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Transformation and fate of 2,4,6-trinitrotoluene (TNT) in anaerobic bioslurry reactors under various aeration schemes: implications for the decontamination of soils

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Abstract Energetic compounds have been used in a variety of industrial and military applications worldwide leading to widespread environmental contamination. Many of these compounds are toxic and resist degradation by oxidative enzymes resulting in a need for alternative remediation methods. It has been shown that trinitrotoluene (TNT)-contaminated soil subjected to treatment in strictly anaerobic bioreactors results in tight binding of TNT transformation products to soil organic matter. The research presented here examined the fate of TNT and its metabolites in bioreactors under three different aeration regimes. In all treatment regimes, the typical metabolites of aminodinitrotoluenes and diaminonitrotoluenes were observed prior to irreversible binding into the soil fraction of the slurry. Significant transformation of TNT into organic acids or simple diols, as others report in prior work, was not observed in any of the treatments and is an unlikely fate of TNT in anaerobic soil slurries. These results indicate that aeration does not dramatically affect transformation or fate of TNT in reactor systems that receive a rich carbon source but does affect the rate at which metabolites become tightly bound to the soil. The most rapid transformations and lowest redox potentials were observed in reactors in which an aerobic headspace was maintained suggesting that aerobes play a role in establishing conditions that are most conducive to TNT reduction.

Keywords Trinitrotoluene · Bioslurry reactor · Aeration · TNT metabolism · Anaerobic · Anoxic · Soil

Abbreviations

ADNT	aminodinitrotoluene
BHI	brain-heart infusion
DANT	diaminonitrotoluene
dpm	disintegrations per minute
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
ESI-MS-MS	electrospray ionization mass spectrometry
FAST	facultative anaerobic soil treatment
HPLC	high-performance liquid chromatography
LC	liquid chromatography
MS	mass spectrometry

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TAT	triaminotoluene
TIC	total ion chromatogram
TNT	trinitrotoluene
TSA	tryptic soy agar
UL	uniformly labeled

Introduction

Energetic compounds play important roles in military and civilian applications, and their production comprises a significant portion of the world's chemical manufacturing industry (Rosenblatt et al. 1991). Soils and waters at a significant number of sites worldwide have become contaminated with energetic organonitro compounds as a result of manufacturing and decommissioning of ordnance (Spain 1995). The high toxicity, mutagenicity, and recalcitrance of these compounds in the environment has led to intensive research for innovative technologies to treat contaminated wastes, soils, and waters (Kaplan 1990, 1992; Lewis et al. 2004; Rosenblatt et al. 1991).

In 1987, researchers at the University of Idaho in conjunction with an industrial partner began development of an anaerobic process to treat soils contaminated with nitroaromatic compounds. The process was accepted for the US EPA's Superfund Innovative Technology Evaluation Program Demonstration in 1995 (USEPA 1995). In this trial, 23 m^3 of soil with an average concentration of 3,000 mg kg^{-1} trinitrotoluene (TNT) was treated. The results showed that the concentration of TNT in the soil was <100 mg kg^{-1} after 50 d of treatment and below detection limits by day 125 (Funk et al. 1995). These results along with data from other studies showed that the anaerobic treatment now termed the facultative anaerobic soil treatment (FAST^{TM}) was a viable technology for treating TNT-contaminated soils (USEPA 1995).

Using strictly anaerobic incubations with glucose-fed sewage sludge cultures, Rieger and Knackmuss showed rapid, nearly stoichiometric reduction of the nitro groups of TNT (Rieger and Knackmuss 1995). These studies showed production of triaminotoluene (TAT), followed by its slow disappearance. The authors described several possible fates of TAT in various chemical environments, noting that biological transformations were only postulated, not characterized. The irreversible binding of TAT to humic substances and charged soil particles was recognized as a highly likely fate for TAT. A remediation process based on this immobilization has been described and tested with soil at a pilot scale (30 tons) (Stolpmann et al. 1995).

The objective of the research presented here was to determine the transformation and fate of TNT and its metabolites in soil slurries under different aeration regimes and specifically to determine if the parent compound is converted to organic acids or aliphatic diols as reported by (Boopathy et al. 1998).

Materials and methods

Chemicals

[UL-^{14}C]glucose (100 μCi g^{-1}) of >98% purity was purchased from Sigma Chemical Company (St. Louis, MO). [Ring-UL-^{14}C]TNT was prepared by two-step nitration from toluene and [ring-UL-^{14}C]toluene (specific activity, 58.9 μCi $mmol^{-1}$; radiochemical purity >99% by high-performance liquid chromatography [HPLC]) (Lewis et al. 1996). Molasses used in the soil slurries was Brer Rabbit^{TM} brand (Nabisco, East Hanover, NJ). Typically, molasses contains 17–25% water, 45–50% sugars (sucrose, glucose, and fructose), and 2–5% polysaccharides (Najafpour and Shan 2003). Nonradioactive TNT metabolites 2,4-diamino-6-nitrotoluene (2,4-DA-6-NT), 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT) and 2,4,6-triaminotoluene (TAT) trichloride were purchased from Chem Service (West Chester, PA). Lactic, acetic, propionic, and butyric acids were purchased from Sigma Chemical Company.

