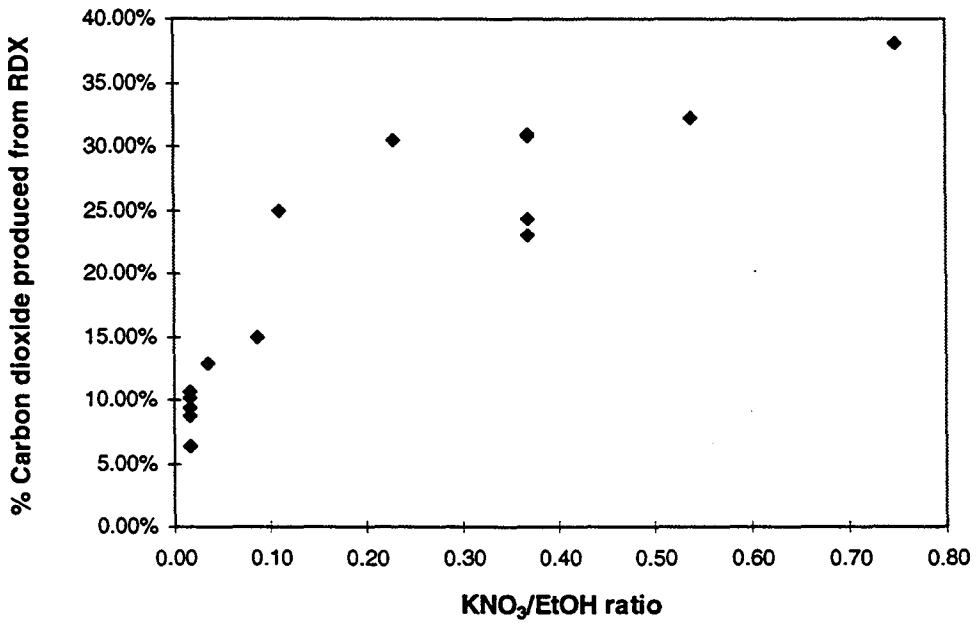

Figure 5. Effect of nitrate/ethanol ratio on the degree of RDX mineralization

DISCUSSION

Varying ethanol concentrations were evaluated to determine the most appropriate concentration for different uses of the culture. For carbon regeneration, very high ethanol concentrations (> 50%) will be used to regenerate the carbon. Therefore it is desirable to use the highest concentration possible in the bioreactors to avoid dilution. For direct treatment of wastewaters, the lowest possible ethanol concentration is most desirable. Two percent (2 % v/v) was the highest ethanol concentration which did not produce inhibition; 5% severely inhibited the RDX degradation. This suggests that the carbon regeneration fluid will have to be diluted 25 to 50 fold before RDX can be degraded. The lowest ethanol concentration producing significant RDX degradation rates was 0.1%. At 0.04% very little degradation occurred.

A study conducted by Rittman and McCarty (1980) discussed the concept of a minimum substrate concentration, Smin, below which no significant biofilm activity should occur at steady-state. Their results illustrate the substrate flux and biofilm activity declined rapidly as the substrate concentration approached Smin. At Smin, the rate of energy captured through cell growth is just equal to the rate of maintenance energy expenditure. This study might explain the result that at less than 0.1 % of ethanol in the feed solution, negligible RDX biodegradation in the continuous flow bioreactor was observed. Another study (Lyman, 1990) showed that several variables can

influence the rate of biodegradation. These factors may be substrate-related, organism-related or environment-related. Environmental variables such as temperature, control microbial metabolic activity, rather than biodegradation specificity. Rates of biological reactions increase with increasing temperature within the tolerable range by the organism. Future work should include a more detailed study of temperature and its effect on RDX degradation. Higher rates may occur if the optimal temperature is determined.

Theoretically, denitrifying bacteria should be the best candidates for use in our treatment concept. Their growth efficiency is the best of all anaerobes. They are able to mineralize organic substances, and they do not produce toxic corrosive H_2S. In an anoxic denitrifying culture, one mole of ethanol can supply five hydrogen equivalents, which exactly equals the theoretical demand of reducing one mole of nitrate to N_2. However the molar ratio of nitrate to ethanol was always less than 1 in our experiments. In attempting to increase nitrate concentration we encountered several problems in our batch cultures. The first was pH control; the denitrifying culture increased in pH until it became rate limited by the high pH. It was difficult to buffer and control the pH in the batch cultures because of their very small size and the need to prevent the release of ^{14}C. We were also concerned about the accumulation of metabolites of the RDX or ethanol metabolism that might be toxic or inhibitory. Finally, we had no way of insuring that essential nutrients did not become rate limiting in the batch experiments.

The maximum RDX mineralization, as indicated by ^{14}C by recovery, was 38.1%, which occurred with a nitrate/ethanol molar ratio that could oxidize only 75% of the ethanol. We anticipate that increasing nitrate relative to ethanol would increase RDX mineralization. Unfortunately it was not possible to increase the nitrate/ethanol ratio in the small batch reactors. A different reactor scheme such as a continuous reactor, might be required to produce suitable conditions. Ideally the reactor would be able to flush out metabolites and insure an adequate nutrient supply. Another solution is to adopt an aerobic polishing step to follow the anaerobic stage as proposed by Roberts *et al.* (1990).

CONCLUSIONS

RDX, an explosive chemical, was treated first by activated carbon adsorption and then by biological degradation in a laboratory scale investigation. "Indirect-off-line-bioregeneration" of the laden-activated carbon was proposed. Results show that RDX can be efficiently degraded in a continuous-flow packed bed bioreactor under anoxic denitrifying conditions. The conclusions based on the four years of study are:

- Polar organic solvents at elevated temperatures markedly enhance the desorption of RDX from activated carbon. For mixtures of ethanol and water, at least 50% (v/v) ethanol must be used to facilitate enhanced desorption.
- Anaerobic biodegradation of RDX is a fortuitous co-metabolism. The mixed bacteria cultures are not able to use RDX as growth substrate. An organic co-substrate is essential for RDX biodegradation.
- Ethanol as co-substrate produces both high growth rates and high biodegradation rates. In addition, ethanol is an effective desorption solvent so it can be used bi-functionally in the proposed indirect-off-line-bioregeneration system.
- Continuous flow, anaerobic biodegradation can effectively reduce inlet concentrations of RDX by more than 90 % in a single pass. This rate is achieved with nitrate as the terminal electron acceptor and ethanol as the co-substrate, at a reactor temperature of 35°C and with a retention time of 3 hours.
- An ethanol concentration of 5% (v/v) produces extreme inhibition of the culture's ability to degrade RDX. This concentration, however, is not lethal as full recovery of the culture is attainable.
- The optimum ethanol concentration in the feeding solution is 2.0 % for continuous flow biodegradation of RDX. RDX transformation efficiency decreases with decreasing ethanol concentration. 0.1 % ethanol is the minimum ethanol concentration required to produce significant RDX degradation.

- The rate of RDX biodegradation increases with increasing temperature and retention time.
- In the first quarter of the reactor, 60-80% of the total degradation occurs. This is the area where more than 50% of the total biofilm mass resides.
- The overall RDX degradation process can be modeled using a pseudo first-order rate equation. The rate constants calculated for glass, silicone and Tygon reactors are 2.7, 4.2, and 4.2 hr^{-1}, respectively.
- Mineralization of RDX increases with increasing nitrate availability as the terminal electron acceptor. 38.1% RDX mineralization is achieved (19 g/L potassium nitrate and 0.5 % ethanol).
- Under anoxic denitrifying conditions, no nitroso derivatives of RDX accumulate.

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