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Effect of food-grade surfactant on bioremediation of explosives-contaminated soil

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Abstract

The use of native soil bacteria to biodegrade explosives-contaminated soil under co-metabolic conditions has been demonstrated. The addition of food-grade surfactants could improve the process by enhancing the rates of explosives desorption from soil, thereby increasing the bioavailability of explosives for microbial degradation. The objective of this study was to decrease residence time in the slurry reactor, thereby increasing output and reducing clean-up costs. In this study, Tween 80 (monooleate), served not only as a surfactant but also as the carbon substrate for the soil microorganisms. Four 2 l soil slurry reactors were operated in batch mode with soil containing 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). The results indicated that TNT and RDX were removed in all reactors except the control (no added carbon source). The reactor enriched with surfactant and molasses performed better than reactors with either molasses or surfactant alone. The TNT and its metabolite, 4-amino-2,6-dinitrotoluene were removed faster in the reactor with surfactant plus molasses (35 days) than in the reactor with molasses alone as carbon source (45 days). A radiolabeling study of the mass balance of TNT in the slurry reactors showed substantial mineralization of TNT to CO_2.

Keywords: TNT; Soil slurry; Bioremediation; Co-metabolism; Surfactant; Tween 80

1. Introduction

The contamination of soil and water with explosives, such as 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), caused by military activities has been known for a long time, but progress in understanding the environmental fate of such compounds has only been made in the last few years [1]. The toxicity of TNT, RDX and other explosives is well documented [2–4]. Basic studies on the degradation of explosives by

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bacteria and fungi have been reported by several investigators [5–16]. Application of microbial processes has been used successfully for the remediation of explosive-contaminated soil [17–23].

Our previous work indicated that native soil bacteria can remove TNT by a co-metabolic process [24]. The most cost-effective co-substrate we used for bioremediation of TNT-contaminated soil was black strap molasses [18,23]. Other workers have reported the removal of TNT by soil bacteria [19,25], through composting [20,26], land farming [21], and by plants [27]. In the present study, an attempt was made to enhance the bioremediation process by using the food-grade surfactant namely Tween 80 (monooleate). Prior treatability work suggested that Tween 80 accelerates biodegradation rates by enhancing the removal of TNT from the soil matrix [28]. In this study, Tween 80 served as the surfactant and also acted as a carbon source for soil bacteria in a soil slurry reactor. A 3% (v/v) Tween 80 concentration was used for the study and performance of the soil slurry reactor was compared to the performance of the reactor that received molasses as carbon source.

2. Materials and methods

2.1. Soil

Contaminated soil was collected at the Joliet Army Ammunition Plant, Joliet, IL, USA. The contaminant concentrations in the soil are given in Table 1. The TNT concentration in the soil ranged from 4000 to 12000 mg/kg. The RDX in the soil was 1100–4000 mg/kg. The concentrations of other explosive contaminants were less than 300 mg/kg of soil. The soil had a total organic matter content of 4–5% which included the contaminants. The nitrogen content of the soil was 7.5 mg/kg as ammonium ion. The nitrate concentration in the soil varied from 6 to 12 mg/kg of soil.

2.2. Soil slurry reactor

Four 2 l laboratory-scale soil slurry reactors were operated in batch mode at ambient temperature (20–22 °C). The reactors were loaded with 20% (w/v) slurry of TNT-contaminated soil in water. One reactor received 0.3% (w/v) molasses (Grandma's molasses, Mott's, USA, Cadbury Beverages Inc., Stanford, CT) as a carbon source every week. Another reactor received a one time addition (on first day) of 3% (v/v) Tween 80 (Sigma, MO) as the carbon

Table 1
Explosives concentrations in the contaminated soil

Explosive[a]	Concentration range (mg/kg of soil)
TNT	4000–12000
TNB	175–300
2,4-DNT	50–200
RDX	1100–4000
HMX	50–100

[a] Abbreviations: TNT, 2,4,6-trinitrotoluene; TNB, trinitrobenzene; 2,4-DNT, 2,4-dinitrotoluene; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; HMX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine.

and surfactant source. The third reactor received weekly additions of 0.3% molasses as a carbon source and a one time (on first day) addition of Tween 80 (3%, v/v) as a surfactant source. The fourth reactor received no carbon and served as a no carbon control. The soil slurry was mixed continuously at the rate of 80 rpm by using a stirring motor (Model RW 20/RW 20DZM; Tekmar Company, Cincinnati, OH). The TNT concentration, its metabolite (4-amino-2,6-dinitrotoluene) concentration, bacterial growth and pH were monitored periodically in all reactors.