Analytical methods

Reactor supernatants were analyzed periodically throughout the experiment for TNT presence and accumulation of metabolites and organic acids. Analysis of organic acids was accomplished using

a Hewlett Packard (HP) HPLC model 1090 (Hewlett Packard, Palo Alto, CA) equipped with a Rezex 7.8 × 300 mm column (Phenomenex, Torrance, CA). The mobile phase was 5 mM sulfuric acid at a flow rate of 0.5 ml min^{-1}. Two detectors were used, a diode array detector set at 210 nm and a radioactivity detector (β-RAM IN/US system, Tampa, FL). The second detector uses a pump to mix the HPLC effluent with scintillation cocktail. In-Flow® scintillation cocktail (β-RAM IN/US system) was used at a flow rate of 3 ml min^{-1}. Injection volumes were 100 μl. A differential refractometer detector model 410 (Waters, Milford, MA) was used in tandem with the radioactivity detector to analyze for radiolabeled organic acids and 2,3-butanediol.

TNT and metabolites were separated by a Rainin Microsorb MV C_{18}, 4.6 × 250 mm reverse phase column (Rainin Instrument Company, Inc., Emeryville, CA). Samples were eluted using a gradient mobile phase containing acetonitrile (A) and 15-mM lithium phosphate at pH 7.1 (B). The method was as follows: flow rate, 1 ml min^{-1}; injection volume 100 μl; 95% B (0–7 min) to 50% B (7–12 min, held for 2 min) to 30% B (14–15 min, held for 5 min), followed by a return to initial conditions (22 min); peak detection was at 210 nm with peak-actuated scanning from 200 to 600 nm with a diode array. The radioactivity detector was used in tandem with the diode array detector to analyze for radioactive metabolites in soil slurry treatments that received [ring-UL-^{14}C]TNT. In both the TNT and organic acid methods, samples were filtered through 0.22-μm Supor syringe filters (Gelman Sciences, Ann Arbor, MI) before chromatographic separation.

Some nonradioactive supernatant samples were analyzed for 2,3-butanediol using a Dionex Liquid Chromatograph (Dionex, Sunnyvale, CA) equipped with a Carbopac™ PA1 4 × 250 mm column. The method was modified from the manufacturer's suggested method with a gradient mobile phase consisting of ultra pure (18 MΩ-cm) water (A) and 100 mM NaOH (B). The flow rate was maintained at 1 min ml^{-1} with an injection volume of 100 μl. The gradient method was as follows: 20% B (0–10 min) to 100% B (10–10.1 min, held for 2.9 min) to 20% B (13–13.1 min, held for 4.9 min). The 2,3-butanediol was quantified with pulsed amperometry followed by electrochemical detection using a standard curve prepared from pure diol.

Culture supernatants were extracted twice with equal volumes of methylene chloride. The methylene chloride extract was dried under a nitrogen stream. The sample was then dissolved in methanol and analyzed with a HP series II 5890 gas chromatograph equipped with a capillary fused-silica column (30 m × 0.25 mm) coated with CP-SIL 8CB MS (Chrompack, Middelburg, The Netherlands). The injector temperature was set at 250°C and the GC-MS interface at 280°C. Chromatographic separations were achieved under a linear temperature gradient from 100 to 200°C at a rate of 5°C min^{-1} and then from 200 to 300°C at 20°C min^{-1}. Samples (1–5 μl) were injected via an automatic injector (HP-7673). An HP quadrupole MS (5989A) controlled by HP MS Chemstation software (PC version) was used for MS analysis under the following conditions: repeller, 7 V; emission, 300 V; electron energy, 70 eV. The source temperature was 250°C and the quadrupole temperature 125°C. The scan parameters were 30–350 or 30–750 *m/z*. Interpretation of the MS spectrum was aided by the Wiley and National Institute of Standards and Technology library of mass spectra stored in the Chemstation database (approximately 200,000 spectra).

A Micromass Quattro II mass spectrometer (Altrincham, England) equipped with an electrospray ionization probe, two quadrupole analyzers, and a hexapole collision cell was used to analyze culture supernatants. The ion source was operated in the negative- or positive-ion mode. Samples were delivered into the source at a flow rate of 5 μl min^{-1} using a syringe pump (Harvard Apparatus, South Natick, MA). A potential of 2.5 kV was applied to the electrospray needle. The sample cone was kept at an average of 15 V and the counter electrode, skimmer, and RF lens potentials were tuned to maximize the ion beam for a given solvent. Argon was used as a collision gas during daughter analysis. The source temperature was kept constant at 80°C. The instrument was calibrated using a polyethylene glycol solution. All spectra represented an average of 10–15 scans.

Transformation and fate of TNT in soil slurries

Soil reactors were established at room temperature in order to examine the effects of aeration on TNT biodegradation in soil slurries. Two studies using different reaction vessels were performed after the first system failed to produce optimal redox conditions. The fate of TNT and glucose was followed during each study.

In the first study, 15 g of an air-dried, munitions-contaminated soil from the Iowa Army Munitions Plant (Burlington, IA) were placed into 150-ml or 120-ml crimp-top serum bottles. The soil contained approximately 600 mg kg^{-1} TNT. The first set of bottles containing air-dried soil received ^{14}C-glucose in water, and the second set received ^{14}C–TNT in acetone at an activity of 5.0×10^6 dpm (disintegrations per minute) per 15 g soil. Soil slurry conditions were established by bringing the total slurry volume to 100 ml with sterile water augmented with molasses (600 g l^{-1}) as a carbon source and NH_4Cl (100 mg l^{-1}) as a nitrogen source.

