SOIL MICROBIAL ACTIVITY BENCHMARKS (PROVISIONAL) FOR ECOLOGICAL RISK ASSESSMENTS AT EXPLOSIVES-CONTAMINATED SITES: PROMOTING RANGE SUSTAINABILITY

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ABSTRACT

The Army Strategy for the Environment applies an ecosystem approach to managing natural resources on Army installations. It incorporates the principles of sustainability across the Army into all functional areas. We conducted investigations to develop critical data required for successful management of Army installations in a sustainable manner and for the knowledge-based decision making. Assessment and protection of the terrestrial environment at Army testing and training ranges is being advanced by developing and applying scientifically based ecotoxicological benchmarks that identify concentrations of energetic materials (EM) in soil that present an acceptable ecological risk for biologically-mediated processes in soil. Without such ecotoxicological benchmarks, the current state of knowledge concerning the nature and extent of residual contamination with EM at Army installations is insufficient to ensure management of training and testing ranges as sustainable resources. We investigated the effects of the nitrogen-based organic EM compounds 2,4-dinitrotoluene (2,4-DNT), 2-aminodinitrotoluene (2-ADNT), 4-aminodinitrotoluene (4-ADNT), and nitroglycerin (NG) on soil function endpoints, including litter decomposition and the enzymatic activity, in Sassafras sandy loam (SSL) soil individually amended with nominal EM concentrations ranging from 10 to 10000 mg/kg. Benchmark data will be made available for use in Ecological Risk Assessment of terrestrial habitats at Army testing and training sites.

1. INTRODUCTION

Sustainability is the foundation for the U.S. Army Strategy for the Environment. This Strategy directs the environmental research to address both present and future Army needs to safeguard the environment (U.S. Army ASAIE, 2004). This Strategy aims at transitioning the Army's compliance-based environmental program to a mission-oriented approach based on the principles of sustainability through the use of innovative technologies and the principles of sustainability to enhance joint operation capability, meet current and future training and testing requirements, improve the Army's ability to operate installations, reduce costs, and minimize the environmental footprint through more sustainable practices. Intensive and highly realistic training of Objective Force Soldiers across the spectrum of military operations leads to an increased release of energetic materials (EM) into the environment at both testing and training sites. The effects of many of these EM on biologically-mediated processes in soil have not previously been investigated.

Maintaining soil quality, fertility, and structure is essential for protecting and sustaining biodiversity and ecological integrity of terrestrial ecosystems. Central to achieving this goal is the need for a greatly improved understanding of the potential effects of EM contaminants on the sustainability of ecosystems at Army installations. Litter decomposition and soil enzymatic activity are among the most integrating processes within the soil ecosystem because they involve complex interactions of soil microbial, plant, and faunal activities with the soil chemical environment. Any disturbance that alters these biologically-mediated processes can result in nutrient losses and a decline in soil fertility. Therefore, an assessment of how a release of selected EMs in soil may alter rates of litter decomposition or soil enzymatic activities, and the subsequent rates of nutrient retention and release, is critical to understanding their potential impacts on the overall functioning of the soil ecosystem at Army testing and training sites.

Integral to achieving sustainable use of current and future training and testing ranges is the development of environmental quality criteria that can be consistently applied in order to gauge the ecotoxicological impacts of the Army operations. Assessment and protection of the terrestrial environment at the Army installations can be advanced by developing and applying scientifically based ecotoxicological benchmarks that can help to identify concentrations of contaminant EM in soil that present an unacceptable ecological risk for microbially-mediated processes in soil. We conducted this research to establish ecotoxicological data that are acceptable for developing such benchmarks for EMs for use in scientifically based Ecological Risk Assessment (ERA).

