Environmental fate and ecological impact of emerging energetic chemicals (ADN, DNAN and its Amino-Derivatives, PETN, NTO, NQ, FOX-7, and FOX-12) and an insensitive formulation

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Environmental Fate and Ecological Impact of Emerging Energetic Chemicals (ADN, DNAN and its Amino-Derivatives, PETN, NTO, NQ, FOX-7, and FOX-12) and an Insensitive Formulation

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EXECUTIVE SUMMARY

The present report for 2013-2014 focuses on the environmental fate and ecological impact of the high explosive pentaerythritol tetranitrate (PETN) and on the PETN-based formulation DM-12. It was dictated by the current willingness of the Canadian Department of National Defense to use formulation DM-12 in replacement of the RDX-based C4. As part of the original CORE proposal, we assessed transformation of 2,4-dinitroanisole (DNAN) and its amino derivatives 2-amino-4-nitroanisole (2-ANAN) and 4-amino-2-nitroanisole (4-ANAN) by the white-rot fungus *Phanerochaete chrysosporium*. *P. chrysosporium* transformed DNAN and both its derivatives, with rates following the order 4-ANAN >2-ANAN >DNAN. For PETN, we determined specific physicochemical properties (aqueous solubility, sorption onto soil, and dissolution under simulated rainfall), and degradation under both abiotic (solar-simulated photolysis, hydrolysis at pH12, and reaction with zero valent iron) and biotic (microbial degradation) conditions. We found that PETN is practically insoluble in water and has relatively high sorption affinities, particularly in organic-rich soil. Water dripping experiment showed significant leaching of PETN from the DM-12 particle under a continuous flow of water. Additionally, PETN slowly photodegraded via hydrolytic denitration, but was completely denitrated using zerovalent iron, and was sensitive to degradation at pH 12. Indigenous soil microbial community efficiently removed PETN under both aerobic and anaerobic conditions, providing that a carbon source was added. Three PETN-transforming strains were isolated from soil and identified as *Enterobacter*, *Sphingomonas* and *Cupriavidus*. PETN toxicity assays showed that, when present in interstitial water of amended soil (with up to 10,000 mg/kg) at concentrations approximating its water solubility limit, PETN did not significantly affect earthworm survival or ryegrass early seedling germination.

Based on our results, we can expect that PETN 1) will not tend to migrate through soil and contaminate groundwater, but 2) will leach from formulation DM-12 (80-90% PETN content) during rain events, however 3) it may not stay
indefinitely on soil surface but rather will undergo bio- and photo-degradation, 4) is amenable to bioremediation and remediation by alkaline hydrolysis, and finally 5) due to its low bioavailability, is not significantly toxic to soil invertebrates and plants.

As for DNAN, relevant environmental data collected to date on both abiotic and biologic transformation were compiled in a comprehensive manuscript (Annex 1). We concluded that hydrolysis under environmental conditions is insignificant whereas photolysis gives photodegradable intermediates 2-hydroxy-4-nitroanisole and 2,4-dinitrophenol. In soil, successive replacement of –NO₂ by –NH₂ in DNAN enhances irreversible sorption and reduces bioavailability under oxic conditions. And finally, although DNAN is more soluble than TNT, its lower hydrophobicity and its tendency to form amino-derivatives that sorb irreversibly to soil contribute to make it less toxic than the traditional explosive TNT (Annex 2).
INTRODUCTION

Since the last century, intensive military activities including training at firing and impact ranges, weapons testing, open burning/open detonation (OB/OD) of obsolete munitions, manufacturing and transport of munitions, have resulted in widespread contamination of soil and water with residues of explosives and related compounds. This is a serious environmental problem that is costing world economy hundreds of millions of dollars to manage and to remediate. Many countries, like Canada, are currently seeking to introduce new insensitive munitions compounds in explosives compositions and applications, e.g., as a replacement for Composition B and TNT in the main explosive charge of insensitive medium and large caliber ammunition. Several new compounds are being tested by the munitions industries to develop insensitive formulations for potential use by the Canadian Army. These compounds include 3-nitro-1,2,4-triazole-5-one (NTO), 2,4-dinitroanisole (DNAN), nitroguanidine (NQ), FOX-7 and FOX-12 (Fig. 1). The U.S. Army has already approved the use of the insensitive munition formulation IMX-101 (Picatinny 2010), which contains 40 to 45% DNAN, 18 to 23% NTO, and 35 to 45% NQ.

Based on past experience on the environmental problems caused by traditional explosives such as nitroglycerine, RDX, HMX and TNT, it is now important to be proactive and assess the environmental risks associated with the manufacturing and use of these new compounds before putting the new insensitive formulations into service. There was a drastic lack of information on the mobility, dissolution rates, abiotic and biotic degradability, and ecological impacts of the chemicals intended for use by the Canadian army. In the last three years, the work from NRC-Montreal has largely contributed to fill some gaps. This project will continue to respond to the Canadian Department of National Defense (DND) program to provide additional data on the environmental fate and ecological impact of NTO, DNAN, NQ, FOX-7 and FOX-12 (Fig. 1). The collected data will hopefully help the munitions suppliers, munitions acquirement
managers, site managers and environmental officers to make informed decisions.

Figure 1. Structures of the insensitive chemicals under study

Both abiotic and biotic reactions readily transform DNAN to its amino derivatives 2-amino-4-nitroanisole (2-ANAN), 4-amino-2-nitroanisole (4-ANAN) and 2,4-diaminoanisole (DAAN). We therefore included these products in the study (Fig. 2).
**Figure 2.** Structures of DNAN and its amino products

**FIGURE 3.** Structure of pentaerythritol tetranitrate (PETN)

In 2013-2014, the traditional explosive pentaerythritol tetranitrate (PETN), as a pure compound and as the major component of an explosive formulation, was added to the list of chemicals to be investigated (Fig. 3). PETN is a nitrate ester chemical (O-NO₂), the same family as nitroglycerin and nitrocellulose. It is one of the most powerful military explosives, but is more sensitive to shock and friction than other secondary explosives like TNT and RDX. PETN is part of many explosive compositions, such as Detasheet, a commercial formulation composed of 63% PETN, Pentolite, a 50/50 mixture of PETN and TNT, and the Semtex plastic explosives.
Also formulation DM-12, composed of PETN (80-90%) and silicone zinc stearate (9-19%), is currently being investigated by the Canadian Army to potentially replace composition C4 (91% RDX).
OBJECTIVES

This year’s work can be divided in five main objectives. Each objective represents a chapter of this report.

Objective 1: Determine environmentally relevant physicochemical properties of PETN
- Determine the solubility of PETN in water
- Determine dissolution of PETN from formulation DM-12
- Determine the effect of simulated rain falls on the dissolution of DM-12

Objective 2: Determine the transport of PETN in soils typical of Canadian DoD ranges

Objective 3: Assess the abiotic degradation of PETN (hydrolysis and photolysis)
- Assess degradability of PETN by alkaline hydrolysis
- Determine the effects of simulated sunlight on PETN

Objective 4: Assess the biologic degradation/transformation of explosives
4a: Assess the biodegradation of PETN
- Determine PETN degradation by soil microbial communities
- Determine PETN degradation by bacterial soil isolates

4b: Assess the biodegradation of DNAN and derivatives by *Phanerochaete chrysosporium*

Objective 5: Assess toxicity and bioaccumulation of PETN for selected organisms
- Determine toxicity of PETN for soil invertebrates (earthworms)
- Determine toxicity of PETN for plants (ryegrass)
SUMMARY OF ACCOMPLISHMENTS

Publications


Annexes

ANNEX 1: the complete manuscript “Environmental fate of 2,4-dinitroanisole (DNAN) and its reduced products” accepted for publication in Chemosphere and reporting environmentally relevant physicochemical properties and transformation processes of DNAN.

ANNEX 2: the manuscript entitled “Ecotoxicological assessment of a high energetic and insensitive munitions compound: 2,4-dinitroanisole (DNAN)”. 
This article by Sabine Dodard et al. was published in 2013 in the Journal of Hazardous Materials 262:143-150.

ANNEX 3: this NATO AVT-197 review chapter summarizes key physico-chemical properties ($S_w$, pH, $K_{ow}$, $K_{dl}$) of high explosives such as RDX and TNT and propellants such as NG, NQ and 2,4-DNT; it discusses how these parameters influence their geo-biochemical interactions with soil and their chemical and microbial transformation routes in the environment. The chapter also contains data on the RDX-based C4 that could be replaced by the PETN-based formulation DM-12.

ANNEX 4: this NATO AVT-197 review chapter explains how ecotoxicological studies can provide relevant baseline toxicity data on the ecological effect of new insensitive munitions and their degradation products for the improved site management at military installations.
1. Physicochemical properties of PETN

1.1 Solubility and dissolution of PETN from DM-12

1.1.1 Introduction

The Canadian Department of National Defense is currently investigating the PETN-based formulation DM-12 for a wider use by the Army to replace RDX-based C4. PETN is very rarely found in munitions-contaminated sites, despite it’s use in energetic formulations. Studies to date have reported the very low solubility of PETN in water, its high solubility in acetone, its decomposition kinetics at high temperatures, its degradation in acidic solutions, etc. However, the largest chunk of literature on the physicochemical properties of PETN concerns its effects as a vasodilator drug to treat certain heart conditions. Here we report some environmentally relevant properties of PETN. We measured its solubility in water, its dissolution from DM-12 particles, and examined its long term dissolution under simulated rain fall.

1.1.2 Materials and methods

Chemicals

PETN, a white powder (> 99%), was provided by Defense Research and Development Canada (Valcartier, QC) and was used as received. DM-12, a pale pink sticky dough formulation, is composed of PETN (80-90%) and silicone zinc stearate (9-19%) and was provided as pieces of 1 g by DRDC-Valcartier. The PETN concentration (86.03 ± 2.69%) of the formulation was measured at NRC-Montreal by dissolving ~15 mg (precisely weighted) in acetonitrile (100 mL), sonicating for 15 min, stirring for 1 h at room temperature (250 rpm), and analyzing the supernatant by high performance liquid chromatography (HPLC) as described below.
Aqueous solubility measurement of PETN

Aqueous solubility of PETN was measured at 25°C by suspending 3.1 mg of the chemical in 10 mL of deionized water. The sample was stirred at 25°C. At days 14, 29 and 64, aliquots (~1 mL) of the mixture was filtered, diluted 1:1 in acetonitrile, and analyzed by HPLC. The solubility was considered reached when the value was stable between two successive measurements. The last measurement was done in triplicate.

Batch experiments for DM-12 dissolution

Leaching experiments were conducted to determine the amount of PETN that would leak from the explosive formulation when in contact with water. Formulation DM-12 was shaped into particles of 20 ± 4 mg to obtain 20 to 25 spherical particles in each replicate. The formulation was stirred for long times in water batches and water was changed after reaching sequential equilibria. Batch experiments were conducted in water at temperatures of 10.0 ± 1, 25.0 ± 1, and 30.0 ± 1°C. A mass (500 mg) of formulation was added to 50 mL of pre-thermostated deionized water (pH 5.5) in a glass bottle. The capped samples were shaken (150 rpm) in a thermostated incubator protected from light. At various time intervals, aliquots (1 mL) of suspension were withdrawn, filtered through a Millex-HV 0.45 μm syringe filter, diluted (1:1 v/v) in acetonitrile and analyzed for PETN by HPLC. Once the dissolved amounts of component reached equilibrium, the solid was isolated from supernatant and stirred again with a fresh batch of deionized water (50 mL) in order to determine the maximal potential of component to dissolve. All experiments were done in duplicate.

Long term dripping experiments

In order to evaluate the leaching of the formulation under a rain event, an experiment was set up where a particle of DM-12 (sphere of 4 mm diameter) was placed on a glass funnel fitted with a nylon mesh and exposed to a continuous flow of water maintained with an HPLC pump at a rate of 0.5 mL min⁻¹,
corresponding to ~19 drops per min. Flow rate was chosen deliberately high to accelerate the dissolution process. Outflow samples were collected in glass flasks covered with aluminum foil and flasks were changed after increasing intervals (from 1 day to 1 week). Only the first five water fractions were analyzed for PETN as PETN was not found after the first two fractions.

HPLC analysis - determining PETN concentration
The system consisted of a Waters HPLC system (Milford, MA, USA) equipped with a model 2996 photodiode array detector, a model 600 pump, a model 717 plus injector, and a temperature control module. PETN samples were analyzed on a Discovery C18 column (25 cm, 4.6 mm, 5 mm) (Supelco, Oakville, Canada), at 35°C. An injection volume of 50 µL was used. The mobile phase (65% methanol: 35% water) was run isocratically at 1 mL min⁻¹. The detector was set to scan from 192 to 450 nm. The detection limit was estimated to be 0.05 mg L⁻¹ at 242 nm.

1.1.3 Results and discussion

PETN aqueous solubility
The solubility of PETN in water was found to be 1.55 ± 0.02 mg L⁻¹ (25°C). The aqueous solubility measured herein is close to the value of 1.9 mg L⁻¹ (25°C) reported by Merrill (1965), 1.5 mg L⁻¹ (20°C) from Chambers et al. (2002) and 2.04 mg L⁻¹ (20°C) from Quinn et al. (2009). Based on its quasi-insolubility, only very small amounts of PETN should dissolve and mix with soil water following deliberate or accidental release in nature.

Batch experiments for DM-12 dissolution
When 500 mg of DM-12 particles was stirred in water at 10, 25 and 30°C, PETN was released until equilibrium after ~3 days (Fig. 1.1). Interestingly, the concentration of PETN leached from DM-12 at 25°C was higher (2.0 mg L⁻¹) than its aqueous solubility (1.55 mg L⁻¹) at the same temperature. This result is
contrary to some other studies that have shown that the dissolution rate of individual explosives was decreased when present in formulations compared to the pure explosives (Lynch et al. 2002; Lever et al. 2005; Taylor et al. 2009). In the case of DM-12, other components from the formulation may have helped increasing PETN solubility. For example, silicone oil is lipophilic and can therefore dissolve lipophilic compounds such as nitrate esters. PETN was released at almost the same rate during the first and the second run suggesting that a continued contact with fresh water would induce a steady release of the chemical in water (Fig. 1.1).

![Graph showing PETN dissolution over time for two runs at different temperatures](image_url)

**Figure 1.1.** Leaching of PETN from formulation DM-12 for two successive runs as a function of temperature
The cumulative dissolved fractions of PETN collected at the second run are reported in Table 1.1. Although PETN release increased with increasing temperatures, only 0.07% of PETN was released after 2 months at 30°C. Data thus show that very small proportions of PETN are extracted from DM-12 after few months of stirring in water. The losses measured correspond to low absolute masses of PETN (0.28 mg at 30°C), suggesting that exposure of chunks of DM-12 to water in the environment would lead to extremely low levels of contamination. It is clear that the low solubility of PETN in water limits its release from DM-12.

**Table 1.1.** Total amounts of PETN released from DM-12 (~500 mg) into water (50 mL) after the second run.

<table>
<thead>
<tr>
<th></th>
<th>10°C</th>
<th>25°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PETN (µg)</td>
<td>101</td>
<td>202</td>
<td>281</td>
</tr>
<tr>
<td>% of initial chemical</td>
<td>0.02</td>
<td>0.05</td>
<td>0.07</td>
</tr>
</tbody>
</table>

_Dripping experiment_

A long-term dissolution experiment was conducted to gain insight on the fate of formulation DM-12 over long periods of time and to be able to predict its behavior when DM-12 particles, dispersed on the soil surface, are exposed to rainfall events. A DM-12 particle was subjected to a continuous flow of dripping water mimicking a rain flow falling on a particle lying on a porous soil where the water would disappear quickly into the ground.

Low concentrations of PETN were found in the first two water collected fractions (1 and 3 days) while PETN was not detected in the subsequent three fractions. Although no PETN could be detected in the eluates, the experiment was continued for 2 months. Then, the remaining particle was dried under air and suspended in acetonitrile for quantification of residual PETN. Analysis of the
acetonitrile solution by HPLC allowed recovery of 28.3 mg PETN, corresponding to 74% of the initial PETN. After 2 months of water dripping, the DM-12 particle had thus lost 9.8 mg in total, corresponding to 26% of its original mass. This result shows that, despite its low solubility, PETN could easily leach from DM-12 towards dissolution under a continuous flow of water.

The dripping assay thus led to a complementary conclusion to the observations made in the batch experiment. Dissolution of PETN from formulation DM-12 is limited by the low solubility in water (batch experiment). However, under a continuous flow of rain, PETN would certainly leach from the formulation, allowing the compound to spread in the surrounding soil to a certain extent. The transport of PETN in soil is also function of its affinity to sorb to soil particles. This is the subject of the next chapter.
2. Transport of PETN in soil

2.1 Sorption

2.1.1 Introduction

Compared to RDX and TNT, the fate and transport of PETN in the environment has received little research attention. The soil-water partition coefficient ($K_d$), used to estimate the extent to which a chemical is sorbed to soils, is one of the most important parameters for estimating the migration potential of contaminants. The partitioning behavior of chemicals and their transformation products in soil also affect their availability to microbial degraders and to receptors (earthworms or plants) used in toxicity assays. Because $K_d$ values are highly site-specific, we evaluated sorption of PETN using two different types of soils from Canadian training range areas.

2.1.2 Materials and methods

Soil characteristics
A soil with low organic content (named DRDC-10) collected in 2010 at a DRDC-Valcartier (QC) training range, and a soil with higher organic content (named Peta-12) collected in 2012 from a DRDC-Petawawa (ON) training range were used in this study. Selected physicochemical properties of the two soils are given in Table 2.1.
Table 2.1. Physicochemical properties of soils DRDC-10 and Peta-12

<table>
<thead>
<tr>
<th></th>
<th>Particle size distribution</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% clay/silt</td>
<td>% sand</td>
<td>TOC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CEC&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(&lt;80 μM)</td>
<td>(&gt;53 μm)</td>
<td>(%)</td>
<td>pH</td>
<td>(mequiv/100g)</td>
</tr>
<tr>
<td>DRDC-10</td>
<td>2.4</td>
<td>97.6</td>
<td>&lt; 0.5</td>
<td>6.0</td>
<td>6.69</td>
</tr>
<tr>
<td>Peta-12</td>
<td>44.1</td>
<td>55.9</td>
<td>2.5</td>
<td>4.9</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

<sup>a</sup> TOC, total organic carbon; <sup>b</sup> CEC, cationic exchange capacity

**Batch sorption experiments**

Batch equilibration was used to quantify the sorption of PETN in the two soils, DRDC-10 and Peta-12, aerobically, and under sterile and non-sterile conditions. To prepare sterile soil, a portion of dry soil was submitted to gamma irradiation from a $^{60}$Co source at the Canadian Irradiation Center (Laval, Quebec) with a dose of 50 kGy over 2 h.

A volume (10 mL) of filtered aqueous solution of PETN was added under sterile conditions to soil (1.5 g) in autoclaved 50-mL tubes. The tubes were kept in the dark at 22 ± 1°C, and were aerated once a week by opening and shaking the tubes. Triplicate tubes were sacrificed at time intervals varying from 1 day to 3 months. The supernatant was withdrawn, filtered through a 0.45 μm Millipore filter (Millipore Corp., Bedford, MA) and analyzed by HPLC as described in section 1.1.2. The sorbed analyte was extracted from soil as described in the EPA SW-846 Method 8330 (USEPA, 1997). Briefly, soil was sonicated with CH$_3$CN (10 mL) at 20°C for 20 h, diluted 1:1 in an aqueous CaCl$_2$ solution and filtered through a Millex-HV 0.45-μm filter prior to HPLC analysis. The percent recovery was calculated by adding the soluble and sorbed fractions of analytes. The soil-water distribution coefficient ($K_d$) was calculated as the soil to water analyte concentration ratio.
2.1.3. Results and discussion

The stability and partitioning of PETN were evaluated in two different soils under sterile and non-sterile conditions. Given the aerobic nature of DRDC soil, the study was carried out under aerobic conditions for both soils. The $K_d$ value for the two soils are presented in Table 2.2. The higher $K_d$ values of PETN in Peta-12 soil suggest that PETN tend to sorb more on organic soil, which is in accordance with the Log $K_{ow}$ reported in the literature (3.7; Rosenblatt, 1991).

Table 2.2. Soil-water partition coefficients ($K_d$) of PETN in DRDC-10 and Peta-12 soils

<table>
<thead>
<tr>
<th></th>
<th>DRDC-10</th>
<th>Peta-12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sterile</td>
<td>Non-sterile</td>
</tr>
<tr>
<td>PETN</td>
<td>$0.17 \pm 0.16$</td>
<td>$0.44 \pm 0.34$</td>
</tr>
</tbody>
</table>

PETN was found to be relatively stable and non-sorbed in DRDC-10 soil, while it tended to sorb and disappear more in the organic-rich Peta-12 soil (Fig. 2.1). The occurrence of abiotic disappearance of PETN in sterile Peta-12 soil was most likely caused by irreversible binding, leading to non-equilibrium situations that did not allow reliable measurement of $K_d$ values. Our results nonetheless suggest that in sandy soil typical of the DRDC-Valcartier training range, small amounts of dissolved PETN will migrate through soil and reach groundwater. PETN disappeared faster in non-sterile than in sterile soils, indicating both biotic and abiotic losses of PETN. Abiotic and biotic transformations of PETN are discussed in the next two chapters.
**Non-Sterile soils**

**Sterile soils**

![Graphs showing sorption and distribution of PETN in non-sterile and sterile soil suspensions.](image)

**Figure 2.1.** Sorption and distribution of PETN in non-sterile (left) and sterile (right) soil suspensions (initial concentration of PETN: 1.8 mg L⁻¹; RT; aerobic).
3. Abiotic degradation (hydrolysis and photolysis) of PETN

3.1 Introduction

Once deposited on the soil surface, unexploded residues of PETN are bound to undergo some sort of degradation caused by hydrolysis, photolysis and metal reduction. Little information is available on the abiotic degradation of PETN in soil under natural conditions. In the present study, we determined the susceptibility of PETN to be degraded by alkaline hydrolysis and photolysis. The data were used to extrapolate on PETN degradation and transformation in the environment.

3.2 Alkaline hydrolysis of PETN

3.2.1 Materials and methods

Hydrolysis experiments were conducted in 125 mL sealed serum bottles incubated statically, in the dark, at 50°C. The pH of the solution (2 mg L\(^{-1}\) PETN in 50 mL deionized water) was adjusted to 12 and 11 with an appropriate amount of 1 M NaOH. A control consisted of 2 mg L\(^{-1}\) PETN in deionized water without pH adjustment. Samples were analyzed by HPLC as described in section 1.1.2. Experiments were done in duplicate.

3.2.2 Results and discussion

When incubated at 50°C and at pH 12, the initial 2 mg L\(^{-1}\) PETN was found to be completely degraded after 49 days (Fig. 3.1). Comparatively, only 25% PETN was degraded after 105 days at pH 11 (50°C). No loss was observed in the control (pH 7.1, 50°C) for the same period of time. PETN rates of degradation were calculated at 0.049 d\(^{-1}\) and 0.003 d\(^{-1}\) at pH 12 and pH 11, respectively (Fig. 3.2).
Figure 3.1. kinetics of the hydrolysis of PETN incubated at 50°C, at pH 12 and pH 11.

Figure 3.2. First order plot for PETN hydrolysis at pH 12 and pH 11.
3.3 Photolysis of PETN

3.3.1 Materials and Methods

Materials
PETN was provided by DRDC-Valcartier (QC, Canada). Denitrated PETN products PETriN (pentaerythritol trinitrate), PEDiN (pentaerythritol dinitrate) and PEMonoN (pentaerythritol mononitrate) were prepared by treating PETN with zerovalent iron under anaerobic conditions as described by Zhuang et al. (2008) and used as references for LC-MS analysis.

Photolysis of PETN
Artificial sunlight generated from a SolSim Solar Simulating Photoreactor (Luzchem Research Inc, Canada) was used to determine the photosensitivity of PETN under solar-simulated conditions. The total power of the Solar Simulator output spectrum was calibrated to the best approximation of ASTM Air Mass 1.5 Global Tilt Standard in the 280-800 nm regions giving a total irradiance of 590,000 W m⁻². Triplicate irradiation assays were conducted in 20-mL quartz tube using 5 mL of aqueous solution of PETN (5.6 µM). Irradiation was run for 21 d at 25°C. Samples were collected at selected time intervals for analysis.

Chemical analysis
PETN was quantified as described in section 1.1.2. Nitrite and nitrate ions were analyzed using an HPLC system (Dionex ICS-3000) equipped with a conductivity detector and Dionex ion chromatography column (IonPac AS15) (250 mm x 4 mm ID). LiOH solution in gradient mode (from 5 mM to 40 mM) was used as eluent. Analysis was carried out at room temperature under a flow rate of 1.5 mL min⁻¹. PETN and its degradation products were analyzed using Bruker Esquire3000 plus mass analyzer attached to an HPLC system (Hewlett Packard 1100 Series) equipped with a DAD detector. Samples (2 µL) were injected into a 5 micron-pore size Zorbax-C18 capillary column (0.5 mm ID × 150 mm; Agilent)
at 25°C. The solvent system was composed of CH$_3$CN and HCOOH (0.05%) at a flow rate of 15 µL min$^{-1}$ using gradient. For mass analysis, negative electrospray ionization mode was used to search for the characteristic mass ion fragment [ONO$_2$]$^-$ at $m/z$ 62 Da, and the adduct mass ions [M+HCOO]$^-$ at $m/z$ 361 Da, 316 Da, 271 Da and 226 Da for PETN, PETrIN, PEDiN and PEMonoN, respectively. Mass range was scanned from 40 to 500 Da.

3.3.2 Results and discussion

PETN contains chromophores that absorb at wavelengths >290 nm and we therefore suspected that it would be prone to direct photolysis by sunlight. Indeed, our results showed that 66% of the initial 5.6 µM PETN was degraded after 21 days of irradiation (Fig. 3.3). The degradation was linear for the duration of the experiment (Fig. 1). PETN disappearance (3.7 µM) was accompanied by the formation of nitrate (8.2 µM) measured after 21 days. Only trace amounts of nitrite were detected. Andrew and Swager (2011) hypothesized that photodegradation of PETN was initiated by the heterolytic cleavage of the O-NO$_2$ bond leading to the initial formation of nitrite, which later photo-oxidize to nitrate.

![Graph showing the degradation of PETN over time](image.png)

**Figure 3.3.** Degradation of PETN in a SolSim photo-reactor simulating solar irradiation.
Because of the low solubility of PETN in water (concentration initial 1.7 mg L\(^{-1}\)) and its slow photo-degradation rate (Fig. 3.3), only traces of the denitrated intermediates PETriN and PEDiN could be detected, still confirming denitration of PETN during photolysis. Denitrated products PETriN and PEDiN were identified by LC-MS by comparison with references materials prepared by treating PETN with zerovalent iron under anaerobic conditions (Figure 3.4) However, the production of 2.2 moles of nitrate for each mole of PETN degraded suggests that denitration of the compound was incomplete over the time studied.

![Image](image.png)

**Figure 3.4.** Extracted (multiple) ions chromatogram of PETN and its denitrated products PETriN (pentaerythritol trinitrate), PEDiN (pentaerythritol dinitrate) and PEMonoN (pentaerythritol mononitrate) prepared by treating PETN with zerovalent iron under anaerobic conditions as described by Zhuang et al. (2008).