Three treatment conditions were utilized: aerated, semi-aerated, and anaerobic. Each treatment contained two sets of triplicate bottles. Aerated bottles were sparged with compressed gas (air) for 2 h per day and continuously shaken at 200 rpm using an orbital shaker. Slightly smaller 120-ml bottles were used in the semi-aerated treatment; the bottles were sparged with compressed gas (air) for 10 min per day and continuously shaken at 100 rpm on an orbital shaker. Anaerobic bottles were sparged with N_2 for 30 min and left static. The anaerobic bottles received an initial headspace of N_2. Treatments were incubated in the dark to minimize light-induced reactions. Each soil slurry received a 1-ml inoculum of a pre-grown anaerobic soil slurry to ensure similar initial conditions. The inoculum culture had been growing on starch in the presence of soil containing nitroaromatic compounds with continuous transfers for about 1 year. The redox potential of this culture was less than –550 mV as measured by a standard electrode. Stoppered 250-ml flasks were run in tandem without added radiolabeled compounds under the same conditions for the purpose of tracking the redox conditions throughout the experiment. Redox was measured using a standard oxidation-reduction potential (ORP) electrode (Microelectrodes, Inc., Bedford, NH).

Headspace samples were captured by flushing the bottles with the appropriate compressed gas (see above). The gases flowed through a series of four liquid traps; two of the traps captured CO_2 and two captured volatile organics. Carbon dioxide was trapped in Carbosorb (Packard Instruments, Meriden, CT). One-ml aliquots of Carbosorb were added to 15 ml of Biosafe scintillation cocktail (Research Products International, Inc., Mount Prospect, IL). Volatile organics were captured in Ready Organics scintillation cocktail (Beckman Instruments, Inc., Fullerton, CA). All samples were analyzed with a Tri-carb 2100TR scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL).

Aliquots of 1.5 ml were taken at each sampling time and analyzed by HPLC for TNT, TNT metabolites, and organic acids using the methods described in the previous section. At the final sampling time, two additional 1.5-ml aliquots were taken for further analysis. At the conclusion of the experiments, 2–5 g soil samples were taken from each reactor and air dried at room temperature for 24 h. One set of samples was extracted successively with acetonitrile three times with a solvent to soil ratio of 4:1. The acetonitrile extract was then analyzed by HPLC for TNT and TNT metabolites as discussed in the previous section. One ml of the acetonitrile was added to scintillation cocktail (see above), and radioactivity was measured using a scintillation counter. One gram of the other soil samples was carefully transferred to ceramic cups for combustion analysis. Each cup was placed into quartz tubes, which were 1/3 packed with Cu mesh. A flow rate of about 60 ml min^{-1} of compressed O_2 was established in the tubes. The tubes were placed in a Sola basic furnace (Lindberg, Watertown, WI) and subjected to a temperature of 600°C for approximately 3 h to completely combust any organic materials residing in the soil pellet. Oxygen passing over the cups of soil carried combusted materials through the Cu where any organics were completely oxidized to CO_2. Carbon dioxide eluted from the quartz tubes was captured in

10 ml Carbosorb. One ml of the Carbosorb was analyzed by liquid scintillation counting as discussed previously. After daily sampling for 30 d, it was determined that no TNT or metabolites were present. The study continued with weekly sampling for 162 d.

After data were analyzed from the first study, experiments were performed to determine an optimized medium and container for soil slurry reactors in order to achieve lower redox values in the anaerobic reactors. Redox readings were slightly higher in the upper 1–2 cm of the aerated microcosms but dropped to lower values immediately below this zone (data not shown). Readings were taken 1–2 cm above the soil–water interface. From these optimization trials, a second study was performed using slightly modified conditions. Reactors were established in 500-ml Erlenmeyer flasks. Due to inconsistent redox values from the first experiment, a mineral salts (MSM) medium was employed and contained the following per liter at pH 7.2: 13 g K_2HPO_4, 4 g KH_2PO_4, 0.1 g NH_4Cl, 2 ml $MgSO_4$ (1 M), and 6 g molasses (Grandma's® unsulfured). The medium was added to 100-g soil samples bringing the volume in the flasks to 400 ml. A preconditioned (see above) soil inoculum of 0.2% (w/v) was added to the flasks. The aeration treatments (sparging time, shaking, and temperature) were the same as in the first study except that the headspace gases were not captured in liquid traps during flushing. Hanging cups filled with 1-N NaOH were used to continuously capture CO_2. The NaOH samples were added to Biosafe scintillation cocktail and analyzed with a liquid scintillation counter. Volatile organic compounds were not captured. Oxidation-reduction potential was measured directly in the flasks. Sampling occurred daily for 30 d after which redox and the NaOH traps were sampled weekly for 300 d.

A NaOH extraction of humic acids modified from Swift (1996) was used in the second study to examine the radiolabel associated with the organic fraction. Five gram portions of soil were air dried overnight at room temperature. The air-dried soil was added to 50-ml Falcon tubes with 25 ml of 0.5-N NaOH solution. The samples were mixed gently on a rocking mixer for 2 h (Swift 1996). The soil was then pelleted by centrifugation and extracted three more times with NaOH. One milliliter of each extract was analyzed by liquid scintillation counting as above.

Analysis of TNT transformation in reactors without soil

Reactors were established in 500-ml Erlenmeyer flasks, but soil was not added. Three reactors contained no carbon source for a control, three reactors received molasses as a carbon source, and three reactors received a mixture of sucrose, glucose, and fructose. Sugars were added in amounts that would be found in molasses, and TNT was added at a level of 120 mg l^{-1} to the reactors. Two separate reactors containing molasses as a carbon source received approximately 250,000 dpm ^{14}C-TNT. Samples were taken and analyzed by HPLC and electrospray ionization mass spectrometry (ESI-MS-MS). Total radioactivity was measured as above. All of the reactors were left static but were mixed gently by hand for 30 s each day, and all were left open to the air.