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2. TECHICAL APPROACH

2.1 Chemicals and Reagents

We obtained EMs 2,4-dinitrotoluene (2,4-DNT; CAS: 121-14-2; Purity: 97%), 2-aminodinitrotoluene (2-ADNT; CAS: 35572-78-2; Purity: 99%), and 4-aminodinitrotoluene (4-ADNT; CAS: 19406-51-0; Purity: 100%) from the Defense Research and Development - Valcartier (Val Bélair, QC, Canada). Nitroglycerin (NG; CAS: 55-63-0; Purity: 99.9%) was obtained from General Dynamics (Salaberry-de-Valleyfield, QC, Canada). We used certified standards of EMs (AccuStandard, New Haven, CT, USA) during all HPLC determinations. All other chemicals were either chromatography grade or reagent grade. American Society of Testing and Materials (ASTM, 2004) Type I water (ASTM, 2004) obtained using Milli-RO® 10 Plus followed by Milli-Q® PF Plus systems (Millipore®, Bedford, MA, USA) was used throughout the studies.

2.2 Test Soil Preparations

These studies were designed to assess the effects of EMs on the microbial activity endpoints in test soil, and thus required the use of freshly-collected soil with a level of microbial activity that was representative of the field conditions. A natural soil, Sassafras Sandy Loam [SSL; Fine-loamy, siliceous, mesic Typic Hapludult] (USDA/ARS, 1999) was used in these studies. The SSL soil was selected for developing ecotoxicological values protective of soil microbial activity endpoints because it has physical and chemical characteristics that support relatively high bioavailability of EM in soil (USEPA, 2005), including low organic matter and clay contents (62% sand; 25% silt; 13% clay; 2.2% organic matter; 7.8 cmol/kg cation-exchange capacity; and pH 5.0). The SSL soil was collected from a grassland field on the property of the U.S. Army Aberdeen Proving Ground (APG), MD.

During soil collection, the root zone of the upper soil layer (O and A Horizons) was retained to ensure sufficient abundance of the indigenous soil organisms. Soil was gently passed through a 5-mm sieve to remove large debris and regularize distribution of soil organisms, then stored in covered plastic containers overnight to preserve the initial field moisture level. A portion of previously collected SSL soil (designated as SSL2007d) was treated by prolonged heating (three days after constant mass was achieved) at 80°C to minimize potential introduction of organisms present in this soil to the overall biological activity (combined microbial and microinvertebrate communities) in the final soil treatments with selected EMs. This heat-treated SSL2007d soil was then sieved through a 2-mm sieve, and used to prepare soil concentrates of individual EMs.

Soil concentrates of 2,4-DNT, 2-ADNT, 4-ADNT, NG, and carrier (acetone) control were prepared using SSL2007d soil to uniformly amend fresh field-moist SSL soil (designated as SSL2007e) without harming soil organisms by exposure to the solvent (acetone). The selected target treatment concentrations were 10, 100, 1000, and 10000 mg/kg for studies with 2,4-DNT or 2-ADNT; and 100, 1000, 5000, and 10000 mg/kg for studies with 4-ADNT or NG. Amended SSL2007d soil batches were mixed for 18 h using a three-dimensional rotary soil mixer.

All final treatments of SSL2007e soil were prepared for testing one day after collecting soil in the field by individually combining and gently mixing the EM soil concentrates with clean SSL2007e soil, in separate plastic bags for each EM treatment. This approach ensured that the amount of fresh SSL2007e soil containing indigenous organisms remained constant throughout the range of treatments. The field soil moisture level of 14% dry soil mass at the time of soil collection was maintained for the duration of the assays by weekly additions of ASTM type I water.

2.3 Extractions and Soil Analyses

Soil samples were collected at the beginning of each enzymatic activity assay after a 24-h moisture equilibration. Soils samples were also collected for analysis on each litter harvest day in the litter decomposition studies. From each treatment soil batch, 2 g dry soil was weighed in triplicate into 50-ml glass tubes, 10 ml acetonitrile was added, samples were vortexed for 1 min, and then sonicated in the dark for 18 h at 20°C. After letting the sonicated samples settled for one hour at room temperature, 5 ml of supernatant were transferred to a glass tube, to which 5 ml of $CaCl_2$ solution (5 g/L) were added as a flocculant. Supernatant was filtered through a 0.45 μm polytetrafluoroethylene syringe cartridge. One ml of this filtered solution was transferred to an HPLC vial. Soil extracts were analyzed and EM concentrations quantified by reversed-phase HPLC using a modified USEPA Method 8330 (USEPA, 1998). Calibration curves were generated before each HPLC run using certified standards of each EM, in a range of concentrations appropriate for each set of determinations. The limits of detection were 0.01, 0.005, 0.005, and 0.05 mg/L for 2,4-DNT, 2-ADNT, 4-ADNT, and NG, respectively, corresponding to 0.1, 0.05, 0.05, and 0.5 mg/kg (dry soil mass).