Figure 3.5 shows the initial denitration route during photo-degradation of PETN by simulated sunlight.
Figure 3.5. Proposed photo-degradation route of PETN by simulated sunlight
4. Biotic degradation and degradation pathway in soil samples relevant to DRDC sites

4.1 Biotransformation of PETN

4.1.1 Introduction

Several studies have been published on the biodegradation and biotransformation of nitroaromatic and nitramine explosives, particularly TNT and RDX, respectively, but much less work has been done on the biodegradation of the nitrate ester explosive PETN. Structurally, the nitrate ester linkage is extremely rare in nature (White et Snape, 1993) and therefore, PETN is considered recalcitrant to biodegradation and represents a major remediation challenge at numerous munitions facilities.

Here we report PETN transformation by the indigenous microbial community of the DRDC-10 soil from DRDC-Valcartier and by pure microbial strains. The fastest PETN removal rate was obtained anaerobically in the presence of 1% molasses. Two soil bacterial isolates (Enterobacter sp. and Cupriavidus sp.) grew anaerobically with PETN as the sole N source. The fungus Phanerochaete chrysosporium transformed PETN to pentaerythritol dinitrate via two denitrification steps. Our results demonstrate the limited potential of natural attenuation of PETN in the DRDC sandy soil, but that bioremediation potential is greatly improved by the addition of a carbon source.

4.1.2 Materials and methods

Materials and culture media
Sandy soil (DRDC-10; pH 6.0, organic carbon 2%, and moisture 7.5%), provided by DRDC-Valcartier, was used for soil microcosms and enrichment cultures. Additionally, a surface soil collected in 2013 from the firing position of the legacy anti-tank training range, described in Bordeleau et al. (2012), was used for soil
enrichment cultures. The white-rot fungus *Phanerochaete chrysosporium* (ATCC 24725) was purchased freeze-dried from the American Type Culture Collection. The mineral salts medium (MSM; pH 7.0) consisted of (per liter of distilled water): 0.38 g K$_2$HPO$_4$, 0.2 g MgSO$_4$.7H$_2$O, 0.05 g FeCl$_3$.6H$_2$O. LB medium consists of 10 g tryptone, 5 g yeast extract and 10 g NaCl per liter of distilled water. The N-limited culture medium for *P. chrysosporium* was (per liter of 20 mM potassium acetate pH 4.2): 56 mM glucose, 1.2 mM ammonium-tartrate, 10 ml trace metals SL-10 and 1 mg thiamine.

*Soil microcosms*

DRDC-10 soil was used for the preparation of microcosms. Soil autoclaved at 121°C for 40 min for three consecutive days was used as abiotic controls. Soil slurries were prepared in 100-mL sterile serum bottles and consisted of 10 g of soil and 20 ml of MSM. PETN was added at a final concentration of 4 mg L$^{-1}$ (12.6 μM). Microcosms were either non-amended or amended with 10 mM glucose and 5 mM succinate (aerobic) or with 1% molasses (anaerobic). Anaerobic conditions were established by degassing with a vacuum pump for 20 min followed by 4 cycles of brief degassing and filling with O$_2$-free argon. Autoclaved controls were prepared for each condition. The microcosm bottles (triplicates) were incubated in the dark at 25°C on a rotary shaker at 150 rpm. Samples were collected at periodic intervals to monitor PETN disappearance over a period of 52 days.

*Isolation of PETN-transforming bacteria*

Serial dilutions of anaerobic soil slurries (DRDC-10 and DRDC-13) enriched with PETN and 1% molasses were plated on R2A agar. Representatives of different morphotypes of colonies were isolated on R2A and LB agar and restreaked twice to ensure purity. Based on size, shape and pigmentation, three or four different types of colonies were obtained. The isolates were tested for anaerobic growth in serum bottles containing MSM with 10 mM each of glucose, fructose and NaNO$_3$.
and 20 mg L\(^{-1}\) PETN and inoculated at an initial optical density at 600 nm (OD\(_{600}\)) of 0.03. The bottles were made anaerobic with argon and incubated at 25\(^\circ\)C and 150 rpm.

16S rRNA gene sequences were analyzed for phylogenetic identification of the strains. Genomic extraction was performed by boiling the cells for 5 minutes and centrifuging at max speed (16,100 x g for 2 min) in a micro-centrifuge. PCR amplification was performed on 1 \(\mu\)l of the supernatant as template. Primers 8F and 1492R were used for amplification of the near-full-length 16S rRNA gene (~1,500 bp). The DNA fragments were partly sequenced on both sides at the McGill University and Génome Québec Innovation Centre (Montreal, QC). The DNA sequences were submitted for comparison to the GenBank databases using the BlastN algorithm.

**PETN transformation by resting cells of strain AP3**

A resting cell assay was performed with strain AP3 grown i) anaerobically in LB medium supplemented with 1% glucose, 10 mM NaNO\(_3\) and 15 mM phosphate buffer pH 7.2, and 2) aerobically in LB plus 10 mM NaNO\(_3\). The cells were harvested at late exponential phase, washed twice in cold ddH\(_2\)O and resuspended in ddH\(_2\)O at OD\(_{600}\) of 2.0. PETN was added from a stock solution in acetonitrile (30 g L\(^{-1}\)) at a final concentration of 20 mg L\(^{-1}\). Cell suspensions without PETN served as controls. The anaerobic conditions were established by briefly degassing (5 min) followed by 4 cycles of degassing and filling with argon. The bottles were incubated at 25\(^\circ\)C in the dark with shaking at 150 rpm. At selected times, samples were collected, centrifuged at max speed for 5 min, and the supernatant was analyzed for PETN and its transformation products.

**PETN transformation by Phanerochaete chrysosporium**

*P. chrysosporium* was grown on potato dextrose agar for 10 days at 30\(^\circ\)C. After 10 days, 20 ml of sterile ddH\(_2\)O with 0.05% Tween 80 was poured onto each plate and a *hockey stick* (bent glass rod) was used to carefully scraped the
conidiospores from the agar; this stock of conidiospores was kept at 4°C until use. Cultures were started at an OD$_{600}$ of 0.07 in N-limited medium (1.2 ammonium) and incubated statically at 37°C for 7 days. At this point, these are considered ligninolytic cultures (defined as 7-day-old cultures grown in a N-limited medium) and are ready to be used for transformation assays. The transformation assays were performed in 120-ml sealed serum bottles containing 10 ml of the ligninolytic *P. chrysosporium* cultures supplemented with 32 μM PETN. The bottles were incubated statically at 37°C. To compensate for consumed oxygen, the headspace air in each bottle was changed every 3-5 days using a 60-ml syringe. Samples were collected at different times for 20 days, centrifuged, and the supernatant was diluted 1:1 with acetonitrile before analysis.

**Chemical analysis**

Analysis of PETN and its transformation products were as described in section 1.1.2 and 3.2.1. Briefly, PETN samples were analyzed with a Waters HPLC system equipped with a model 2996 photodiode array detector. The detector was set to scan from 192 to 450 nm. The detection limit was estimated to be 0.05 mg L$^{-1}$ at 242 nm. PETN and its degradation products were also analyzed using Bruker Esquire3000Plus mass analyzer attached to an HPLC system equipped with a DAD detector. For mass analysis, negative electrospray ionization mode was used to search for the characteristic mass ion fragment [ONO$_2$]$^-$ at m/z 62 Da, and the adduct mass ions [M+HCOO]$^-$_2 at m/z 361 Da, 316 Da, 271 Da and 226 Da for PETN, PETriN, PEDiN and PEMonoN, respectively. Mass range was scanned from 40 to 500 Da.

4.1.3 Results and discussion

**Biotransformation of PETN in DRDC soil**

The indigenous microbial community of the DRDC-10 soil transformed PETN under all conditions tested, i.e., aerobic and anaerobic, with or without nutrients (Fig. 4.1). After 2 days, we observed the loss of 10% PETN under both aerobic
and anaerobic conditions in unamended soil. In comparison, 22% and 34% PETN was transformed in aerobic and anaerobic nutrient-amended soils, respectively. On day 7, while a large amount of PETN was still present in unamended soils (75%) and in aerobic amended soil (66%), PETN was completely transformed in anaerobic molasses-amended soil. Except for the anaerobic amended soil where transformation was considerably faster, the other transformation rates were quite similar over time regardless of the conditions,

![Graph showing biotransformation of PETN in DRDC-10 soil.](image)

**Figure 4.1.** Biotransformation of PETN in DRDC-10 soil. Amendments were 10 mM glucose and 5 mM succinate in aerobic soil and 1% molasses in anaerobic soil. There was no significant loss of PETN in killed controls (not shown).

We did not find any other reports in the literature that evaluated biotransformation of PETN in soil microcosms. A few studies have focused on the transformation of PETN by bacterial strains obtained from soil enrichments. Zhuang et al. (2012) published a study using an anaerobic microbial consortia isolated from PETN-contaminated soil. They demonstrated that PETN could be transformed all the way to pentaerythritol via the mono-, di- and tri-denitrated intermediates under nitrate-reducing conditions.
**PETN-transforming bacteria**

We did not succeed to isolate PETN-transforming bacteria from the DRDC-10 soil. We obtained one or two colony morphotypes difficult to isolate in pure culture because of the invasive growth of fungal colonies. Once colonies were isolated in pure cultures and tested, they showed to be negative for growth with PETN as an N source. However, bacterial colonies were obtained on R2A agar from anaerobic, molasses-amended DRDC-13 soil slurries. Four of them were selected based on the colony size, shape and color and isolated in pure cultures. The four isolates were originally named AP1, AP2, AP3 and AP4. Each strain was tested for its ability to grow in mineral medium MSM supplemented with sugars, 10 mM NaNO₃ (as an electron acceptor) and PETN as an N source. Strains AP1, AP3 and AP4 grew anaerobically in the mineral medium with PETN as an N source to an OD₆₀₀ of ~0.22. No growth was observed in controls without PETN. HPLC analysis showed the formation of denitrated products in all cases. All the strains were also showed to metabolize PETN in the rich medium, where denitrated products were also formed. Strain AP2 did not show significant growth under the tested conditions and additional tests (e.g., using different carbon sources or electron acceptors) are needed to conclude if the strain is a PETN-degrader or not.

Explosives such as PETN are anthropogenic compounds with very few naturally occurring analogs. Natural microbial populations are thus not acclimated and explosives tend to persist in the environment. Although the total microbial community from the DRDC-10 soil (not contaminated) was able to transform PETN, we were not able to enrich and isolate a bacterium capable of transforming PETN from this soil. On the other hand, the indigenous microbial community of the DRDC-13, which have been exposed to nitrate ester contaminants (NG) for a long period of time, showed a promising bioremediation potential.
**Phylogenetic identification**

16S rRNA gene analysis (Table 4.1) revealed that the strains belong to the phylum *Proteobacteria*, with members of the *Alpha-, Beta- and Gamma-* subdivisions. Alignment of the DNA sequences of strains AP3 and AP4 showed that the two are identical. Visual observation also showed that the colonies of the two strains are indeed identical.

**Table 4.1.** Phylogenetic identification of the PETN-transforming bacteria based on the 16S rRNA gene analysis

<table>
<thead>
<tr>
<th>strain</th>
<th>identity</th>
<th>best match</th>
<th>class; order</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP1</td>
<td>100%</td>
<td><em>Cupriavidus metallidurans</em></td>
<td><em>Betaproteobacteria</em>; <em>Burkholderiales</em></td>
</tr>
<tr>
<td>AP2</td>
<td>100%</td>
<td><em>Sphingomonas</em> sp.</td>
<td><em>Alphaproteobacteria</em>; <em>Sphingomonadales</em></td>
</tr>
<tr>
<td>AP3</td>
<td>100%</td>
<td><em>Enterobacter</em> sp.</td>
<td><em>Gammaproteobacteria</em>; <em>Enterobacteriales</em></td>
</tr>
<tr>
<td>AP4</td>
<td>100%</td>
<td><em>Enterobacter</em> sp.</td>
<td><em>Gammaproteobacteria</em>; <em>Enterobacteriales</em></td>
</tr>
</tbody>
</table>

Strain AP1 shared 100% DNA identity with *Cupriavidus metallidurans*, a heavy-metal resistant bacterium, capable of degrading a variety of xenobiotics. Because heavy metals are often found as co-contaminants with explosives, strain AP1 may be an important player for bioremediation of contaminated sites at DRDC locations.

Strain AP2 produced red-pigmented colonies and was identified as a *Sphingomonas*. Several strains of *Sphingomonas* have been isolated from contaminated environments with the ability to transform toxic compounds such as TNT (Maeda et al. 2006).
Strain AP3, and consequently AP4, classified as *Enterobacter* spp. Binks et al. (1996) previously reported the isolation of *Enterobacter cloacae* PB2, a bacterium that can grow aerobically with PETN, NG or TNT as its sole N source (French et al 1998; Binks et al 1996). An enzyme, designated PETN reductase, was purified from cell extracts of PB2 and shown to reductively liberate nitrite ion from PETN to form pentaerythritol trinitrate and then pentaerythritol dinitrate, which is not a substrate for this enzyme. In our study, we demonstrated the ability of an *Enterobacter* sp. to transform PETN under both aerobic and anaerobic conditions. The presence of PETN was not required to induce the enzyme(s) involved in PETN reduction. Resting cells of *Enterobacter* strain AP3, grown aerobically in a rich medium in the absence of PETN, transformed PETN aerobically and anaerobically to pentaerythritol dinitrate via the intermediary formation of pentaerythritol trinitrate. Likewise, resting cells of strain AP3 grown anaerobically in rich medium transformed PETN anaerobically to pentaerythritol dinitrate. PETN transformation rates followed the order (growth/resting cells assay): anaerobic/anaerobic > aerobic/anaerobic > aerobic/aerobic, with 0.09, 0.59 and 1.13 mg L\(^{-1}\) PETN remaining after 2.5 h of incubation, respectively.

*Transformation of PETN by P. chrysosporium*

*P. chrysosporium*, member of the *Basidiomycetes*, is known to transform various xenobiotics under ligninolytic conditions. When incubated with PETN (30 \(\mu\)M, higher than solubility), the ligninolytic culture of *P. chrysosporium* transformed \(~25\ \mu\)M PETN in the first 5 days (Fig. 4.2). LC-MS analysis showed that the compound was transformed to pentaerythritol trinitrate, which was then transformed to pentaerythritol dinitrate as the final product. Servent et al. (1991) previously reported that *P. chrysosporium* transforms NG to its di- and mononitrate. Therefore, both nitrate esters undergo double denitration by the fungus, leaving intact one nitro-group in NG and two in PETN.
Figure 4.2 Transformation of PETN by a ligninolytic culture of *Phanerochaete chrysosporium*. The results are the means of triplicate assays.

Aerobic and anaerobic bacterial assays and the fungal assay all resulted in the formation of pentaerythritol dinitrate as final product of PETN transformation. Abiotic transformations of PETN by photolysis (Section 3.3) and by neutral and acidic hydrolysis at 100-125°C (Aubertein and Rehling, 1953) led to the same result. Although a few studies such as the one by Zhuang et al. (2012) demonstrated that pentaerythritol dinitrate was further transformed to pentaerythritol mononitrate, it appears that the stability of pentaerythritol dinitrate is very high. It will likely persist on soil surface and further studies on the physicochemical properties and ecotoxicity of this metabolite are suitable.
4.2 Transformation of DNAN and its derivatives by *P. chrysosporium*

4.2.1 Introduction

Manganese peroxidase (MnP), an enzyme secreted by ligninolytic fungi, is known to participate in the degradation of recalcitrant compounds including aromatic pollutants such as TNT (Hawari et al., 1999), 2,4-dichlorophenol (Valli et al., 1991) and 2,4-dinitrotoluene (2,4-DNT) (Valli et al., 1992). Last year we reported that when 4-amino-2-nitroanisole (4-ANAN) was incubated with MnP of *P. chrysosporium*, methanol was produced at a molar fraction of 0.25 (±1%), formaldehyde at 0.08 (±11%) and ammonia at 0.33 (±12%). The formation of these products suggested that 4-ANAN underwent demethylation and deamination. This year we assessed the transformation of 2,4-dinitroanisole (DNAN) and its two mono-amino derivatives, 2-amino-4-nitroanisole (2-ANAN) and 4-amino-2-nitroanisole (4-ANAN) by the fungus itself.

Our study on the environmental behavior and ecological risk associated with DNAN, partially reported in previous reports, was completed during this fiscal year to produce a comprehensive document entitled “Environmental fate of 2,4-dinitroanisole (DNAN) and its reduced products” (Annex 1).

4.2.2 Materials and methods

*Chemicals*

2,4-DNAN (98.4%) was provided by Defense Research and Development Canada (Valcartier, QC). 2-ANAN (99%) and 4-ANAN (98%) were purchased from MP Biomedicals LLC and Apollo Scientific Ltd, respectively.

*Biotransformation assays*

Biotransformation assays with cultures of *P. chrysosporium* (ATCC 24725) in N-limiting conditions were as described in Section 4.1.2. Ten ml of a 7-day-old
culture of *P. chrysosporium* was supplemented with DNAN, 2-ANAN or 4-ANAN at a concentration of 119 µM. Samples were collected at different times for 20 days, centrifuged, and the supernatant was diluted 1:1 with acetonitrile before analysis.

**Chemical analysis**

DNAN, 2-ANAN and 4-ANAN were analyzed by reversed-phase high-performance liquid chromatography (HPLC)-UV with a system that consisted of a W600 pump (Waters), a 717 plus autosampler and a 2996 Photodiode-Array Detector. 50-µL injection volumes were separated with a Discovery C18 column (25 cm x 4.6 mm x 5 µm) (Supelco, Oakville, Canada) at 35°C. The mobile phase (50% aqueous methanol) ran isocratically at 1 mL min⁻¹ for 15 min. The detector was set to scan from 192 to 450 nm. Detection of DNAN was performed at 298 nm and its limit of detection at this wavelength was 0.010 mg L⁻¹. Liquid chromatography–mass spectrometry (LC-MS) analyses were performed using a mass spectrometer (MS, Bruker MicroTOFQ mass analyzer) attached to an HPLC system (Hewlett Packard 1200 Series) equipped with a DAD detector. Aliquots (10 µL) were injected into a 3.5 micron-pore size Zorbax SB-C18 column (2.1 mm ID x 150 mm; Agilent, Mississauga, Canada) at 25°C. The solvent system was composed of a CH₃OH/H₂O gradient (40 to 90% v/v) at a flow rate of 0.15 mL min⁻¹. For mass analysis, negative electrospray ionization (ES-) was used to produce deprotonated molecules (M-H)⁻ and characteristic mass ion fragments. Mass range was scanned from 40 to 1000 Da.

4.2.3 Results and discussion

*P. chrysosporium* transformed DNAN and its two mono-amino derivatives under N-limiting conditions. DNAN was transformed at an average rate of ~4.1 µM/day during the 20 days of incubations. Both 2-ANAN and 4-ANAN were formed during the transformation of DNAN, which could be explained by the non-stereospecific nature of the lignin-degrading system of *P. chrysosporium*.
(Nakatsubo et al. 1982), presumed to participate in DNAN transformation like demonstrated for other nitroaromatic compounds. Two other peaks with retention times of 4.96 min and 5.63 min were observed on the HPLC chromatograms (not shown). 2-ANAN and 4-ANAN disappeared at an average rate of ~8.5 μM/d and ~5.7 μM/d, respectively. As seen for DNAN, peaks at 4.96 and at 5.63 min were observed on the chromatograms of 4-ANAN and 2-ANAN, respectively. The compound with a retention time of 5.63 min is suspected to be an hydroxylamino intermediate (not shown), while the one at 4.96 was identified as a putative formamido derivative by LC-MS analysis (Fig. 4.3). Another compound with a retention time of 5.27 min was produced during the transformation of 4-ANAN and tentatively identified as an acetamido derivative by LC-MS (Fig. 4.3).

![HPLC-UV chromatogram showing the putative formamido and acetamido derivatives of 4-ANAN.](image)

**Figure 4.3.** HPLC-UV chromatogram showing the putative formamido and acetamido derivatives of 4-ANAN.
5. Assessment of PETN toxicity to soil organisms (earthworms and plants)

5.1 Determination of the bioavailability of PETN by soil equilibration studies

5.1.1 Introduction

Bioavailability is important to understand the toxicity of a chemical on ecological receptors such as earthworms and plants. Soil equilibration studies were designed to determine the bioavailability of PETN following its addition in soil. PETN bioavailability was evaluated using a single amendment of PETN in DRDC-10 soil. DRDC-10 soil was chosen for the assay since our previous studies with other energetic materials showed that this soil, with its low biotransformation activity and low sorption to organic carbon, allows relatively high chemical bioavailability.

5.1.2 Materials and methods

PETN received from DRDC-Valcartier was in suspension in water (approximately 50-60% dry weight). For the equilibrium study, PETN was added directly to non-sterile DRDC-10 soil to reach a nominal concentration of 2000 mg PETN/kg dry soil. The soil was mixed using a three-dimensional rotary soil mixer for 18 h, the day before the experiment. ASTM type I water was then added to reach 75% of the DRDC-10 water holding capacity (WHC). Soil was left tightly covered at room temperature in the dark. Three aliquots of soil were taken at 0, 1, 2, 3, 7, 14, and 21 d following soil hydration. Concentrations of PETN in soil extracts were determined at each sampling date using the acetonitrile extraction procedure described in section 2.1.2.

In addition to acetonitrile extracts, the soil interstitial water (IW) was recovered by coupled filtration-centrifugation as described by Savard et al. (2010). In this method, 10 g of each soil sample was placed into a separate filter (10.8 cm long, 9 mL capacity). Each filter was inserted into a separate conical
polypropylene tube for subsequent centrifugation at 1800xg for 45 min at 20°C. Approximately 5 mL of filtrate was collected and passed through a 0.45 μm cartridge to eliminate the precipitate, and then was mixed with acetonitrile (1:1, v/v) before HPLC analysis (as described in section 1.1.2) for water-soluble PETN concentrations at the designated time periods.

5.1.3 Results and discussion

Figure 5.1 showed that PETN (2000 mg PETN/kg dry soil nominal concentration) was stable in moist soil for up to 21 d. No transformation products were observed in these samples. These data indicate that PETN is not sorbed in DRDC-10 soil.

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**Figure 5.1.** PETN recovered in soil acetonitrile extracts after up to 21-day equilibrium in DRDC-10 soil.
Interstitial water (IW) analyses of the equilibrated soils are presented in Figure 5.2. PETN was found in the IW samples at concentrations close to PETN water solubility (1.50 to 2.04 mg/L at 20 °C (this work; Merrill 1965; Chambers et al. 2002; Quinn et al. 2009)) (Fig. 5.2). These data indicate that the interstitial water is saturated at the concentration used (2000 mg PETN/kg soil). Statistical analyses of the IW results showed no significant differences between any of the time period ($p \geq 0.11$). For the subsequent toxicity studies using earthworms and plants, an equilibration time of seven days was chosen.

![Graph showing maximum solubility in water (1.5 to 2.04 mg/L)]

**Figure 5.2.** PETN recovered in interstitial water of soil equilibrated for up to 21 days in DRDC-10 soil.

The equilibrium study confirmed that PETN is not sorbed in DRDC-10 soil. Therefore, PETN bioavailability is limited to its water solubility limit.
5.2 Terrestrial toxicity of PETN to soil invertebrates (earthworms) and plants (ryegrass) exposed to amended DRDC-10 soil

5.2.1 Introduction

The above studies showed that PETN added in soil was present in the soil interstitial water (IW). The bioavailability of PETN in the soil IW indicates that this compound may affect soil eco-receptors. Therefore, experiments were conducted to evaluate the toxic effects of PETN on earthworms and on plants. PETN is a nitrate ester similar to nitroglycerin. However, PETN is less soluble in water than nitroglycerin (1.5-2.0 compared to 1800 mg/L). Despite its low solubility, PETN could still be toxic to soil receptors; our hypothesis was that PETN will cause adverse effects on earthworm and plants.

5.2.2 Materials and methods

Range-finding experiments were performed by adding PETN in DRDC-10 soil at nominal concentrations from 10 to 10,000 mg/kg using the same soil preparation as for the equilibrium study described above. Water was added to reach 75% of the soil WHC. Hydrated soils were left to equilibrate for 7 d prior to the beginning of the experiment. Soil was extracted with acetonitrile at the beginning of earthworms or plants tests to confirm soil concentrations. In addition, soil IW was extracted from these PETN-amended soils to quantify the PETN bioavailable fraction at each nominal concentration. The same batch of amended soil was used for both earthworms and plants toxicity assays.

The 14-d earthworm lethality assay (USEPA, 1989) was performed using *Eisenia andrei*, originally obtained from Carolina Biological Supply (Burlington, NC, USA), and cultured in our lab for at least one year prior to their use as a test species. Twenty four hours prior to the initiation of the assay, the earthworms were acclimated in a moist ‘clean’ soil using DRDC-10 without amendment. An environment-controlled incubator (20 ± 1°C, humidity set at 70%, photoperiod
cycle of 16 h-light (800 ± 400 lux) and 8 h-dark) was used. Ten *E. andrei* earthworms (with well-developed clitellum, weighing from 0.35 to 0.52 g) were exposed to PETN-amended soil for up to 14 d. Intermediate measurements were taken after 7 d. At the end of the assay, the surviving earthworms were counted, rinsed and weighed.

Phytotoxicity of PETN was determined using ASTM and U.S. Environmental Protection Agency (USEPA) methods as described by Rocheleau et al. (2008). The test species perennial ryegrass *Lolium perenne* Express was obtained from Pickseed Canada Inc. (St-Hyacinthe, QC). All treatments including 0 (control) were carried out in triplicate and were incubated in sealed plastic bags to maintain soil moisture for the duration of the test. Plant toxicity tests were performed in a temperature and light-controlled growth chamber (Conviron, Winnipeg, MB, Canada). Seedling emergence was determined after 7 d, whereas shoot growth (dry mass) was determined after 19 d.

5.2.3 Results and discussion

PETN was recovered from soil in both acetonitrile extracts and IW samples prior to earthworms addition (Table 5.1). PETN concentrations in acetonitrile soil extracts were consistent with the nominal concentrations, except for 100 mg/kg that was less than 70% of the theoretical value. This difference may be attributed to difficulties associated to the weighing of the moist PETN starting material.

The concentrations of PETN in soil IW increased with increasing nominal concentrations in soil, and seem to reach a plateau around 2.4 mg/L. A similar effect was observed by Savard et al. (2010), where RDX concentration in soil IW was shown to approach its water solubility. The values obtained here are consistent with the PETN concentrations obtained in the equilibrium study, in which PETN was found at 1.74 mg/L in soil amended with 2000 mg/kg for 7 d.
Table 5.1. PETN measured in acetonitrile (ACN) and interstitial water (IW) extracts of amended soil prior to the beginning of toxicity tests

<table>
<thead>
<tr>
<th>Nominal PETN at start of study (mg/kg)</th>
<th>PETN measured in ACN extracts (mg/kg)</th>
<th>PETN measured in soil IW (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>10</td>
<td>13.4 ± 3.9</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>30</td>
<td>26.7 ± 6.7</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>100</td>
<td>67.9 ± 22.0</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>1000</td>
<td>1091.7 ± 196.6</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>10000</td>
<td>9425.9 ± 427.7</td>
<td>2.4 ± 0.1</td>
</tr>
</tbody>
</table>

PETN did not significantly affect earthworm survival at any of the concentration tested ($p > 0.05$). All earthworms survived after the 14 d exposure period in DRDC-10 soil. However, Figure 5.3 shows that PETN has sub-lethal effects reflected by a significant decrease in earthworm wet weight at the end of the exposure period for earthworms exposed to PETN concentrations of 1,000 and 10,000 mg/kg ($p \leq 0.018$) compared to control (no chemical added). This sub-lethal effect may be associated to other biological effects such as decrease in reproduction, accumulation, or ingestion of soil by the worms. Further experiments should be done to verify this statement. The present results are in agreement with Quinn et al. (2009) who reported no mortality of rats following oral exposure to PETN.
Figure 5.3. Earthworms loss weight following the 14-day lethality assay.

