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Characterization and remediation of soil contaminated with explosives: *development of practical technologies.*

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14. ABSTRACT
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Chapter 1. The fate of the cyclic nitramine explosive RDX in natural soil.

Tamara W. Sheremata, Annamaria Halasz, Louise Paquet, Sonia Thiboutot, Guy Ampleman, and Jalal Hawari. 2001. Environ. Sci. Technol. 35: 1037-1040.

A copy of the article is enclosed in the appendix.

1.1 Abstract

The sorption-desorption behavior and long-term fate of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) was examined in sterilized and nonsterilized topsoil. Results of this study indicate that although RDX is not extensively sorbed by the topsoil ($k^s{}_d$ of 0.83 L/kg), sorption is nearly irreversible. Furthermore, there was no difference in the sorption behavior for sterile and nonsterile topsoil. However, over the long-term, RDX completely disappeared within 5 weeks in nonsterile topsoil, and hexahydro-1-nitroso-3,5,-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5,-triazine (TNX) metabolites formed in the aqueous phase. Over the same period, recovery of RDX from sterile topsoil was high (55-99%), and the nitroso metabolites were not detected. Only traces of RDX were mineralized to CO_2 and N_2O by the indigenous microorganisms in nonsterile topsoil. Of the RDX that was mineralized to N_2O, one N originated from the ring and the other from the nitro group substituent, as determined using N^{15} ring-labeled RDX. However, N_2O from RDX represented only 3% of the total N_2O that formed from the process of nitrification/denitrification.

Chapter 2: Biotransformation routes of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by municipal anaerobic sludge.

Jalal Hawari, Annamaria Halasz, Sylvie Beaudet, Louise Paquet, Guy Ampleman, and Sonia Thiboutot . 2001. Environ. Sci. Technol. 35: 70-75.

A copy of the article is enclosed in the appendix.

2.1 Abstract

Recently we demonstrated that hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a trimer of methylene nitramine (CH_2=N-NO_2) undergoes spontaneous decomposition following an initial microbial attack using a mixed microbial culture at pH 7 in the presence of glucose as carbon source. The present study describes whether the second cyclic nitramine octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), a more strained tetramer of CH_2=N-NO_2, degrades similarly using sludge of the same source. Part of HMX biotransformed to give products that are tentatively identified as the nitroso derivatives octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine (mNs-HMX) and octahydro-1,3-dinitroso-5,7-dinitro-1,3,5,7-tetrazocine and its isomer octahydro-1,5-dinitroso-3,7-dinitro-1,3,5,7-tetrazocine, (dNs-HMX). Another fraction of HMX biotransformed, apparently *via* ring cleavage, to produce products that are tentatively identified as methylenedinitramine ($O_2NNHCH_2NHNO_2$) and bis(hydroxymethyl)nitramine, ($(HOCH_2)_2NNO_2$). None of the above intermediates accumulated indefinitely, they disappeared to predominantly form nitrous oxide (N_2O) and formaldehyde (HCHO). Formaldehyde biotransformed further to eventually produce carbon dioxide ($^{14}CO_2$). Nitrous oxide persisted in HMX microcosms containing glucose but denitrified rapidly to nitrogen in the absence of glucose. The presence of nitrous oxide was accompanied by the presence of appreciable amounts of hydrogen sulfide, a known inhibitor of denitrification.

Chapter 3: Effect of Tween 80 on the degradation pathway and the mineralization of 2,4,6-trinitrotoluene (TNT) using the fungus *Phanerochaete chrysosporium*.

Diane Fournier, Annamaria Halasz, Louise Paquet, and Jalal Hawari

3.1 Introduction

TNT is an energetic compound that is bio-transformed by many microorganisms. However mineralization of TNT (degradation to CO_2) is only significantly achieved by several fungal species, such as the white-rot basidiomycete *Phanerochaete chrysosporium* (Fernando et al. 1990; Scheibner et al. 1997). Mineralization of TNT have been markedly enhanced by adding the surfactant Tween 80 to the culture medium of *P. chrysosporium*, indicating that some intermediates were degraded into mineral carbon instead of being transformed into dead-end products (Halasz et al. 1997; Hodgson et al. 1997; Hodgson et al. 2000). However the exact role of Tween 80 remains unclear.