2.4 Bioassays

Assessments of EM effects on litter decomposition were initiated in November 2007 using Orchard grass (*Dactylis glomerata*) straw. The duration of each test was eight months and included six harvests after 1, 2, 3, 4, 6,

and 8 months of exposure. Orchard grass was collected from grassland on the property of APG, dried at 60°C until constant mass was achieved, and cut into 5-cm long internodular sections. Three straw sections were used to form a straw cluster of approximately 0.2 g each. The mass of each cluster was recorded and an identification tag was attached to each cluster with a nylon string.

Individual studies of 2,4-DNT, 2-ADNT, 4-ADNT or NG were conducted using four replicates per treatment per harvest date. Approximately 300 g of prepared SSL soil treatments was loosely packed into individual test containers (glass jars 900 mL volume, 90 mm diameter). Six randomly-selected straw clusters were placed in the soil in each test container. The total mass of each test container with soil and straw clusters was recorded. Clear plastic film was stretched over the top of each container, secured with a rubber band, and the plastic film was perforated with three pinholes to facilitate air exchange. All containers were randomly placed in an environment-controlled incubator under a 16 h-light:8 h-dark photoperiod cycle with a mean photosynthetically active radiation (PAR) light intensity of 12.8±0.7 µM/m/sec (985±52 lux), mean relative humidity of 86±2%, and mean temperature of 21.6±0.1°C.

Litter mass loss data from each harvest date was used to compare litter decomposition rates among individual EM treatments of SSL soil. Annual decay rate constants (k) for litter residues, and corresponding standard errors (SE) and regression coefficients (r^2) will be determined using the single exponential decay model $m_t/m_0 = e^{-kt}$, where m_t/m_0 = fraction mass remaining at time t, t = time elapsed in years, and k = the annual decay constant, after completion of all ongoing assays.

The potential nitrification (PN) activity and the enzymatic activities of dehydrogenase (DH), acid phosphatase (AP), and N-acetyl-glucosaminidase (NAG) were quantified in each individual soil treatment of 2,4-DNT, 2-ADNT, 4-ADNT or NG during the 8 to 25-hour assays. The PN activity was assessed using the Griess-Illosvay technique where nitrites react with the primary aromatic amine, sulfanilamide, to form salts. These salts are further coupled with the aromatic compound N-1-naphthyl-ethylenediamine dihydrochloride to produce a colored compound measured spectrophotometrically. The DH activity was determined using 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazoliumchloride (INT) as substrate and by the spectrophotometric quantification of the produced formazan. The AP and NAG activities were determined using method described in Sinsabaugh et al. (2005). Standard solutions of 4-methylumbelliferyl (MUB) phosphate for AP, or 4-MUB-N-acetyl-beta-glucosaminide for NAG, were prepared in sterile ASTM type I water. Each microplate was incubated at room temperature (20-22°C). Assays were terminated by addition of 10 µL 0.5 N NaOH to each well of the microplate. Fluorescence was determined after 15 min for AP, and 40 min for NAG, using a Bio-Tek Synergy HT-I fluorimeter set at 360-nm excitation and a 460-nm emission. Enzyme activity (expressed as nmol/h/g dry soil) was calculated as the rate of accumulation of product equivalents.

2.5 Data Analyses

Nonlinear regression analyses to derive ecotoxicological benchmark values were conducted on untransformed data from assays based on the concentration-response relationships for quantitative endpoint data, using regression models described in Stephenson et al. (2000). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select best-fit models. The 95% confidence intervals (CI) and regression coefficients (R^2) associated with the point estimates were determined.