Results of the ryegrass toxicity assay are presented in Table 5.2. Ryegrass seeds showed growth after 7 d in DRDC-10 soil amended with PETN. The seed germination was 88% or higher at all concentrations tested although the control group showed higher but not significantly different (using paired t-test) germination rate (96.6%) than the treated groups. However, plant weights were significantly diminished compared to the control group at PETN concentrations greater than 26 mg/kg (Fig. 5.4). Nevertheless this growth inhibition effect was only less than 20% of the control group, and would not be considered as an adverse effect. A hormetric effect (growth stimulation) was observed at the lowest concentration tested (13 mg/kg). Unfortunately, these data could not be modelled because only one concentration showed growth stimulation. Additional tests will have to be performed at concentrations lower than 13 mg/kg, and matching water solubility, to estimated ECx values using hormetric model.
Table 5.2. Phytotoxicity parameters measured in the 19-day ryegrass assay

<table>
<thead>
<tr>
<th>Concentration (mg/kg)</th>
<th>Early Seedling 7 d (% germinated)</th>
<th>Seedling 19 d (% germinated)</th>
<th>Growth (mg wet weight)</th>
<th>Growth (mg dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>96.7</td>
<td>96.7</td>
<td>114.3 ± 13.7</td>
<td>16.4 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>95.0</td>
<td>95.0</td>
<td>120.6 ± 5.0</td>
<td>17.2 ± 0.4</td>
</tr>
<tr>
<td>30</td>
<td>90.0</td>
<td>90.0</td>
<td>100.4 ± 5.9</td>
<td>14.3 ± 1.0</td>
</tr>
<tr>
<td>100</td>
<td>88.3</td>
<td>90.0</td>
<td>93.3 ± 5.5</td>
<td>13.9 ± 1.0</td>
</tr>
<tr>
<td>1,000</td>
<td>90.0</td>
<td>91.7</td>
<td>96.8 ± 7.9</td>
<td>14.1 ± 0.9</td>
</tr>
<tr>
<td>10,000</td>
<td>91.7</td>
<td>95.0</td>
<td>103.9 ± 8.8</td>
<td>15.1 ± 1.1</td>
</tr>
</tbody>
</table>

Figure 5.4. Phytotoxic effect of PETN to ryegrass after 19-day growth in soil

5.2.4 Conclusions
The present study showed that PETN is present in interstitial water of amended soil (with up to 10,000 mg/kg) at concentrations approximating its water solubility limit (maximum 2.4 mg/L). The PETN concentrations (up to 10,000 mg/kg) used
in the present soil toxicity study did not significantly affect earthworm (*Eisenia andrei*) survival or ryegrass (*Lolium perrenne*) early seedling germination. However, sub-lethal effects (such as decreased earthworm body weight after 14 days and diminution of ryegrass biomass after 19 days) were observed at concentrations of 1092 mg/kg and higher for earthworms or 27 mg/kg and higher for plants.
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ANNEXES
ANNEX 1

Environmental fate of 2,4-dinitroanisole (DNAN) and its reduced products

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ABSTRACT

Several defense departments intend to replace 2,4,6-trinitrotoluene (TNT) in munitions formulations by the less sensitive 2,4-dinitroanisole (DNAN). To help understand environmental behavior and ecological risk associated with DNAN we investigated its key initial abiotic and biotic reaction routes and determined relevant physicochemical parameters (pKₐ, log Kₐw, aqueous solubility (Sₐ), partition coefficient (Kₐ)) for the chemical and its products. Reduction of DNAN with either zero valent iron or bacteria regioselectively produced 2-amino-4-nitroanisole (2-ANAN) which, under strict anaerobic conditions, gave 2,4-diaminoanisole (DAAN). Hydrolysis under environmental conditions was insignificant whereas photolysis gave photodegradable intermediates 2-hydroxy-4-nitroanisole and 2,4-dinitropheno!l. Physicochemical properties of DNAN and its amino products drastically depended on the type and position of substituent(s) on the aromatic ring. Sₐ followed the order (TNT < DNAN < 2-ANAN < 4-ANAN < DAAN) whereas log Kₐw followed the order (DAAN < 4-ANAN < 2-ANAN < DNAN < TNT). In soil, successive replacement of –NO₂ by –NH₂ in DNAN enhanced irreversible sorption and reduced bioavailability under oxic conditions. Although DNAN is more soluble than TNT, its lower hydrophobicity and its tendency to form aminoderivatives that sorb irreversibly to soil contribute to make it less toxic than the traditional explosive TNT.

Keywords: 2,4-dinitroanisole; 2,4-diaminoanisole; regioselectivity; insensitive munition
1. Introduction

The insensitive munition compound 2,4-dinitroanisole (DNAN) is currently under evaluation by the defense industry as a replacement for more sensitive explosives such as 2,4,6-trinitrotoluene (TNT). It has been reported that DNAN requires a higher temperature than TNT for detonation thus making it safer to manufacture, transport and store (Davies and Provatas, 2006). Before large scale production and deployment in the field, the fate and ecological risk associated with DNAN and its potential transformation products, especially the amine-derivatives, require investigation. Few studies have been reported on the biotic or abiotic transformation of DNAN (Platten III et al., 2010; Ahn et al., 2011; Perreault et al., 2012; Olivares et al., 2013; Rao et al., 2013; Salter-Blanc et al., 2013). Several of these studies identify the reduced products, 2-amino-4-nitroanisole (2-ANAN) and 2,4-diaminoanisole (DAAN), as key initial or final products (Platten III et al., 2010; Ahn et al., 2011; Perreault et al., 2012; Olivares et al., 2013). Currently, there is only limited knowledge available on the transport, transformation, and toxicity of DNAN and its amine derivatives. (Figure 1).

Other nitroaromatic compounds (NACs) such as TNT and dinitrotoluenes (DNTs) are known to undergo sequential reduction to their corresponding amino derivatives with selectivities depending on redox conditions used and on the position of the –NO₂ group on the aromatic ring (Barrows et al. 1996). A number of papers reveal that aromatic amines, if formed in a soil environment, tend to irreversibly bind to soil organic matter through covalent bonding, e.g. -CO-NH- linkages (Haderlein and Schwarzenbach, 1995; Rieger and Knackmuss, 1995; Elovitz and Weber, 1999; Thorn and Kennedy, 2002). Once bound to soil, the amines are considered to represent a lower ecotoxicological risk due to their lack of bioavailability. One might exploit past observations on TNT environmental behavior, i.e. transformation, transport and ecotoxicity, to gain some insights into the environmental behavior of DNAN but the immediate challenge that one may face is how to deal with the dramatic differences in the reaction products of the two nitroaromatic compounds. In most cases TNT is regioselectively reduced at the para-position (Funk et al., 1993; Rugge et al., 1998; Elovitz and Weber, 1999; Wang et al., 2000), whereas DNAN seemingly favors reduction at the ortho-position (Olivares et al., 2013; Perreault et al., 2012). For example, Barrows et al. (1996) reported that abiotic reduction of TNT with bisulfite is 100% regioselective towards forming the para isomer 4-amino-2,6-dinitrotoluene as opposed to DNAN that under the same conditions produces
only the ortho isomer 2-ANAN. Different products mean different physicochemical properties (aqueous solubility ($S_w$), $pK_a$, $K_{ow}$) which will strongly affect the partitioning of chemicals between water, soil, and environmental receptors, and consequently the ecological impact of these chemicals.

The present study was thus undertaken to help understand the behavior of DNAN under natural environmental conditions and to compare its behavior to that of TNT, which it may replace in the near future. We first investigated relevant transformation processes likely to occur under environmental conditions. Then we determined key relevant physicochemical and transport parameters ($K_{ow}$, $pK_a$, $S_w$, $K_d$) of DNAN and its observed products to predict their respective ecotoxicological risk and therefore provide an answer to the key question of whether DNAN represents an environmental risk higher or lower than that induced by TNT.

2. Materials and methods

2.1. Chemicals

DNAN (98.4%) was provided by Defense Research and Development Canada (Valcartier, QC). DAAN (99%), 2,4-dinitrophenol (DNP) (85%) and 1-octanol were purchased from Sigma-Aldrich (Oakville, ON). 2-ANAN (99%) was purchased from MD Biomedicals (Santa Ana, CA) and 4-ANAN (98%) was purchased from Apollo Scientific, Bradbury, UK. Zero valent iron (ZVI) was from Fisher Scientific (Nepean, ON). All solvents and reagents were used as received.

2.2. Soils

Two soils were used in this study. Table 1 lists their relevant properties. PETAWAWA soil was a silty soil sampled (5-15 cm depth) at Petawawa training range (ON, Canada), and TOPSOIL soil was a gardening top soil purchased from a local gardening company (Fafard, QC, Canada). Each soil was stored in a cold room (4°C) until use, passed through a 2-mm sieve, air dried, and sterilized by gamma irradiation from a $^{60}$Co source at the Canadian Irradiation Center (Laval, Quebec) with a dose of 50 kGy over 2 h.

2.3. Biological transformation of DNAN

Anaerobic biotransformation of DNAN was performed with resting cells of Enterobacter strain DM7 (our lab), Shewanella oneidensis strain MR-1 (ATCC 700550), Pseudomonas fluorescens I-C (NRRL B-59269) and Burkholderia cepacia strain JS872 (ATCC 700450). Cells were grown anaerobically, in LB (MR-1, I-C and JS872) or M9
minimal medium (Maniatis et al., 1982) plus 10 mM NaNO₃ (DM7), in the presence of 50 μM DNAN. The cells were harvested at late exponential phase, washed twice in sterile double-distilled water (ddH₂O), and resuspended at an optical density (at 600 nm) of ~3.3 in ddH₂O containing 200 μM DNAN. The reactions were performed in triplicate in serum bottles that were sealed and made anaerobic by briefly degassing (1 min) and then purging with argon for 10 min at 5 psi. The bottles were incubated at 25°C in the dark. Samples were collected at selected times (1-22 h) for analysis of DNAN and its products as described below.

2.4. Reaction of DNAN with zero valent iron, ZVI
Experiments were carried out in 60-mL serum bottles each containing granular iron (ca 40 mesh) (0.5 g) and 50 mL aqueous solution of DNAN (50 mg L⁻¹) at room temperature. The bottles were sealed with Teflon coated septa under argon. Aliquots (2 mL) of the reaction mixture were withdrawn at selected times ranging from 15 to 360 min and analyzed by LC-MS as described below. Experiments were conducted in duplicate.

2.5. Photolysis of DNAN
Artificial sunlight (total irradiance of 590,000 mW m⁻²) generated from a SolSim photoreactor (Luzchem Research Inc., Canada) was used to photolyze DNAN under solar-simulated conditions. Irradiation assays were conducted in duplicate at 25°C in 20 mL quartz crucibles (25 mm ID) containing 5 mL of aqueous solutions of DNAN (50 mg L⁻¹). Samples were withdrawn at selected times ranging from 0.7 to 21 d and analyzed by HPLC and LC-MS as described below. DNP (70 mg L⁻¹) was also photolyzed using the same conditions. A control containing DNAN covered with aluminum foil was also prepared.

2.6. Solubility measurements
Aqueous solubility of DNAN, 2-ANAN, 4-ANAN, and DAAN was measured at 25°C, in triplicate, as described previously (Monteil-Rivera et al., 2004). Briefly, suspensions of DNAN, 2-ANAN, or 4-ANAN were agitated at 150 rpm and analyte concentration was measured in the supernatant until constant values were measured. Solubility was reached within 2 weeks for DNAN and 2-ANAN, and 3 months for 4-ANAN. For DAAN, an anoxic suspension (0.4 g/10 mL) was prepared under argon to minimize decomposition and polymerization, then sonicated for 5 min, capped and sealed with an aluminum crimp and stirred at 150 rpm. At each sampling event, the suspension was
deaerated with argon and sonicated for 5 min. Aliquots of the aqueous phase were analyzed by HPLC as described below.

2.7. Octanol-water coefficient (K_{ow}) measurements

Log K_{ow} for DNAN, 2-ANAN, 4-ANAN, and DAAN was measured in triplicate at 23 ± 2 °C using the traditional flask shaking method as described previously (Monteil-Rivera et al., 2004). The aqueous phase was analyzed directly by HPLC whereas the octanol phase was diluted five times with a solution containing 70% methanol in water prior to HPLC analysis.

2.8. Measurement of dissociation constants, pKₐ's

pKₐ of 2-ANAN, 4-ANAN, and DAAN was measured spectrophotometrically as described by Albert and Serjeant (1971) using 10⁻² M chloroacetic, formic, and chloroacetic and acetic buffers, respectively.

2.9. Sorption assays

Sorption and fate of DNAN and its amino-derivatives were studied in long term batch experiments using the soils described in Table 1. Sorption experiments were conducted in borosilicate tubes (50-mL) containing either DNAN, 2-ANAN, 4-ANAN, or DAAN (50 mg L⁻¹ each) and sterile soil (1.5 g) in 10 mL water. Samples were incubated aerobically, statically and away from light at room temperature (23 ± 2°C). Samples were not tightly closed and were shaken twice a week to ensure a good aeration level. At time intervals varying from 1 day to 2 months, three replicates were sacrificed. The supernatant was withdrawn, filtered through a 0.45 μm PVDF Millipore filter (Millipore Corp., Bedford, MA), diluted 1/1 in acetonitrile (CH₃CN) and analyzed by HPLC as described below. Sorbed analyte was extracted from soil by sonication in acetonitrile as described in the EPA SW-846 Method 8330 (USEPA, 1997). The soil water distribution coefficient (K_d in L kg⁻¹) was calculated as the ratio [DNAN]_s/[DNAN]_eq, where [DNAN]_s is the concentration of DNAN adsorbed on soil (mg kg⁻¹) and [DNAN]_eq is the concentration of DNAN in the aqueous phase at equilibrium (mg L⁻¹). The normalized distribution coefficient (K_{oc}) was calculated as the ratio K_d/f_OC where f_OC represents the fraction of organic carbon in soil. A percent recovery was calculated as ((DNAN_s + DNAN_i)/DNAN_{ini}) × 100, where DNAN_s, DNAN_i and DNAN_{ini} represent the amount (µg) of DNAN sorbed, soluble, and initially introduced, respectively.
2.10. Stability of DAAN in water
An aqueous solution of DAAN (100 mg L\(^{-1}\)) was prepared using water deaerated with 20-
min argon bubbling and split into two bottles. One bottle (oxic) was vigorously aerated under air, screw capped and kept at room temperature in the dark. The second one (anoxic) was capped with a rubber stopper, crimped with aluminum seal, bubbled with argon for 10 min and incubated statically at room temperature in the dark. Aliquots of the oxic and anoxic solutions (3 mL) were periodically sampled and analyzed by HPLC-UV, UV-Vis, and LC-MS, as described below. The oxic sample was stirred for 10 min under air at each sampling event.

2.11. Analytical methods
UV-Vis spectra of aqueous solutions of DNAN or its products were collected using a UV1 Thermo Spectronic spectrophotometer.

DNAN and its reduced products were quantified by HPLC as described previously, using 50% methanol as mobile phase and a C18 column for separation (Perreault et al., 2012). Detection limits were estimated to be 0.010 mg L\(^{-1}\) at 298 nm for DNAN, 0.005 mg L\(^{-1}\) at 245 nm for 2-ANAN and 4-ANAN, and 0.010 mg L\(^{-1}\) at 245 nm for DAAN.

Unknown products were analyzed using a Bruker MicroTOFQ mass analyzer attached to an HPLC system (Hewlett Packard 1200 Series) equipped with a DAD detector. Samples (10 μL) were injected into a 2.5 micron-pore size Synergi-Polar column (2 mm ID × 100 mm; Phenomenex) at 25°C. The solvent system was composed of CH\(_3\)OH and HCOOH (0.05%) at a flow rate of 0.15 mL min\(^{-1}\) using a gradient. For mass analysis, positive and negative electrospray ionization modes were used to produce protonated [M+H]\(^+\) and deprotonated [M−H] molecular mass ions. Mass range was scanned from 40 to 500 Da.

Nitrite, nitrate, formate and ammonium were quantified by ion chromatography as described previously (Balakrishnan et al. 2004). Formaldehyde derivatized with 2,4-
pentadione was quantified by HPLC (Bhatt et al. 2006).
3. Results and discussion

3.1. Transformation of DNAN

When we investigated the aerobic biotransformation of DNAN by *Bacillus* sp., DNAN appeared to give regioselectively the *ortho* isomer, 2-ANAN, as a terminal reduced amine product (Perreault et al., 2012). No DNAN ring cleavage products were observed. Recently, Olivares et al. (2013) also reported regioselective reduction of DNAN to 2-ANAN in sludge that subsequently was reduced to DAAN under both aerobic and anaerobic conditions. In the present study, transformation of DNAN by resting cells of four strains of DNAN-reducing bacteria was investigated anaerobically. Resting cells of *Enterobacter* strain DM7 incubated anaerobically transformed DNAN at the rate of 9.4 ± 0.2 μmol min⁻¹ g⁻¹ protein with the formation of 2-HA-NAN (2-hydroxylamino-4-nitroanisole) that subsequently reduced to 2-ANAN (0.42 moles for each mole of DNAN degraded). Similarly, *B. cepacia* JS872 reduced the –NO₂ of DNAN at the *ortho*-position only. Resting cells of *S. oneidensis* MR-1 transformed DNAN into 2-ANAN at a rate of 2.1 ± 0.2 μmol min⁻¹ g⁻¹ protein. However in this case, 2-ANAN further transformed to DAAN. Anaerobic cells of *P. fluorescens* I-C followed the same pathway as MR-1, transforming DNAN to DAAN via 2-ANAN. In no case was 4-ANAN detected. This differs significantly from TNT, which was shown to be biologically reduced to its *para* amine derivative, 4-amino-2,6-dinitrotoluene (Funk et al., 1993).

Abiotically, Ahn et al. (2011) reported reduction of DNAN with ZVI to DAAN with the formation of intermediates such as 2-ANAN and 4-ANAN without any reference to regioselectivity. Koutsospyros et al. (2012) used a Fe/Cu system to degrade DNAN but no products were shown. In the present study, treatment of DNAN with ZVI gave the *ortho*-hydroxylamine (2-HA-NAN) in few min; the latter was further reduced to the *ortho*-amine derivative, 2-ANAN, which was subsequently reduced to the diamine DAAN product (Fig. 2). The nitroso-nitroanisole detected in samples at 15 and 80 min by LC-MS was presumably the *ortho*-nitroso derivative (2-NO-NAN) of the DNAN (Fig. 2). After 6 h of reaction DAAN was detected as the only product. The *para*-isomer, 4-ANAN, was not detected. Regioselectivity of Ar–NO₂ reduction in substituted NACs usually depends on the reducing agent used and the type and position of the substituent relative to the nitro group. In DNAN, regioselectivity might be controlled by two competing factors, one driven by steric effects favoring reduction at the *para* –NO₂ group and another driven by electronic effects favoring reduction at the more electronegative –NO₂ group, *i.e.*, the
ortho position (Davey et al., 1994, Barrows et al., 1996). This analysis is best exemplified by the products distribution observed during reduction of DNAN and its sulfur analogue 2,4-dinitrothioanisole using Baker's yeast. In the first case, the reduction was 80% regioselective at the ortho position (electronically favored) while in the second case the more sterically hindered S atom directs reduction mostly at the para position (Davey et al. 1994). Terpko and Heck (1980) found that triethyl ammonium formate reduces 2,4-DNT at the least hindered para –NO₂ group but reduces DNAN and other dinitroaromatics including 2,4-dinitroaniline and 2,4-dinitrophenol (DNP) at the more sterically hindered ortho- position. In addition to the electronic rationale given above to explain regioselective ortho reduction of DNAN, we attribute the ortho regioselective reduction to the stability gained through intramolecular H-bonding between the -OMe group and the ortho –NH₂ which are absent in the case of the para-isomer 4-ANAN. In summary, experimental evidences gathered from the present work and from literature reports indicate that reduction of DNAN occurs regiosselectively at the ortho position.

When a DNAN aqueous solution was photolyzed under solar simulated conditions, DNAN disappeared following a first order kinetics at a rate of 0.22 d⁻¹. After complete disappearance of DNAN (t = 21 d), we detected nitrate anion (0.7 mol), ammonium (1.0 mol), and formaldehyde and formic acid (total of 0.9 mol) for each mole of DNAN degraded. 2-hydroxy-4-nitroanisole (2-HONAN) together with smaller amounts of DNP were detected (Fig. 3) but neither the ortho- nor the para- ANAN isomer was observed. Production of DNP started quickly and reached a maximum at 7 d of irradiation. In a separate experiment, we found DNP to be unstable under the same photolytic conditions giving nitrocatechol as a major degradation product. This finding is in line with an earlier study by Rao et al (2013) who found the same products and neither 2-ANAN nor 4-ANAN during photolysis of DNAN. Interestingly, we detected several formamide derivatives of both aminonitroanisole and aminonitrophenol, which most likely resulted from the reaction of formaldehyde (or formic acid) originally generated from the demethylation of the ArO-Me group in DNAN with the amine groups of the amino derivatives.

As for hydrolysis, Hill et al. (2012) and Salter-Blanc et al. (2013) indicated the much slower hydrolysis of DNAN compared to TNT. Although DNAN has been shown to hydrolyze to give mainly DNP (Murto and Tommila, 1962; Rochester, 1963; Davies and
Provatas, 2006), the reaction occurred only under severe alkaline conditions (≥ pH 12). Under natural environmental conditions, DNAN does not hydrolyze.

3.2. Physicochemical properties of DNAN and its products

Table 2 summarizes the physicochemical properties ($S_w$, $pK_a$, and $K_{ow}$) measured herein for DNAN and its amino derivatives 2-ANAN, 4-ANAN, and DAAN. Relevant parameters of the more traditional explosive TNT are also gathered in Table 2 for comparison.

The aqueous solubility of DNAN and its major products follows the order: DNAN < 2-ANAN < 4-ANAN < DAAN (Table 2). Sequential reduction of the nitro groups into amino groups increased the water solubility of the aromatic chemical. However, while the solubility of 4-ANAN (4.43 ± 0.06 g L⁻¹) was twenty times higher than that of DNAN (0.213 ± 0.012 g L⁻¹), that of 2-ANAN (0.252 ± 0.008 g L⁻¹) was only slightly higher than DNAN. Two chemical phenomena might explain these different solubilities: 1/ the para isomer might have an ability to form solute-solvent intermolecular H-bonding that the ortho isomer cannot form due to the intramolecular H-bonding mentioned above, or 2/ the para isomer is more easily protonable than the ortho isomer. In water, substituted aminoaromatics (ArNH₂) equilibrate with their acidic protonated forms (ArNH₃⁺) and the dissociation constant ($pK_a$) of the latter depends on the relative position of the NH₂ group and other substituents, -MeO and -NO₂ in the present case, on the aromatic ring. The $pK_a$ values measured herein show that 4-ANAN ($pK_a = 3.50$) will be more protonated and hence more soluble than 2-ANAN ($pK_a = 2.55$) in distilled water (pH 5.5). As for DAAN ($pK_{a1} = 2.61$; $pK_{a2} = 5.46$) half of the chemical is expected to be monoprotonated at the water pH of 5.5, thus explaining its markedly higher water solubility. TNT was the least soluble of all chemicals (0.15 g L⁻¹).

The octanol/water partition coefficient, log $K_{ow}$ for DNAN and its reduced products followed the order: DNAN > 2-ANAN > 4-ANAN > DAAN (Table 2), which is the exact opposite of the observed solubility trend. Successive replacement of the –NO₂ groups by –NH₂ groups in DNAN therefore reduces the hydrophobicity (Table 2). The precursor, DNAN, which is less soluble than any of its amine derivatives, exhibited a higher log $K_{ow}$ value (1.58). The fully reduced DAAN, which partially ionizes in water, is very polar and has a marked preference for water over organic solvents (log $K_{ow} < -1$). TNT, with its three –NO₂ groups, was the most hydrophobic chemical of all (1.8 < log $K_{ow} < 2.0$).
With significantly higher solubility in water and equal or lower hydrophobicity than DNAN, DNAN products including 2-ANAN, 4-ANAN, and DAAN have a high potential to migrate through subsurface soil unless their migration is slowed down by immobilization mechanisms different from simple hydrophobic partitioning. The next section is aimed at elucidating soil-water interactions for DNAN and its products.

3.3. Sorption and fate of DNAN and its products in soil

Sorption and fate of the four chemicals were measured individually in the two sterile soils under aerobic conditions (Fig. 4). The amounts of chemicals sorbed on soil were obtained by extraction in CH$_3$CN, which should only desorb chemicals sorbed by weak (electrostatic, electron donor-acceptor) and stronger (hydrophobic partitioning) reversible interactions but not the ones irreversibly chemisorbed by covalent binding (Elowitz and Weber, 1999). While DNAN sorbed reversibly on the two soils, the monoamines sorbed both reversibly and irreversibly on both soils, and DAAN sorbed or reacted (see stability of DAAN below) irreversibly in the two soils (Fig. 4). $K_d$ values corresponding to reversible non-covalent binding are provided in Table 3 along with normalized $K_{oc}$ values. The concurrent reversible and irreversible processes observed with 2-ANAN and 4-ANAN led to non-equilibrium situations that forced us to measure $K_d$ values for both monoamines at day 2, when irreversible binding was at its minimum.

Nitroaromatics (NACs) can sorb reversibly to organic matter, as dictated by their $K_{ow}$, or to clay minerals through electron donor-acceptor complexes with the oxygen of the siloxane surface(s) of the clays or through complexation with exchangeable cations (Haderlein et al., 1996; Zhang et al., 2005; Qu et al., 2011). The large $K_d$ values measured in TOPSOIL soil despite its low clay content and the overall correlation observed between $K_d$’s and $K_{ow}$’s confirm the binding of DNAN, 2-ANAN and 4-ANAN with organic matter. On the other hand, the markedly larger $K_{oc}$ values measured for all three amino compounds in PETAWAWA soil (Table 3) suggest the occurrence of interactions between the three amines and clay. The concurrent sorption of nitroaromatics to both organic matter and clay in natural soil was also recently demonstrated by Qu et al. (2011) using 2,4-DNT. As previously reported for other NACs (Haderlein et al., 1996), in the present study, reversible sorption decreased with the number of nitro groups and followed the order (DNAN > 2-ANAN > 4-ANAN > DAAN ≈0).
Opposite to reversible sorption, irreversible sorption increased with the number of amino groups and was more pronounced for 4-ANAN than for 2-ANAN. While DNAN was fully recovered after two months in sterile PETAFAWA and TOPSOIL soils, less than 40% of 2-ANAN and 20% of 4-ANAN was recovered after 2 months in either soil, and DAAN did not persist after 3 days in either soil. The increasing irreversible binding observed for DNAN amine products with increasing number of amino groups has previously been observed when contacting other NACs, e.g. TNT, and their reduced products with organic rich soils under oxic conditions (Haderlein and Schwarzenbach, 1995; Rieger and Knackmuss, 1995; Elovitz and Weber, 1999). In the absence of catalyst, the –NH₂ group of aromatic amines undergo nucleophilic addition with quinones and other C=O groups of the natural organic matter (Thorn and Kennedy, 2002). Using ¹⁵N NMR and labeled chemicals, Thorn et al. (2002 & 2008) showed that amino-derivatives of TNT or DNTs bind covalently to soil organic matter through aminohydroquinone, aminoquinone, imine, and amide linkages. The increasing pKₐ values measured for 2-ANAN, 4-ANAN, and DAAN are indicative of an increase of nucleophilicity of the –NH₂ in the order 2-ANAN < 4-ANAN < DAAN, which supports a similar order for the increase of binding capacity of the chemical with electrophilic sites (C=O, COOH). The most common products of DNAN, 2-ANAN and DAAN, will be less mobile than DNAN or completely immobilized, respectively, in soil under oxic conditions. If non transformed, DNAN will be slightly more mobile than TNT in soil (Table 2).