Tween 80 is typically used as a surfactant allowing an increased pollutant bioavailability in various bioremediation treatment processes. In the particular case of TNT degradation using *P. chrysosporium*, it was suggested that Tween 80 could play other important roles than desorbing and solubilizing TNT. For example it may act as a carbon source or may enhance the permeability of the biological membrane of the fungi, allowing better exchanges of the contaminant and its mebabolites between the exterior and the interior of the cells (Hodgson et al. 1997). Literature also indicates that Tween 80, as well as a mix of Tween 80 and oleic acid, increased the production of lignin peroxidase (LiP) by *P. chrysosporium* (Asther et al. 1987). A recent study showed that linoleic acid plays a role of co-oxidant for the extracellular manganese-dependent peroxidase (MnP) (Kapich et al. 1999). According to these authors, the fatty acid molecules would be oxidized into reactive radicals that enhance the MnP-catalyzed mineralization processes. Interestingly, we previously reported that the addition of Tween 80 to fungi cultures resulted in the detection of large amounts of oleic acid and other smaller fatty acids in the culture medium (Halasz et al. 1997). It was also shown that degradation of TNT was accompanied by the production of LiP and MnP (Hawari et al. 1999) and that mineralization was correlated with the increase in MnP (Fritsche et al. 2000). We are here hypothesizing that following the first nitroreduction attack of TNT, Tween 80 and the fatty acids released from its hydrolysis are promoting an oxidative degradation pathway via the action of MnP.

The aim of this study was to better understand the role of Tween 80 in the enhancement of TNT mineralization, a process that implies the breakdown of the aromatic ring. First, we focussed our efforts on the optimization of TNT mineralization using Tween 80. Since the production of the ligninolytic enzymes LiP and MnP depends on the physiological stage of the fungus, we investigated this parameter on TNT degradation. In a second part,

we studied the putative role of fatty acids in the enhancement of TNT mineralization. Finally we performed an extensive characterization of the metabolites formed in the presence and the absence of Tween 80. The comparison of the metabolites may provide insights into the degradation pathway used in the presence and the absence of the surfactant.

3.2 Materials and Methods

3.2.1 Chemicals

2,4,6-Trinitrotoluene (TNT) was obtained from the Defense Research Establishment of Valcartier (Quebec). Acetonitrile was from Fisher Scientific (Nepean, Ontario). Tween 80 (polyoxyethylenesorbitan monooleate), linoleic and oleic acid were purchased from Aldrich (Milwaukee, Wis.). Uniformly labeled [U-^{14}C]-TNT was synthesized as described previously (Ampleman et al. 1995). 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT), 2,6-diamino-4-nitrotoluene (2,6-DANT), 2-hydroxylamino-4,6-dinitrotoluene (2-HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT), 2,2',6,6'-tetranitro-4,4'-azoxytoluene, 4,4',6,6'-tetranitro-2,2'-azoxytoluene, 2,2',6,6'-tetranitro-4,4'-azotoluene and 4,4',6,6'-tetranitro-2,2'-azotoluene were obtained from AccuStandard inc. (New Hawen, CT). As HADNTs degrade rapidly in water, an equivalent amount of sodium metabisulfite was added to prevent their oxidation. These HADNTs calibration solutions were injected right after their preparation.

3.2.2 Organism and Growth Conditions

P. chrysosporium ATCC 24725 was used throughout this work. The strain was maintained on YPD (per liter: yeast extract 5 g, peptone 10 g, dextrose 20 g, agar 20 g, pH 5.5 adjusted with H_2SO_4) or malt extract (per liter: malt extract 20 g, yeast extract 1 g, agar 20 g, pH 5.5 adjusted with H_2SO_4). Conidiospores were produced according to the following procedure. The surface of a ten days old solid culture was scraped with a 10 ml pipet and the mycelium was mixed with MC-1 medium (Hodgson et al. 1997) and 0.2% (vol/vol) Tween 80. The suspension was then filtered through a sterile cheese cloth and the spore concentration was determined spectrophotometrically (an optical density of 0.5 corresponds to 2.5 X 10^6 spores/ml). The spore suspension was kept at 4°C.