Analysis of variance (ANOVA) and Fisher's least significant difference pairwise comparison tests were applied to litter mass loss and enzymatic activity data for NOEC and LOEC determinations. A significance level of $p \leq 0.05$ was accepted for all statistical analyses. All toxicity benchmarks were established using untransformed data and analytically determined (USEPA Method 8330) EM concentrations in soil. Statistical analyses were performed using SYSTAT 11 (SYSTAT® Software, Inc., Point Richmond, CA, USA).

3. RESULTS

3.1 Effects of Energetic Materials on Litter Decomposition in SSL Soil

Provisional results showed that exposure to 2,4-DNT in SSL significantly ($p=0.032$) inhibited litter decomposition in the 8830 mg/kg treatment after one month compared with carrier control (Figure 1). Inhibition in this treatment remained significant ($p \leq 0.001$) throughout the eight-month study. Litter decomposition was also significantly ($p \leq 0.003$) inhibited in the 1200 mg/kg treatment after six and eight months compared with carrier control (Figure 1), thus indirectly suggesting an adverse effect on microbial activity at these 2,4-DNT concentrations in soil. However, the rates of litter decomposition were significantly ($p<0.015$) increased in the 4 and 62 mg/kg treatments after four and six months compared with carrier control but were not significantly ($p \geq 0.507$) different by the end of the eight-month study (Figure 1).

In contrast with the results of exposures to 2,4-DNT, litter decomposition rates were significantly ($p \leq 0.042$) greater in and above the 100 mg/kg 2-ADNT treatments compared with carrier control after two and four months, and remained significantly ($p=0.001$) greater in the 10000 mg/kg treatment after six months. Decomposition rates were not statistically ($p \geq 0.279$) different among any treatments by the end of the eight-month study (Figure 2). Also, litter decomposition rates were not significantly ($p \geq 0.10$) different among any of the 4-ADNT treatments throughout the first four months of the ongoing eight-month study (Figure 3).

Exposure to NG significantly ($p \leq 0.001$) inhibited litter decomposition in 1000 mg/kg and greater treatments compared with carrier control during the first four months of the ongoing eight-month study (Figure 4). Litter decomposition in the 30 mg/kg treatment was not significantly ($p \geq 0.134$) different from carrier control during the same exposure period (Figure 4). Table 1 summarizes ecotoxicological benchmarks for the effects of the four EMs on litter decomposition.

Table 1. Provisional Ecotoxicological Benchmarks for 2,4-DNT, 2-ADNT, 4-ADNT, and NG Determined for Litter Decomposition Endpoint in Individual Tests with Orchard Grass (*Dactylis glomerata*) Straw in Sassafras Sandy Loam.

Energetic Material	NOEC (mg/kg)	LOEC (mg/kg)
2,4-DNT	62 ($p \geq 0.507$)	1200 ($p \leq 0.003$)
2-ADNT	11 ($p > 0.050$)	100 ($p \leq 0.042$)*
4-ADNT	13000 ($p \geq 0.10$)	>13000
NG	30 ($p \geq 0.134$)	1000 ($p \leq 0.001$)

Table notes: Concentration values are based on acetonitrile extraction from soil (USEPA Method 8330); Probability values for significant effects are the greatest among all litter harvest dates within each treatment compared with carrier control; *statistically significant increase compared with carrier control.

3.2 Effects of Energetic Materials on Enzymatic Activities in SSL Soil

Preliminary results showed that soil treatment with 2,4-DNT inhibited the NAG and DH activities with the respective EC_{20} values of 122 and 114 mg/kg, and the PN activity with the LOEC of 4 mg/kg, but did not affect ($p=0.574$) the AP activity up to and including 8830 mg/kg. Soil treatment with 2-ADNT have established the EC_{20} values of 175, 406, and 830 mg/kg for the PN, DH, and AP activities, respectively; and the Maximum Allowable Toxic Concentration (MATC; geometric mean of the NOEC and LOEC values) of 355 mg/kg for the NAG activity.