2.3. Analyses

TNT and RDX in the soil were extracted by the method recommended by the US Army Environmental Research Center [29]. Soil slurry was oven dried, then 1 g of the oven-dried soil was extracted with 10 ml acetonitrile. The whole mixture was sonicated for 18 h with a sonicator (Solid State/Ultrasonic Cleaning System Model FS-7652, Fisher Scientific, Itasca, IL). After sonication, 5 ml of the soil slurry was mixed with 5 ml of calcium chloride (5 g/l) and the mixture filtered through a 0.45 μm filter. The filtrate was analyzed for TNT and RDX using a Waters Associate (Milford, MA) liquid chromatograph equipped with two model 6000A solvent pumps, a Model 490E programmable multi-wavelength detector set at 254 nm, a data module and a Model 600 E system controller. The mobile phase was methanol:water (50:50, v/v) with a flow rate of 1.5 ml/min. Aliquots of 50 μl were injected into a C-18 Supelco column (25 cm × 4.6 mm and 5 μm) at 4 °C.

The gas chromatography/mass spectrometry (GC/MS) analyses were performed in the electron ionization mode on a Finnigan Model INCOS 50 system. The initial chromatography temperature was 80 °C held for 1 min, then increased to 250 °C at 20 °C/min and held at 250 °C for 10 min. The column used was a SPB-5 (Supelco, 30 m × 0.25 mm, 0.25 μm film). The injection temperature was 250 °C and the transport line kept at 200 °C. The helium flow rate was 25 cm/s and the injection volume 2 μl.

Bacterial activity in the reactor was monitored by total plate counts using tryptic soy agar (TSA) plates [21]. Nitrate and ammonia concentrations were analyzed by colorimetric methods using Hach water analyses reagent kits (Hach Company, Loveland, CO). The pH in the soil slurry sample was measured using a pH meter (Accumet pH meter, Model 50, Denver, CO).

2.4. [^{14}C]-TNT mineralization studies

After 2 months of reactor operation, 100 ml of the soil slurry was taken from each reactor. The soil slurry was incubated with uniformly labeled TNT to provide mass balance and determine metabolite production including [^{14}C]-CO_2. The [^{14}C]-TNT was added to the soil slurry at the level of 20,000 dpm/ml in respirometer flasks. The various treatments of the reactors were maintained by adding molasses or surfactant to the respirometer flasks. In the no-carbon treatment, no carbon was added to the respirometer. Samples were withdrawn periodically and the quantity of TNT converted to biomass was determined as trichloroacetic acid (TCA) precipitable material [30] by using a liquid scintillation spectrometer (Beckman Model LS 5000 TD). To make sure that TNT is not converted to dimer or polymeric compounds, we extracted the soil slurry with a series of solvents, namely, ethanol, methanol,

acetonitrile and ether as suggested by Kaplan and Kaplan [10] and the extracted materials were analyzed for azoxy polymeric compounds using HPLC.

Respirometer flasks [31] containing [^{14}C]-TNT were used to monitor the CO_2 evolved by the soil bacteria. KOH (0.5N) was added to the side arms. The flasks were incubated at ambient temperature in a shaker at 50 rpm. The respirometers were sampled periodically by withdrawing the KOH, measuring the radioactivity with a liquid scintillation spectrometer and replacing the KOH. The percentage of [^{14}C]-TNT mineralized as [^{14}C]-CO_2 was calculated. The experiment was conducted in duplicate.

The TNT metabolites were analyzed by collecting the fractions every 30 s after passage through the HPLC column. The radioactivity in each fraction was measured by using a liquid scintillation counter. Soil bound radioactive TNT was analyzed by using the soil extraction procedure described above and the radioactivity in the acetonitrile/sonication extract was measured by using a liquid scintillation counter.

2.5. Chemicals

Radiolabeled TNT (uniformly labeled, specific activity 21.5 mCi/mM, 98% pure) was purchased from Chemsyn Science laboratories, Lenexa, KS. The non-radioactive TNT was obtained from Chem Service Inc., West Chester, PA. Trinitrobenzene (TNB), 2,4-dinitrotoluene (DNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) were obtained from the Naval Surface Warfare Center, Indian Head, MD. The rest of the chemicals were of reagent grade.