Enrichment and isolation of aerobic and anaerobic 2,3-butanediol degraders

Enrichments for 2,3-butanediol-degrading bacteria were performed using 1-kg samples of a Westlake–Latahco silt loam (cumulic ultic haploxeroll), a Latahco silt loam (argiaquic xeric argialboll), and a garden-variety soil. The Westlake–Latahco silt loam and the Latahco silt loam were most recently planted with pea and wheat crops, respectively. Soils from two contaminated sites were also used for enrichments. The contaminated soils were collected from the Burlington Iowa site and from the Nebraska Ordinance Plant (Mead, NE). The soils contained approximately 575 mg kg^{-1} TNT. Two 100-g subsamples of each soil were placed into 500-ml Erlenmeyer flasks. Enrichments were initiated by the addition of 250 ml of sterile MSM medium at pH 7.0 containing 10 g l^{-1} 2,3-butanediol as a carbon source. Per liter of medium, the MS medium consisted of: 4.25 g $K_2HPO_4 \cdot 3H_2O$; 1.0 g $NaH_2PO_4 \cdot 3H_2O$; 2.0 g NH_4Cl; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.012 g $FeSO_4 \cdot 7H_2O$; 0.0003 g $MnSO_4 \cdot H_2O$;

0.003 g $ZnSO_4$; 0.001 g $CoSO_4$; and 0.2 g nitroacetic acid. The aerobic enrichment flasks were shaken at 150 rpm at room temperature; the anaerobic enrichment flasks were left static. One milliliter aliquots were taken daily to analyze for the disappearance of 2,3-butanediol using liquid chromatography (LC), as described above.

After 2 weeks of shaking, a 0.1-ml aliquot of each aerobic enrichment was transferred to 5 ml of fresh 2,3-butanediol/MS media. Enrichments that were positive for 2,3-butanediol degradation in the fresh media were streaked for isolation of pure cultures on basal salts agar containing 2,3-butanediol as the sole carbon source and ammonium as a nitrogen source. Isolated colonies were checked for purity by streaking on tryptic soy agar (TSA). Isolated organisms were then transferred to fresh 2,3-butanediol/MS media, and the supernatant was analyzed over time by LC to confirm the disappearance of 2,3-butanediol.

Flasks containing the enrichment medium (above) were flushed with N_2, stoppered, and allowed to incubate under anaerobic conditions at room temperature. Aliquots of 0.1 ml were taken from anaerobic enrichments that were positive for 2,3-butanediol degradation and placed into 5 ml of fresh sulfide-reduced anaerobic medium. The medium was similar to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) medium 293 and consisted of (per liter of medium): 0.2 g $K_2HPO_4{\cdot}3H_2O$; 0.25 g NH_4Cl; 2 g NaCl; 3 g $MgCl_2{\cdot}6H_2O$; 0.5 g KCl; 0.15 g $CaCl_2$; 1 ml of a trace element solution (Widdel and Pfennig 1981); and 0.36 g Na_2S. Subcultures showing growth were streaked for isolation of pure cultures anaerobically on brain-heart infusion (BHI) agar. Isolated colonies were placed back into 5 ml of fresh anaerobic medium and incubated at 30°C. Samples were analyzed over time by LC for the disappearance of 2,3-butanediol accompanying growth.

Results and discussion

Fate and degradation of ^{14}C-glucose in soil slurry systems under various aeration conditions

To determine if the carbons in the organic acids were derived from sugars or from TNT, ^{14}C radiolabeled glucose was used in soil slurries. Mineralization rates of glucose were significantly higher in the aerated soil slurries. At 13 d, 45.5% of the original glucose was captured as CO_2 (data not shown). By the end of 162 d, 64.7% of the glucose was mineralized (Table 1). The anaerobic slurries initially exhibited a greater glucose mineralization rate as compared to other treatments, but by the end of the experiment, the two systems were not significantly different (Fig. 1). After 162 d, 22.1% of the added glucose was mineralized in the semi-aerated slurries and 25.3% in the anaerobic slurries (Table 1).

The accumulation of volatile compounds other than CO_2 accounted for less than 3% of the total glucose added. The aerated slurries converted 1% of the total glucose into volatile compounds, while the semi-aerated and anaerobic slurries converted 2.1% (Table 1).

Table 1 Summary of the results from the first radiolabeled soil treatment study after 162 d of incubation

Treatment[a]	CO_2 (%)	Volatile compounds (%)	Soluble (%)	Reversibly sorbed (%)	Irreversibly sorbed (%)	Total recovery (%)	(±) (%)
Aer Glu	64.7	1.0	2.0	0.8	30.1	99.0	5.3
Semi Glu	22.1	2.1	11.9	3.3	61.0	100.4	5.4
Anaer Glu	25.3	2.1	17.6	6.5	51.9	103.4	2.7
Aer TNT	0.2	0.0	0.9	1.6	95.7	98.5	1.4
Semi TNT	0.4	0.1	15.4	33.3	49.2	98.4	5.0
Anaer TNT	0.2	0.2	1.1	1.3	96.9	99.7	2.0

[a] Abbreviations used: Aer, aerated; Semi, semi-aerated; Anaer, anaerobic; Glu, radiolabeled glucose added; TNT, radiolabeled TNT added; ±, standard deviation of the mean of three replicate treatments

