E-20-L03
#1

Title: Laboratory Investigation of biological strategies for destruction and decontamination of organic contaminants at Air Force and DoD sites

Problem: Manufacturing, testing and training activities at Air Force and Department of Defense (DoD) sites has led to the contamination of soil and groundwater at industrial sites and bases with chlorinated solvents and the contamination of training ranges with munitions components. Additionally, with the implementation of new defense technologies designed to maintain global competitiveness comes the risks associated with emerging contaminants.

Background: Over the last 20 years significant advances have been made in the use of Monitored Natural Attenuation (MNA), biostimulation and bioaugmentation for the remediation of contaminated sites, particularly with regard to chlorinated solvents, explosives and propellants. MNA and enhanced *in situ* bioremediation remedies hold the promise of reducing the costs associated with the remediation of DoD sites impacted by chlorinated solvents. However, there are a significant number of DoD sites where tetrachloroethylene (PCE) and trichloroethylene (TCE) undergo dechlorination to *cis*-dichloroethylene (c-DCE), but where the c-DCE persists and migrates uncontrolled in the groundwater, rather than undergoing further dechlorination to ethene (the desired end product of chlorinated solvent bioremediation). We currently lack the understanding of how to bring about the complete degradation of the contaminants in such plumes. Similarly, there are gaps in our knowledge regarding the environmental fate, behavior and transport of munitions components and emerging contaminants. Our previous research under SERDP and AFOSR-sponsorship investigated the biodegradation of mono- and dinitrotoluenes in soil and ground water that was contaminated as the result of large-scale explosives manufacturing, processing, and disposal activities. Such activities gave rise to chronic contamination of sufficient concentration and duration to allow the evolution of bacteria that are able to grow on such compounds. Based on our findings of biodegradation of dinitrotoluenes at Badger Army Ammunition Plant, bioremediation systems involving both active treatment and MNA have been put into operation to clean up Propellant Burning Ground, Deterrent Burning Ground, and Spoils areas at the plant. What was not addressed in the previous studies was whether the much more heterogeneous contamination found at the training ranges is amenable to biological treatment. As of December, 2002, the Air Force had 268 operational training ranges within CONUS covering 6.42 million acres (1), while overall the DoD is responsible for 30 million acres within CONUS (7). To comply with reporting requirements to Congress with regard to civilian and environmental impacts of sustainable training ranges, DoD has begun assessments of operational ranges to determine if munitions constituents migrate off range in concentrations that are an imminent and substantial danger to human health or the environment (7).

The enormous acreage encompassed by training ranges, and the heterogeneous distribution of contaminants on the ranges distinguishes contamination of the ranges from contamination of manufacturing sites. Heterogeneity of contamination at the ranges extends in both temporal and spatial dimensions; that is, the ranges are used episodically and contamination comes from numerous point sources. Unlike the manufacturing and processing plants, most of the munitions components are destroyed during use on the ranges, and what remains where depends upon the type of munition and type of detonation (3). 2,4-Dinitrotoluene (DNT) is a major contaminant at the Army Ammunition Plants because it is an intermediate in the production of 2,4,6-trinitrotoluene (TNT), and because it was used as a deterrent to control the burn rate of single-base propellants (11). On the ranges, DNT occurs as the predominant munitions residue on the

soil surface around artillery firing points and as an occasional component at the site of low order detonations (3). Studies at military and industrial manufacturing sites with high concentrations of DNT in the soil and large ground water plumes containing DNT, have shown that disappearance of DNT from soil/water microcosms accompanied by the stoichiometric accumulation of nitrite is linked to the presence of bacteria with the ability to grow on DNT as the sole carbon and nitrogen source. Similar information is lacking for the DoD ranges. Munitions contamination at the ranges is highly heterogeneous (4) but there are significant concentrations of DNT in the surface soil at howitzer firing points where DNT is used as a deterrent. The concentrations of DNT in the subsurface and in the groundwater are negligible at such sites. It is unknown whether the heterogenous distribution of DNT at low concentrations can lead to the development of DNT-mineralizing microbial populations in soils. A second question is whether such microbial populations can degrade the DNT in surface soils and thus prevent the deeper migration of the contaminant.