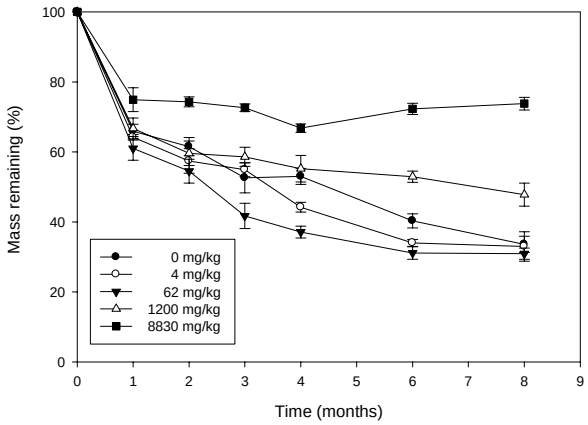

Fig. 1. Effect of 2,4-DNT on litter decomposition in Sassafras Sandy Loam soil.

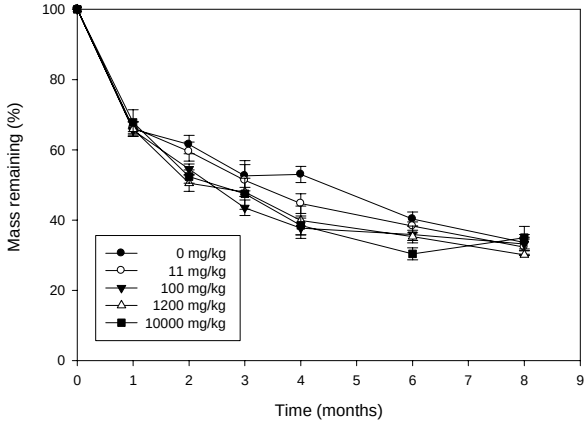

Fig. 2. Effect of 2-ADNT on litter decomposition in Sassafras Sandy Loam soil.

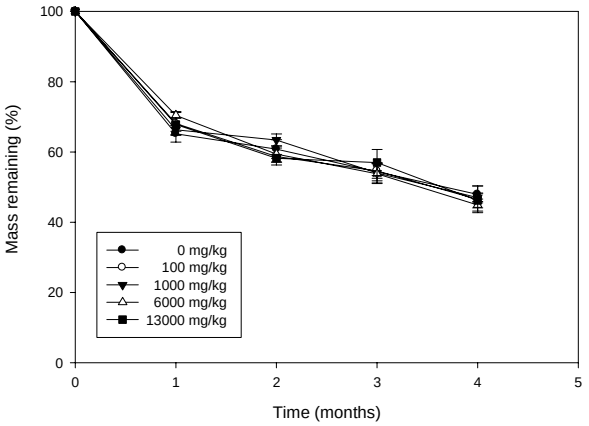

Fig. 3. Effect of 4-ADNT on litter decomposition in Sassafras Sandy Loam soil.

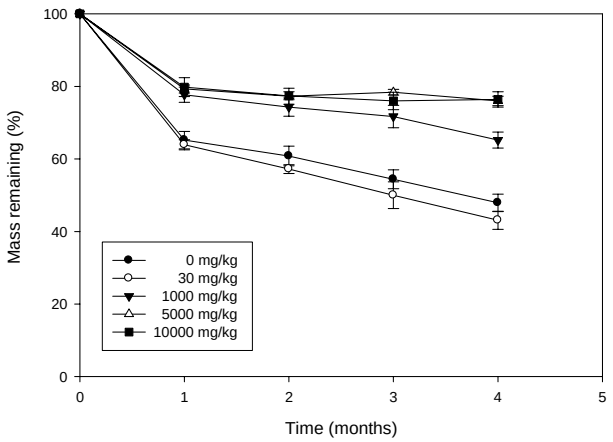

Fig. 4. Effect of NG on litter decomposition in Sassafras Sandy Loam soil.