3.4. Stability of DNAN products in water

We found both monoaminated products of DNAN to be stable over six months in water, under air, at ambient temperature (23 ± 2 °C), and away from light. Under the same conditions, DAAN remained stable under argon but disappeared under air at a rate of approximately 0.08 d⁻¹. Disappearance of DAAN under air was accompanied by the formation of several products as indicated by the broad shoulder appearing between 250 and 500 nm in UV-Vis spectra (Fig. 5). LC-MS (ES⁺) analysis of the oxic solution after 18 d showed two peaks with protonated molecular mass ions [M+H]⁺ at m/z 273.116 and m/z 259.101 matching the empirical formulae C₁₄H₁₈N₄O₂ and C₁₃H₁₄N₂O₂, respectively, which were tentatively identified as the azo dimer of DAAN and its demethylated azo derivative. Previously, Platten III et al. (2010) observed a product with an m/z 259 after exposing DAAN to air in the effluent of an anaerobic digester fed with DNAN and attributed the mass ion to a mass fragment of the azo dimer of DAAN with
[CH$_2$]$^+$ cleaved off. Our study suggests that both the azo dimer and the demethylated azo dimer of DAAN were formed when exposing an aqueous solution of DAAN to air.

Instability of DAAN in water under oxic conditions is similar to what we observed earlier with the autooxidation of diamino derivatives of 2,4-DNT (Yang et al., 2008) and triamino derivatives of TNT (Hawari et al 1998).

3.5. Implication on toxicity of DNAN and its products

In a recent study, Dodard et al. (2013) compared the toxicity of DNAN and TNT and found that TNT was more toxic than DNAN when either aqueous (Vibrio fischeri, green algae) or terrestrial (earthworms) receptors were investigated. Donlon et al. (1995) reported that NACs were approximately 500-fold more toxic towards methanogens than their corresponding amines. Recently, Liang et al. (2013) confirmed this trend for DNAN but showed that only complete reduction to DAAN decreased the toxicity of DNAN to Aliivibrio fischeri, used in the Microtox assay. From literature data it appears that the toxicity of DNAN and its products in aqueous media follows the order TNT > DNAN > (or =) 2-ANAN > DAAN. This trend is perfectly in line with the decreasing hydrophobicity (log $K_{ow}$) measured herein (Table 2) and is likely related to the higher ability of hydrophobic compounds to bioaccumulate in receptors. Based on the $K_{ow}$ measured in the present study (Table 2), 4-ANAN should exhibit a toxicity markedly lower than that of DNAN, between that of 2-ANAN and DAAN. In soil, the amino-derivatives will be less bioavailable than their nitro analogous due to possible irreversible binding, so that the lower toxicity observed in water should be confirmed in soil.

4. Conclusion

To help understand the environmental behavior and ecological risk associated with DNAN we investigated its key initial abiotic and biotic reaction routes and determined relevant physicochemical parameters ($S_w$, $pK_a$, log $K_{ow}$, $k_d$) for the chemical and its products. 2-ANAN and DAAN were identified as DNAN major products, abiotically and biotically. DNP was found to form under photolysis conditions and photodecompose. $S_w$ followed the order (TNT < DNAN < 2-ANAN < 4-ANAN < DAAN) whereas log $K_{ow}$ followed the order (DAAN < 4-ANAN < 2-ANAN < DNAN < TNT). In soil successive replacement of –NO$_2$ by –NH$_2$ in DNAN enhanced irreversible sorption and reduced
bioavailability under oxic conditions. Toxicity of DNAN was found to be lower than that of TNT and toxicity of DNAN amino products was found comparable to or lower than that of DNAN (Dodard et al., 2013; Liang et al., 2013). The present findings therefore favor the use of DNAN against that of TNT in munitions manufacturing.

Acknowledgements

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References


Science and Technology Organization, DSTO-TR-1904 Department of Defense, Commonwealth Australia.


Murto, J., Tommila, E., 1962. The influence of the solvent on reaction velocity XXI. The reaction of 2,4-dinitrophenyl alkyl ethers with hydroxyl ion in water and in methanol-water and ethanol-water mixtures. Acta Chem. Scand. 16, 63-70.


Table 1. Physicochemical properties of soils investigated

<table>
<thead>
<tr>
<th></th>
<th>Particle size distribution</th>
<th>CEC*</th>
</tr>
</thead>
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<tr>
<td></td>
<td>% Clay/Silt (&lt;80 μm)</td>
<td>% Sand (&gt;80 μm)</td>
</tr>
<tr>
<td>PETAWAWA</td>
<td>44.1</td>
<td>55.9</td>
</tr>
<tr>
<td>TOPSOIL</td>
<td>0.6</td>
<td>99.4</td>
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</tbody>
</table>

*CEC = Cationic Exchange Capacity
Table 2. Properties of TNT, DNAN, and DNAN major products

<table>
<thead>
<tr>
<th>Compound</th>
<th>( S_w ) at 25°C (g L(^{-1}))</th>
<th>Log ( K_{ow} ) at 25°C</th>
<th>pK(_a) at 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value(^a)</td>
<td>Reference</td>
<td>Value(^a)</td>
</tr>
<tr>
<td>TNT</td>
<td>0.150</td>
<td>Rosenblatt et al. 1991</td>
<td>1.86-2.00</td>
</tr>
<tr>
<td>DNAN</td>
<td>0.213 ± 0.012</td>
<td>This work</td>
<td>1.58 ± 0.01</td>
</tr>
<tr>
<td>2-ANAN</td>
<td>0.252 ± 0.008</td>
<td>This work</td>
<td>1.47 ± 0.01</td>
</tr>
<tr>
<td>4-ANAN</td>
<td>4.43 ± 0.06</td>
<td>This work</td>
<td>0.80 ± 0.01</td>
</tr>
<tr>
<td>DAAN</td>
<td>&gt; 40</td>
<td>This work</td>
<td>&lt; -1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\(^a\) Values are given as mean ± standard deviation; \(^b\) NA = non applicable.
Table 3. Soil-water partition coefficients, $K_d$ and $K_{oc}$, for DNAN and its amino derivatives

<table>
<thead>
<tr>
<th>Component</th>
<th>PETAWAWA</th>
<th>TOPSOIL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (L kg$^{-1}$)</td>
<td>$K_{oc}$ (L kg$^{-1}$)</td>
</tr>
<tr>
<td>DNAN</td>
<td>9.1 ± 0.7</td>
<td>364 ± 28</td>
</tr>
<tr>
<td>2-ANAN</td>
<td>7.9 ± 0.8</td>
<td>316 ± 32</td>
</tr>
<tr>
<td>4-ANAN</td>
<td>6.0 ± 0.3</td>
<td>240 ± 12</td>
</tr>
<tr>
<td>DAAN</td>
<td>&lt; 0.01</td>
<td>&lt; 0.5</td>
</tr>
</tbody>
</table>

$^a$ Values are given as mean ± standard deviation
Figure 1. Structures of DNAN and its amino products 2-amino-4-nitroanisole (2-ANAN), 4-amino-2-nitroanisole (4-ANAN), and 2,4-diaminoanisole (DAAN)
Figure 2. UV chromatograms (Diode Array signals between 200 and 400 nm) of DNAN and its products after various times of ZVI reduction
Figure 3. LC-MS (ES-) extracted ion chromatogram (m/z 153) of DNAN and its products photolyzed after 1 d of simulated solar irradiation
**Figure 4.** Overall mass balance for the reaction of DNAN and its major products in sterile aerobic soils (Error bars represent standard deviation)
**Figure 5.** UV-Vis monitoring of DAAN transformation in water, away from light
ANNEX 2

Ecotoxicological assessment of a high energetic and insensitive munitions compound: 2,4-Dinitroanisole (DNAN)

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Keywords: 2,4-dinitroanisole; ecotoxicity; environmental risk assessment; explosives; Microtox

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ABSTRACT

The high explosive nitroaromatic 2,4-dinitroanisole (DNAN) is less shock sensitive than 2,4,6-trinitrotoluene (TNT), and is proposed as a TNT replacement for melt-cast formulations. Before using DNAN in munitions and potentially leading to environmental impact, the present study examines the ecotoxicity of DNAN using selected organisms. In water, DNAN decreased green algae *Pseudokirchneriella subcapitata* growth (EC$_{50} = 4.0$ mg/L), and bacteria *Vibrio fischeri* bioluminescence (Microtox, EC$_{50} = 13.9$ mg/L). In soil, DNAN decreased perennial ryegrass *Lolium perenne* growth (EC$_{50} = 6.9$ mg/kg), and is lethal to earthworms *Eisenia andrei* (LC$_{50} = 47$ mg/kg). At sub-lethal concentrations, DNAN caused an avoidance response (EC$_{50} = 31$ mg/kg) by earthworms. The presence of DNAN and 2-amino-4-nitroanisole in earthworms and plants suggested a role of these compounds in DNAN toxicity. Toxicity of DNAN was compared to TNT, tested under the same experimental conditions. These analyses showed that DNAN was equally, or even less deleterious to organism health than TNT, depending on the species and toxicity test. The present studies provide baseline toxicity data to increase the understanding of the environmental impact of DNAN, and assist science-based decision makers for improved management of potential DNAN contaminated sites.
1. Introduction

Highly energetic chemicals used for explosives and propellants can be found in soil at military training sites as well as at munitions production and disposal facilities. Contamination of soil, and ground and surface water by these chemicals is a serious health and environmental problem, and could result in high costs for managing and mitigation of contaminated sites [1,2]. There is a recent interest in the introduction of new shock-insensitive munitions compounds such as 2,4-dinitroanisole (DNAN) (Fig. 1) for use in various munitions compositions and applications (e.g., PAX-21, IMX-101, and IMX-104) [3-6]. Fig. 1 shows the structures of DNAN and its transformation products including 2-amino-4-nitroanisole (2A-4NAN) and 4-amino-2-nitroanisole (4A-2NAN) together with 2,4,6-trinitrotoluene (TNT). As Fig.1 shows, these compounds share strong electron –NO₂ withdrawing groups, which are important in determining the environmental fate, transport, transformation, and ecological impact of the explosive [7]. Because DNAN is a nitroaromatic compound like TNT, it might then share similar acute and chronic toxic effects in humans and ecological receptors.

Like TNT, DNAN has been reported to transform easily under abiotic and biotic conditions to initially produce amino-reduced products (Fig. 1). For example, DNAN in water can undergo photo-transformation and can biologically reduce under anaerobic and aerobic conditions to form 2A-4NAN and 2,4-diaminoanisole [8-11]. These DNAN degradation studies suggest that DNAN can undergo abiotic and biotic transformation in the environment. Some of these pathways could be used for DNAN environmental remediation. For example, a reductive technology based on Fe/Cu bimetallic particles was proposed for the treatment of aqueous effluents contaminated with DNAN [12]. However, DNAN may also decrease the performance of microbial degradation

Currently, there is a lack of published information on the toxicity of DNAN and its related amine products on ecological species. For toxicity, DNAN has been used by the armed forces as a lice egg pesticide in MYL formula [14] and has been found to be mutagenic in the Salmonella/mammalian microsome test [15]. Recent studies conducted by the U.S. Army described a 90-d toxicity study of DNAN using rats [16]. Toxicological effects included organ-specific effects, including neurotoxicity. Another study reported the acute and chronic toxicity of DNAN to aquatic vertebrate and invertebrate species including the larval fish Pimephales promelas and the water flea Ceriodaphnia dubia [17]. The 48-h median lethal concentration (LC$_{50}$) ranged from 37 to 42 mg/L DNAN, whereas the sublethal and chronic effects (EC$_{50}$, median effect concentration) ranged from 11 to 15 mg/L DNAN, using these two species. Effects on unicellular primary producers (bacteria, algae) were not reported, and detailed information on the terrestrial toxicity of DNAN is scant. Preliminary unpublished studies [18,19] reported that DNAN was toxic to earthworms Eisenia fetida exposed for 28 d in amended field soil (100% mortality at 300 mg/kg). DNAN is toxic to wheat exposed to a 0.01 M DNAN solution on filter paper for 7 d [20]. These studies indicate that more information on the ecotoxicological effects of DNAN is needed for environmental hazard and risk assessment.

The objectives of the present study were to determine the toxicity of DNAN to various test species representing different trophic levels including bacteria (Vibrio fischeri), freshwater green algae (Pseudokirchneriella subcapitata, formerly known as Selenastrum capricornutum), earthworms (Eisenia andrei), and plants (Lolium perenne). Results will be compared with TNT, and finally, we will examine the behavioral effects of DNAN amended soil on earthworms.
because in earlier studies, earthworms avoided TNT-contaminated soils [21]. Chemical analyses of soil fractions as well as test organism tissue residues were used to confirm DNAN exposure concentrations and the presence of DNAN transformation products such as 2A-4NAN and 4A-2NAN in the test systems. Bioavailability of DNAN in soil was monitored using chemical analyses of the soil interstitial water (IW) fractions, based on the soil (sediment) water equilibrium partitioning [22,23].

2. Materials and methods

2.1. Chemicals and reagents

2,4-Dinitroanisole (purity 98.4%) was obtained from Defense Research Development Canada (DRDC) – Valcartier, whereas TNT (purity >99.9 %) was obtained from ICI explosives Canada (McMasterville, Qc, Canada). Reference standards including DNAN, TNT, 2A-4NAN, 4A-2NAN, and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), were purchased from AccuStandard (New Haven, CT, USA) or Sigma-Aldrich Chemical (Canada). Acetone and acetonitrile (HPLC grade) were obtained from Caledon Laboratories (Georgetown, ON, Canada). ASTM type I water [24] was obtained using a MilliQ system (Millipore, Canada).

2.2. Preparation of exposure media for toxicity testing

For the aquatic toxicity tests, a stock solution of DNAN (30 mg in 100 mL water) was prepared and kept in the dark at room temperature. Aliquots were taken every other day for up to two weeks to verify the DNAN concentration using HPLC analysis (described below). For terrestrial toxicity testing, a natural sandy soil (DRDC2010; 0.7% clay, 2.0% organic matter, 97.6% sand, 1.6% silt, and pH 5.5 – 6.0) provided by DRDC Valcartier in 2010, was used. This soil was amended with DNAN using acetone as the carrier solvent as described previously [25], and was
used throughout the study. Following solvent evaporation, three replicates from each dry soil batch were hydrated individually to 75% of the soil water holding capacity. Soil samples amended with different DNAN concentrations (0 (control), 10 to 1,000 mg/kg) were taken at days 1, 3, 7, and 14, following soil hydration.

Soil equilibration studies were then performed in the dark to estimate the minimum time required for DNAN to equilibrate in the hydrated soil and to maximize its bioavailability prior to terrestrial toxicity testing. Concentrations of DNAN and transformation products in soil acetonitrile extracts (total extractable fraction) and in soil IW (bioavailable fraction), were determined separately at each sampling time. The total DNAN added in soil was confirmed using the acetonitrile extraction procedure, whereas the amount of dissolved DNAN in soil IW was determined using the coupled filtration-centrifugation method [26,27]. The collected IW (about 1 mL) was analyzed using HPLC.

2.3. Toxicity to bacteria, algae, and terrestrial plants

Toxicity assays conducted in the current study complied with our in-house laboratory control charts, and results for known reference toxicants (as suggested in the cited standard toxicity protocols) are reported in Tables 1 and 2. The standard 15-min Microtox toxicity test was performed on the aqueous samples as previously described [28] using the following nominal test concentrations of DNAN: 0 (negative control), 0.1, 1.5, 6.0, 13, 63, and 126 mg/L, or TNT: 0 (negative control), 0.1, 0.2, 0.4, 0.8, 2.0, and 6.5 mg/L based on our earlier studies with TNT [29,30]. Microtox data were expressed as the average percentage of light emission inhibition compared with the negative control. To express the toxic response, data were analyzed using the maximum likelihood probit method, and effective concentration that causes 50 % reduction of bioluminescence compared to the negative control (EC$_{50}$) was determined. The test was done in
triplicate; negative control was 2% NaCl (no DNAN or TNT added) in water. Vibrio fisheri had optimal bioluminescence at pH values between 6.0 and 8.0, therefore pH was adjusted when necessary with NaOH to verify if effects are due to pH toxicity. Both Microtox measurements (with and without pH adjustment) are reported.

The chronic toxicity of DNAN and TNT to freshwater green algae was tested using the 72-h growth inhibition of P. subcapitata, described elsewhere [31]. Algae were exposed to different nominal concentrations of aqueous DNAN solution (0 (negative control), 0.1, 0.2, 0.3, 0.6, 1.3, 2.0, 3.0, 5.0, 7.0, and 10 mg/L), or aqueous TNT solution (0 (negative control), 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, and 51.2 mg/L in a 96-well microplate and under continuous lighting at 24 ± 2°C. These concentrations were chosen based on earlier studies [29,30]. Following exposure to aqueous samples, the number of algae cells was measured using a Coulter Z2 (Beckman Coulter) cell counter, and the percentage of growth inhibition was calculated compared with the negative control. Data were plotted, and linear regression and interpolation were used to determine the inhibitory concentration that causes 50% reduction of cell growth compared to a control (EC50).

The phytotoxicity of DNAN in soil was assessed using previously described methods [25,32,33]. The test species perennial ryegrass L. perenne Express was obtained from Pickseed Canada Inc. (St-Hyacinthe, Quebec, Canada). Nominal concentrations of DNAN were 0 (negative control), 0.5, 1.0, 2.0, 3.0, 5.0, and 10 mg DNAN/kg soil. All treatments were carried out in triplicate.

Tests were performed in a temperature and light-controlled growth chamber (Conviron, Winnipeg, Manitoba, Canada). Seedling emergence was determined after 7 d, whereas shoot growth (dry mass) was determined after 19 d. Shoots were cut just above the soil line, and dry mass per treatment groups (mg tissue) was determined after lyophilizing the shoot tissue in a
freeze-dryer for 24 h. Dried tissues were stored at 4°C until extraction and HPLC analyses, described below.

2.4. Toxicities of DNAN and TNT to earthworms

*E. andrei* were originally obtained from Carolina Biological Supply (Burlington, NC, USA) and were cultured in our laboratory, as described elsewhere [27]. Clitellated earthworms weighing from 307 to 626 mg were acclimated to laboratory conditions in the DRDC2010 soil for 24 h prior to testing. The 14-d earthworm lethality test was done according to earlier methods [34,35] and included the following concentrations: 0 (negative control), 10, 20, 30, 60, 100, 200, and 300 mg DNAN/kg dry soil, or 10, 30, 60, 100, and 200 mg TNT/kg dry soil. After 7-d exposure, earthworms were counted and dead individuals were removed, and the experiment was continued for another week. After 14-d exposure, earthworms were counted, rinsed, weighed, purged on filter paper, and stored in -80°C for tissue residue determination. Data were expressed as the total number of survivors in the different treatment groups compared with the control. The concentration that causes 50% mortality compared to control (LC50) was calculated using the linear interpolation method, and was used as the toxicity endpoint value.

The 48-h acute avoidance test measures the behavior of earthworms in stainless steel circular test units containing amended soil (Fig. 2A,B), as described by others [35,36]. Separate test units were used for each DNAN concentration. The nominal DNAN concentrations in soil were 0 (control), 10, 20, 30, 100, and 1000 mg/kg (Fig. 2B). Worms were allowed to move between compartments (Fig. 2A). At the end of the study, each compartment was carefully emptied and earthworms were counted, recorded, rinsed, purged, and weighed on filter paper for tissue residue determination. Soil samples were also taken for later extraction and chemical analysis for DNAN and its products.
The avoidance response was expressed as percentage of the average ratio of earthworms found in the control compared with treated sections of each test unit, according to the following equation:

\[
\text{Avoidance (\%) = } \frac{\text{(\# worms in clean soil)} - \text{(\# worms in test soil)}}{\text{(total \# worms)}} \times 100.
\]

where, at the end of the study, (\# worms in clean soil) is the number of live worms found in all compartments containing unamended soil, (# worms in test soil) is the number of live worms found in all compartments containing DNAN-amended soil, and (total # worms) is the total number of live worms found in all compartments. The avoidance behavior was estimated using a criterion of ≥ 60% avoidance response [35]. Earthworms from controls and treated compartments were analyzed separately for the presence of DNAN or its metabolites.

2.5. Analyses of DNAN and its transformation products

Triplicate soil samples (2 g each) were collected from each soil amendment and were extracted using USEPA Method 8330A [37], prior to quantification by HPLC. Aliquots of the Microtox and green algae toxicity test media were taken at the beginning of the experiment for chemical analyses to confirm the DNAN exposure concentrations. Acetonitrile was then added to these and the soil IW samples (1:1, v/v) for HPLC analyses of DNAN and its degradation products.

Plant and earthworm tissue samples were extracted according to others [38-40]. For plants, 500 μL of HMX solution (1 mg/L in acetonitrile, used as an internal standard) and was added to the dry material in a 15 mL glass centrifugation tube. Plant tissue was further extracted for determination of acid-extractable 2A-4NAN using 8 μL formic acid per 150 μL plant extract, prior to HPLC analyses.
All extracts were filtered through a 0.45 μm Millex™ HV cartridge (Millipore, Nepean, ON, Canada) prior to HPLC analyses for TNT, DNAN, 2A-4NAN, and 4A-2NAN. The HPLC system consisted of a Waters 600 pump (Waters Corp, Milford, MA), a 717 plus autosampler, and a 2996 photodiode-Array Detector. Samples (50 μL) were separated with a Discovery C18 column (25 cm, 4.6 mm, 5 mm) (Supelco, Oakville, Canada) at 35°C. The mobile phase (50% aqueous methanol) was run isocratically at 1 mL/min for 15 min. The detector was set to scan from 192 to 450 nm. HPLC peak detection was set for TNT at 254 nm, or 298 nm for the other compounds, with a detection limit of 0.005 mg/L for TNT and 0.010 mg/L for all other compounds. The limits of quantitation were 0.1, 0.5, and 1.0 mg/kg dry weight for soil, earthworm, and plant, respectively.

2.6 Statistical analyses

For each toxicity assay, the data were plotted as concentration-response curves. The toxicity endpoints were expressed as EC$_{50}$ or LC$_{50}$ values, as determined using best curve fitting of data, and described elsewhere [41,42]. Uncertainty of these endpoint values was expressed as 95% confidence intervals (CI). Different software packages (TOXCALC v5.0, 1997; SYSTAT 11.00, SPSS Inc., Chicago, IL) were used to analyze the toxicity test data. The Student’s two tailed t-test was used to analyze the changes in earthworm wet weights. For avoidance test results, analysis of variance was done using SYSTAT. Fisher least significant difference (LSD) test was performed for means separations among treatments. A significance level of $p \leq 0.05$ was accepted for all statistical tests.
3. Results and discussion

3.1. Aqueous toxicities of DNAN and TNT

Concentration-response studies showed that DNAN and TNT inhibited the luminescence of *Vibrio fischeri* (Table 1). The 30-min Microtox EC₅₀ value for DNAN was 55.9 (95% CI 44.2 – 112.0) mg/L at pH 5.2 (unadjusted), and did not change greatly when the solution pH was adjusted (pH 6-8). Microtox toxicity studies of TNT yielded 30-min EC₅₀ value of 0.7 (0.2 – 1.3) mg/L at pH 5.3 (unadjusted), a similar value was found after pH adjustment (Table 1). These data show that *Vibrio fischeri* bacteria is more sensitive to TNT than DNAN. Exposure to DNAN decreased growth of freshwater green algae *P. subcapitata* using the 72-h growth inhibition test. Table 1 shows the 72-h EC₅₀ values of 2.0 (1.7 – 2.2) at pH 5.2 (unadjusted), and 4.0 (3.5 – 4.2) mg/L after pH adjustment (pH 6-8). *P. subcapitata* was more sensitive to TNT with 72-h EC₅₀ value of 0.7 (0.6 -0.7) mg/L at pH 5.3 (unadjusted) than DNAN described above (Table 1). Adjustment of pH (from pH 6 – 8) did not greatly change this response.

3.2. Soil water equilibration studies

Confirmation of DNAN equilibration in the soil toxicity test systems and verification of the nominal exposure concentrations were done using acetonitrile extracts of hydrated soil samples left for up to 14 d. Data showed that from 90 to 97% of the DNAN was recovered in extracts of soil amended from 10 to 1000 mg/kg (Fig. 3; data not shown for 1000 mg/kg treatment). Fig. 3A shows that the initial concentration of DNAN measured in the acetonitrile extracts of amended soil confirmed the nominal soil concentrations. The soil IW was also used to quantify the concentrations of bioavailable DNAN in the freshly amended sandy soil samples. Concentrations of DNAN recovered from the soil IW were linear with nominal DNAN concentrations in soil (Fig. 3B; \( R^2 = 0.991 \); 3 points) until a maximum of 160 mg/L was attained that corresponded to
40 – 60 mg/kg dry soil. This maximum value is slightly less that the reported aqueous solubility of DNAN (191 mg/L in salted water) at 25°C [43]. No DNAN degradation products could be quantified in the soil acetonitrile extracts, whereas only traces (< 0.5 mg/kg) of 2A-4NAN were observed sporadically in the IW extracts (data not shown). Analysis of DNAN from acetonitrile extracts (Fig. 3A) and soil IW (Fig. 3B) were similar (p > 0.05) in samples taken between 1, 3, 7, and 14 d; therefore, a one-day equilibration period was chosen before starting the earthworm and plant toxicity studies.

3.3. Terrestrial toxicity of DNAN and TNT

Chemical analyses showed that control DRDC2010 soil did not contain quantitative levels of explosives, or unacceptable concentrations of environmental contaminants [44]. Earthworm toxicity assays were done using DNAN- and TNT-amended separately in DRDC2010 soil (from 0 (control) to 300 mg/kg). Data shows that both TNT and DNAN-amended soils were toxic to earthworms. The LC₅₀ values for earthworm mortality were 38 (27 – 43) and 38 (28 – 42) for TNT, and 98 (60 – 141) and 47 (32 – 81) mg/kg soil for DNAN, for the 7-d and 14-d exposures, respectively (Table 2). Exposure to DNAN amended soils also caused a minor but significant decrease in worm wet weights (p ≤ 0.05) at concentrations ≥ 20 mg/kg (Fig. 4A).