This report summarizes preliminary studies to determine the presence of DNT-degrading bacteria and their potential for destruction of the contaminant in contaminated soil samples collected from DoD range sites.

Objective: The objective of this effort was to determine whether biological destruction of DNT occurs in contaminated soil at training ranges.

Materials and Methods:

Thomas Jenkins of the Army Corps of Engineers Cold Regions Research Environmental Laboratory (CRREL) collected and characterized soil samples from a variety of training and artillery ranges. Three sets of soil samples were provided by Jenkins for analysis for DNT-degradation activity. The initial set of 5 samples were archived samples that had been dried and sieved and found to contain DNT when previously analyzed by CRREL (Table 1). A second set of samples was collected from an artillery firing point at Ft. Richardson, AK (Table 2), and sent on ice by overnight courier to Georgia Institute of Technology (GaTech). A third set of samples was collected from an artillery firing point in Ft. Lewis, WA (Table 3), and sent on ice by overnight courier to GaTech.

Upon receipt, soil samples were analyzed for DNT by extracting the soil with a volume of acetonitrile equal to 5 times the mass of the soil with gentle shaking for 15 min. The acetonitrile was clarified by centrifugation then analyzed by high performance liquid chromatography (HPLC). No significant DNT was found in any soil sample, therefore all microcosms constructed with the soil samples were supplemented with DNT (100 μM). Microcosms were constructed with 10% (w/v) soil in nitrogen-free minimal medium (BLK) (2). The microcosms were incubated at room temperature (20 – 25 °C) with shaking (200 rpm). Control cultures were killed by autoclaving the soil before aseptic addition of BLK.

Plant roots dominated soil from Ft. Lewis. Soil was weighed before and after the soil was washed from the roots with a measured volume of sterile water. The plant root mass was weighed and the amount of soil in the root washings was calculated in order to produce a 10 % soil slurry for the Ft. Lewis microcosms.

DNT in microcosms was analyzed by HPLC using an HP 1100 system with diode array detector (Hewlett-Packard Co., Palo Alto, CA). The column was a 50 mm Chromolith SpeedROD RP18e (Merck KGaA, Darmstadt, Germany) and the mobile phase consisted of a mixture of part A (13.5 mM trifluoroacetic acid in water), and part B (6.75 mM trifluoroacetic acid in acetonitrile). Initial conditions were 98% A and 2% B at a flow rate of 1 ml/min for 1 min. Between 1 and 7 min a linear gradient changed the proportions of A and B to 50% A and 50% B and the flow rate to 1.5 ml/min. The final conditions were held for 1 min. Column and autosampler temperatures were 35 and 5 °C, respectively. Samples from microcosms constructed with Ft. Richardson soils sorbed significant amounts of DNT. Therefore the soil pellet was extracted with acetonitrile and the total DNT was corrected for the sorbed DNT. DNT in initial soil extracts was analyzed using a Hypercarb porous graphite column as previously described (6).

Nitrite was analyzed by standard colorimetric methods (8) or with a Dionex DX100 ion chromatograph in highly colored samples. The stationary phase was an IonPac AS14A (4 x 250 mm) column with an ASRS Ultra II membrane suppressor. The eluent consisted of 8 mM Na_2CO_3 with 1 mM $NaHCO_3$.

Most probable number (MPN) analyses were carried out on the Ft. Richardson and Ft. Lewis soil samples. Soil slurries (10% w/v) in BLK were serially diluted with 100µM 24DNT in BLK in duplicate 96-well microtiter plates and incubated at room temperature. After several weeks, samples were taken from each well and analyzed for nitrite release as a presumptive indicator of DNT degradation (5, 12).

Table 1 Characterization of first set of soils by CRREL. Concentrations are in mg/kg. 2,4-DNT values in parentheses were measured at GaTech.