3. Results and discussion

3.1. Desorption of TNT by Tween 80

An experiment was conducted to study the effect of Tween 80 concentration on the desorption of TNT and RDX from the contaminated soil. A 20% (w/v) of explosives-contaminated soil was added to a series of six flasks and the flasks were autoclaved (sterilized) in order to prevent biodegradation activities. Various Tween 80 concentrations ranging from 0 to 5% (v/v) were added to the flasks. After the flasks were shaken for 10 days in a shaker at 80 rpm, aqueous TNT and RDX concentrations were determined. Fig. 1 shows the aqueous TNT concentrations increased almost linearly with the increase in Tween 80 concentration. In the absence of Tween 80, aqueous TNT concentration was at a minimum (50 ppm). In contrast, at 5% Tween 80, water soluble TNT increased to 620 ppm. A similar trend was observed with regard to water soluble RDX. These results clearly demonstrate the effect of Tween 80 on the desorption of TNT and RDX from soil into the aqueous medium. The concentration of 3% Tween 80 was selected for use in further experiments mainly to avoid overloading the system with surfactant.

3.2. Removal of TNT in the soil slurry reactors

The concentrations of TNT in the slurry reactors are given in Fig. 2. The soil-TNT concentration in the no carbon control reactor remained high (around 4000 mg/kg of soil) through-

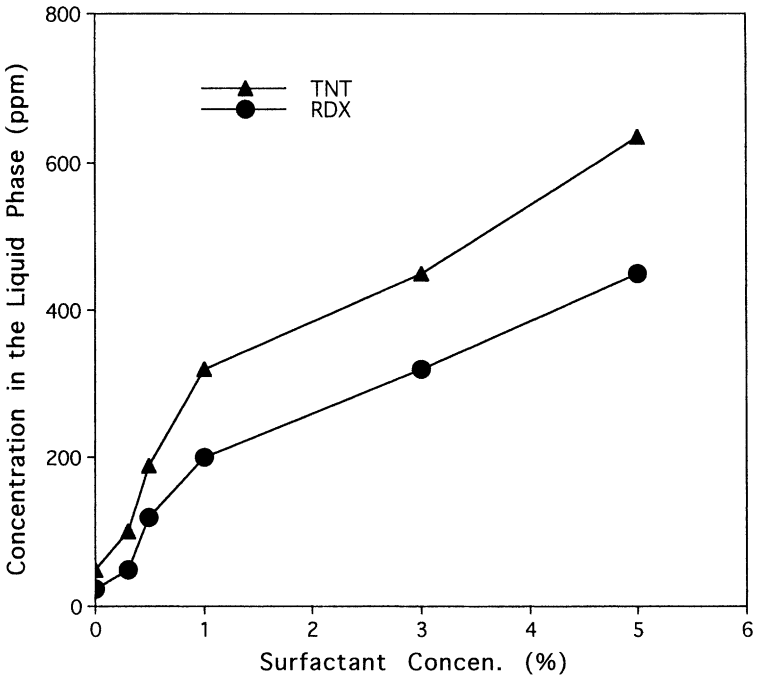

Fig. 1. Increase in aqueous TNT and RDX with increase in surfactant concentration.

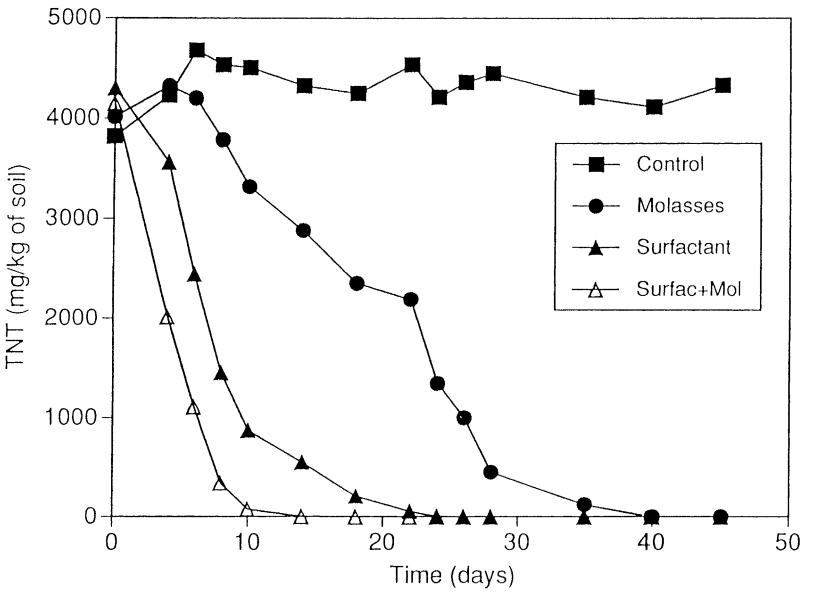

Fig. 2. Concentration of TNT in the soil slurry reactors.