These data show that earthworms were up to 2.6-times more sensitive to TNT (192 µmoles/kg) than DNAN (495 µmoles/kg), under the same experimental conditions. The EC₅₀ results reported here (using a sandy soil) are lower than those reported in other studies using TNT-amended forest soils [45-47]. However, the difference in relative toxicities of DNAN and TNT to the earthworm may also be attributed to the differences in soil properties (e.g., clay and organic material) and associated microbial degradation potential [7,48].
Chemical analyses of test soil (Fig. 4B,C) and earthworm tissue (Fig. 4D) showed that addition of earthworms to soil aided in transformation of DNAN to 2A-4NAN. Concentrations of DNAN and 2A-4NAN in earthworm tissues increased with increasing soil concentrations up to 28 mg DNAN/kg dry soil (Fig. 4D). At this soil concentration, 152 ± 13 (SEM) mg/kg DNAN and 531 ± 8 mg/kg 2A-4NAN were recovered in the earthworm tissues. The tissue concentrations of 2A-4NAN were always higher than DNAN for all soil concentrations tested. The DNAN transformation product 4A-2NAN was not quantified, suggesting that DNAN biotransformation is regio-selective and probably involves enzymes associated with the earthworms, as discussed below.

Additional studies explored the behavior of earthworms exposed to sub-lethal concentrations of DNAN in soil, based on the earthworm toxicity results reported above. Using the 48-h avoidance test, earthworms were allowed to choose between compartments containing control or DNAN amended soils (Fig. 4A). Results showed that earthworms could detect and avoid exposure to DNAN, and a concentration-dependent avoidance effect was observed. Earthworms did not show preferences (or avoidance) to groups having 20 mg/kg or less DNAN amended soil. The EC$_{50}$ value for avoidance was 31 (14 – 147) mg/kg (Table 2). Earthworms have a similar avoidance response to TNT-contaminated field soils at comparable concentrations (29 mg/kg) [21]. It is not known whether the worm avoidance response to DNAN reflects sensorimotor effects [49] that may lead to neurotoxicity, because DNAN-induced neurobehavioral effects were reported in rat toxicity studies [16].

Tissue residue analyses (Fig. 5A) shows that earthworms recovered from the control soil compartments at the end of the study had low or similar DNAN levels (36 to 67 µg/g dry tissue) compared with up to 201 µg/g in earthworms recovered from the 91 mg/kg treated soil ($p \leq 0.05$).
compartment. Only one earthworm was found in the 996 mg/kg treatment group and could not be analyzed due to insufficient tissue. Earthworms found in the control soil compartments at the end of the study had from low to similar 2A-4NAN concentrations (20 to 48 μg/g), whereas those recovered from DNAN treated compartments had from 38 to 120 μg/g of 2A-4NAN (Fig. 5B). Notably, worms in the 91 mg/kg compartment had a significantly higher \( p \leq 0.05 \) concentration of 2A-4NAN (120 μg/g) compared with the other treatment groups. These residues are most likely explained by the passage of earthworms through at least one of the DNAN-containing soil compartments during the bioassay, before choosing and remaining in the control soil compartments at the end of the study. Accordingly, these earthworms probably carried DNAN from the amended soil compartments, and eventually excreted the DNAN and 2A-4NAN into the control soil compartments.

Determination of the DNAN toxicity and behavioral endpoints expressed as concentrations (such as \( LC_{50} \) or \( EC_{50} \) values) for earthworms can be very challenging because the soil DNAN concentrations were not stable, and decreased during these earthworm tests. This decrease could result from transformation (as evidenced by 2A-4NAN formation) and sorption to soil components. This suggests that the observed toxic effects of DNAN amended soil in earthworms might be caused by DNAN, its transformation products such as 2A-4NAN, or both.

Transformation of nitroaromatic compounds by earthworms was reported earlier in studies with TNT amended soils [46,47].

Chemical analyses of earthworm tissue showed the presence of 2A-4NAN. These results are consistent with findings of Perreault et al. [10] who also detected 2A-4NAN as the major end-product formed via arylnitroso and aryl-hydroxylamino intermediates, using an isolated *Bacillus* strain (13G) from aerobic soil microcosms incubated with DNAN. Microbial transformation of
DNAN that results in formation of products toxic to earthworms is supported by earlier studies showing that soil invertebrates contain microflora that have nitroaromatic reducing activity (e.g., a nitroreductase) as described by Dodard et al. [50]. These workers reported that white potworm (*Enchytraeus albidus*) homogenates could transform TNT in vitro to amino-dinitrotoluenes and diamino-nitrotoluenes, an effect that is completely inhibited by addition of broad spectrum antibiotics. A similar transformation mechanism for DNAN might be occurring in earthworms, and link the formation of 2A-4NAN to the observed DNAN toxicity in earthworms. Further investigations to determine the relative toxicities of DNAN and 2A-4NAN in earthworms are in progress.

Ryegrass emergence was inhibited in DNAN amended soil at > 5 mg/kg. Table 2 shows similar EC$_{50}$ values for both 7-d seedling emergence and 19-d shoot growth, i.e., 6 (5 – 7) and 7 (6 – 7) mg/kg, respectively. These effects are similar to others [19] who studied DNAN phytotoxicity on wheat (*Triticum aestivum*) using filter paper tests. Our data here showed that DNAN can be more phytotoxic than TNT (EC$_{50}$ = 137 mg/kg), based on earlier studies using the same ryegrass species and a Sassafras sandy loam, which had a higher clay content (17%) and lower pH (5.2) [25] compared to the soil used in our studies. The present results also show that ryegrass toxicity tests are more sensitive to DNAN than earthworm tests (lethality and avoidance responses) for similar durations of exposure. Tissue residue analyses (using HPLC) showed the presence of DNAN (up to 14.9 ± 1.5 µg/g dry tissue at 4.7 mg/kg dry soil; Fig. 6A) and an acid-extractable form of 2A-4NAN (up to 37.6 ± 4.5 µg/g dry tissue at 4.7 mg/kg dry soil; Fig. 6B) in DNAN exposed ryegrass shoots. No other DNAN transformation products were quantified (< 0.5 µg/g) in the plant samples.
3.4. Comparison of TNT and DNAN toxicities and insight to modes of DNAN toxicity

Comparing the toxicities of TNT and DNAN to earthworms and bacteria under the same test conditions showed that TNT was equal to, or even more toxic than DNAN, depending on the species and toxicity test (Tables 1 and 2). On an equimolar basis, the EC_{50} ratios of DNAN/TNT (indicates how many times TNT is more toxic than DNAN) were \sim 100 for bacteria, 3-8 for algae, and 1.3-2.6 for earthworms. The exact reason for the interspecies differences in nitroaromatic ecotoxicity is not known [1]. Presently there is no consensus on the mode of DNAN toxicity. A recent toxicity report hypothesized that DNAN toxicity to rats is related to 2,4-dinitrophenol (DNP) that may be formed by DNAN metabolism in vivo [16]. DNP is a known mitochondrial uncoupler of oxidative phosphorylation leading to increased respiration and decreased ATP generation. Dumitras-Hutanu et al. [20] also speculated that DNP is underlying the toxicity of DNAN to wheat. It is unlikely that DNP is the toxicant underlying DNAN toxicity in the present Microtox studies. Firstly, if the toxic effects of DNAN on luminescent bacteria (30-min EC_{50} = 282 \, \mu\text{mol/L}; Table 1) is caused by DNP toxicity (15-min EC_{50} is 215 \, \mu\text{mol/L} [51]), then nearly all of the DNAN added to the Microtox test media should have been transformed to DNP. Our chemical analysis showed no change in DNAN concentration in the 30-min Microtox test media. Secondly, DNP was not quantified in the incubation media, although internal DNP concentrations (within Vibrio fischeri) were not measured. Therefore, it is unlikely that DNAN toxicity was caused by DNP, at least using the Microtox test.

4. Conclusions

Despite its benefits as a shock insensitive energetic material, DNAN has deleterious effects on bacteria, algae, earthworms, and plants, using standard toxicity tests applicable for
ecotoxicological hazard and risk assessment. Also, earthworms can detect and avoid sub-lethal concentrations of DNAN in soil. Toxicity endpoint data reported here can be used for ecotoxicological risk assessment, and to assess the relative toxicity of DNAN and TNT. The DNAN transformation product 2A-4NAN was quantified in exposed earthworms and plants suggesting that biotransformation of DNAN might occur in these organisms, and may be related to the observed DNAN toxicity.

**Acknowledgements**

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**References**


Figure legends:

Fig. 1  Structures of DNAN, TNT, and some transformation products related to DNAN.

Fig. 2  Stainless steel circular test units showing chambers empty (left) and filled with soil samples (right) used for the earthworm avoidance test.

Fig. 3  2,4-Dinitroanisole (DNAN) concentrations recovered from acetonitrile extracts (A) and interstitial water (B), of DNAN-amended soil

Fig. 4  Changes in earthworm wet weights following 14-d exposure to DNAN-amended soil (A). DNAN and 2A-4NAN recovered from DNAN-amended soil (B), DNAN-amended soil interstitial water (C), and earthworm tissue residues (D), at the end of the 14 d earthworm lethality test.

Fig. 5  Tissue concentrations of DNAN (A) and 2A-4NAN (B) recovered from the 48-h earthworm avoidance test. Common letters designate no significant difference ($p > 0.05$) between treatment groups.

Fig. 6  DNAN and its product tissue concentrations in ryegrass grown in DNAN-amended soil for 19 d. Limit of quantification is 1 μg/g.
Table 1.

Toxicity of DNAN, TNT, and reference toxicants to luminescent bacteria (*Vibrio fischeri*) and freshwater green algae (*Pseudokirchneriella subcapitata*)

<table>
<thead>
<tr>
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<th>30-min Microtox EC$_{50}$ (95% CI)</th>
<th>72-h Algal growth inhibition EC$_{50}$ (95% CI)</th>
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<tr>
<td></td>
<td>(mg/L) (µM)</td>
<td>(mg/L) (µM)</td>
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<tr>
<td>DNAN (pH 5.2)</td>
<td>55.9 (282.0)</td>
<td>2.0 (9.9)</td>
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<td>(44.2 – 112.0) (223.1-565.11)</td>
<td>(1.7 – 2.2) (8.8 – 10.9)</td>
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<td>DNAN (pH &gt; 6)</td>
<td>60.3 (304.5)</td>
<td>4.0 (20.2)</td>
</tr>
<tr>
<td></td>
<td>(45.8 – 118.9) (231.0-600.3)</td>
<td>(3.5 – 4.2) (11.6 – 17.7)</td>
</tr>
<tr>
<td>TNT (pH 5.3)</td>
<td>0.7 (2.9)</td>
<td>0.7 (3.0)</td>
</tr>
<tr>
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<td>(0.2 – 1.3) (1.0 – 5.6)</td>
<td>(0.6 – 0.7) (2.7 -3.1)</td>
</tr>
<tr>
<td>TNT (pH &gt; 6)</td>
<td>0.7 (2.9)</td>
<td>0.6 (2.6)</td>
</tr>
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<td></td>
<td>(0.5 – 0.8) (2.2 – 3.6)</td>
<td>(0.6 – 0.6) (2.5 -2.7)</td>
</tr>
<tr>
<td>Reference toxicants:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol (Microtox test)</td>
<td>24.5 (20.5 – 29.2)</td>
<td></td>
</tr>
<tr>
<td>Zinc sulphate (algae growth inhibition test)</td>
<td>0.050 (0.049 - 0.051)</td>
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Table 2.
Toxicity of DNAN and TNT to earthworms and plants following exposure to amended soil

<table>
<thead>
<tr>
<th>Species and assays used</th>
<th>EC$<em>{50}$ or LC$</em>{50}$ (mg/kg) (95% CI)</th>
<th>EC$<em>{50}$ or LC$</em>{50}$ (μmole/kg) (95% CI)</th>
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<tr>
<td>Earthworm (<em>Eisenia andrei</em>) tests</td>
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<td></td>
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<tr>
<td>DNAN study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 d Survival</td>
<td>98 (60 - 141)</td>
<td>495 (303 – 711)</td>
</tr>
<tr>
<td>14 d Survival</td>
<td>47 (32 – 81)</td>
<td>237 (162 – 409)</td>
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<tr>
<td>48 h Avoidance behavior</td>
<td>31 (14 – 147)</td>
<td>157 (71 -742)</td>
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<tr>
<td>TNT study</td>
<td></td>
<td></td>
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<tr>
<td>7-d earthworm survival</td>
<td>38 (27 – 43)</td>
<td>192 (136 – 219)</td>
</tr>
<tr>
<td>14-d earthworm survival</td>
<td>38 (28 – 42)</td>
<td>190 (139 – 211)</td>
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<td>Perennial ryegrass (<em>Lolium perenne</em>) tests</td>
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<tr>
<td>7 d Seedling emergence</td>
<td>6 (5 – 7)</td>
<td>31 (27 – 37)</td>
</tr>
<tr>
<td>19 d Growth inhibition</td>
<td>7 (6 – 7)</td>
<td>35 (32 – 37)</td>
</tr>
<tr>
<td>Reference toxicants:</td>
<td></td>
<td></td>
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<tr>
<td>KCl (14-d earthworm survival test)</td>
<td>6423 (5869 - 6677)</td>
<td></td>
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<tr>
<td>Boric acid (48-h earthworm avoidance test)</td>
<td>1482 (528 - 6268)</td>
<td></td>
</tr>
<tr>
<td>Boric acid (plant toxicity test)</td>
<td>208 (156 – 287)</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1.  Structures of DNAN, TNT, and some transformation products related to DNAN
Fig. 2  Stainless steel circular test units showing chambers empty (left) and filled with soil samples (right) used for the earthworm avoidance test.
Fig. 3. 2,4-Dinitroanisole (DNAN) concentrations recovered from acetonitrile extracts (A) and interstitial water (B), of DNAN-amended soil
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**Fig. 6.** DNAN and its product tissue concentrations in ryegrass grown in DNAN-amended soil for 19 d. Limit of quantification is 1 μg/g.
ANNEX 3

FATE AND TRANSPORT OF MUNITIONS CONSTITUENTS

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List of Acronyms and Definitions

2ADNT 2-amino-4,6-dinitrotoluene
4ADNT 4-amino-2,6-dinitrotoluene
2,4DANT 2,4-diamino-6-nitrotoluene
2,6DANT 2,6-diamino-4-nitrotoluene
2,4-DNT 2,4-dinitrotoluene
CEC Cation Exchange Capacity
Comp B Composition B a high explosive composed of 60-39-1, RDX-TNT-wax
DoD Department of Defense
Double-base propellant = NC+NG
DNX Hexahydro-3,5-dinitroso-1-nitro-1,3,5-triazine
EDAX Energy Dispersive X-ray Spectrometer
ERDC Engineer Research and Development Center
ER Environmental Restoration
HMX Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC High Performance Liquid Chromatography
K_{d} Soil partitioning coefficient
k Reaction rate constant
K_{ow} octanol/water partition coefficient
MC munitions constituents
MNX Hexahydro-1-nitroso-3,5-dinitro-triazine
NC Nitrocellulose
NDAB Nitro-2,4-diazabutanal
NG Nitroglycerin
NQ Nitroguanidine
OC Organic carbon
OM Organic matter
RDX Hexahydro-1,3,5-trinitro-1,3,5-triazine
Single-base propellant = NC or NC+2,4-DNT
SEM Scanning electron microscope
S_{w} Aqueous solubility
TAT 2,4,6-triaminotoluene
TNT 2,4,6-trinitrotoluene
TNX Hexahydro-1,3,5-trinitroso-1,3,5-triazine
Triple-base propellant = NC+NG+NQ
Tritonal Explosive made from ~80%TNT and 20% aluminum.

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ABSTRACT

Live-fire military training scatters energetic compounds onto range soils. Once deposited on soil the explosives and propellants ingredients can dissolve in water, experience complex interactions with soil constituents, and migrate through subsurface soil leading to ground water contamination. While in contact with soil these chemicals are also subject to several abiotic (hydrolysis, photolysis, and reaction with metals) and biotic (biotransformation) reactions both in the solid and in the aqueous state.

In this review we summarize the current state of knowledge on how energetic residues are deposited on range soils, what the residues look like and how quickly they dissolve. We also describe the key physiochemical properties ($S_w$, pH, $K_{ow}$, $K_d$) of high explosives and propellants and discuss how these parameters influence their geo-biochemical interactions with soil and their chemical and microbial transformation routes in the environment. Knowing the reaction routes of these chemicals will help us understand their fate, their ecological impact, and how to enhance in situ remediation. This review also identifies knowledge gaps and highlights future research needs.
Chapter X.1- INTRODUCTION

Poly-nitro-organic compounds are highly energetic chemicals that rapidly release large amounts of gaseous products and energy upon detonation. Because of their explosive properties these chemicals are extensively used by the military and in the construction and mining industry [1]. In the US alone close to 313 million kg of N-containing explosives had been released to the environment as of 1992 [2]. The military in many NATO countries commonly use the aromatic TNT (2,4,6-trinitrotoluene), and the cyclic nitroamines RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) as explosives, and NQ (nitroguanidine), NG (trinitroglycerine) and DNT (2,4-dinitrotoluene) in propellants (Fig. 1).

![Chemical structure of munitions constituents](image.png)

**Figure 1.** Chemical structure of munitions constituents

Live-fire military training scatters energetic compounds onto range soils. Once deposited on soil, the explosives and propellants ingredients can dissolve in water (precipitation), experience complex interactions with soil constituents, and migrate through subsurface soil leading to ground water contamination (Fig. 2). While in contact with soil these chemicals are also subject to several abiotic (hydrolysis, photolysis, and reaction with metals) and biotic (aerobic and anaerobic biotransformation) reactions both in the solid and in the aqueous state.
Deposition, dissolution and transformation of explosives and energetic compounds are of interest for two reasons. First, these chemicals are toxic and can be deleterious to human, animal and plant health. Second, these labile compounds can migrate through subsurface soil and contaminate groundwater. If contaminated groundwater migrates off a military base, political and regulatory actions can close the base to training.

![Figure 2](image.png)

**Figure 2.** Schematic showing the processes that affect the environmental fate of explosives deposited on training range soils.

This review summarizes key physiochemical properties ($S_w$, pH, $K_{ow}$, $K_d$) of high explosives and propellants and discusses how these parameters influence their geo-biochemical interactions with soil and their chemical and microbial transformation routes in the environment. Understanding the reaction routes of these chemicals will help understand their fate, their ecological impact, and help design strategies to enhance *in situ* remediation. In this review, we discuss how the highly oxidized explosives react in various redox systems with particular emphasis on the initial steps involved in their decomposition. We also summarize the current state of knowledge and identify knowledge gaps to highlight future research needs.

**X.2. Field deposition**

Military training scatters explosive and propellant compounds onto the soil surface. The mass of the scattered materials depends on the type of round fired and the manner in which it
detonated: high-order, low-order (partial), or blow-in-place detonations. Table 1 lists compounds found in commonly used explosives and propellants.

Table 1. Energetic chemicals found in military propellants and explosives. Note that military grade RDX contains ≈ 10% HMX and military-grade TNT contains ≈ 1% other TNT isomers and DNTs.

<table>
<thead>
<tr>
<th>Compound</th>
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<td></td>
<td><strong>Propellant formulations</strong></td>
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<tr>
<td>Single base</td>
<td>Artillery</td>
<td>NC, 2,4-DNT</td>
</tr>
<tr>
<td>Double base</td>
<td>Small arms, mortar, artillery</td>
<td>NC, NG</td>
</tr>
<tr>
<td>Triple base</td>
<td>155 mm howitzer</td>
<td>NC, NG, NQ</td>
</tr>
<tr>
<td></td>
<td><strong>Explosive formulations</strong></td>
<td></td>
</tr>
<tr>
<td>Comp. B</td>
<td>Artillery; mortar</td>
<td>60% RDX, 39% TNT</td>
</tr>
<tr>
<td>C4</td>
<td>Demolition explosive</td>
<td>91% Military-grade RDX</td>
</tr>
<tr>
<td>Tritonal</td>
<td>Bombs</td>
<td>TNT, aluminum</td>
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<tr>
<td>Comp. A4</td>
<td>40-mm grenades</td>
<td>RDX</td>
</tr>
<tr>
<td>TNT</td>
<td>Artillery</td>
<td>TNT</td>
</tr>
<tr>
<td>Comp. H-6</td>
<td>Bombs</td>
<td>RDX and TNT, aluminum</td>
</tr>
<tr>
<td>Octol</td>
<td>Antitank rockets</td>
<td>HMX and TNT</td>
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</tbody>
</table>

x.2.1 Propellants

Propellants are generally composed of nitrocellulose (NC) impregnated with either 2,4-dinitrotoluene (2,4-DNT single-base), nitroglycerin (NG double-base) or nitroglycerin and nitroguanidine (NQ triple-base). Propellant residues are partially burned and unburned particles of the solid propellant deposited on top of the soil surface. The shape of the original propellant grain and the presence or absence of holes (perforations made to increase the burn rate) dictates the appearance of the residue [3]. For example, propellants grains with a single perforation leave rings or crescent shaped residues (Fig. 3a), those with multiple perforations leave slivers (Fig. 3b) and those without perforations leave residues that are smaller versions of the original propellant (Fig. 3c). The size range of propellant residues is limited because most propellant grains are mm in size.
Figure 3. Propellant grains and their residues: a) single-perforated M45 propellant; b) multi-perforated M1 propellant; c) M9 propellant.

Tests, where small arms, mortars, artillery, and shoulder-fired antitank rockets were fired and the residues quantified, show that the mass of NG and 2,4-DNT deposited varies substantially for different munitions (Table 2). For example, the mass of NG deposited for a 155-mm howitzer was estimated at 1.2 mg per round fired, while the NG deposition for an 84-mm AT4 shoulder-fired rocket was 20,000 mg per rocket fired. Most of this deposition is as nitrocellulose particles, with NG or 2,4-DNT in the NC matrix [4]. NQ-containing propellants appear to leave little residue [5].
At fixed firing positions the propellant residues decrease downrange, except for residues from shoulder-fired rockets, where residues are deposited rearward from the firing positions. For small arms, propellant residues are generally within 5 to 30 m of the firing position and up to 75 m downrange for artillery. For shoulder-fired rocket the residues reside largely in a zone about 30 m behind the firing position. For antitank rockets, propellant residues are also present at impact areas, because all the propellant has not been expended before the rocket hits the target. Often pieces of propellant are visible on the surface. Although the particles of NC are usually not transported, the 2,4-DNT, NG, and NQ, initially within the NC matrix, can be dissolved by precipitation and travel with the water into the soil. The energetics near the surface of the propellant are readily dissolved but it can take a long time for energetics to diffuse through the NC matrix [6]. Propellants have been found to contain energetics even after being in the ocean for 50 years.

x.2.2 High explosives

Pieces of high explosives are scattered onto soils when rounds are detonated. For high order detonations these pieces are μm in size. For partial detonations, pieces range from μm to cm in size (Fig. 4). If the round does not detonate it becomes a UXO that will eventually release all of its explosives into the environment if it is not removed or destroyed (Fig. 5).

Experiments, conducted to estimate the mass of explosives residues deposited when munitions detonate at impact areas, show that for high-order detonations 99.99% of the mass of Comp B in these rounds was destroyed (Table 3). The small amount of residue deposited is in micrometer-size particles [7,8].

Low-order or partial detonations, on the other hand, deposit some fraction of the fill in the rounds (Table 3) as particles and chunks of different size. The percentage of fired rounds that undergo low-order detonations varies from one munitions type to another [9]. Thus, partial detonations can deposit from 10,000 to 100,000 times more residue
per round than high-order detonations. Also, the residues from partial detonations are particles that are much larger than those from high-order detonations, sometimes in the centimeter size range [5,7,8].

Table 2. Mass of propellant energetic constituent deposited during firing.

<table>
<thead>
<tr>
<th>Weapon System</th>
<th>Propellant</th>
<th>Constituents</th>
<th>Rounds fired</th>
<th>Residues/Round (mg)</th>
<th>Deposition Distance (m)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Howitzer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>105-mm</td>
<td>M1-1&amp;2</td>
<td>2,4-DNT</td>
<td>71</td>
<td>34</td>
<td></td>
<td>[10]</td>
</tr>
<tr>
<td>105-mm</td>
<td>M1</td>
<td>2,4-DNT</td>
<td>22</td>
<td>6.4</td>
<td></td>
<td>[4]</td>
</tr>
<tr>
<td>155-mm</td>
<td>M1</td>
<td>2,4-DNT</td>
<td>60</td>
<td>1.2</td>
<td></td>
<td>[11]</td>
</tr>
<tr>
<td>Mortars</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60-mm</td>
<td>Ignition cartridge</td>
<td>NG</td>
<td>40</td>
<td>0.09</td>
<td>12</td>
<td>[12]</td>
</tr>
<tr>
<td>81-mm</td>
<td>M9</td>
<td>NG</td>
<td>61</td>
<td>1000</td>
<td>50</td>
<td>[12]</td>
</tr>
<tr>
<td>120-mm</td>
<td>M45</td>
<td>NG</td>
<td>40</td>
<td>350</td>
<td></td>
<td>[13]</td>
</tr>
<tr>
<td>Shoulder-fired rocket</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>84-mm Carl Gustov</td>
<td>AKB204/0</td>
<td>NG</td>
<td>39</td>
<td>1055</td>
<td>30*</td>
<td>[14]</td>
</tr>
<tr>
<td>84-mm AT4</td>
<td>AKB204</td>
<td>NG</td>
<td>5</td>
<td>20,000</td>
<td>50*</td>
<td>[10]</td>
</tr>
<tr>
<td>Tank (Leopard)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>105-mm</td>
<td>M1</td>
<td>2,4-DNT</td>
<td>90</td>
<td>6.7</td>
<td></td>
<td>[15]</td>
</tr>
<tr>
<td>Grenades</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40-mm HEDP</td>
<td>M2</td>
<td>NG</td>
<td>144</td>
<td>76</td>
<td>5</td>
<td>[16]</td>
</tr>
<tr>
<td>40-mm TP</td>
<td>F15080</td>
<td>NG</td>
<td>127</td>
<td>2.2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Small Arms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.56-mm rifle</td>
<td>WC844</td>
<td>NG</td>
<td>100</td>
<td>1.8</td>
<td>10</td>
<td>[17]</td>
</tr>
<tr>
<td>5.56-mm MG</td>
<td>WC844</td>
<td>NG</td>
<td>200</td>
<td>1.3</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>7.62-mm MG</td>
<td>WC846</td>
<td>NG</td>
<td>100</td>
<td>1.5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>9-mm Pistol</td>
<td>WPR289</td>
<td>NG</td>
<td>100</td>
<td>2.1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>12.7-mm MG (.50 Cal)</td>
<td>WC860 &amp; WC857</td>
<td>NG</td>
<td>195</td>
<td>11</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

*Major deposition is behind the firing line for shoulder-fired rockets. MG=machine gun
When fired, some rounds do not detonate and result in UXO. These UXO are sometimes destroyed using blow-in-place practices by military EOD or contractor UXO technicians. Currently, military EOD personnel use C4 demolition explosive that is 91% RDX. Thus, even if RDX was not present in rounds fired, it is often present in soils when rounds have been destroyed using C4. In general, the masses of residues deposited from high-order, blow-in-place operations are slightly higher than high-order detonations. A blow-in-place detonation, however, can also result in a partial detonation that deposits a significant fraction of its explosive fill (Table 3).