Soil	Date	2,4-DNT	2,6-DNT	RDX	HMX	NG
Ft. Lewis	13 Jul 04	~40 (0)				
Pet-20	Oct 2004	~5 (1.64)				~40
Pet-2	Oct 2004	13 (2.23)	0.4	0.15		1.0
Pet-3	Oct 2004	7.0 (0.31)	0.2	2.4	0.13	0.32
Pet-4	Oct 2004	13 (0.42)	0.5	86	3.0	1.8

Table 2. Initial characterization of soils from Ft. Richardson AK.

Sample	pH	2,4-DNT
OP1	5.72	0.0
Pt. 123	5.54	0.0
Pt. 125	6.36	0.0
Pt. Crane	6.51	0.0

Table 3. Initial characterization of soils from Ft. Lewis, WA.

Sample	pH	2,4-DNT
Firing Point 1	6.12	0.0
Firing Point 2	6.62	0.0
Firing Point 3	5.52	0.0
Background	5.60	0.0

Results and Discussion:

Archived dried soils.

2,4-DNT disappeared from initial microcosms constructed with dried soils in three weeks. No disappearance occurred in the autoclaved controls.

Sample	2,4-DNT (μM), initial	2,4-DNT (μM), 3 weeks
Pet 2	84.1	0.0
Pet 3	86.3	0.0
Pet 4	84.7	0.0
Pet 20	99.2	30.8
Ft. Lewis	89.6	0.0

Five ml of each of the experimental microcosms was transferred to duplicate 250 ml shake flasks containing 100 ml of fresh medium spiked with 2,4-DNT. As before, one flask in each pair was autoclaved. 2,4-DNT was degraded in all microcosms, but the lag periods varied and the behavior after respikes was unpredictable (Figure 1). For each soil, 2,4-DNT disappearance was accompanied by stoichiometric nitrite accumulation. No 2,4-DNT degraded and no nitrite accumulated in any control flask. Traces of 2-amino-4-nitrotoluene were detected in some microcosms. The results show that 2,4-DNT disappearance is biologically mediated and that the predominant mechanism is by aerobic oxidation in which bacteria mineralize 2,4-DNT with the release of nitrite (6, 10). The traces of 2-amino-4-nitrotoluene were most likely the product of the action of non-specific nitroreductases in the 2,4-DNT-degrading bacteria or other microorganisms present in the soils. In no case was the amount of 2-amino-4-nitrotoluene significant, and it appeared to undergo no further transformation.

Samples from the transfer microcosms were serially diluted in 96-well microplates with minimal medium containing 2,4-DNT (100 μM). After 3-4 weeks incubation at room temperature, individual wells were analyzed for nitrite. The remaining contents of the wells that were positive for nitrite were spread on agar plates containing 3 mM 2,4-DNT (5). Positive wells were detected for all soil samples. Growth appeared on the selective plates after 3 weeks incubation at 30 °C.

The results showed that 2,4-DNT is biodegraded in soils from DoD ranges. Because the soils had been collected at various times and subjected to different post-collection treatments, it was not possible to project or compare *in situ* degradation rates. However it is evident that 2,4-DNT-degrading bacteria can develop in soils with a patchy 2,4-DNT distribution and low (compared to manufacturing sites) 2,4-DNT concentrations. Moreover, the rate of degradation of 2,4-DNT was much faster than the rate of nonspecific reduction. The study did not address the question of whether the presence of other explosives in the soils affects the rate or extent of 2,4-DNT biodegradation.

Fort Richardson, AK

Because degradation rates are likely to be faster in freshly collected soils, new samples from Fort Richardson, AK were shipped by overnight courier to the Georgia Institute of Technology on 20th September 2005. The soils were collected around an artillery firing point that was expected to be contaminated with 2,4-DNT. The soils consisted of fine silt with a large organic component consisting of fine plant roots.

All analyses of the Ft. Richardson microcosms were complicated by the high organic content of the soil and by precipitation when culture medium contacted acetonitrile used in the HPLC mobile phase and for pellet extraction. Analyses were highly variable from day to day but general trends are evident.