TNT degradation assays were performed in 120 ml serum bottles with 10 ml of culture. The composition of the MC-1 medium was the following: per liter: KH_2PO_4 2 g, $CaCl_2.2H_2O$ 0.14 g, $MgSO_4.7H_2O$ 0.70 g, $FeSO_4.7H_2O$ 0.07 g, $ZnSO_4.7.H_2O$ 0.046 g, $MnSO_4.H_2O$ 0,035 g, $CuSO_4.7H_2O$ 0,004 g, disodium tartrate 2.3 g (pH 4.5). NAT 89 and veratryl alcohol are facultative supplements and were here omitted. It was previously shown that glucose supports high level of H_2O_2 production (Kelley and Reddy 1986) so 10 g/l glucose was then used as the carbon source. In order to cultivate the strain under

nitrogen deficient conditions that optimize the induction of ligninolytic enzymes (Kirk et al. 1978) yeast extract was omitted and a concentration of 0.23 g/l of diammonium tartrate (1.2 mM) was used instead of 1.84 g/l (10 mM). The fungi were inoculated at a concentration of 2 X10^5 spores/ml and the cultures were incubated in the dark, at 39°C, without agitation. In order to maintain aerobic conditions, the headspace of the microcosms was flushed with oxygen gas each 3 to 4 days. The addition of TNT (40 ppm) from an acetone stock solution (40 000 ppm), was done after 4 days of pre-incubation (Hodgson et al. 1997) or after 6 days of pre-incubation.

To allow the monitoring of TNT degradation and metabolites formation, the cultures were sacrificed. The mycelium mat was disrupted using a 3 ml syringe; then 50 % acetonitrile was added and the mixture was sonicated for 10 min. The suspension was then filtered through 0.45 µm Millex-HV filter (Millipore, Bedford, MA) for subsequent HPLC and LC/MS analysis without any delay.

3.2.4 Mineralization assay

The mineralization was determined as previously described (Whyte et al. 1996). The cultures were performed as described above. After 4 or 6 days of pre-incubation, 100 000 dpm [U-^{14}C]-TNT and unlabelled TNT were added to give a total TNT concentration of 40 ppm. A 5 ml tube containing 1 ml of 0.5 M KOH was placed in the serum bottle as a CO_2 trap. The serum bottles were closed with Teflon-coated butyl rubber seals, crimped with aluminum caps and were incubated in the same conditions described above. The KOH traps were regularly sampled and $^{14}CO_2$ values were monitored using a Tri-Carb 4530 liquid scintillation counter (LSC, model 2100 TR, Packard Instrument Company, Meriden, CT).

3.2.5 Analytical method for TNT and metabolites

TNT and metabolites were analyzed with a Sulpelcosil C8 column (25cm, 4.6mm, 5µm) that was held at 35°C. The solvent system consisted of an acetonitrile/water gradient at a flow rate of 1 ml/min. The initial solvent composition was 30% acetonitrile and 70% water. A first linear gradient was run from 30% to 40% acetonitrile over 20 min. A second linear gradient was run from 40% to 80% acetonitrile over the next 10 min. and then maintained for 2 min. This solvent ratio was changed to the initial conditions over 2 min. and then held for an extra 6 min. for a total of 40 min. A volume of 50 µl was injected. Photodiode array detector was used to detect and quantify the metabolites (wavelength of 254nm, with a wavelength range of 200-350nm).

Liquid chromatography/mass spectrometry was performed on a Micromass Platform II benchtop single quadrupole mass detector fronted by a Hewlett Packard 1100 Series HPLC system equipped with a Photodiode Array Detector. Samples were either analyzed under positive electronspray ionization (ES+) using a cone voltage of 30V and a source

temperature of 150 °C or negative electronspray ionization (ES-) using 30 V and 90 °C. The chromatographic conditions used were as above.

3.3 Results and Discussion

3.3.1 Effect of Tween 80 and the physiological stage of *P. chrysosporium*

Fig.1 shows that TNT was transformed by 6 days old *P. chrysosporium* cultures without any lag period. Compared to the culture where no surfactant was added, the cultures amended with 1% Tween 80 reduced of about 10 hours the time required for the complete disappearance of TNT. Similar results were obtained using 4 days old cultures indicating that the enzymatic steps involved in nitro-reduction of TNT do not depend on the physiological stage of the culture.