out the experiment. This observation suggests that the indigenous microflora from the contaminated site would not degrade TNT without the addition of nutrients or co-substrates. The soil-TNT concentration in the reactor that received molasses as co-substrate dropped gradually and fell below the detection limit (<0.1 mg/kg of soil) on day 40 of the study. In the reactor with Tween 80 alone, TNT was removed faster than in the molasses amended reactor. The TNT removal rate was fastest in the reactor operated with both Tween 80 and molasses. The soil-TNT concentration in this reactor dropped from 4120 mg/kg on day 0 to <0.1 mg/kg of soil on day 12 of the experiment.

3.3. Production of TNT metabolites

In any investigation of the biological degradation of TNT, it is also important to track the production, accumulation and removal of metabolic intermediates within the experimental system. The predominant intermediate (by mass concentration) observed in this study was 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT). Fig. 3 illustrates the concentration of this intermediate in the soils of the four slurry reactors. The production of 4-A-2,6-DNT was significant in the reactors that received molasses alone, Tween 80 alone and both and it ranged from 1260 to 2989 mg/kg. In the reactor that received both molasses and Tween 80, the concentration of 4-A-2,6-DNT peaked on day 9 of the experiment (2989 mg/kg) and dropped to <0.1 mg/kg on day 35. In the molasses supplemented reactor, the increase and decrease in the concentration of 4-A-2,6-DNT were gradual with 45 days requirement to completely remove this metabolite. The 4-A-2,6-DNT metabolite accumulated in the soil

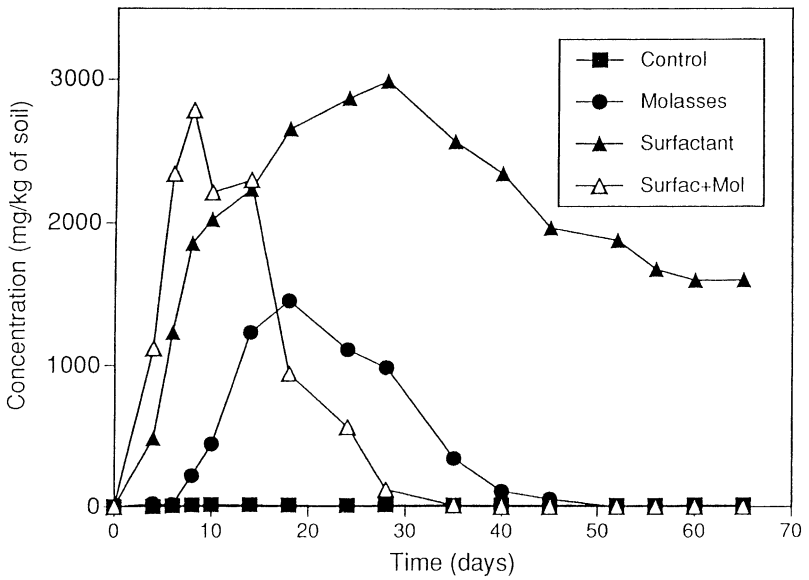

Fig. 3. Concentration of 4-amino-2,6-dintrotoluene in the soil slurry reactors.

slurry reactor that received Tween 80 alone, remaining at 2000 mg/kg of soil at the end of 60 days of reactor operation. However, repeated addition of molasses to this reactor, caused the concentration of 4-A-2,6-DNT to eventually fall below the detection limit (data not shown). A very minimal amount of 4-A-2,6-DNT was produced in the no carbon control reactor (Fig. 3).