DNT disappeared in all active microcosms, but not in the killed controls (Figure 2). DNT disappeared most rapidly in the microcosm constructed with OP1 soil. On day 7, a new microcosm was started with a 10 % inoculum from the original OP1 microcosm. DNT degradation in the transfer microcosm was rapid and accompanied by nitrite release.

No nitrite accumulated in any other microcosm. Low levels of nitrate were seen in most of the microcosms after ion chromatography was adopted as the analytical method for nitrite and nitrate. High concentrations of ammonia (150 to 735 μM) were measured in the active microcosms but not in the killed controls when the microcosms were terminated. Only traces of 2-amino-4-nitrotoluene were detected in some microcosms.

MPN plates did not accumulate nitrite.

Ft. Lewis, WA

Of the initial dried soil samples, the greatest activity with 2,4-DNT was seen in the microcosms constructed with soil from Ft. Lewis. Fresh soil samples were obtained from Ft. Lewis, WA on 10 February 2006. The Ft. Lewis samples differed from all others obtained for this study by the predominance of plant material in the soil samples. Even after the washing procedure described above, a significant amount of root matter remained in the microcosms.

DNT disappeared in all active microcosms, but not in the killed controls within the first week of inocubation (Figure 3). No nitrite release was detected in any microcosm. On day 7, new microcosms were started with 10% transfers from the original microcosms (including killed controls). After varying lag periods, DNT disappeared in the majority of the transfer microcosms with stoichiometric release of nitrite. Second additions of DNT were degraded with stochiometric release of nitrite. One each of the duplicate microcosms constructed with soil from Firing Point 1 and Background failed to degrade DNT, even after extended incubation periods of 107 days.

Samples from all transfer microcosms that degraded 2,4-DNT were spread on 2,4-DNT selection plates. After 3-4 weeks colonies were picked and patched into 96-well plates with 2,4-DNT (100 μM in BLKN). After 2 weeks incubation, wells that were positive for nitrite release were spread onto 2,4-DNT plates and onto non-selective plates (1/4-strength tryptic soy agar) to test for purity. Bacteria that use 2,4-DNT as the sole carbon, nitrogen and energy source have now been isolated from each soil sample from Ft. Lewis. Representative isolates are currently being identified.

All of the MPN plates were negative for nitrite. The contents of 8 wells were analyzed by HPLC and only half of the original DNT remained and no 2-amino-4-nitrotoluene was detected. The result suggests that DNT degradation did occur, but the nitrite was somehow bound or converted by other organic or biological components of the cultures.

No nitrite was detected in any of the initial microcosms constructed with high organic content soils from Ft. Richardson or Ft. Lewis. Nitrite release was stoichiometric in the microcosms transferred to fresh nitrogen-free medium. High ammonia was measured in microcosms in which DNT was degraded with no accumulation of nitrite. Taken altogether the results suggest that the DNT-degrading bacteria use the known 2,4-DNT degradation pathway that involves initial dioxygenation and release of nitrite, but that the high organic content of the soil or the presence of (micro)organisms that use nitrite released from DNT prevents the accumulation of nitrite.

It is clear that there are DNT-degrading bacteria in soils surrounding artillery firing points on DoD ranges. The heterogeneous DNT distribution has not prevented the development of DNT-degrading bacterial populations at such sites. The different lag periods might reflect the relative abundance of DNT-degrading organisms in the soil populations or it might reflect the time it takes for the DNT-degradation pathway to be fully expressed (9). In all cases, once the DNT-degradation pathway was expressed, the rate of degradation was sufficient to destroy 100 μM 24DNT within a few days. Therefore the answer to the first question, whether heterogenous distribution of DNT at low concentrations can lead to the development of DNT-mineralizing microbial populations in soils, is an unqualified yes. The presence of such bacteria at range sites suggests that there is considerable potential for natural attenuation of DNT on the DoD ranges.