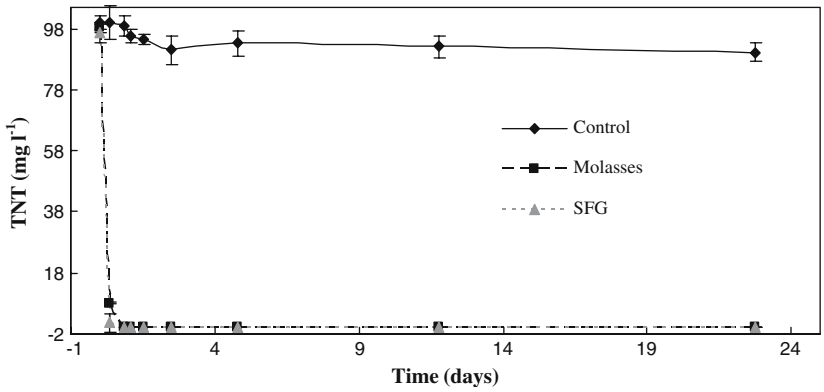

Fig. 1 TNT concentration over time in an anaerobic reactor with no added soil. Control reactors received no carbon source while the others received molasses or a mixture of sucrose, glucose, and fructose (equal to that found in molasses; see Materials and methods). Error bars represent one standard deviation of the mean of three replicates

Compounds extractable by acetonitrile were considered to be noncovalently bound to the soil-humus matrix. The anaerobic treatment at 8.3% produced the largest noncovalently bound fraction, while the semi-aerobic produced 4.3% and the aerated 1.1%. Compounds not extractable by acetonitrile were considered covalently bound to the soil-humus matrix (Achtnich et al. 1999a). The covalently bound fraction was determined by subtracting the dpm of the acetonitrile extraction from the dpm counted during combustion analysis. The semi-aerated treatment produced the highest amount of unextractable compounds that originated from glucose (61.0%). The anaerobic and aerated treatments maintained tightly sorbed products from glucose at 51.9% and 30.1%, respectively (Table 1).

Radiolabeled compounds were found in the soluble fraction of the soil slurries. In the aerated reactors, 2% of the total radiolabeled glucose added was in the soluble fraction (Table 1). HPLC analysis of the soluble fraction throughout the time course of this experiment confirmed that soluble radioactive organic acids did not accumulate in the aerated treatments. In the semi-aerated treatment, 11.9% of the total radiolabel added existed in the soluble form (Table 1). HPLC analysis confirmed the accumulation of radiolabeled acetic and butyric acid in these slurries throughout the course of the experiment. In the anaerobic treatment, the soluble components accounted for 17.6% of the total radiolabel added (Table 1). HPLC analysis showed similar results to those observed for the semi-aerated treatment in that radiolabeled acetic and butyric acid were present in the soluble fraction throughout the experiment. Anaerobic reactors contained radiolabeled propionic acid in addition to acetic and butyric acid. In summary, during study one HPLC/radioactivity analysis showed that propionic, butyric, and acetic acids were present in the soil slurries and resulted from glucose metabolism.

Results from the second study are listed in Table 2. In general, trends were similar to the first study, but a few notable differences did occur. The majority of the label (>85%) was evolved as CO_2 in all of the treatments. The rate of CO_2 evolution occurred at a fairly steady rate over the course of the experiment (data not shown).

Significance of organic acids and diols during treatment

Since the transient accumulation of organic acids commonly associated with bacterial fermentations was observed, it is conceivable that these compounds could have served as substrates for anaerobic populations such as methanogens. Since 2,3-butanediol, another commonly produced bacterial carbohydrate fermentation product (Biebl et al. 1998; Jansen and Tsao 1983), was not detected in any of the treatments, enrichments were used to isolate bacteria that could

Table 2 Summary of the results from the second radiolabeled soil treatment study after 300 d of incubation

Treatment[a]	CO_2 (%)	Soluble (%)	Acetonitrile extract (%)	NaOH extract (%)	Total recovery (%)	(±) (%)
Aer Glu	91.7	4.1	1.9	1.7	99.4	1.9
Semi Glu	85.8	14.2	2.3	2.5	104.8	5.8
Anaer Glu	80.5	11.9	1.9	4.2	98.5	2.6
Aer TNT	2.6	10.0	7.2	70.2	90.0	3.7
Semi TNT	0.5	17.2	15.5	58.5	91.7	2.6
Anaer TNT	0.4	23.0	21.1	50.4	95.0	3.4

[a] Abbreviations used: Aer, aerated; Semi, semi-aerated; Anaer, anaerobic; Glu, radiolabeled glucose added; TNT, radiolabeled TNT added; ±, standard deviation of the mean of three replicate treatments

utilize this compound. All aerobic soil enrichments were positive for 2,3-butanediol degradation after the 2-week incubation time. When 0.1-ml aliquots of the enrichment slurries were transferred to 5 ml of fresh medium, 2,3-butanediol was not detectable within 24 h via LC analysis. When samples were streaked onto 2,3-butanediol/MSM agar, at least two colony types were isolated from each soil enrichment. After the colonies were checked for purity by streaking onto TSA plates, they were inoculated into 5 ml of fresh 2,3-butanediol/MSM medium and analyzed over 24 h by LC for disappearance of 2,3-butanediol. All isolates from the aerobic soil enrichment were able to degrade 2,3-butanediol within 24 h.

The enrichment for anaerobic organisms capable of growing in fresh medium with 2,3-butanediol as the only carbon source was also examined. The pea field soil (Westlake–Latahco silt loam) enrichment produced three different colony types when plated onto anaerobic BHI plates. The TNT-contaminated soil from the Iowa Munitions Plant produced two colony types on BHI plates. When placed into fresh anaerobic 2,3-butanediol/MSM media, each of these colonies showed growth within 3 d.

During the entire experiment, 2,3-butanediol did not accumulate and certainly was not detected as a metabolite of TNT degradation as has been reported in other research (Boopathy et al. 1998). Other organic acids were detected but were associated with byproducts of glucose degradation. Aerobic 2,3-butanediol degraders are ubiquitous in the soils examined, and aerobic enrichments for the bacteria were highly successful. Anaerobic degraders of 2,3-butanediol were isolated but were not as ubiquitous as the aerobic degraders. The results from the enrichment study show that 2,3-butanediol would most likely be degraded in any reactor system containing soil.