![Figure 4. Comp B pieces from a single partial detonation.](image)

![Figure 5. Explosive fill exposed by corrosion of UXO casings.](image)

At impact areas, the largest mass of explosives is scattered by partial detonations (low-order), UXO ruptured from other live-fire detonations, or blow-in-place activities designed to destroy UXO. These residues are not widely distributed and produce a localized zone of high concentrations. The resulting distribution of residues at impact areas is described as one of distributed point sources. Because these concentrations come from individual events, the concentrations across the impact area are not correlated. The short-range heterogeneity of explosives residues is very large, making it
difficult to detect any gradient in concentration. Unlike propellants, it is difficult to estimate the mass of HE deposited at an impact area even if the number and type of rounds fired are known. This is because the largest HE mass comes from partial detonations and the number of these occurrences is poorly known. The values provided by [9] can be used as a guide, but the mass of residues deposited needs to be determined by sampling.

**Table 3.** Mass of explosives residues deposited from high-order and partial detonations of TNT and Composition B-filled rounds.

<table>
<thead>
<tr>
<th></th>
<th>Analyte</th>
<th>Average mass deposited (µg)</th>
<th># Rounds sampled</th>
<th>% of the HE fill deposited</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mortars-Comp B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60mm</td>
<td>RDX</td>
<td>94</td>
<td>11</td>
<td>3×10^{-5}</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>TNT</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>81mm</td>
<td>RDX</td>
<td>8500</td>
<td>5</td>
<td>2×10^{-3}</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>TNT</td>
<td>1100</td>
<td>5</td>
<td>3×10^{-4}</td>
<td></td>
</tr>
<tr>
<td>120-mm</td>
<td>RDX</td>
<td>4200</td>
<td>7</td>
<td>2×10^{-4}</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>TNT</td>
<td>320</td>
<td>7</td>
<td>2×10^{-5}</td>
<td></td>
</tr>
<tr>
<td><strong>Hand grenade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M67</td>
<td>RDX</td>
<td>25</td>
<td>7</td>
<td>2×10^{-5}</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>TNT</td>
<td>ND*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Howitzer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>105-mm</td>
<td>RDX</td>
<td>95</td>
<td>9</td>
<td>7×10^{-6}</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>TNT</td>
<td>170</td>
<td>9</td>
<td>2×10^{-5}</td>
<td></td>
</tr>
<tr>
<td>155-mm</td>
<td>RDX</td>
<td>300</td>
<td>7</td>
<td>5×10^{-6}</td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td>TNT</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**PARTIAL-DETONATIONS**

|                |         |                             |                  |                            |      |
| **Mortars-Comp B** |         |                             |                  |                            |      |
| 60-mm          | RDX+TNT |                             |                  | 35                         | [20] |
| 81-mm          | RDX+TNT |                             |                  | 42                         |      |
| 120-mm         | RDX+TNT |                             |                  | 49                         |      |
| **Howitzer-Comp B and TNT** |         |                             |                  |                            |      |
| 105-mm         | RDX+TNT |                             |                  | 27                         | [20] |
| 155-mm         | TNT     |                             |                  | 29                         |      |

*ND – Not Detected

At antitank ranges, the distance from firing position to target is only a few hundred meters and, hence, most rounds detonate, or rupture, within a small distance of targets. The gradient in residue concentrations is evident with the highest concentrations
present next to targets [21]. For artillery targets, the concentrations of residues are much lower than for antitank rocket targets, and there is no concentration gradient evident as a function of distance away from these targets. The reason is the much greater distance between firing positions and targets, which results in some detonations being far from targets. This same situation is true for mortar targets that often are used for both artillery and mortar practice. Deposition patterns for energetic residues and how to sample for these on different types of ranges are discussed in Hewitt et al. [22,23]. However, other types of analytes (e.g., metals) may have different depositional patterns or fate and transport characteristics that would need to be considered for determining their likely spatial distribution.

X.3. Physicochemical Properties of constituents

Different chemicals have different physicochemical properties and thus different environmental interactions, i.e. partitioning and transport between water, soil, and other biological receptors, and different transformation routes, i.e. abiotic and biotic degradation. The main physicochemical properties (aqueous solubility ($S_w$), octanol/water partition coefficients ($K_{ow}$), and soil sorption constants ($K_d$)) can provide insight into the fate of chemicals in the environment and the risk associated with their open-field use and applications.

Table 4 summarizes key physicochemical parameters, molecular weight, $S_w$, and log $K_{ow}$, of studied explosives. The aqueous solubility is a measure of how much of a compound can dissolve in water given its temperature, pH and ionic strength. The solubility of the compound is related to the size and polarity of the molecule, with smaller more polar molecules having higher solubility.

Energetics in propellants, 2,4-DNT, NG, and NQ, have varying degrees of solubility in water (Table 4). These three chemicals are soluble and have low health screening levels for drinking water. NG, for example, has a solubility limit of around 1500 mg/L at 20°C in water [29] and a screening level of 1.5 μg/L in residential water [32].
Nitrocellulose, NC, on the other hand, is not soluble and as it has no known health or environmental risks, the mass of NC deposited for various weapons is usually not estimated [14]. Energetics in the high explosives, RDX, HMX and TNT have low solubility. HMX is almost insoluble in water (4 mg/L) and does not migrate to the subsurface, while RDX and TNT, which are more soluble, can be detected in subsurface environments and in the groundwater of known contaminated sites [33].

Log $K_{ow}$ indicates the potential of an explosive to partition into soil organic matter (OM) [34]. The higher the log $K_{ow}$, the higher the lipophilicity of the chemical, which affects its geo-biochemical interactions in the environment, e.g. diffusion through a cellular membrane causes damage to the biological receptor and migration through subsurface (sorption/desorption) causes groundwater contamination. TNT, with its three $\text{–NO}_2$ groups, is the most hydrophobic chemical of all ($1.8 < \text{log } K_{ow} < 2.0$). Therefore transformation of TNT to its amine derivatives, i.e. replacing $\text{–NO}_2$ groups by $\text{–NH}_2$ groups, reduces its hydrophobicity and affinity for soil organic matter and increases its water solubility. Reduced TNT amine products, therefore tend to migrate through subsurface soil unless their migration is slowed down by immobilization mechanisms, e.g. chemisorption by forming $\text{–NH–C}(O)–$ covalent bonds.

Sorption to soil, $K_d$, is governed by the soil composition, i.e. content of organic matter, clay, iron and aluminum oxides and hydroxides. As $K_d$ increase, the chemical tends to reside mostly on the solid surface and little is transported downward in the moving pore water. As the majority of adsorption of organic contaminants in soils are attributed to soil organic carbon, $K_d$ values are often normalized to soil organic carbon content. The resulting $K_{OC}$ parameter (soil organic carbon partition coefficient) can then be used to calculate adsorption to other soils based on their carbon content. For example, RDX tends to partition to organic carbon [35, 36], while TNT, which undergoes relatively rapid transformation to its amine derivatives, exhibits very complex sorption behavior. In addition to being adsorbed in clay interlayer surfaces [37], TNT’s amine products can undergo irreversible adsorption to organic matter through formation of covalent bonds [38]. As Table 4 shows, solubility changes drastically depending on if the chemical is a nitroaromatic (TNT, DNT), a cyclic nitroamine (HMX, RDX), or a nitrate ester (NG).
Table 4. Physico-chemical properties of explosives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>formula</th>
<th>Mol Wt.</th>
<th>Density (g cm^-3)</th>
<th>S_w (g L^-1)</th>
<th>Log K_{OW} at 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNT</td>
<td>C\textsubscript{7}H\textsubscript{5}N\textsubscript{3}O\textsubscript{6}</td>
<td>227</td>
<td>1.65</td>
<td>0.150\textsuperscript{[24]}</td>
<td>1.86-2.00\textsuperscript{[24]}</td>
</tr>
<tr>
<td>2,4-DNT</td>
<td>C\textsubscript{7}H\textsubscript{5}N\textsubscript{2}O\textsubscript{4}</td>
<td>182</td>
<td>1.52</td>
<td>0.28\textsuperscript{[24]}</td>
<td>1.98\textsuperscript{[24]}</td>
</tr>
<tr>
<td>RDX</td>
<td>C\textsubscript{3}H\textsubscript{6}N\textsubscript{6}O\textsubscript{6}</td>
<td>222</td>
<td>1.82</td>
<td>0.42\textsuperscript{[25a]} 0.60</td>
<td>0.87\textsuperscript{[26]} 0.81-0.87</td>
</tr>
<tr>
<td>HMX</td>
<td>C\textsubscript{4}H\textsubscript{4}N\textsubscript{6}O\textsubscript{8}</td>
<td>296</td>
<td>1.81</td>
<td>0.0045\textsuperscript{[27]}</td>
<td>0.17\textsuperscript{[27]}</td>
</tr>
<tr>
<td>NG</td>
<td>C\textsubscript{3}H\textsubscript{5}N\textsubscript{3}O\textsubscript{9}</td>
<td>227</td>
<td>1.6</td>
<td>1.8\textsuperscript{[28]} 1.5\textsuperscript{[29]}</td>
<td>1.62\textsuperscript{[28]}</td>
</tr>
<tr>
<td>NQ</td>
<td>CH\textsubscript{4}N\textsubscript{4}O\textsubscript{2}</td>
<td>104</td>
<td>1.71</td>
<td>3.2\textsuperscript{[30]} 2.6\textsuperscript{[30]} 4.4\textsuperscript{[31]}</td>
<td>-0.68\textsuperscript{[30]} -0.89\textsuperscript{[28]} 0.148\textsuperscript{[30]}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} at 20°C; \textsuperscript{b} at 18°C. The references are in brackets.

X.4. Dissolution of energetic compounds

Dissolution is thought to be the rate-limiting step for aqueous transport to groundwater. Fig. 6 shows experimental techniques that measure the dissolution of individual HE particles\textsuperscript{[39]}. Each technique isolates dissolution from the confounding effects of soil interaction and scales dissolution directly with dripping (rainfall) rate. These tests mimic field conditions on training ranges, where residues are scattered on the soil surface and whose constituents are dissolved by precipitation and were similar to those successfully used to measure and model dissolution of explosives\textsuperscript{[40]}.

Fig. 6. Laboratory drip tests (left) and outdoor dissolution tests (right) from\textsuperscript{[41]}. 
x.4.1. Propellants

Single-base propellants containing 2,4-DNT are used to fire artillery, mainly 105- and 155-mm rounds from Howitzers. Fig. 7a shows the % of DNTs dissolved during drip tests on a variety of M1 propellants [3]. Note that for the unfired grains the dissolution rate was quite linear and that even after 500 days the maximum DNT loss is only 10%. The larger propellant grains lost more DNT than the smaller grains but a smaller percentage of what they contained. The residues from M1 seven-perforation propellant (12 fibers) lost the highest percentage of their 2,4-DNT owing the their large surface to volume ratios, although the total mass was small. The mass loss curve for the residues was not linear. It rose rapidly initially but after day 20 it becomes more linear but still had a positive slope (Fig. 7a).

Double-base propellants are the most common and are used to fire small arms, mortars, and rockets. In the drip tests unfired double-base propellants show initial rapid dissolution of NG followed by much slower dissolution. Most double base propellants lost NG in proportion to how much NG they contained. For example, the M9 propellant with 40% NG lost a greater percentage of its NG than a propellant that contained less. Taylor et al. [3] found that the mass of NG dissolved was a function of the NG/NC ratio in the propellant and the data are plotted this way (Fig. 7b). Clustering of the data using this normalization suggests that NC binds 10 to 20% of the NG and that any extra NG is not well retained.

Exceptions from this trend are the ball propellants, used to fire small arms, and the M45 propellant, used to fire mortars from the Stryker. The ball propellants all contain ~10 NG, yet variable amounts of NG are dissolved independent of their NC content. The M45 propellant also contains 10% NG yet it loses <1% during the drip tests (Fig. 7b). The M45 is a squat grain with a central perforation. The central perforation should increase its surface area and, therefore, increase its NG loss. This result was observed suggesting that the nitrocellulose in M45 was not fully nitrated when it was manufactured so that the NG was effectively bound to the NC.
Both fired residue and unfired grains were collected from double-base ball propellants used to fire small arms. Concentration of NG in the unfired grains were within the variability given in the technical manual whereas the fired residues contained about 80% of the NG on a mass basis. **Fig. 8a** shows that more NG was dissolved from the unfired propellants (15-20%) than from their residues (3 to 7%). For the unfired propellants the shape of the cumulative mass loss versus time curves were consistent with rapid loss of the NG from the surface of the grain followed by slower diffusion of the NG from the interior of the grain. The high aqueous solubility of NG suggests that NG could be rapidly dissolved by contact with water. If NG existed as fine liquid droplets within an NC matrix rather than as dispersed molecules, droplets at the grain surface would be in direct contact with water whenever the grain was wet. Once this outer layer of liquid NG was depleted, NG would need to diffuse through the NC matrix to reach the water, with considerably lower diffusivity ($\sim 10^{-14} \text{ cm}^2\text{s}^{-1}$) [6]. Late time dissolution would thus be limited by molecular diffusion.

This concept also qualitatively accounts for the much lower dissolution rates observed for fired grains. Firing likely burns or volatilizes surface NG droplets, and we estimate that volatilizing NG from a surface layer about 5% of the thickness of the fired particle would lower the NG concentration by 20%, the difference we measured between fired and unfired propellants.

Scanning electron microscopy images of the small arm propellants show pits whose 5μm depth (**Fig. 8b**) could produce the decrease in concentration. All dissolution of NG from fired grains would thus be limited by molecular diffusion. The linear shape of the cumulative mass loss curves, the slower dissolution rate of NG from the fired residues and their 20% lower NG concentration compared to unfired grains are explained if NG near the surface is burned during firing.

Triple base propellants are also used to fire artillery and contain NQ. Although there is more NQ than NG in the M31 propellant (55% vs. 20%), and NQ is more soluble than NG (Table 4), both by mass and by percentage more NG was dissolved than NQ (**Fig. 8a**).
This results because during manufacture of the propellant, NG is added as a liquid, whereas NQ is mixed in as a solid.

Tests to measure how well the components in triple-based propellants were mixed [42] showed that NG is not as well mixed as NC and NQ and that there is more NG near the surface and less in the interior of the grain. These authors suggest that after a certain threshold (27%NG for a 12.2% nitrated NC) the NG does not effectively bond to the NC and it migrates to the propellant surface as a low viscosity fluid. This migration would make liquid NG available near the surface of the propellant where it would be removed when in contact with water. The NQ on the other hand would have to dissolve to leave the propellant.

**x.4.2. High explosives**

The dissolution of pieces and particles of TNT, Comp B, and Tritonal were measured both in the laboratory [39] and in the field [41, 43]. The TNT particles became smoother and smaller but retained their original shapes as they dissolved. The Comp B particles became noticeably bumpier and “sugary-looking” as dissolution of the surface TNT revealed the larger (~ 0.1 mm), slower-dissolving RDX crystals. The Tritonal particles became smaller and slightly bumpier as TNT dissolved exposing the aluminum grains (Fig. 9a & b).

The outdoor dissolution tests showed that Comp B, Tritonal, and TNT particles all turned rust red with occasional shiny, almost iridescent, black patches. Following heavy rains, the reddish product washed off some surface areas, exposing the lighter-colored explosive beneath. Four of the 34 HE chunks split naturally during the tests and others spalled small flakes or cracked (Fig. 9c, 10b).
Fig. 7. (a) Percent of 2,4-DNT and 2,6-DNT dissolved into water from unfired seven perforation M1 propellant grains (black) and from fired residues (red). (b) Plot showing the % NG dissolved normalized by the NG/NC ratio versus time. All of the data from NG containing propellants, except for the small arms, are plotted on this figure. (c) Percent NG and NQ dissolved (Ave. ± 1 sigma, n=8) versus time for unfired M31 single-perforation propellants, from [3].
**Fig. 8.** Shown on the left is the percent NG dissolved versus time for unfired (top) and fired (bottom) small arms propellants. On the right are images of 9-mm propellant grains showing progressive heating; a) an unfired grain; b) pits visible on surface; c) bubbly surface; d) gas bubbles open holes on surface; e) metals and other elements within the propellant become concentrated on the surface; f) breakdown of nitrocellulose structure.

**Figure 9.** (a) Photograph of particle and (b) close-up of its surface for TNT, Comp B and tritonal (c) Number of HE chunks that cracked, spalled off a 1 mm piece, or split into multiple millimeter-size pieces over the three-year test. For example, the circled triangle indicates that 3 of the 34 chunks had split by day 810 of the test.
The cumulative mass losses for TNT pieces are shown in Fig. 10a. Data for Comp B, Tritonal and C4 are in [41] and a dissolution model for explosives and validation studies are presented in [40, 41, 43]. Except for the chunks that split (TNT 3 and 5) the shapes of the cumulative mass loss curves are similar among all the chunks. Although the largest chunks lost the most mass, the small HE chunks lost a larger percentage of their initial mass due to a larger ratio of surface area to mass. Figure 11 summarizes the mass loss for each HE chunk after three years. Note that two types of measurements are plotted against each other: mass loss measured by electronic balance, and cumulative dissolved mass obtained via HPLC analysis. Mass losses measured with the electronic balance were larger than dissolved masses, and the losses grew with time. These results were unexpected because the two measurement methods had low uncertainties, and there was very good mass balances for TNT, Tritonal, and Comp B in the laboratory tests [39, 40].

![Cumulative HE Loss vs Time](image)

**Fig. 10.** Left, cumulative mass loss of TNT (mg) versus time as measured by HPLC; right, images of TNT 3 over the course of the experiment.

Mass balance discrepancies could be attributed to several factors including abiotic degradation caused by photolysis of the particle’s surface or by hydrolysis on the wetted surface of the particle. Photo-transformation of TNT in solution has been well documented, but the transformation measured occurred on solid pieces of all the explosives, including C4 that contains only RDX. Solid RDX does photo-transform to
other compounds [44] however, anecdotal accounts suggest that RDX is much more stable in sunlight than TNT, so that the rate may not be high enough to account for missing mass.

Fig. 11. Over 60% of the mass lost from the HE pieces, as measured by electronic balance, were not measured as dissolved explosives by the HPLC. The unaccounted for masses were larger than dissolved explosive masses, they scaled closely with surface area of each particle and they grew with time.

Photo-transformation is inherent to outdoor weathering of explosives and the lack of mass closure for these tests indicates that photoproducts are not quantified by Method 8330B [45]. Taylor et al. [41] noted that TNT, Tritonal and Comp B pieces, all containing TNT, changed color to red upon exposure to light, possibly due to the formation of Meisenheimer complexes and azo derivatives. Also found was 2-amino-4,6-dinitrobenzoic acid in quantities similar to those measured for TNT. The poor mass balance of RDX was attributed to photo-destruction of the high explosive when formaldehyde and nitrate were detected in photo-degradation studies [41]. Interestingly,
photo-degradation of solid HEs differs from photo-degradation of these chemicals in aqueous solutions both in the kinetics and in the products formed. On training ranges, these transformation products likely constitute additional HE-based contaminant influx into range soils.

**X.5. Soil interactions**

Explosives are N-based organics that are rich in functional groups, a property that makes them susceptible to biogeochemical interactions in the environment. Soil constituents, including organic matter, phyllosilicate clays, iron and aluminum oxides and hydroxides, can all adsorb energetic compounds due to their high surface areas [e.g. 36] and their various functionalities. The soil type can, therefore, drastically influence the extent of soil/contaminant interactions (Table 5). Interactions of explosive constituents with soil provide insights into their environmental fate and the risk associated with their use in the field.

The-NO₂ functional group(s) in explosives can transform to the corresponding –NH₂ (amino-) group(s) under various environmental redox conditions [2, 46]. Different products have different physicochemical properties (Sₘ, pKₐ, Kₐw) and thus partition differently between water and soil [38, 47, 48, 49]. For example, Haderlein et al. [37] reported that reversible sorption to montmorillonite decreased with the number of nitro groups and followed the order TNT > DNT > NT. Clearly sorption depends on the explosive compound and on the type and composition of the soil (Table 5).

**x.5.1. Nitroaromatic compounds and their transformation products**

Nitroaromatic compounds, such as TNT and DNT, interact both with organic matter and phyllosilicate clays in the soil. Haderlein et al. [37] showed that K-saturated clays have very high affinities for both TNT and DNT but that their affinities decreased by several orders of magnitude when saturated with other cations. Although K⁺ saturation is not common in soils, experiments indicate that clays do adsorb nitroaromatics, but that the affinities are lower due to mixed clay mineralogy, the cation composition and organic and oxide coatings on soil clays [36, 50].
### Table 5. Soil-water partition coefficients, $K_d$, between energetic compounds and soils and minerals.

<table>
<thead>
<tr>
<th>Soils</th>
<th>Clay (%)</th>
<th>TOC (%)</th>
<th>$K_d$ (L kg$^{-1}$)</th>
<th>2,4DNT</th>
<th>RDX</th>
<th>HMX</th>
<th>NG</th>
<th>NQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newport</td>
<td>5.6</td>
<td>3.5</td>
<td>2.3$^{[46]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lonestar</td>
<td>10.0</td>
<td>0.06</td>
<td>2.5$^{[46]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cornhuskers</td>
<td>20.0</td>
<td>0.83</td>
<td>4.1$^{[46]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Crane</td>
<td>20.6</td>
<td>2.8</td>
<td>3.7$^{[46]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Joliet</td>
<td>23.8</td>
<td>3.6</td>
<td>6.8$^{[46]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Holston B</td>
<td>43.8</td>
<td>1.2</td>
<td>3.0$^{[46]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sharkey Clay</td>
<td>54.4</td>
<td>2.4</td>
<td>11$^{[46]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>K$^+$- LAAP D$^a$</td>
<td>32</td>
<td>0.20</td>
<td>167$^{[58]}$</td>
<td>12.5</td>
<td>0.66$^{[58]}$</td>
<td>1.73$^{[58]}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aqua-gel</td>
<td>&gt; 87</td>
<td>ND</td>
<td>130$^{[59]}$</td>
<td>130$^{[59]}$</td>
<td>6.6$^{[59]}$</td>
<td>8.9$^{[59]}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sassafras loam</td>
<td>11$^{[27]}$</td>
<td>0.33$^{[27]}$</td>
<td>16.4$^{[3]}$</td>
<td>1.30$^{[3]}$</td>
<td>2.34$^{[3]}$</td>
<td>0.7$^{[27]}$</td>
<td>0.26$^{[3]}$</td>
<td>0.60$^{[3]}$</td>
</tr>
<tr>
<td>Catlin silt loam</td>
<td>15.7$^{[3]}$</td>
<td>3.75$^{[3]}$</td>
<td>18$^{[36]}$</td>
<td>4.23$^{[36]}$</td>
<td>17.9$^{[36]}$</td>
<td>15.30$^{[36]}$</td>
<td>2.03$^{[36]}$</td>
<td>1.27$^{[3]}$</td>
</tr>
<tr>
<td>Kenner muck</td>
<td>55</td>
<td>35.4</td>
<td>285.2$^{[36]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Benndale fine sandy loam</td>
<td>20</td>
<td>0.89</td>
<td>1.77$^{[36]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Adler silt loam</td>
<td>4.5$^{[60]}$</td>
<td>0.29</td>
<td>2.4$^{[60]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Plymouth sandy loam</td>
<td>14.4$^{[3]}$</td>
<td>1.72$^{[3]}$</td>
<td>5.0$^{[60]}$</td>
<td>1.6$^{[60]}$</td>
<td>5.06$^{[3]}$</td>
<td>0.28-2.01$^{[61]}$</td>
<td>0.65$^{[60]}$</td>
<td>0.43$^{[60]}$</td>
</tr>
<tr>
<td>Yokena/Sharkey Clay</td>
<td>48.7</td>
<td>2.4</td>
<td>10$^{[63]}$</td>
<td>12.5$^{[65]}$</td>
<td>9.43$^{[57]}$</td>
<td>3.5$^{[46]}$</td>
<td>12.1$^{[66]}$</td>
<td>0.43$^{[64]}$</td>
</tr>
<tr>
<td>Picatinny</td>
<td>5</td>
<td>0.63</td>
<td>2.06$^{[57]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Grange Hill</td>
<td>10</td>
<td>0.29</td>
<td>0.43$^{[57]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Varennes</td>
<td>4</td>
<td>8.4</td>
<td>4.2$^{[55]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>LAAP Ab</td>
<td>6</td>
<td>0.31</td>
<td>26$^{[58]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>LAAP Cb</td>
<td>12</td>
<td>0.08</td>
<td>64$^{[58]}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>LAAP Db</td>
<td>32</td>
<td>0.2</td>
<td>167$^{[58]}$</td>
<td>1.67$^{[65]}$</td>
<td>0.7$^{[58]}$</td>
<td>0.7$^{[58]}$</td>
<td>2$^{[58]}$</td>
<td></td>
</tr>
</tbody>
</table>

**Minerals**

| K$^+$-mont.  | NA | ND | 21500$^{[37]}$ | 7400$^{[37]}$ | 1.2$^{[37]}$ |
| K$^+$-illite | NA | ND | 12500$^{[37]}$ | 3650$^{[37]}$ |    |
| K$^+$-kaolinite | NA | ND | 1800$^{[37]}$ | 690$^{[37]}$ |    |
| Ca$^{2+}$-mont. | NA | ND | 1.7$^{[37]}$ |    |    |
| Ca$^{2+}$-illite | NA | ND | 1.2$^{[37]}$ |    |    |
| Ca$^{2+}$-kaol.  | NA | ND | 0.3$^{[37]}$ |    |    |
| K$^+$-mont.  | NA$^b$ | ND$^c$ | 414$^{[58]}$ | 3.17$^{[58]}$ | 22.1$^{[58]}$ |

$^a$ LAAP, Louisiana Army Ammunition Plant; $^b$ NA: Non applicable; $^c$ ND: Not determined. The references are in brackets.
Fig. 12. Linear correlation between a) measured TNT absorption coefficients ($K_d$) and cation exchange capacity (CEC) that accounts for clay and OM in the soil ($P = 1.5 \times 10^{-10}$), data are from [58]; and b) measured 2,4DNT $K_d$s and percent organic carbon (TOC) in the soil ($P = 7.61 \times 10^{-8}$) data are from [3, 58]. If P values are smaller than 0.01 this indicates a highly significant correlation.

Organic matter also affects adsorption of TNT in soils [36]. Octanol-water partitioning coefficients for nitroaromatic compounds (Table 4) suggest nonspecific hydrophobic partitioning to OM [51]. This mechanism was thought to be important for particulate organic matter [52] while more polar soluble organic carbon interacts with TNT and its
transformation products through ionic bonds with functional groups present in humic substances.

Pure iron oxides (magnetite, hematite, lepidocrocite, and goethite) do not adsorb TNT or other nitroaromatics [53]. Ainsworth et al. [54] showed a negative relationship between dithionite-citrate extractable iron (Fe_d) in soils and TNT adsorption. Removal of poorly crystalline iron oxides (oxalate extractable) increased adsorption of TNT by soil clays [36]. The likely reason for the negative effect of iron oxides on TNT adsorption is that they cover clay surfaces and interfere with adsorption onto the clay minerals.