Summary:

2,4-DNT was degraded in microcosms constructed with soil samples from a variety of DoD ranges. Preliminary results show that bacteria in the soil mineralize 2,4-DNT using the known aerobic pathway for 2,4-DNT biodegradation. Live microcosms degraded 2,4-DNT accompanied by the stoichiometric accumulation of nitrite while killed controls did not. Subsamples from all microcosms in which DNT-degradation occurred have yielded bacterial isolates that grow on 2,4-DNT-selection plates. Nonspecific nitroreduction of 2,4-DNT accumulated trace amounts of 2-amino-4-nitrotoluene in the microcosms, but no further transformation of the amino compound was detected. The presence of DNT-mineralizing bacteria at a variety of DNT contaminated sites suggests that the development of DNT-degrading populations occurs even when there is considerable temporal and spatial heterogeneity of DNT distribution. The widespread presence further suggests that there is considerable potential for natural attenuation of DNT on the DoD ranges.

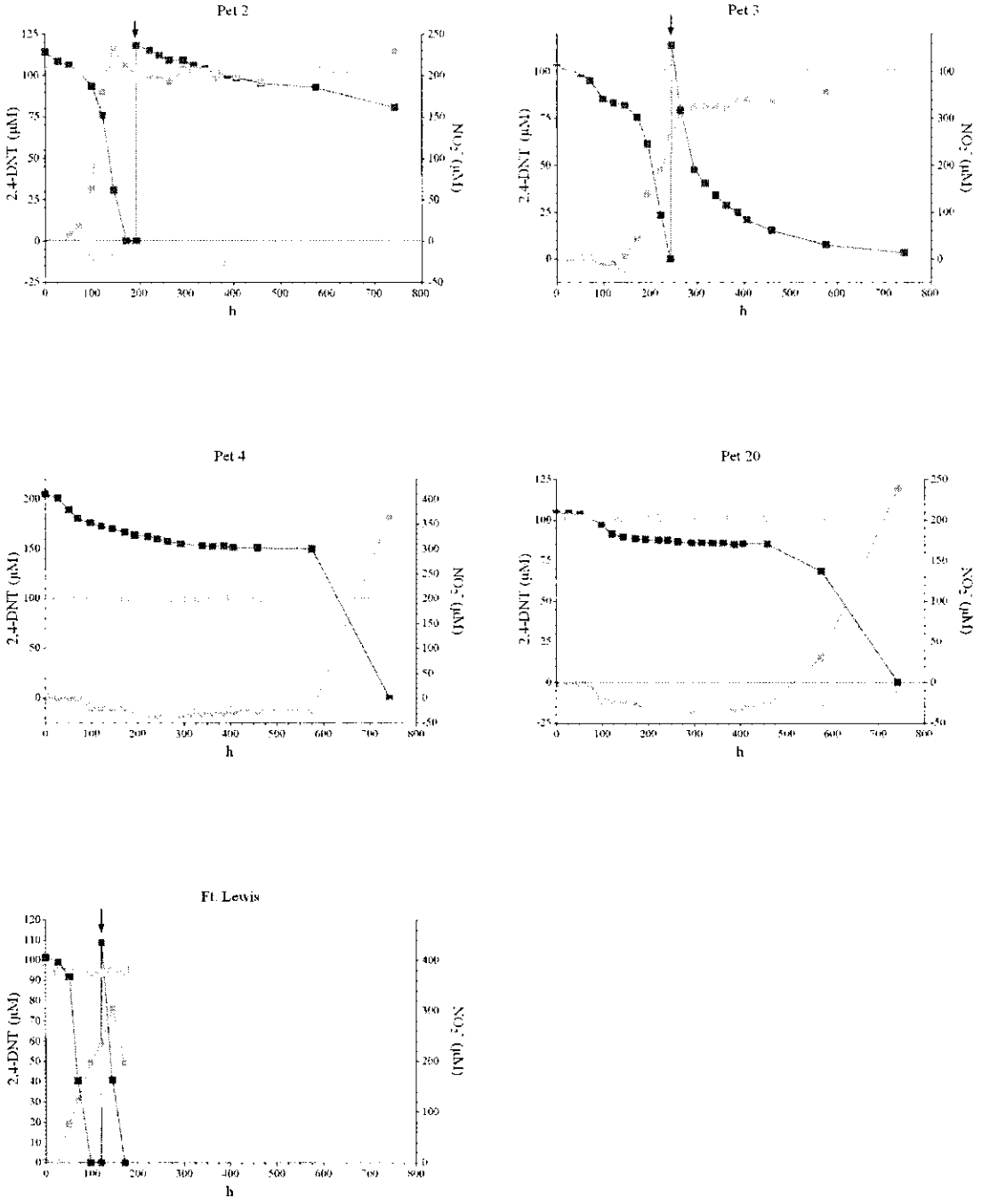

Figure 1. Degradation of 2,4-DNT in soil microcosms. Symbols: magenta squares, 2,4-DNT; green circles, NO_2^-. Solid symbols, live flasks; open symbols, killed controls. Arrows indicate respike with 2,4-DNT.

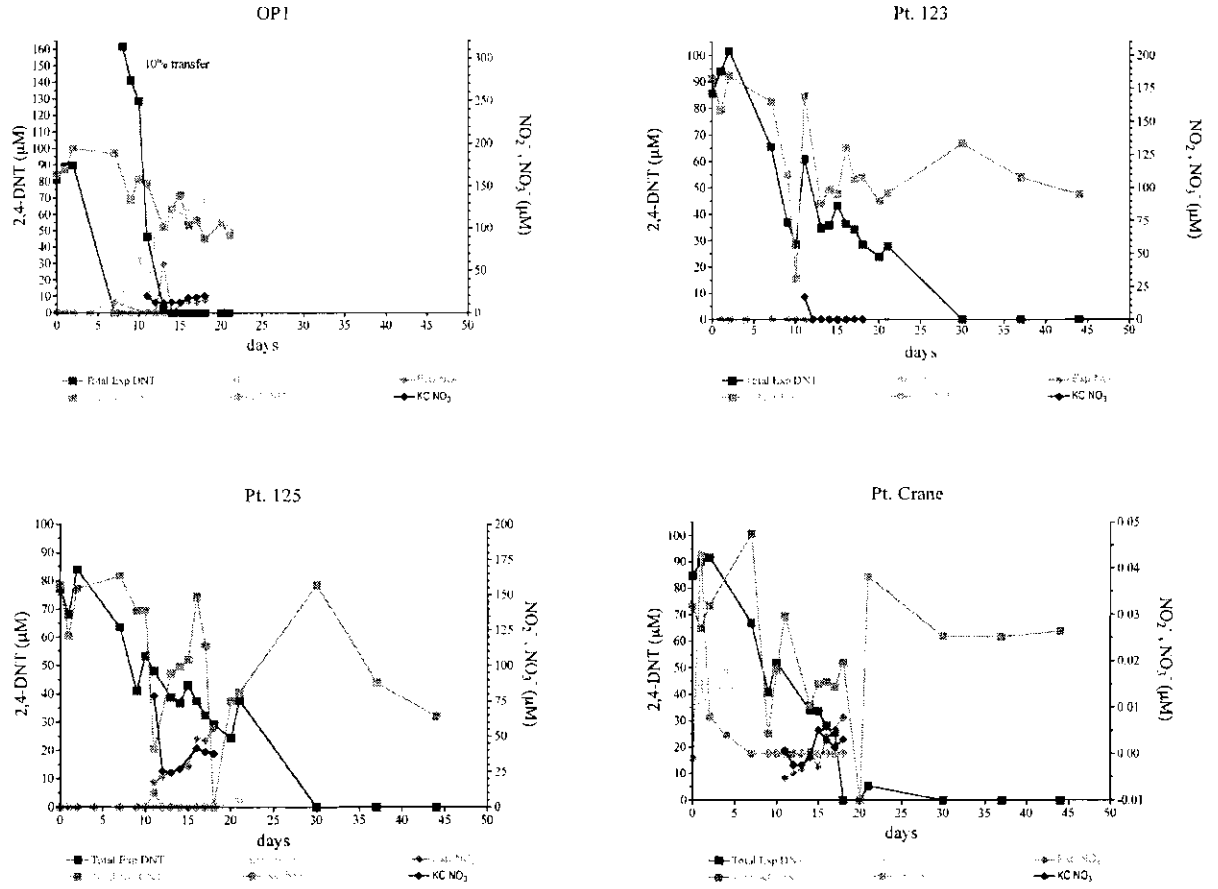

Figure 2. DNT degradation in microcosms constructed with soils from Ft. Richardson.