Fate and degradation of TNT in soil slurry systems under various aeration conditions

The resistance of TNT to degradation is due in part to its biological toxicity. The electrophilic nature of the nitro group causes TNT to readily oxidize biological reductants, causing toxicity directly or by formation of other reactive products such as nitroarene radicals (Mason and Josephy 1985). In addition, the nitro groups draw electrons from the aromatic π bonds, effectively reducing the electron density of the conjugated aromatic system. As a result, TNT is resistant to degradation via electrophilic attack by oxygenase (Rieger and Knackmuss 1995; Vorbeck et al. 1994, 1998). Thus, in order for TNT to be mineralized, organisms must first remove or transform the nitro groups. Rapid reduction at low redox potentials and minimization of oxidative polymerization reactions due to the absence of oxygen may allow more time for complete reduction of TNT to TAT and for cleavage of the aromatic ring (Lewis et al. 1997). TAT has been described as a metabolite in anaerobic enrichments (Funk et al. 1993, 1995; Hwang et al. 2000; Preuss et al. 1993). These results suggested that highly reductive conditions may be optimal for the destruction of TNT; however, the ultimate fate of the metabolites and especially of TAT is still in question.

TNT and its metabolites were not mineralized or volatilized at a rapid rate in any of the slurries

during either study (Tables 1 and 2). In the first study, CO_2 fractions represented less than 0.5% of the total radioactivity. In study two, 2.6% of the radiolabel was captured as CO_2 in the aerated treatments, while in the other two treatments CO_2 represented less than 0.5% (Tables 1 and 2). When soils were extracted with an organic solvent, most of the radiolabeled components in these slurries were tightly bound to the soil and humic acids indicated as the covalently bound fraction in Tables 1 and 2. In the aerated treatment, 95.7% and in the anaerobic treatment 96.9% of the total added ^{14}C was found in the tightly bound fraction (Table 1). The semi-aerated treatment maintained the greatest amount (33.3%) of the total radiolabel in the noncovalently bound fraction. HPLC analysis of the acetonitrile extract revealed that aminodinitrotoluene (ADNT) made up a significant portion of the noncovalently bound compounds. 2,4-DA-6-NT was also present in the acetonitrile extract but in a lesser amount than ADNT. In the second study, the majority of the label was associated with the humic acid fraction. In the aerated treatment, 70.2% of the label was bound to the humics, while in the semi-aerated and the anaerobic treatments, 58.5% and 50.4% were bound, respectively (Table 2).

In the first study, soluble radiolabeled compounds were found in significant amounts in the semi-aerated treatment, but in the second study the components were present in all the treatments (Tables 1 and 2). During the first study, HPLC analysis did not show a significant accumulation of radiolabeled organic acids; however, this soluble fraction contained radiolabeled 4-A-2,6-DNT and 2,4-DA-6-NT.

Conventional activated sludge and bioslurry systems have been used to treat TNT-contaminated soils and waters. Carpenter et al. found that after 3–5 d of incubation in an aerated reactor, no significant amount of ^{14}C–TNT was detectable (Carpenter et al. 1978). The ^{14}C was determined to be associated with macromolecular structures of the polyamide type, which precipitated in the lipid and protein components of the microbial flora. They suggested that partially reduced metabolites of TNT reacted with the organic components of the reactor. In evaluating the fate of ^{14}C–TNT in soil slurry conditions, Schmelling et al. determined that reduced products of TNT were tightly associated with the soil fraction of the slurries, and $^{14}CO_2$ was not readily evolved (Schmelling et al. 1998). Likewise, Kreslavski et al. found that rhizosphere soils catalyzed a more rapid transformation of TNT and formed a more unextractable bound residue than control soils (Kreslavski et al. 1999). Even if the metabolites were bound tightly to the soil matrix during these experiments, they may biodegrade and so their long-term fate remains unclear.

Fate of TNT in reactors without added soil

To determine the main transformation routes without interference from soil, reactors were established with no added soil. The transformation of TNT in reactors that did not contain added soil proceeded as in reactors with soil (Figs. 1, 2, 3). However, the amounts of ADNT and 2,4-DA-6-NT detected in reactors without added soil (Fig. 3) were much greater than in reactors with soil (Fig. 4). Most of the radiolabel (95%) remained in the soluble fraction. When samples were centrifuged to remove cells from the supernatant, 52% of the label remained. This result suggests that the label was bound to cellular material. ESI-MS-MS analysis confirmed the degradation of TNT in the reactors. However, analysis of the fate of metabolites proved difficult and inconclusive even in the reactors containing a defined medium. This result confirms that soil plays a significant role in the removal of TNT metabolites from the aqueous fraction of reactors.

The pattern of TNT transformation in anaerobic systems has been studied extensively. The metabolites are well known and have been the subject of numerous reviews (Bruce 1998; Hawari et al. 1998, 2000a, b; Lewis et al. 1997, 2004; Preuss and Rieger 1995; Rieger and Knackmuss 1995). Until the last decade, the behavior and fate of TNT metabolites in soils treated by anaerobic biological systems was in question. Researchers have recently determined that intermediates in the transformation of TNT under anaerobic conditions interact and bind with soil humic materials (Achtnich et al. 1999a, b; Daun et al.