These results for TNT and metabolite, 4-A-2,6-DNT, in the slurry reactors demonstrate that Tween 80 can desorb TNT from soil, and can help to make the contaminant available for bacterial action. The addition of Tween 80 alone did not allow the bacteria to completely remove TNT and its metabolite. Molasses supported well balanced degradation with removal of both TNT and 4-A-2,6-DNT within 45 days. The complex composition of molasses which contains sugars, amino acids, organic nitrogen, proteins, vitamins and minerals is conducive to the growth of many types of bacteria in soil [18] and helps in the total degradation of TNT. On the other hand, Tween 80 mainly contains four long chain fatty acids, including palmitic acid, oleic acid, stearic acid and linoleic acid, [1] and lacks essential minerals, vitamins, and a nitrogen source for bacterial growth. This difference in nutrients could explain the accumulation of 4-A-2,6-DNT in the slurry reactor that received only Tween 80. The use of Tween 80 as a co-substrate for the bioremediation of TNT-contaminated soil was studied in detail by Boopathy and Manning [28] and they concluded that the Tween 80 is biodegradable by aerobic bacteria because of the presence of various fatty acids. Addition of both, Tween 80 and molasses, increased the removal rates of both TNT and 4-A-2,6-DNT, as shown in Figs. 2 and 3. The time required to completely remove TNT and its metabolite was reduced by 10 days by adding a one time Tween 80 dose of 3% (v/v) to the soil slurry reactor. The fate of 4-A-2,6-DNT was monitored in the reactor by using GC/MS analysis. As explained in detail by Boopathy et al. [18], we observed the production of a non-aromatic metabolite namely, 2,3-butanediol in the slurry reactors that was converted to CO_2. The mass balance of TNT metabolism is explained below.

3.4. Reactor pH and microbial concentration

The pH in the reactors was monitored throughout the experiment. The pH remained approximately neutral in the no carbon control and in the reactor with Tween 80. However, the molasses containing reactor tended to be acidic with pH values of 6–6.5 throughout the experiment. In the reactor, supplemented with Tween 80 and molasses, the pH remained at about 6.8 (data not shown).

Bacterial plate counts were performed several times over the course of the experiment. The bacterial plate counts in the reactors receiving molasses were consistently higher by four to five orders of magnitude than those in the no carbon control reactor and higher by two orders of magnitude than those in the Tween 80 reactor (Table 2). These results also show the value of the molasses addition which helps to increase the population of soil bacteria in the reactor. Molasses is the best among various substrates studied, such as succinate, glucose, acetate and citrate [24], it is well balanced with nutrients including carbon, nitrogen, phosphorus, vitamins and minerals for bacterial activity [24].

[1] Sigma Catalog, Sigma Chemical Company, St. Louis, MO, 1995, p. 1226.

Table 2
Bacterial counts in the soil slurry reactors (CFU/ml of soil slurry)

Day	Bacterial count (colony forming unit per ml of slurry) in the reactor			
	Control	Surfactant	Molasses	Surfactant plus molasses
0	36×10^3	30×10^4	36×10^4	32×10^4
10	22×10^3	160×10^6	56×10^6	160×10^6
20	26×10^3	186×10^6	254×10^8	180×10^8
30	25×10^4	120×10^6	210×10^8	196×10^8
40	28×10^4	114×10^6	190×10^8	144×10^8

3.5. Radiolabeling experiment

The addition of radiolabeled TNT to the reactor biomasses provided evidence for the mineralization of TNT (Fig. 4). Radiotracer studies with the reactor biomasses revealed various intermediates, including 4-A-2,6-DNT, and 2,3-butanediol after 3 weeks of starting the radiolabeled studies. In the no carbon control, 96% of ^{14}C-TNT was recovered as TNT which did not undergo degradation and only 2% was converted to 4-A-2,6-DNT (Fig. 4A). This result again shows the importance of adding a co-substrate like molasses for complete degradation of TNT. In the reactor with Tween 80 alone, ^{14}C-TNT was converted to CO_2 (14%), biomass (19%), 2,3-butanediol (20%) and 4-A-2,6-DNT (42%) (Fig. 4B). In the reactor supplemented with molasses as the carbon source, 25% of the TNT was mineralized to CO_2, 28% converted to biomass, nearly 30% metabolized to 2,3-butanediol and 8% remained as 4-A-2,6-DNT (Fig. 4C). The reactor with both molasses and Tween 80 had results similar to those of the reactor with molasses alone, with the conversion of radiolabeled TNT to CO_2 (29%), biomass (30%) and 2,3-butanediol (28%) (Fig. 4D). The extraction of soil slurries with various solvents did not show the formation of dimer or polymeric compounds either from the TNT or from its intermediate, 4-A-2,6-DNT and this further indicates that the TCA-precipitable materials reported in Fig. 4 are representation of biomass. The radiolabeling study showed a very reasonable mass balance for TNT metabolism, confirming the earlier results of Boopathy et al. [18].