Soil treatments with 4-ADNT significantly ($p<0.0001$) inhibited the DH activity at and above the lowest concentration tested compared with pooled data from carrier and negative controls, and established an unbounded LOEC of 100 mg/kg. Nonlinear regression analysis (exponential model) established the EC_{20} and EC_{50} values (and corresponding 95% CI) of 28 (15-42) and 88 (47-130) mg/kg, respectively. Similar to the effects on the DH activity, 4-ADNT significantly ($p<0.0001$) inhibited the PN activity at and above the lowest concentration tested compared with acetone control, and established an unbounded LOEC of 100 mg/kg after the 25-h exposure. Nonlinear regression analysis (exponential model) of the PN data established the EC_{20} and EC_{50} values of 113 (84-142) and 350 (261-440) mg/kg, respectively. Soil treatments with 4-ADNT did not significantly ($p>0.1$) affect the NAG activity at any concentration tested compared with data from carrier control, and established an unbounded NOEC of 13000 mg/kg. The AP activity was affected by 4-ADNT, with a maximum of 56 percent inhibition at 1000 mg/kg (LOEC; $p<0.0001$) and 43 percent inhibition at the greatest tested concentration 13000 mg/kg, compared with pooled data from carrier and negative controls. Nonlinear regression analysis (exponential model) of the AP activity data established the EC_{20} and EC_{50} values of 90 (0-335) and 278 (0-1040) mg/kg, respectively. These preliminary results suggested that 4-ADNT can adversely affect the activity of three out of four soil enzymes tested in this project.

Soil treatments with NG significantly ($p<0.0001$) inhibited the DH activity at and above the lowest concentration tested compared with pooled data from carrier and negative controls, and established an unbounded LOEC of 64 mg/kg. Nonlinear regression analysis (exponential model) established the EC_{20} and EC_{50} values of 34 (23-45) and 105 (70-139) mg/kg, respectively. The PN activity could not be quantified due to low activity in control treatment. Soil treatments with NG did not significantly ($p>0.05$) affect NAG activity at any concentration tested compared with data from carrier control, and established an unbounded NOEC of 9800 mg/kg. The only significant ($p=0.002$) effect on the AP activity was a 27 percent inhibition at 5800 mg/kg compared to carrier control. All other NG treatments of SSL soil had no significant ($p>0.11$) effect on the AP activity compared to carrier control.

4. DISCUSSION

The Army Strategy for the Environment represents a major advancement in the Army's appreciation of the interdependence among the mission, the community, and the environment. It builds on the four pillars of conservation, restoration, pollution prevention, and compliance, defined in the Army's Environmental Strategy published in 1992. This Strategy applies an ecosystem approach (plus others) to managing natural resources on Army installations. It incorporates the principles of sustainability across the Army and into all functional areas. Our investigations were designed to address the critical data needs required for successful management of Army installations in a sustainable manner, and for the knowledge-based decision making.

Nitroaromatic EMs introduced into soil during testing and training activities at Army installations can undergo rapid transformation to the amino-nitro intermediates. Frequent co-occurrence of trinitrotoluene (TNT), trinitrobenzene (TNB), DNTs, and ADNTs in soils of contaminated sites or in experimentally contaminated soil treatments have precluded investigators from partitioning the effects of the parent materials and their transformation products on soil microorganisms. As a result, the toxicity benchmarks for TNT established in previous studies should not be accepted unequivocally. We designed our investigations to definitively resolve the toxicity of individual nitroaromatic EMs and NG to the soil microbial activity endpoints and to critical processes in the soil ecosystem regulated by this community.

Preliminary results showed that soil contamination with 2,4-DNT, 2-ADNT, 4-ADNT, and NG can alter the rates of biologically-mediated processes in soil by either inhibiting or stimulating the soil microbial activity at the affected sites. Ecotoxicological benchmark values determined in our studies were generally comparable to those reported for the effects of TNT, which was the most investigated nitroaromatic EM. However, the majority of ecotoxicological data for soil microorganisms determined in the TNT studies was

derived from amended growth media or soil slurries; therefore, those data cannot be used directly to infer the exposure effects in aerobic upland soil used in our studies because the differences in the bioavailability and the fate of EMs in amended media can be substantial.

Estimates of the impact of TNT on the carbon cycle performed in different laboratories such as an EC_{50} value of 376 mg/kg for substrate-induced respiration reported by Gong et al. (2001) were generally similar to preliminary results of our litter decomposition studies, which determined the LOEC values for 2,4-DNT and 2-ADNT of 100 and 1000 mg/kg, respectively (Table 1). The EC_{50} values for inhibition of DH activity ranging from 139 to 493 mg/kg (Gong et al., 1999) compare favorably with the results of our studies. However, using the more sensitive indicators of carbon cycle impairment, such as the metabolic quotient, the EC_{50} and EC_{20} values of 35 and 3 mg/kg, respectively, have been established for TNT (Frische and Hoper, 2003).