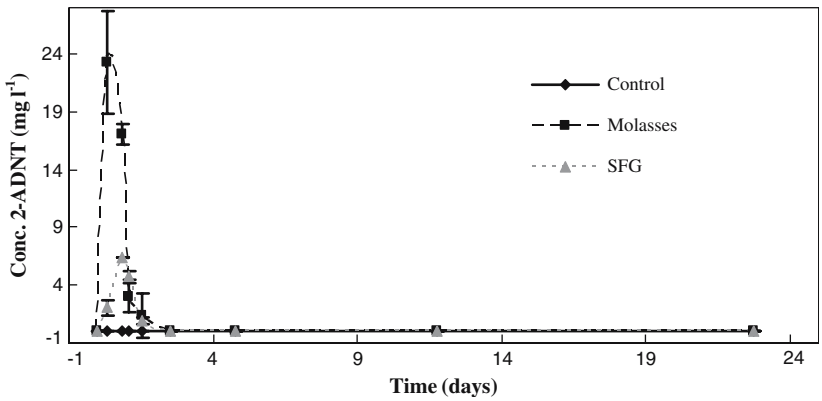

Fig. 2 Concentration of aminodinitrotoluenes over time in anaerobic reactor with no added soil. Control reactors received no carbon source while the others received molasses or a mixture of sucrose, glucose, and fructose (equal to that found in molasses; see materials and methods). Error bars represent one standard deviation of the mean of three replicates

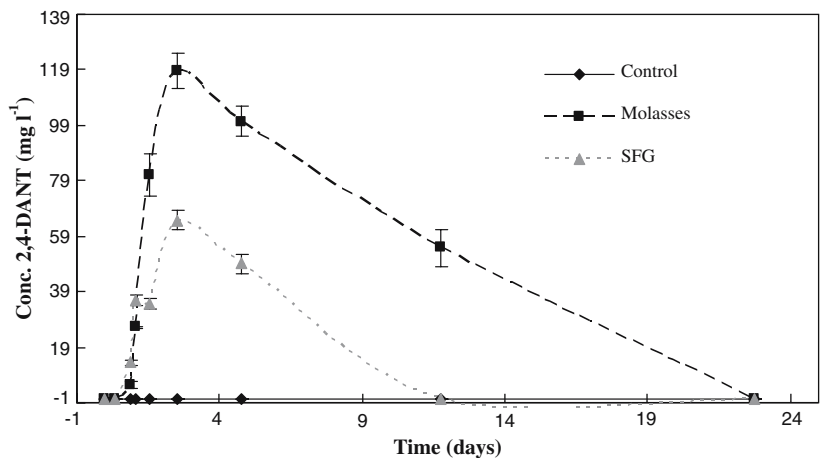

Fig. 3 Concentration of 2,4-DA-6-NT over time in anaerobic reactor with no added soil. Control reactors received no carbon source while the others received molasses or a mixture of sucrose, glucose, and fructose. Error bars represent one standard deviation of the mean of three replicates

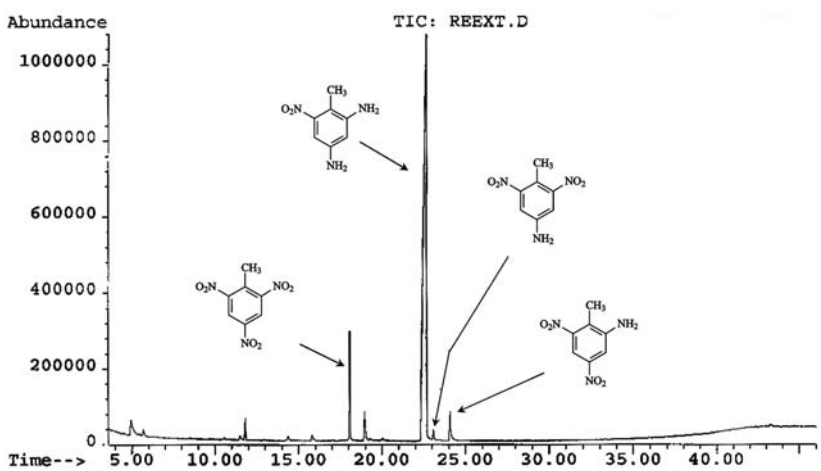

Fig. 4 GC-MS total ion chromatogram (TIC). The structures of the compounds are shown with arrows pointing to their corresponding peaks. This sample was extracted on day 9 from reactors containing soil. The approximate amounts of each compound in mg l^{-1}: TNT ~4; 4ADNT <1; 2ADNT <1; 2,4-DANT ~19

1998; Knicker et al. 2001; Lenke et al. 1998). Furthermore, these researchers have determined that if oxygen is introduced after anaerobic treatment, then metabolites bind more tightly to soil and soil organic matter in the long-term and even under severe leaching conditions (Achtnich et al. 1999a, b, 2000; Achtnich and Lenke 2001; Knicker et al. 2001).