Once the nitro groups in TNT and DNT are reduced to amino groups, the latter can irreversibly adsorb to OM through covalent bonds [38, 67]. The mechanism involves the amino-transformation products of TNT, (2ADNT; 4ADNT; 2,4DANT; 2,6 DANT and TAT), undergoing nucleophilic addition reactions with quinone and other carbonyl groups in the soil humic acid to form both heterocyclic and nonheterocyclic condensation products. Earlier studies also reveal that monoamino- and diamino derivatives of TNT, ADNT and DANT, experience reversible adsorption in soils [58, 60]. The strongest indicator of TNT adsorption is the cation exchange capacity (CEC) that accounts for both OM and clay content in the soil (Fig. 12). For 2,4DNT, adsorption to the clays is lower (Table 5) and OC is a better predictor of adsorption to soils (Fig. 12).

**5.2. Cyclic nitroamines.**

RDX and HMX are heterocyclic compounds. They are more polar compared to nitroaromatic TNT and DNT, with smaller K_{ow} values (Table 4). This affects their interactions in soils. They have not been shown to adsorb specifically to clay minerals [37], and have lower affinity for soils that is influenced primarily by OM only (Fig. 12).

Measured soil adsorption coefficients (K_{d}s) were reviewed by [35, 58]. Significant linear regression between RDX K_{d} values and soil OC content was observed by Tucker et al. [35] indicating that adsorption to organic matter is the main mechanism of RDX interaction with the soils. Adsorption isotherms for RDX are generally linear and
reversible [35] confirming partitioning as the principal sorption mechanism. Haderlein et al. [37] showed that RDX does not exhibit specific adsorption to clay surfaces as shown for nitroaromatic compounds; however, it can participate in hydrogen bonding with clays [54]. HMX has similar behavior to RDX but higher measured \( K_d \) values (Table 5).

While presence of iron oxides that coat clay particles was hypothesized to affect sorption and [54] used amount of dithionite-extractable iron as one of several predictors of RDX adsorption, it has not been confirmed in other studies. Szecsody et al. [68] observed no dependence of RDX adsorption on iron oxide content in studied sediments. When soil clays were treated to remove iron oxides (both amorphous and crystalline), it also did not affect adsorption of RDX [36]. Therefore it can be concluded that similarly to nitroaromatics, RDX does not adsorb to iron oxides.

**x.5.3. Nitroglycerine.**

Reported nitroglycerin soil adsorption coefficients range from 0.08 to 3.8 cm\(^3\) g\(^{-1}\) (Table 5). These are lower than the ones determined for 2,4-DNT but similar to TNT that have similar \( K_{ow} \) values (Table 4) indicting that \( K_{ow} \) may not be a strong predictor of soil behavior for energetics. Reported \( K_{ow} \) values for NG (Table 4) can vary depending on the method used to estimate them [69], but generally indicate a preference for non-polar interactions. However, NG adsorption coefficients are not correlated with organic matter content (\( P=0.4945 \)), suggesting that other mechanisms are responsible for adsorption. NG is a polar molecule [70] and may form dipole-dipole and hydrogen bonds with polar moieties in the soils.

**x.5.4. Nitroguanidine**

NQ is a highly polar compound. However, reported pKa values (12.8) indicate that it is not protonated in environmental pH range [71]. It has low sorption and degradation in soils and is very mobile. Batch studies report \( K_d \) values between 0.15 and 0.60 cm\(^3\) g\(^{-1}\) [3, 20]. Column transport studies also showed limited potential for NQ adsorption, with \( K_d \) values ranging from 0 to 0.14 cm\(^3\) g\(^{-1}\) [62]. Log of NQ adsorption coefficient values normalized for soil organic carbon content (\( K_{oc} \)) were similar between the previously
mentioned studies: 1.25–2.12 for [64], 0.82-1.66 for [3] and 1.83–2.22 for [62]. However, NQ adsorption coefficients do not correlate with OC content in the soil (P=0.1585) indicating lack of partitioning behavior. This is likely related to the polar nature of the NQ molecule with negative log K_{ow} values (Table 4), which results in low affinity for non-polar organic matter in the soils.

X.6. Transformation pathways of munitions constituents

N-based organic explosives are rich in functional groups, most notably –NO_{2}, and thus are prone to abiotic and biotic degradation in the environment. Nitroaromatic explosives such TNT and DNT as well as cyclic and acyclic nitro-organic explosives such as RDX, HMX, NG and NQ can degrade abiotically by photolysis, hydrolysis, and reaction with zero valent iron (ZVI) an important element that is abundant in the environment [72] and be degraded by soil microorganisms and biota isolates under both aerobic and anaerobic conditions [73].

x.6.1 Propellants constituents

Nitroguanidine decomposes by photolysis to guanidine, hydroxyguanidine, urea, cyanoguanidine, ammonia and nitrosoguanidine [30, 71, 74]. When in contact with ZVI nano particles, NQ reduces to produce mainly aminoguanidine (Hawari, personal communication). Others reported that NQ can be reduced by catalytic hydrogenation to nitrosoguanidine and aminoguanidine [75].

Biotically, soil microbes degraded NQ aerobically in the presence of a supplementary carbon but no degradation occurred under anaerobic conditions [76]. An aerobic NQ-degrading bacterium, Variovorax strain VC1, was then isolated from the soil and degraded NQ when NQ was the sole nitrogen source. The products produced were NH_{3}, nitrous oxide (N_{2}O) and CO_{2}. A key intermediate was also detected that was identified as nitrourea by comparing it with a reference material [76]. Nitrourea is unstable in water and decomposes to NH_{3}, N_{2}O and CO_{2} (Fig. 13).
Fig. 13. Degradation pathway of nitroguanidine (NQ) with *Variovorax paradoxus*.

NG was degraded to glycerol and ammonium using ZVI nano particles [77]. The disappearance of TNG was accompanied by the transition formation of the denitrated compounds 1,3-DNT, 1,2-DNT and MNGs and NO$_2^-$- Also Oh et al. [78] reported the reduction of NG with cast iron (200 mg mL$^{-1}$) to produce glycerol and nitrite that was further reduced to ammonium ions.

The hydrolysis of nitroglycerine (NG) has been studied [79, 80, 81], but little information is available on the eventual fate of the nitrate ester. Under mild alkaline conditions (pH 9) using microwave heating at 50 °C the trinitrate ester, NG, was easily denitrated [82]. The environmental significance of the reaction lies in the transformation of the xenobiotic and hazardous chemical to simple more ubiquitous ones such as nitrite, nitrate, HCOOH, glycolic acid and compound I, O=CHCH(OH)HC=O (Fig. 14).

Photo-degradation of 2,4-DNT in aqueous solution was reported under photo-oxidative conditions [83, 84] and in the presence of cationic surfactant [85], humic acids, or sodium salt [86]. Zero valent iron reduced 2,4-DNT’s -NO$_2$ group to -NH$_2$ at the *para* position, while graphite-mediated reduction favored reduction of the *ortho* nitro group [87]. 2,4-DNT was found to resists hydrolysis under alkaline conditions in soil [88]. As for biodegradation, 2,4-DNT was found to undergo biotransformation under both aerobic and anaerobic conditions as summarized in Table 6.
**x.6.2 High explosives compounds**

Transformation pathways for the two cyclic nitroamines, RDX and HMX, and for the nitroaromatic TNT have been published [2, 89, 73, 72]. RDX and HMX degrade abiotically (with ZVI, hydrolysis, photolysis) and biotically under laboratory and field conditions [90, 91, 92, 93, 94]. Degradation rates are higher for RDX and occur via 1) initial denitration followed by ring cleavage and 2) stepwise reduction of RDX N-NO₂ functional groups leading to the formation of the corresponding nitroso derivatives MNX, DNX, and TNX [95, 96]. During RDX transformation in soils several key intermediate ring cleavage products were detected which helped construct the degradation pathways of the cyclic nitroamine explosive. One of these key products, 4-nitro-2,4-diazabutanal (NDAB), has been detected in the field at known RDX contaminated sites thus providing vivid experimental evidence that *in situ* natural attenuation of RDX occurs [33].

Nitroaromatic compounds such as TNT undergo sequential reduction to their corresponding amino derivatives that depend on redox conditions used and on the position of the –NO₂ group on the aromatic ring. TNT is known to (bio)transform [89, 2, 73]. In the majority of cases TNT undergoes reductive reactions that form mono- and
di-amino derivatives. This process can occur abiotically and biotically under both aerobic and anaerobic conditions. Only under strictly anaerobic conditions can the third –NO₂ groups reduce further giving the triamine derivative 2,4,6-triaminotoluene (TAT) [97, 48, 98]. TNT can also degrade via the formation of an unstable Meisenheimer complex, whose denitrification leads to the decomposition of the aromatic ring [99, 100].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Microorganisms</th>
<th>[ref]</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-DNT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Burkholderia sp.                 | [101] | Consortium                       | [102]                              |
|           | Pseudomonas sp.                  | [103, 104] | Pseudomonas aeruginosa           | [105]                              |
|           | Pseudomonas aeruginosa           | [105] | Clostridium acetobutylicum       | [106]                              |
|           | Consortia                        | [107] | Indigenous microflora            | [108]                              |
| NG        | Consortium                       | [109] | Consortia                        | [110]                              |
|           | Pseudomonas sp.                  | [111] |                                  |                                    |
|           | Arthrobacter sp.                 | [112] |                                  |                                    |
|           | Agrobacterium radiobacter        | [113] |                                  |                                    |
|           | Pseudomonas putida               | [114] |                                  |                                    |
|           | Rhodococcus sp.                  | [114] |                                  |                                    |
| NQ        | Variovorax strain VC1            | [76]  | Consortium                       | [115]                              |

X.7. FUTURE WORK

Generally speaking, explosives are labile molecules that are subject to various abiotic and biotic transformation routes in the environment. For example, in the case of RDX and HMX, we found that the cyclic nitroamine degrades via initial attack on one of its chemical bonds, i.e., -N–N-, -N–C-, -N–O. Information on the type and order of bond cleavage would thus be useful in understanding the degradation pathway(s) of the chemical and also in identifying and isolating bacteria capable of degrading the explosive. Two techniques provide this type of information, namely, compound specific isotope analysis (CSIA) [116] and stable isotope probing (SIP) [117]. In the case of CSIA, the method relies on the fact that bacteria break the chemical bond with the lighter isotope faster than the bond with heavier isotope (e.g., cleavage of $^{14}\text{N} - ^{14}\text{N}$ rather than $^{15}\text{N} - ^{14}\text{N}$) leading to enrichment in the heavier isotopes within the residual parent molecules as biodegradation proceeds. By comparing measured stable isotope ratios of a contaminant in a field sample and in the original chemical one should be able
to get useful information on the source of contamination, the occurrence of natural attenuation, and the mechanisms of degradation. CSIA analysis has been used to quantify RDX biodegradation in groundwater [118] and to quantify aerobic biodegradation of 2,4-DNT and TNT [119].

The second technique, SIP, uses microorganisms that can utilize the energetic chemical, e.g., RDX, TNT, or DNT, enriched with either $^{13}$C– or $^{15}$N–atoms. The $^{13}$C–DNA or $^{15}$N–DNA produced during the growth of the microorganism on a spin-labeled $^{13}$C– or $^{15}$N– is then resolved from $^{12}$C–DNA and $^{14}$N–DNA, respectively, by density-gradient centrifugation [120]. The isolated spin-labeled DNA can be used as a biomarker to identify and isolate microorganisms responsible for in situ degradation of the explosive. Recently Roh et al. [121] and Gallagher et al. [122] identified microorganisms responsible for RDX biodegradation and TNT-utilizing anaerobic bacteria, respectively, using the SIP technique. SIP and CSIA are showing promise for identifying and isolating RDX degraders and more research and optimization of these techniques is needed.

**X.8. CONCLUSIONS**

We have summarized how energetic compounds used by NATO countries are deposited in the field, the characteristics of these explosive and propellant particles and how they migrate and transform in the environment. We discussed key environmental physicochemical parameters (dissolution, $S_w$, log $K_{ow}$, $k_d$) that give insight into the migration potential of TNT, RDX, HMX, 2,4-DNT, NG, and NQ through subsurface soil. We also presented the primary routes involved in the degradation of these chemicals. We hope the data presented in this report will be useful to other researchers and help support sustainable training at military ranges.

**Chapter X- REFERENCES**


ANNEX 4

ECOTOXICOLOGY OF INSENSITIVE ENERGETIC MATERIALS

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ABSTRACT

There is global interest in many NATO countries (including Canada) to introduce new insensitive munitions (IM) compounds for use in various explosives compositions and applications. Several new munitions compounds are presently being considered for use by the Canadian Army, including dinitroanisole (DNAN), 3-nitro-1,2,4-triazole-5-one (NTO), nitroguanidine (NQ), 1,1-diamino-2,2-dinitroethylene (FOX-7), and N-guanylurea-dinitramide (FOX-12). Here we will provide examples showing how the ecotoxicological studies can provide relevant baseline toxicity data on the ecological effect of IM and their degradation products for the improved site management at military installations. Understanding the environmental fate and impact of new IM compounds will help site managers and environmental officers to make science-based decisions to manage their operational activities and training practices.

1.0 GENERAL BACKGROUND

The present chapter provides background information to a presentation given on May 13, 2013 at the NATO STO meeting in Karlstad, Sweden (AVT-197). In this chapter, we will focus on the scientific advancements in the soil ecotoxicology of emerging energetic materials (EM) including insensitive munitions (IM) and how this information can be used for ecotoxicological assessment of sites contaminated with highly energetic chemicals. The reader is encouraged to consult “Ecotoxicology of Explosives” by Sunahara et al. [1] for more detailed information about the effects of traditional explosives on ecological receptors and how this information can be used for environmental risk assessment.
Sustainable training is a necessary component of readiness for the Canadian Armed Forces. But this exercise requires use of various munitions, which often lead to severe environmental contamination. Consequently, contamination of soil and water by explosives is a serious environmental problem that is costing world economy (including Canada) hundreds of millions of dollars to manage and to remediate. Recently there has been a global interest in many NATO countries to introduce new IM compounds for use in various explosives compositions and applications, e.g., as a replacement for Composition B and TNT in the main explosive charge of insensitive medium and large caliber ammunition.

Several new IM chemicals (Fig. 1) are presently being considered for use by the Canadian Army, including 2,4-dinitroanisole (DNAN), 3-nitro-1,2,4-triazole-5-one (NTO), nitroguanidine (NQ), 1,1-diamino-2,2-dinitro-ethylene (FOX-7) and N-guanylurea-dinitramide (FOX-12). Based on the lessons learned from consequences of environmental contamination associated with the use of traditional explosives such as TNT, RDX, HMX, and NG (see [1]), it is important to assess the potential environmental risks associated with the manufacturing and use of these new IM compounds. Better understanding of the environmental fate and effects of new IM compounds will help site manager and environmental officers to make the knowledge based decisions for managing training and testing at military sites. The present discussion provides examples how the Canadian Department of National Defense (DND)-funded program on ecotoxicology of explosives provides relevant baseline toxicity data on the ecological impact of IM chemicals and their degradation products for the improved site management at military installations across Canada.

Figure 1: Chemical structures of different insensitive munitions chemicals

Highly energetic chemicals used for explosives and propellants can be found in soil at military training sites as well as at munitions production and disposal facilities. Contamination of soil, and ground and surface water by these chemicals is a serious health and environmental problem, and could result in
high costs for managing and mitigation of contaminated sites [1,2]. There is a recent interest in the introduction of new shock-IM compounds. The following section describes our recent laboratory studies on the ecotoxicology of DNAN, a chemical used in various munitions compositions and applications (e.g., PAX-21, IMX-101, and IMX-104) as described by others [3-6]. In water, DNAN can undergo phototransformation and can be biologically reduced under anaerobic and aerobic conditions to form 2-amino-4-nitroanisole (2A-4NAN) and 2,4-diaminoanisole [7-10]. These DNAN degradation studies suggest that DNAN can undergo abiotic and biotic transformation in the environment. DNAN can also decrease microbial degradation of other munitions. DNAN can inhibit perchlorate degrading bacterial activity in batch studies of PAX-21 biodegradation [11].

2.0 ECOTOXICOLOGY OF DNAN

Figure 2 shows the structures of DNAN and its transformation products including 2A-4NAN and 4-amino-2-nitroanisole (4A-2NAN) together with 2,4,6-trinitrotoluene (TNT). These compounds share strong electron withdrawing groups (-NO₂), which are important in determining the environmental fate, transport, transformation, and ecological impact of the explosive [12]. Like TNT, DNAN can initially produce mono-amino-reduced product isomers [8].

![Figure 2: Chemical structures of different energetic nitroaromatic compounds, and some transformation products related to 2,4-dinitroanisole (DNAN).](image)

Currently, there is a lack of published information on the toxicity of DNAN and its related amine products, on ecological species. For toxicity, DNAN has been used by the armed forces as a lice egg pesticide in MYL formula [13] and has been found to be mutagenic in the Salmonella/mammalian microsome test [14].
Recent studies conducted by the U.S. Army described a 90-d toxicity study of DNAN using rats [15]. Toxicological effects included organ-specific effects, including neurotoxicity. Another study reported the acute and chronic toxicity of DNAN to aquatic vertebrate and invertebrate species including the larval fish *Pimephales promelas* and the water flea *Ceriodaphnia dubia* [16]. The 48-h median lethal concentration (LC$_{50}$) ranged from 37 to 42 mg/L DNAN, whereas the sublethal and chronic effects (EC$_{50}$, median effect concentration) ranged from 11 to 15 mg/L DNAN, using these two species. Effects on unicellular primary producers are not reported, and detailed information on the terrestrial toxicity of DNAN is scant.

Preliminary unpublished studies [17,18] reported that DNAN was toxic to earthworms *Eisenia fetida* exposed for 28 d in amended field soil (100% mortality at 300 mg/kg). DNAN is toxic to wheat exposed to a 0.01 M DNAN solution on filter paper for 7 d [19]. These studies indicate that more information on the ecotoxicological effects of DNAN is needed for environmental hazard and risk assessment. Therefore, we examined the hypothesis that DNAN, a nitroaromatic compound like TNT, might have similar acute and chronic toxicity to selected ecological receptors competed to TNT.

### 2.1 TOXICITY OF DNAN TO SELECTED AQUATIC AND TERRESTRIAL ORGANISMS

Aquatic toxicity tests were performed to compare the toxicity of DNAN to TNT. DNAN was obtained from DRDC Valcartier (98.4% pure). A stock solution of DNAN (30 mg in 100 mL water) was prepared and kept in the dark at room temperature. Standard 15- and 30-min Microtox toxicity tests [20] used aqueous samples containing the test compound dissolved in American Society for Testing and Materials (ASTM) type I water. The nominal test concentrations ranged from 0 (negative control) to 126 mg/L, based on our earlier studies [21,22]. Data were expressed as the average percentage of light emission inhibition compared with the negative control. The test was done in triplicate; negative control was 2% NaCl (no DNAN added) in ASTM type I water. The pH was adjusted between 6.0 and 8.0 when necessary with either HCl, or NaOH to avoid toxicity associated with pH. Chemical analysis indicated that the average measured DNAN concentration of the stock solution was 230 ± 6 (SE) mg/L, and no transformation products (2A-4NAN or 4A-NAM) were detected by HPLC analysis (data not shown). Each prepared solution was yellow and slightly acidic (pH = 5.6). The pH was adjusted to pH 6-8, using 0.01 M NaOH. HPLC analyses of water-based incubation media confirmed the DNAN target concentrations.

Concentration-response studies examined the aquatic toxicity of DNAN to luminescent bacteria and green algae. DNAN inhibited the luminescence of *Vibrio fischeri* in a concentration-dependent manner (Table 1). Studies showed that DNAN and TNT inhibited the luminescence of photobacterium *V. fischeri*. The effects of DNAN and TNT on green algae are discussed below.

The 15-min Microtox EC$_{50}$ value for DNAN was 14 (95% CI 12-16) mg/L. The 30-min Microtox EC$_{50}$ value for DNAN was 56 (44-112) mg/L at pH 5.2 (unadjusted), and did not change greatly when the solution pH was adjusted (pH 6-8). Microtox toxicity studies of TNT yielded 30-min EC$_{50}$ value of 0.7 (0.2-1.3) mg/L at pH 5.3 (unadjusted), a similar value was found after pH adjustment. These data show that *V. fischeri* are more sensitive to TNT than DNAN. Chemical analyses indicated that the DNAN transformation products 2A-4NAN and 4A-2NAN were not detectable in the incubation medium after the Microtox test.
Table 1: Selected aquatic and terrestrial toxicities of DNAN and TNT

<table>
<thead>
<tr>
<th></th>
<th>DNAN mg/L (95% CI)*</th>
<th>TNT mg/L (95% CI)</th>
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<tbody>
<tr>
<td><strong>Aquatic tests</strong></td>
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<tr>
<td>Microtox (V. fischeri)</td>
<td></td>
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<tr>
<td>15 min</td>
<td>EC₅₀ 14 (12-16)</td>
<td>NA</td>
</tr>
<tr>
<td>30 min</td>
<td>EC₅₀ 60 (46-119)</td>
<td>0.7 (0.5-0.8)</td>
</tr>
<tr>
<td>Algal (P. subcapitata)</td>
<td></td>
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<tr>
<td>growth inhibition (72 h)</td>
<td>EC₅₀ 4 (3.5-4.2)</td>
<td>0.6 (0.6-0.6)</td>
</tr>
<tr>
<td><strong>Terrestrial invertebrate tests</strong></td>
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<tr>
<td>Earthworm (E. andrei)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-d survival</td>
<td>LC₅₀ 98 (60-141)</td>
<td>38 (27-43)</td>
</tr>
<tr>
<td>14-d survival</td>
<td>LC₅₀ 47 (32-81)</td>
<td>38 (28-42)</td>
</tr>
<tr>
<td>48-h avoidance</td>
<td>EC₅₀ 31 (14-147)</td>
<td>NA</td>
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<tr>
<td><strong>Terrestrial plant tests</strong></td>
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<tr>
<td>Perennial ryegrass (L. perenne)</td>
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<tr>
<td>7-d seedling emergence</td>
<td>EC₅₀ 6 (5-7)</td>
<td>137 (110-165)**</td>
</tr>
<tr>
<td>19-d growth inhibition</td>
<td>EC₅₀ 7 (6-7)</td>
<td>86 (70-103)**</td>
</tr>
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</table>

* CI, confidence intervals, N = 3 replicates, ** Data from [24]. NA, not available.

It is difficult to compare the relative toxicities of DNAN, TNT, and dinitrotoluene (DNT) on ecological species if the experimental conditions differ among the toxicity tests. To address this issue, we compared our Microtox toxicity data with the peer-reviewed Microtox literature using studies that reported both 15 min-EC₅₀ and 15 min-EC₂₀ values (converted to µmole/L equivalents) [21,22]. Figure 3 summarizes this analysis and shows that the DNAN transformation product 2A-4NAN had a similar toxicity as DNAN, whereas 4A-2NAN was about four-times less toxic than the parent compound.
The toxicity of DNAN was then compared with TNT, 2,4-DNT, 2,6-dinitrotoluene (2,6-DNT) and some of their reduced transformation products. DNAN was clearly less toxic (at least 20-times) than TNT. However, DNAN was more toxic than 2-amino-dinitrotoluene (2-ADNT), 4-amino-dinitrotoluene (4-ADNT), 2,4-diamino-dinitrotoluene (2,4-DANT), 2,6-diamino-nitrotoluene (2,6-DANT), and triamino-nitrotoluene (TAT). The toxicity of DNAN was similar to 2,4-DNT, 2-amino-4-nitrotoluene (2A-4NT), and 4-amino-2-nitrotoluene (4A-2NT). DNAN was about 10-times more toxic than 2,4-diaminotoluene (2,4-DAT), and 2,6-diaminotoluene (2,6-DAT). DNAN was < 10-times less toxic than 2,6-DNT, 2-amino-6-nitrotoluene (2A-6NT) and 4-amino-nitrotoluene (4A-NT). These results indicate that the order of toxicity (from most to least toxic) using 15-min Microtox test data is: TNT > 2,6-DNT and its mono-amino transformation products (2A-6NT) > DNAN and its mono-amino transformation products (2A-4NAN and 4A-2NAN) ≥ 2,4-DNT and its mono-amino transformation products (2A-4NT), and the reduced products of TNT transformation (2ADNT, 4ADNT, 2,4-DANT, and 2,6-DANT).

Presently there is no consensus on the mode of DNAN toxicity. A recent toxicity report hypothesized that DNAN toxicity to rats is related to 2,4-dinitrophenol (DNP) that may be formed by DNAN metabolism in vivo [15]. DNP is a known mitochondrial uncoupler of oxidative phosphorylation leading to increased respiration and decreased ATP generation. Dumitras-Hutanu et al. [19] also speculated that...
DNP might be underlying the toxicity of DNAN to wheat. It is unlikely that DNP is the toxicant underlying DNAN toxicity in the present studies. Firstly, if the toxic effects of DNAN on luminescent bacteria (15-min EC$_{50}$ = 70 µmol/L; Table 1) is caused by DNP toxicity (Microtox 15-min EC$_{50}$ is 215 µmol/L) [23], then nearly all of the DNAN added to the Microtox test media should have been transformed to DNP. Our chemical analysis showed no change in DNAN concentration in the 15-min Microtox test media. Secondly, DNP was not detected in the incubation media, although internal DNP concentrations (within _V. fischeri_) were not measured. Therefore, it is unlikely that DNAN toxicity was caused by DNP, at least using the Microtox test. Other modes of toxicity include direct effects of DNAN on the organism following dermal absorption or ingestion of DNAN in soil, or by indirect and secondary toxic effects, e.g., caused by the formation of DNAN transformation product 2A-4NAN.

Individual chronic toxicities of DNAN and TNT to freshwater green algae were tested using the 72-h growth inhibition of _Pseudokirchneriella subcapitata_, as described elsewhere [22,24]. Algae were exposed to different nominal concentrations of aqueous DNAN solutions ranging from 0 (negative control) to 10 mg/L, or aqueous TNT solutions from 0 (negative control) to 51.2 mg/L. The number of algae cells was measured, and the percentage of growth inhibition was calculated compared with the negative control. Data were used to determine the inhibitory concentration that causes 50% reduction of cell growth compared to a control (EC$_{50}$). Results indicate that DNAN was toxic to freshwater green algae _P. subcapitata_ using the 72-h growth inhibition test. Table 1 shows the 72 h-EC$_{50}$ values (95% CI) of 2 (1.7-2.2) at pH 5.2 (unadjusted), and 4 (3.5-4.2) mg/L after pH adjustment (pH 6-8). _P. subcapitata_ was more sensitive to TNT with 72 h-EC$_{50}$ value of 1 (0.6-0.7) mg/L at pH 5.3 (unadjusted) than DNAN described above. These data indicate that _P. subcapitata_ was approximately three-times more sensitive to DNAN than the _V. fischeri_.

For terrestrial toxicity testing, a natural sandy soil (DRDC2010; 0.7% clay, 2.0% organic matter, 97.6% sand, 1.6% silt, and pH 5.5-6.0) provided by DRDC Valcartier in 2010, was used. This soil was amended with DNAN using acetone as the carrier solvent as described previously [25]. _E. andrei_ were originally obtained from Carolina Biological Supply (Burlington, NC, USA) and were cultured in our laboratory, as described elsewhere [26]. Clitellated earthworms (from 307 to 626 mg) were acclimated to laboratory conditions in the DRDC2010 soil for 24 h prior to testing. The 7- and 14-d earthworm lethality tests were done according to earlier methods [27,28] and included the following EM concentration ranges from 0 (negative control) to 300 mg/kg dry soil for DNAN, or from 10 to 200 mg/kg dry soil for TNT. After 7-d exposure, earthworms were counted and dead individuals were removed, and the experiment was continued for another week. After 14-d exposure, earthworms were counted, rinsed, weighed, purged on filter paper, and stored in -80°C for tissue residue determination. Data were expressed as the total number of survivors in the different treatment groups compared with the control. The concentration that causes 50% mortality compared to control (LC$_{50}$) was calculated using the linear interpolation method, and was used as the toxicity endpoint value.