An average EC_{50} value of 7.8 µg/ml was reported for TNT by Fuller and Manning (1997) for cell growth inhibition of 14 different Gram-positive bacterial isolates. Exposure to TNT was reported to lead to wide spread changes in microbial community composition (Siciliano and Greer, 2000) and decreased diversity (Siciliano et al., 2000a). An increase in TNT concentration from 10 to 80 µg/ml resulted in lower diversity as measured by a decrease in the number of identifiable phospholipid fatty acids (PLFAs) from 34 to 14 (Fuller and Manning, 2004). Actinomycetes were reported to have a substantially lower EC_{50} value of 362 mg/kg compared to Gram-positive organisms having an EC_{50} value of 4,177 mg/kg, as assessed by the prevalence of PLFA under *in situ* soil conditions of Joliet Army Ammunition Plant (Joliet, IL, USA; Fuller and Manning, 1998) but the precise reason why TNT is more toxic to actinomycetes is unclear at the present time.

Sensitive techniques routinely detected differences in generic microbial community functions at low TNT concentrations. A study of functional diversity of microbial community based on utilization of 32 different substrates by a cultured indigenous community established an EC_{10} value of 0.2 µg/ml (Siciliano et al., 2000b). The EC_{20} values for inhibition of microbial respiration ranged from 70 mg/kg in a forest soil (4.1% C_{org}) to 530 mg/kg in a garden soil (8.7% C_{org}) (Gong et al., 2000). These selective impacts have been assessed using the principle of Pollution Induced Community Tolerance (PICT) in several investigations. The PICT principle states that a community exposed to a toxicant will become tolerant to that toxicant. As a result, when a tolerant (*i.e.*, exposed) community is further exposed to that toxicant it will retain more of its functionality at a given concentration of a toxicant compared to a non-exposed community. Exposure to TNT resulted in exactly this pattern of tolerance acquisition (*i.e*., adaptation), as assessed by *in vitro* (Siciliano et al., 2000b) or *in situ* (Gong et al., 2000) techniques. Recently, a non-PICT-based study has suggested that not only does TNT cause selective disruption of the microbial community, but that these changes are irreversible (Fuller and Manning, 2004).

Nitrification is the commonly used term that combines the activities of two distinctly different groups of microorganisms, ammonia oxidizing bacteria and nitrite oxidizing bacteria. In combination, these two groups of organism convert ammonia to nitrate and derive energy from this process. Our studies showed that the effects of 4-ADNT on the PN activity were comparable with those established for TNT by Gong et al. (1999; 2001) based on the reported EC_{50} values of 39 and 227 mg/kg.

Overall, the results of our studies and those reported in literature show that assessment of the soil microbial activity endpoints provides valuable information on the EM effects on critical ecosystem-level processes such as energy and nutrient cycling, and compliment and expand upon the ecotoxicological significance of data from the standardized single-species toxicity tests, thereby meeting DoD stewardship goals plus promoting sustainable use of Army ranges.

5. CONCLUSIONS

The Army Strategy for the Environment incorporates the principles of sustainability across the Army and into all functional areas. Our investigations were designed to develop critical benchmark data required for successful management of Army installations in a sustainable manner and for the knowledge-based decision making. Assessment and protection of the terrestrial environment at the Army installations will be advanced by applying scientifically based ecotoxicological benchmarks for microbially-mediated processes in soil established in our studies. These benchmarks will allow screening of site soil data to identify those EM contaminants that are of potential ecological concern, and should be considered in baseline ecological risk assessment (BERA). These benchmarks for the soil microbial activity will provide a useful tool for the Army installation managers to gauge the ecotoxicological impacts of the Army operations that involve the use of explosives, thus ultimately promoting the sustainable use of testing and training ranges by today's and future Warfighters.

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