This research examined the effect of aeration on the fate of TNT in biological reactors. In addition, the research examined whether organic acids or diols originate from TNT or glucose under the various aeration schemes. Study one showed that the starting conditions and aeration of soil slurries had little effect on the fate of TNT during biological treatment. HPLC analysis revealed that in all the treatments the degradation of TNT proceeded through ADNT to 2,4-DA-6-NT (Figs. 1–3). Radiolabeled TAT was not detected in any of the treatments. Since the formation of TAT only occurs under strict anaerobic conditions (Lewis et al. 1997; McCormick et al. 1976; Preuss et al. 1993), this result was not surprising for the aerated or the semi-aerated treatments. TAT has been shown to accumulate only under strict anaerobic conditions in previous experiments (Funk et al. 1993; Hawari et al. 1998; Preuss et al. 1993; Rieger and Knackmuss 1995). Recent studies on the binding of TNT during anaerobic treatment show that the less-reduced metabolites may bind tightly to soil and humic acids, explaining the lack of TAT in our system (Achtnich et al. 1999a, b). The fate of TNT during this study was similar to these previous results, with metabolites bound tightly to soil and soil organic matter (Table 1). The semi-aerated treatment did produce an anomalous result when <50% of the label was tightly bound to the soil. Analysis of the redox conditions in these reactors revealed that the potential in the semi-aerated treatment did not drop as low nor sustain values as low as the other reactors. The aerated treatment resulted in a more rapid transformation rate of TNT to 2,4-DA-6-NT than with the other two treatments (data not shown). Furthermore, 2,4-DA-6-NT disappeared from the soluble phase more rapidly than in the other two treatments. It has been shown previously that aerobic processes can carry out limited transformations of TNT to 2,4-DA-6-NT under aerobic conditions in soils (Boopathy et al. 1994; Bradley et al. 1994; Kaplan and Kaplan 1982; Pennington et al. 1995). However, the rate of transformation to these products is usually slower than anaerobic processes (Funk et al. 1993). Additionally, in the semi-aerated treatment, radiolabeled ADNT and 2,4-DA-6-NT persisted in the soluble phase longer than in the other two treatments as shown by HPLC analysis. This could indicate that the conditions in this treatment did not allow establishment of an active microbial community, a hypothesis supported by the fact that of the added radiolabel, 15.4% was in the soluble phase and 33.3% was extractable by acetonitrile during the semi-aerated treatment.

The second study was set up to take advantage of a slurry system optimized for low oxidation-reduction potentials in the anaerobic treatment. The results were similar to the first study with a few notable differences. Glucose was mineralized to a significant extent in all of the treatments indicating the presence of a robust microbial community. In the aerated treatments, most of the radiolabel was associated with the organic matter fraction (Table 2). This result correlates with previously published results showing that an aerobic polishing step following anaerobic treatment resulted in the binding of TNT metabolites tightly to the soil (Achtnich et al. 1999a, b; Achtnich and Lenke 2001; Knicker et al. 2001). Thus in lagoons or other treatment systems that are open to the air, a sequential aerobic step may not be necessary.

It is important to note that the lowest sustained redox values were observed in the aerated treatments, a result that was reproduced in all experiments. In fact, when a rich nutrient such as molasses was added to treatments, oxic conditions were unachievable initially and only returned to semi-oxic after approximately day 50 (Fig. 5). After these time periods, all of the TNT had been transformed and metabolites were no longer observed (Figs. 1–3). Implications for the full-scale Fermentative Anaerobic Soil Treatment (FAST^{TM}) process or any lagoon system is that rapid reduction of TNT does occur while metabolites bind tightly to the soil and humic materials.

Metabolite analysis of reactors without soil proceeded through the typical reductive pathway (Figs. 1–3). ESI-MS-MS analysis of the liquid failed to show any significant accumulation of condensation products from TNT metabolites and other organic compounds. In this system it appeared that cellular material bound a significant amount of the metabolites. This binding

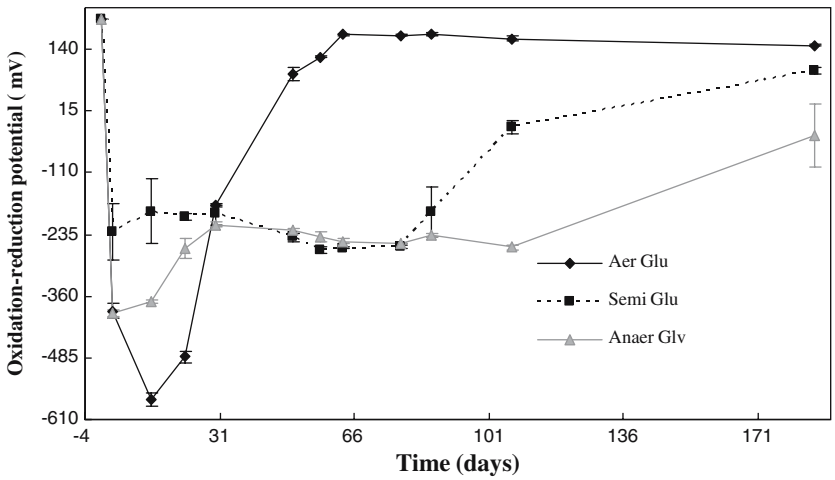

Fig. 5 Oxidation-reduction potential over time during the second study. Aer = aerated, Semi = semi-aerated, Anaer = anaerobic. Glu indicates measurements taken from reactors that received radiolabeled glucose. Error bars represent the standard deviation of the mean of three replicates

may have decreased concentrations of metabolic products to the extent that they could not be resolved with ESI-MS-MS directly. Future work should include extraction techniques to concentrate potential metabolic products or use of alternate analytical methods to detect the more labile metabolites (Maeda et al. 2006).

This work demonstrates that aeration does affect the fate of TNT in reactor systems that receive a rich nutrient source. Recently researchers observed that oxygen concentrations drop to near-zero values in slurry reactors where air was delivered by a diffuser when exogenous carbon was added to the system (Park et al. 2003). The lowest redox values observed during these experiments were associated with reactors that maintained an aerobic headspace. The data collected during the current studies agree with previous work in that the fate of TNT metabolites in an anaerobic soil slurry system is closely regulated by soil and soil organic matter (Achtnich et al. 1999a, b; Achtnich and Lenke 2001; Hawari et al. 2000a; Knicker et al. 2001; Lenke et al. 2000). While various aeration regimes did not affect the fate of TNT metabolites in this system, they do appear to affect the rate at which metabolites covalently bind to the soils.

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