Data indicated that both TNT and DNAN-amended soils were toxic to earthworms (Table 1). The LC$_{50}$ values (mg/kg; 95% CI in parentheses) for earthworm survival were 38 (27-43) and 38 (28-42) for TNT, and 98 (60-141) and 47 (32-81) for DNAN, for the 7-d and 14-d exposures, respectively. Exposure to DNAN amended soils also caused a minor but significant decrease in worm wet weights ($p \leq 0.05$) at
concentrations ≥ 20 mg/kg (data not shown). These data show that earthworms were up to 2.6-times more sensitive to TNT (192 μmoles/kg) compared to DNAN (495 μmoles/kg), under the same experimental conditions. The EC50 values reported here (using a sandy soil) are lower than those reported in other studies using TNT-amended forest soils [29-31]. The difference in relative toxicities of DNAN and TNT, as well as their degradation products, to the earthworm may be attributed to the differences in soil properties (e.g., clay and organic matter contents) leading to irreversible sorption to soil, and associated microbial degradation potentials of the two soils [12,32].

Chemical analyses of test soil (Fig. 4A) and earthworm tissue (Fig. 4B) showed that addition of earthworms to soil aided in transformation of DNAN to 2A-4NAN. Soil samples without added worms did not show DNAN degradation products. These results are consistent with findings of Perreault et al. [8] who also detected 2A-4NAN as the major end-product formed via arynitroso and aryl-hydroxylamino intermediates, using an isolated Bacillus strain (13G) from aerobic soil microcosms incubated with DNAN. The tissue concentrations of 2A-4NAN were always higher than DNAN for all soil concentrations tested. The DNAN transformation product 4A-2NAN was not detected, suggesting that DNAN biotransformation is regio-selective and probably involves enzymes associated with the earthworms, as discussed below.

![Graph A: Final concentration of compounds in interstitial water vs. DNAN concentration in soil (mg/kg)](image)

![Graph B: Final concentration of compounds in earthworm tissue vs. DNAN concentration in soil (mg/kg)](image)

**Figure 4:** Final concentrations of DNAN (O- in A, unfilled bars in B) and 2A-4NAN (▲- in A, filled bars in B) recovered from soil acetonitrile extracts of DNAN-amended soil (A) and earthworms (B), at the end of the 14 d earthworm lethality test. Data are expressed as mean ± SE (n=3) of DNAN or 2A-4NAN. In the 52 mg DNAN/kg dry soil treatment group, all earthworms died in one of the three replicates (N=2).

Behavioral studies of earthworms exposed to sub-lethal concentrations of DNAN in soil were conducted, based on the earthworm toxicity results reported above. The earthworm 48-h acute avoidance test measures the behavior of earthworms in stainless steel circular test units containing amended soil, as
described by others [28,33]. Separate test units were used for each DNAN concentration. The nominal DNAN concentrations in soil were from 0 (control) to 1000 mg/kg. Worms were allowed to move between compartments. At the end of the study, each compartment was carefully emptied and earthworms were counted, recorded, rinsed, purged, and weighed on filter paper for tissue residue determination. Soil samples were also taken for later extraction and chemical analysis for DNAN and its products. The avoidance response was expressed as percentage of the average ratio of earthworms found in the control compared with treated sections of each test unit, according to the following equation:

\[
\text{Avoidance (\%)} = \frac{(# \text{ worms in clean soil}) - (# \text{ worms in test soil})}{(total \ # \text{ worms})} \times 100,
\]

where, at the end of the study, \((# \text{ worms in clean soil})\) is the number of live worms found in all compartments containing unamended soil, \((# \text{ worms in test soil})\) is the number of live worms found in all compartments containing DNAN-amended soil, and \((total \ # \text{ worms})\) is the total number of live worms found in all compartments. Earthworms from controls and treated compartments were analyzed separately for the presence of DNAN or its metabolites.

Results showed that earthworms could detect and avoid exposure to DNAN, and a concentration-dependent avoidance effect was observed (Table 1). Earthworms did not show preferences (or avoidance) to groups having 20 mg/kg or less DNAN amended soil. The EC
\(_{50}\) value for avoidance was 31 (95% CI, 14-147) mg/kg. Earthworms have a similar avoidance response to TNT-contaminated field soils at comparable concentrations (29 mg/kg) [34]. It is not known whether the worm avoidance response to DNAN reflects sensorimotor effects [35] that may lead to neurotoxicity, because DNAN-induced neurobehavioral effects were reported in rat toxicity studies [15].

Determination of the DNAN toxicity and behavioral endpoints expressed as concentrations (such as LC
\(_{50}\) or EC
\(_{50}\) values) for earthworms is very challenging, because the soil DNAN concentrations were not stable and decreased during both earthworm tests. Decreased DNAN concentrations could result from DNAN transformation (as evidenced by 2A-4NAN formation) and sorption to soil components. It is possible that the observed effects in earthworms are caused by DNAN or by its transformation products such as 2A-4NAN. Transformation of nitroaromatic compounds by earthworms was reported in studies with TNT amended soils [30,31]. Metabolism studies should be conducted to examine the soil toxicity of 2A-4NAN, and the effect of earthworms and their microflora on DNAN transformation.

The phytotoxicity of DNAN in soil was assessed using perennial ryegrass *Lolium perenne* Express and test methods previously described [25,27,36]. Nominal concentrations of DNAN ranged from 0 (negative control) to 10 mg DNAN/kg soil. All treatments were carried out in triplicate. Tests were performed in a temperature and light-controlled growth chamber. Seedling emergence was determined after 7 d, whereas shoot growth (dry mass) was determined after 19 d. Shoots were cut just above the soil line, and dry mass per treatment groups (mg tissue) was determined after lyophilizing the shoot tissue in a freeze-dryer for 24 h.
Ryegrass emergence was inhibited when seeds were sown in DNAN amended soil at concentrations greater than 5 mg/kg. Table 1 shows similar EC₅₀ (95% CI) values for both 7-d seedling emergence and 19-d shoot growth, i.e., 6 (5-7) and 7 (6-7) mg/kg, respectively. The phytotoxicity reported here is consistent with Lotufo et al. [16] who studied DNAN phytotoxicity to wheat (*Triticum aestivum*) using filter paper tests. Our results also show that ryegrass is more sensitive to DNAN than earthworms (lethality and avoidance responses) for similar durations of exposure. DNAN can be more phytotoxic than TNT (EC₅₀ = 137 mg/kg) [25] or 2,4-DNT (EC₅₀ = 16 mg/kg), based on other studies using the same ryegrass species and a Sassafras sandy loam, which had a higher clay content and lower pH [37] compared to the soil used in our studies. Further studies should be carried out to confirm the relative order of these phytotoxicities.

### 2.2 BIOACCUMULATION OF IM USING EARTHWORMS EXPOSED TO DNAN-AMENDED SOIL

Earthworm uptake experiments were performed using DNAN amended into DRDC2010 sandy soil. DNAN concentration in soil of 30 mg/kg was chosen as a non-lethal exposure [38] for the bioaccumulation study. Six earthworms were placed into each replicate (n = 3) test unit containing 60 g (dry wt.) of moist soil. Earthworm wet weights were recorded at the start of the experiment. Measurements of DNAN in tissue and soil samples were taken by a time-series sampling in which earthworms were sampled destructively from 0.25 to 14 d of exposure.

On each sampling, the earthworms were collected, counted, rinsed with ASTM type I water, and depurated for 24 h on a moistened filter paper. The earthworms were then rinsed blotted-dry, placed into glass tubes, weighed, and were immediately frozen at -80°C. Soil samples from each replicate container were homogenized and stored at -20°C until processed for HPLC analysis. Chemical analyses were conducted on triplicate samples of soil or tissue collected from each treatment group on the designated sampling days.
The accumulation of DNAN by earthworms in soil at different exposure durations was investigated, as described by Sarrazin et al. [26]. All the earthworms (DNAN-exposed, and controls) survived in this experiment and had healthy appearance. DNAN accumulated in earthworms as early as 2 h of exposure (Fig. 5). The DNAN tissue concentration was maximal (337 mg/kg tissue) at 1 d of exposure, and then decreased steadily until the end of the test (14 d). As DNAN concentration increased, 2A-4NAN was formed and its concentrations steadily increased in the worm tissue until the end of the 14-d exposure test (Fig. 5, Table 2). The bioaccumulation factor (BAF) was calculated as the ratio of DNAN concentrations in tissue compared to soil. DNAN is accumulated in soil invertebrates (BAF > 1) using the earthworm as an example. Table 2 shows that the BAF value increased from 6 to 13 during 0.08 to 1 d exposure, and then decreased to BAF of 2, at 14 d exposure. During this study period, the soil DNAN concentration decreased from 24 to 4 mg/kg.

Figure 5: Uptake of DNAN and formation of 2A-4NAN in earthworms following exposure to amended soil for up to 14 d. Symbols used: DNAN ——; 2A-4NAN ——; Total ——. Data expressed as mean ± SE (n = 3 replicates)
Table 2: DNAN and metabolite concentrations in earthworms (Eisenia andrei) exposed to DNAN-amended soil*

<table>
<thead>
<tr>
<th>Exposure (d)</th>
<th>Final DNAN concentration in soil (mg/kg)</th>
<th>DNAN in earthworm (mg/kg tissue)**</th>
<th>2A-4NAN in earthworm (mg/kg tissue)***</th>
<th>BAF***</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08 (2 h)</td>
<td>24</td>
<td>159 ± 10</td>
<td>33 ± 4</td>
<td>6</td>
</tr>
<tr>
<td>0.25 (6 h)</td>
<td>23</td>
<td>204 ± 20</td>
<td>50 ± 1</td>
<td>8</td>
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<tr>
<td>1</td>
<td>18</td>
<td>337 ± 32</td>
<td>111 ± 24</td>
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<tr>
<td>2</td>
<td>16</td>
<td>300 ± 9</td>
<td>158 ± 8</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>210 ± 49</td>
<td>170 ± 2</td>
<td>8</td>
</tr>
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<td>7</td>
<td>7</td>
<td>132 ± 19</td>
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</tr>
<tr>
<td>9</td>
<td>5</td>
<td>83 ± 4</td>
<td>221 ± 5</td>
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<tr>
<td>14</td>
<td>4</td>
<td>41 ± 11</td>
<td>262 ± 21</td>
<td>2</td>
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</table>

*: nominal concentration at beginning of test was 30 mg/kg. ** Mean ± SD (n = 3 replicates). ***: the isomer 4A-2NAN was not detected. ****: Bioaccumulation factor (BAF) was the ratio of the tissue DNAN concentration in tissue (mg/kg) to the soil DNAN concentration (30 mg/kg, measured prior to addition of earthworms to test soils).

3.0 ECOTOXICOLOGY OF NTO, NQ, FOX-7, AND FOX-12

There is limited information available on the toxicity of other IM such as NTO, NQ, FOX-7, and FOX-12 were obtained from DRDV Valcartier. Their chemical structures are shown in Figure 1. Almost no information is available on the impact of these compounds on ecological receptors. Smith and Cliff [39] reported that NTO as well as its synthetic precursor 1,2,4-triazol-5-one are not toxic when tested in vivo using mice, rats, and rabbits. In addition, Sarlauska et al. [40] examined the toxicity of NTO by looking at selected enzymes isolated from pig, or a virus-transformed lamb kidney fibroblasts line (FLK cells). These authors have shown that NTO is less toxic than TNT because NTO is a weaker electron acceptor than TNT (LC50 of NTO > 3500 μM compared to 25 μM for TNT). It is thought that even if NTO has low toxicity, the release of this compound in the environment may represent a potential threat because it may be degraded with microbes [41]. Recent in vitro and in vivo studies indicated that NTO was not genotoxic, and suggest a low risk of genetic hazards associated with exposure [42].

3.1 AQUATIC TOXICITIES OF NTO, NQ, FOX-7, AND FOX-12

NTO, NQ, FOX-7, and FOX-12 were dissolved separately in water to their maximum water solubility limits, and the toxicity was then evaluated using the standard toxicity assays described above. These assays were designed to assess the toxicity of the compound dissolved in water at half of its maximum water solubility and lower. Table 3 shows the preliminary results of individual exposures of these compounds to V. fischeri for 15 min.
<table>
<thead>
<tr>
<th></th>
<th>EC&lt;sub&gt;20&lt;/sub&gt;*</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;*</th>
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<th>NOEC</th>
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<tr>
<td></td>
<td>(mg/L)</td>
<td>(mg/L)</td>
<td>(mg/L)</td>
<td>(mg/L)</td>
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<tr>
<td>NTO</td>
<td>2405</td>
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<td>611</td>
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<td></td>
<td>(940-3399)</td>
<td>(3533-26625)</td>
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<td>NQ</td>
<td>1253</td>
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<td>406</td>
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<td></td>
<td>(1032-1544)</td>
<td>(2647-7351)</td>
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<tr>
<td>FOX-7</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>128</td>
</tr>
<tr>
<td>FOX-12</td>
<td>874</td>
<td>1816</td>
<td>843</td>
<td>421</td>
</tr>
<tr>
<td></td>
<td>(588-1067)</td>
<td>(1532-2406)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* EC<sub>x</sub> values were obtained using Maximum Likelihood-Probit and are expressed mg/L, with 95% confidence intervals in parentheses. N = 3 replicates.

Compound FOX-12 was more soluble (3405 mg/L) than FOX-7 (259 mg/L) and probably explains why FOX-12 is more toxic than FOX-7. The EC<sub>50</sub> of FOX-12 was 1816 mg/L, whereas no toxicity was observed for FOX-7 at or below 128 mg/L. These two compounds (FOX-7 and FOX-12) are less toxic than DNAN (EC<sub>50</sub> = 14 mg/L, see Table 1). Data indicates that within their solubility limits of IM compounds, the relative toxicity profile (most to least toxic) based on 15 min-EC<sub>50</sub> values for Microtox was DNAN > FOX-12 > NQ ≈ NTO > FOX-7.

Individual exposures of NTO, NQ, FOX-7, and FOX-12 inhibited *P. subcapitata* growth in the 96-h exposure test. The results presented in Table 4 show a similar toxicity for both FOX-7 and FOX-12 (EC<sub>20</sub> of 9 and 10 mg/L, respectively). In addition FOX-12 is 100 times more toxic to algae (EC<sub>50</sub> = 17 mg/L) than *V. fisheri* (EC<sub>50</sub> = 1816 mg/L). DNAN was 8 to 10 times more toxic to algae (EC<sub>50</sub> = 2 mg/L, see Table 1) than FOX-7 or FOX-12. Compared to the latter IM chemicals, NTO and NQ were less toxic, having EC<sub>50</sub> values 2324 and 1094 mg/L, respectively. The relative toxicity profile (most to least toxic) based on 72 h-EC<sub>50</sub> values for green algae growth inhibition was DNAN > FOX-7 = FOX-12 > NQ > NTO.
### Table 4: Toxicity of selected insensitive munitions using the 96-h algal (Pseudokirchneriella subcapitata) growth inhibition test

<table>
<thead>
<tr>
<th></th>
<th>EC$_{20}$* (mg/L)</th>
<th>EC$_{50}$* (mg/L)</th>
<th>LOEC (mg/L)</th>
<th>NOEC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTO</td>
<td>1587 (1535-1652)</td>
<td>2324 (2130-2579)</td>
<td>4489</td>
<td>2245</td>
</tr>
<tr>
<td>NQ</td>
<td>760 (0.0-924)</td>
<td>1094 (925-1195)</td>
<td>1491</td>
<td>746</td>
</tr>
<tr>
<td>FOX-7</td>
<td>9 (3-12)</td>
<td>22 (17-25)</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>FOX-12</td>
<td>10 (9.5-10.4)</td>
<td>17 (17-18)</td>
<td>12</td>
<td>6</td>
</tr>
</tbody>
</table>

* EC$_x$ values are obtained from linear interpolation analyses and are expressed as mg/L. N = 3 replicates.

### 3.2 TERRESTRIAL TOXICITIES OF NTO, NQ, FOX-7, AND FOX-12

Terrestrial toxicities of NTO, NQ, FOX-7, and FOX-12 were assessed using earthworms and plants (Tables 5 and 6) according to standard protocols described above. Table 5 indicates that NQ and FOX-12 were not lethal to the earthworm at the highest concentrations tested. In contrast, NTO (LC$_{50}$ = 2768 mg/kg) and FOX-7 (LC$_{50}$ = 6463 mg/kg) were lethal to earthworms, but their toxicities (higher LC$_{50}$ values) were much less than DNAN (LC$_{50}$ = 47 mg/kg) or TNT (LC$_{50}$ = 38 mg/kg) (see Table 1). The relative toxicity profile (most to least toxic) based on 14 d-LC$_{50}$ values for earthworm survival was DNAN >NTO > FOX-7 > NQ ≈ FOX-12.

### Table 5: Lethality of selected insensitive munitions to earthworms (Eisenia andrei)

<table>
<thead>
<tr>
<th></th>
<th>14 d-LC$_{20}$ mg/kg</th>
<th>14 d-LC$_{50}$ mg/kg</th>
<th>BAF*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTO</td>
<td>1687**</td>
<td>2768**</td>
<td>0.2</td>
</tr>
<tr>
<td>NQ</td>
<td>&gt;4768</td>
<td>&gt;4768</td>
<td>0.4</td>
</tr>
<tr>
<td>FOX-7</td>
<td>1108***</td>
<td>6463****</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>(541-2129)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOX-12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Guanylurea</td>
<td>&gt;3187</td>
<td>&gt;3187</td>
<td>0.3</td>
</tr>
<tr>
<td>- Dinitramide</td>
<td>&gt;2742</td>
<td>&gt;2742</td>
<td>Nd</td>
</tr>
</tbody>
</table>

* Preliminary bioaccumulation factor (BAF) estimated using the concentration ratio of tissue to soil after 14 d exposure in amended soil at sub-lethal conditions. ** Only one concentration with toxic effect (LOEC). *** LC$_x$ values were obtained using Maximum Likelihood-Probit and are expressed mg/kg, with 95% confidence intervals in parentheses (N = 3 replicates). ****Above the maximum concentration tested. Nd: not determined.
The effect of NTO, NQ, FOX-7, and FOX-12 on the perennial ryegrass seedling emergence and growth of perennial ryegrass (*Lolium perenne*) are summarized in Table 6. A preliminary experiment with NTO showed that soil were acidic (pH 3.7 to 5.1) following amendment with NTO at concentrations up to 5000 mg/kg. Therefore, ryegrass seedlings did not emerge in soils amended with ≥ 10 mg/kg. In the second study, initial concentrations of NTO measured in soil were from 0 (control) to 7.9 mg/kg. The initial soil pH values were similar to control soil (pH around 5.6). NTO amended soil inhibited ryegrass seedling emergence and the growth (Table 5). EC$_{50}$ values of 6 (5-6) mg/kg and 2 (0-4) mg/kg were estimated for emergence and growth, respectively.

Initial measured concentrations of NQ in soil ranged from 0 (control) to 4768 mg/kg. Preliminary data indicated that NQ-amended soil did not inhibit ryegrass seedling emergence or growth (Table 6). The initial measured concentrations of FOX-7 in soil were from 0 (control) to 4755 mg/kg. These data indicated that FOX-7 amended soil did not inhibit ryegrass seedling emergence, but effects on the 19 d growth were observed at 79 mg/kg and higher. Additional experiments using concentration higher than 4755 mg/kg should be conducted to estimate the EC$_{50}$ value.

**Table 6: Toxicity of selected insensitive munitions to perennial ryegrass (*Lolium perenne*)**

<table>
<thead>
<tr>
<th></th>
<th>EC$_{20}$*</th>
<th>EC$_{50}$*</th>
<th>LOEC</th>
<th>NOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td>mg/kg</td>
<td>mg/kg</td>
<td>mg/kg</td>
</tr>
<tr>
<td>7 d seedling emergence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTO</td>
<td>5 (4-5)</td>
<td>6 (5-6)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>NQ</td>
<td>&gt; 4768</td>
<td>&gt; 4768</td>
<td>&gt; 4768</td>
<td>4768</td>
</tr>
<tr>
<td>FOX-7</td>
<td>&gt; 4755</td>
<td>&gt; 4755</td>
<td>&gt; 4755</td>
<td>4755</td>
</tr>
<tr>
<td>FOX-12</td>
<td>93 (68-154)</td>
<td>36 (10-53)</td>
<td>100</td>
<td>4.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>LOEC</th>
<th>NOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td>mg/kg</td>
</tr>
<tr>
<td>19 d growth inhibition (dry weight)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTO</td>
<td>1 (0-3)</td>
<td>2 (0-4)</td>
</tr>
<tr>
<td>NQ</td>
<td>&gt; 4768</td>
<td>&gt; 4768</td>
</tr>
<tr>
<td>FOX-7</td>
<td>23 (0-61)</td>
<td>&gt; 4755</td>
</tr>
<tr>
<td>FOX-12</td>
<td>15 (0-45)</td>
<td>82 (51-119)</td>
</tr>
</tbody>
</table>

*EC$_x$ values were obtained from linear interpolation analyses and are expressed as mg/kg (N = 3 replicates.)
Initial measured soil concentrations of FOX-12 ranged from 0 (control) to 5929 mg/kg. Preliminary data indicated that FOX-12 amended soil inhibited ryegrass seedling emergence and growth. The EC$_{50}$ (95% CI) values of 93 (68-154) mg/kg and 82 (51-119) mg/kg were observed for the 7-d early seedling emergence and the 19-d growth, respectively. The relative toxicity profile (most to least toxic) based on 19 d-EC$_{50}$ values for ryegrass growth inhibition was DNAN $\approx$ NTO $>$ FOX-12 $>$ NQ $\approx$ FOX-7.

4.0 SUMMARY AND FUTURE RESEARCH

We have described the aquatic and terrestrial ecotoxities of DNAN, NTO, NQ, FOX-7, and FOX-12, based on our preliminary experiments. Different experimental and conceptual approaches were used to compare the toxicity of an IM such as DNAN (used as a TNT replacement in melt-cast formulations) to that of TNT. Microtox studies indicated that DNAN was less toxic than TNT or DNT. Future studies should confirm the relative toxicities of DNAN and TNT using other aquatic and terrestrial species.

Ecotoxicity of DNAN can be compared to other IM chemicals such as NTO, NQ, FOX-7, and FOX-12. Table 7 is a compilation of EC$_{50}$ and LC$_{50}$ values from Tables 1 to 6. These results show the IM concentrations that cause toxic effects in different aquatic (bacteria and algae), as well terrestrial (plants and invertebrates) species. Relative IM toxicity data analyses (i.e., comparing the columns) indicate that DNAN appears to be the most toxic IM to many of the test species, as indicated by the red squares. The toxicity of TNT to most of our test organisms (except algae) [see 43,44] was greater than that of the test IM chemicals.

For relative sensitivity analysis (i.e., comparing the rows), there is no consensus on the sensitivity of species to any particular IM as indicated by different colored circles; although, freshwater green alga appears to be very sensitive to most (but not all) of the test IM chemicals. Further studies should be conducted to fill in the data gaps, and should include toxicity testing using additional freshwater and terrestrial test species, definitive dose-response studies with multiple concentration treatments to replace those using only one exposure concentration (NTO, 14 d earthworm survival), as well as to consider sub-lethal responses (e.g., reproduction) in earthworms.

Laboratory soil toxicity studies indicated that IM compounds (e.g., DNAN) can be transformed (by biotic or abiotic processes) to reduced products (e.g., 2A-4NAN). DNAN and 2A-4NAN were measured in both the test soil (with worms added) and test organism (worm) at the end of the toxicity study. Ecotoxicology risk assessors are cautioned that toxicity is not necessarily caused by the parent test compound. In fact, the toxicity observed in the present studies could be due to the formation of a toxic metabolite, as shown earlier by Lachance et al. [31] using earthworms exposed to TNT-amended soil. The toxicity of the 2A-4NAN in earthworms should be studied further.
### Table 7: Comparative ecotoxicities of selected insensitive munitions*

<table>
<thead>
<tr>
<th></th>
<th>DNAN</th>
<th>NTO</th>
<th>NQ</th>
<th>FOX-7</th>
<th>FOX-12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aquatic Species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacteria</strong> (Microtox, mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min inhibition</td>
<td>14 (12-16)</td>
<td>5100 (3533-26625)</td>
<td>3727 (2647-7351)</td>
<td>&gt; 128</td>
<td>1816 (1532-2406)</td>
</tr>
<tr>
<td><strong>Algae</strong> (green algae, mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96 h growth</td>
<td>4 (3.5-4.2)</td>
<td>2324 (2130-2579)</td>
<td>1094 (925-1195)</td>
<td>22 (17-25)</td>
<td>17 (17-18)</td>
</tr>
<tr>
<td><strong>Terrestrial species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plants</strong> (ryegrass, mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 d Seed emergence</td>
<td>6 (5-7)</td>
<td>6 (5-6)</td>
<td>&gt; 4768</td>
<td>&gt; 4755</td>
<td>36 (10-53)</td>
</tr>
<tr>
<td>19 d Growth</td>
<td>7 (6-7)</td>
<td>2 (0-4)</td>
<td>&gt; 4768</td>
<td>&gt; 4755</td>
<td>82 (51-119)</td>
</tr>
<tr>
<td><strong>Invertebrates</strong> (earthworms, mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 d Survival</td>
<td>47 (32-81)</td>
<td>2768**</td>
<td>&gt; 4768</td>
<td>6463***</td>
<td>GU &gt;3187, Di-NA &gt;2742</td>
</tr>
</tbody>
</table>

* Data taken from Tables 1 to 5 of this article. ** Only one concentration with toxic effect.
*** Above the maximum concentration tested. GU: Guanylurea, DiNA-Dinitramide

Relative species sensitivity to a single IM (○, comparing rows): **Red:** highest; **Blue:** moderate; **Green:** lowest

Relative IM toxicity to a single species (□, comparing columns): **Red:** highest; **Blue:** moderate; **Green:** lowest

Colored boxes signify common degrees of relative toxicity and sensitivity.

Tissue residue analyses of IM in bioaccumulation studies can provide evidence of potential trophic transfer in the food chain. Preliminary data indicate that DNAN has potential to be bioaccumulated in soil; however, this effect may be limited by the rate of DNAN degradation in soil and its biota. The reader should appreciate that the toxicity and bioaccumulation of IM described here represent a worst case scenario. Our soil toxicity studies tested IM chemicals freshly amended in a sandy soil (from DRDC Valcartier), which has a relatively high IM bioavailability. Weathering and aging of test compounds in soil should also be included in the experimental designs to improve our understanding of potential exposure conditions in the field with concomitant alterations in exposure effects on terrestrial receptors.
5.0 CONCLUDING REMARKS
This chapter reviews the adverse effects of different IM chemicals on different ecological receptors (bacteria, algae, terrestrial plants, and soil invertebrates). These data and those from future ecotoxicity studies can be used for the ecotoxicological risk assessment and science-based management of sites contaminated with emerging environmental pollutants such as IM chemicals.

6.0 ACKNOWLEDGMENTS
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