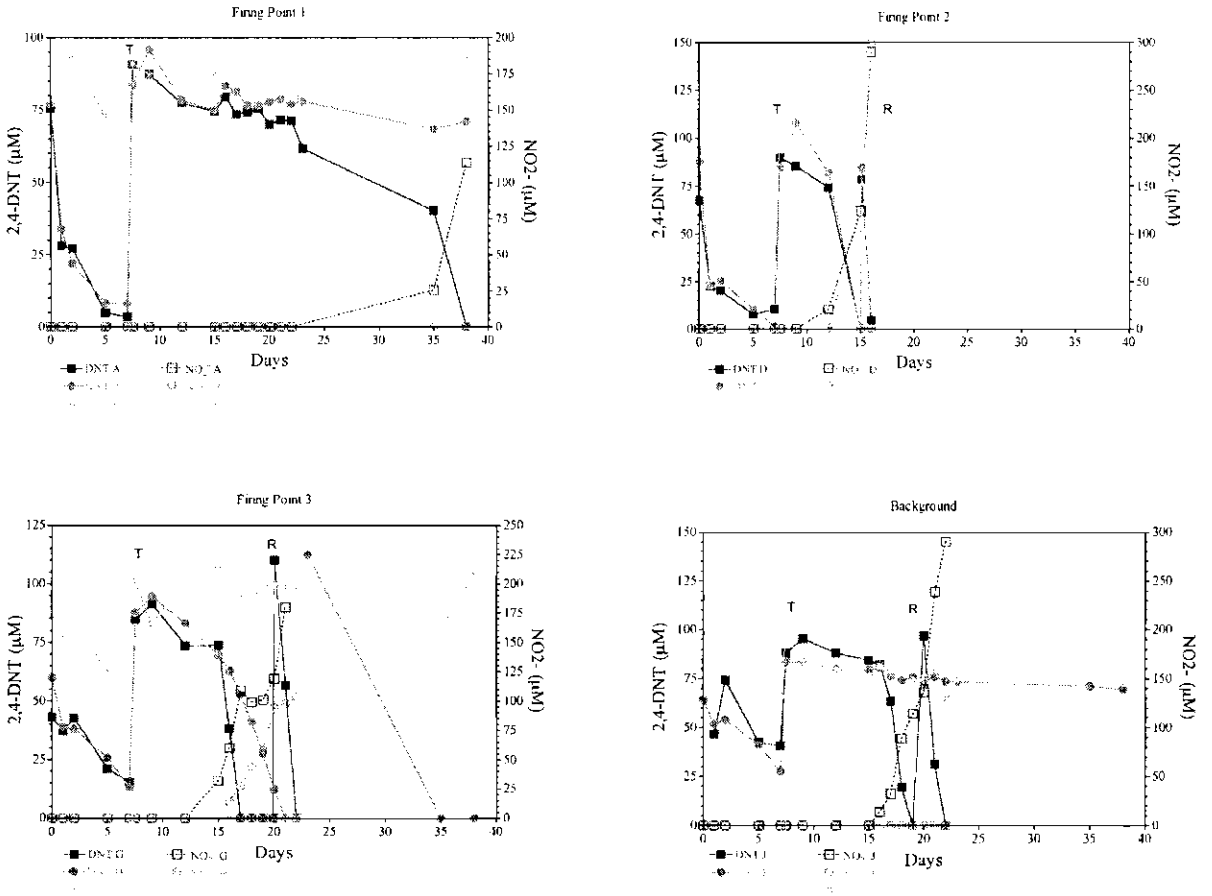

Figure 3. Degradation of DNT in microcosms constructed with soils from Ft. Lewis, WA. T = 10% transfer to new microcosm. R= respike with 2,4-DNT.

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