3.6. Removal of RDX in the soil slurry reactor

The concentration of RDX in the soil was monitored throughout the study and the result is given in Fig. 5. In the control reactor, the RDX concentration was approximately 1600 mg/kg and this concentration did not change throughout the study. In the reactor with molasses as co-substrate, the RDX concentration remained at approximately 1500 mg/kg during the first 21 days of reactor operation. After 3 weeks, the RDX concentration started to decrease steadily and reached 20 mg/kg on day 77 of the experiment. This result showed that RDX metabolism by soil bacteria needs an initial acclimation period of 21 days before bacteria start to metabolize this compound. Identification of the metabolites of RDX was unsuccessful in this study, however, the multiple metabolite peaks that appeared during the gas chromatography analyses disappeared toward the end of the experiment, indicating possible further degradation of RDX metabolites by the soil bacteria. In the reactors, where

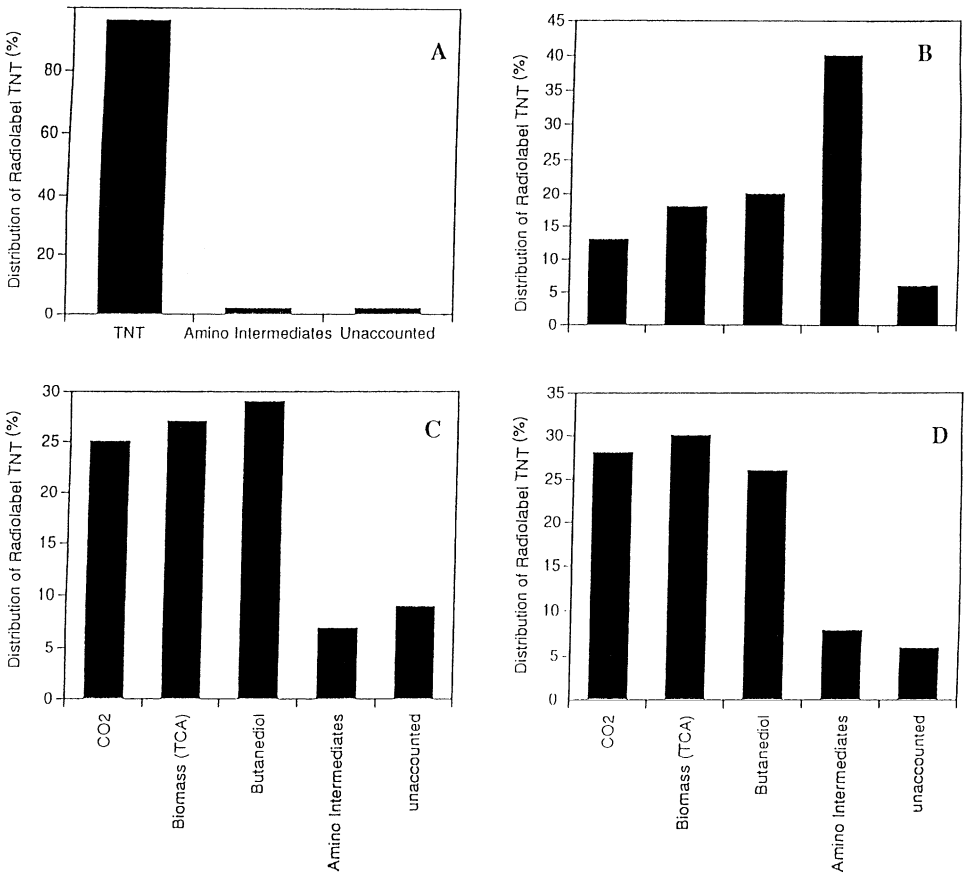

Fig. 4. Mass balance of radiolabel TNT. (A) No carbon control; (B) samples from the surfactant reactor; (C) samples from the molasses reactor and (D) samples from the surfactant + molasses reactor.

Tween 80 served either as sole carbon source or a surfactant additive with molasses as a co-substrate, the RDX concentration started to decrease within 2 weeks, indicating a shorter acclimation period. In the Tween 80 supplemented reactors, the RDX concentration reached below 20 mg/kg of soil on day 60. This study demonstrated a reduction in treatment time by 17 days for RDX contaminated soil when the surfactant is used.

This study showed that the soil slurry reactor can effectively remediate soil contaminated with TNT and RDX. Operation of laboratory-scale soil slurry reactors in a batch mode achieved 100% removal of TNT and 99% of RDX. Addition of the surfactant Tween 80 to the reactor system reduced the treatment time by 10 days for TNT and 17 days for RDX, which will prove to be cost-effective in a large-scale clean-up operation. The complete degradation of TNT was demonstrated by mineralization of ^{14}C-TNT, metabolite formation, and the presence of ^{14}C in the cell biomass as TCA-precipitable material. Each of the biological systems reported in the literature as acting on TNT catalyzes the reduction of

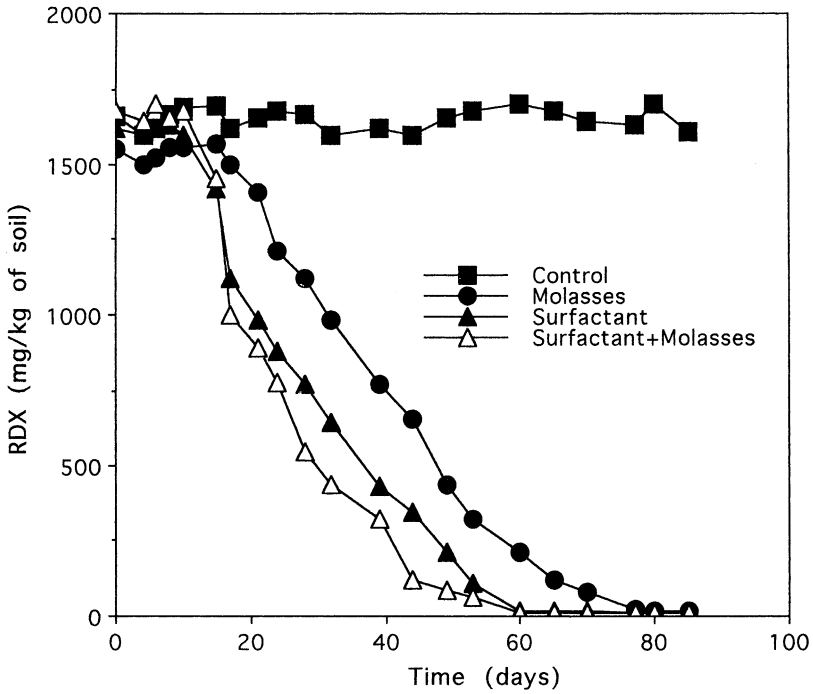

Fig. 5. Concentration of RDX in the soil slurry reactors.

at least one nitro group [5,7,9]. In the present work, the microbes produced 4-A-2,6-DNT as the major metabolite, which was further mineralized to CO_2 via the production of the non-aromatic metabolite, 2,3-butanediol. Because the ring carbons of TNT were uniformly labeled, conversion to CO_2 clearly denotes ring cleavage. The mechanism of TNT ring cleavage is not clear. However, the first step was a reduction process, as reported previously by others [9,16]. By taking into account the samples taken out for the analyses from the reactors, and the molasses and Tween 80 added to the systems, the degradation of TNT would require about 7 mg chemical oxygen demand (COD) in the form of co-substrate either Tween 80 or molasses per mg TNT.

Because of the non-availability of radiolabeled RDX, the mass balance for RDX metabolism by the soil bacteria was not investigated in this study. Future work will be directed toward addressing this problem and the mass balance for RDX metabolism will be investigated.

Among the different bioremediation methods reported for ammunition compounds, the soil slurry reactor seems to be very promising. The composting method described by Williams et al. [20] removed TNT under thermophilic and mesophilic conditions. The disadvantage of composting is that it needs large quantities of additives. The soil slurry reactors demonstrated by Boopathy et al. [18] showed promising results in complete degradation of TNT in the contaminated soil. The studies described in the present paper conclusively

proved that a one time addition of Tween 80 at 3% (v/v) concentration to a soil slurry reactor operated with molasses increased the TNT degradation rates significantly. The treatment time was reduced by 10 days for TNT and 17 days for RDX compared to the reactor with no surfactant addition (molasses treatment only). The advantage of the slurry reactor is its simple operating conditions. The method needs only mixing and a carbon source. Molasses is an inexpensive carbon source that could be used in a large-scale operation at low cost.

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