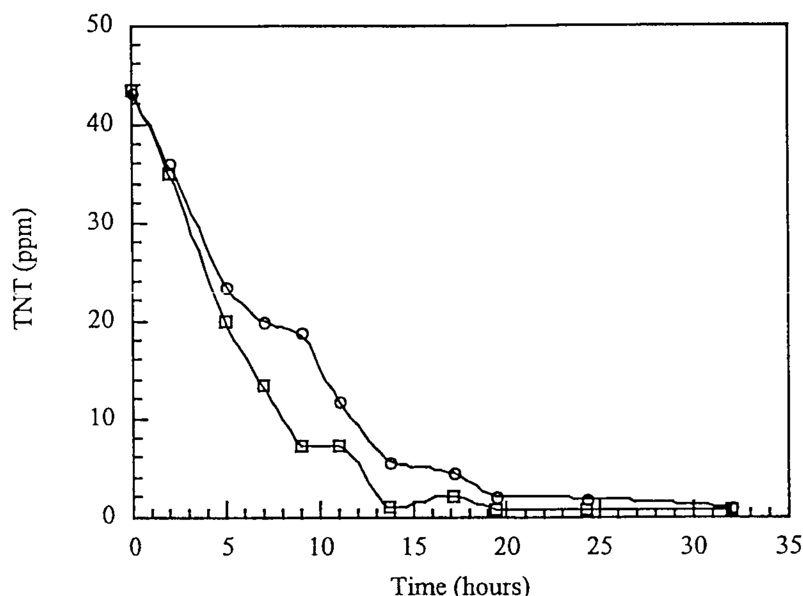

Figure 1. Time course of TNT transformation using six days old cultures of *P. chrysosporium* at 39 °C. Mineral MC-1 medium was amended with the following compounds: no surfactant added (○); 1% (vol/vol) Tween 80 (□).

However Fig. 2 shows that mineralization of TNT involves reactions that depend on the physiological stage of *P. chrysosporium*. When Tween 80 was used at a concentration of 0.2%, 6 days old cultures mineralized 32% of TNT in 20 days compared to 24% using 4 days old cultures (Fig. 2). The same effect was observed when Tween 80 was used at a concentration of 1%. In this condition, 6 days old cultures mineralized 47% of TNT in 20 days of incubation and 4 days old cultures mineralized 37% TNT. These results indicate

that the enhancing effect of Tween 80 is more important during the ligninolytic phase of *P. chrysosporium* which is reached after 6 to 7 days of incubation, supporting that ring cleavage of TNT would be catalyzed by non-specific ligninolytic enzymes such as LiP or MnP.

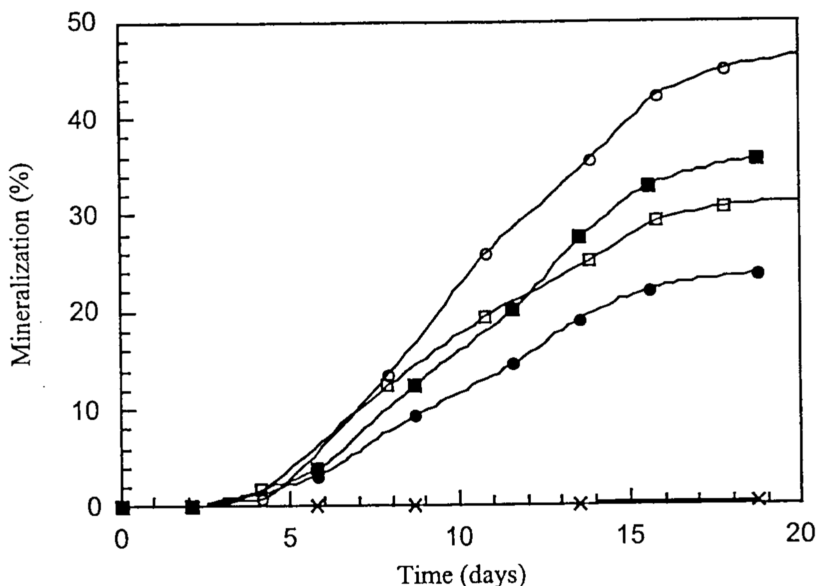

Figure 2. Effect of the physiological stage and surfactant on mineralization of TNT using 4 days old (closed symbols) and 6 days old (opened symbols) cultures of *P. chrysosporium*. The cultures were the following: 4-days old culture + 0,2% Tween 80 (●), 4-days old culture + 1% Tween 80 (■), 6-days old culture + 0,2% Tween 80 (□), and 6-days old culture + 1% Tween 80 (○). Control without fungi (X).

3.3.2 Effect of fatty acids

As mentioned previously, fatty acids are enhancing the production (Asther et al. 1987) and the activity (Kapich et al. 1999) of ligninolytic enzymes. In this work we studied the effect of oleic and linoleic acids on the mineralization of TNT. Using 4 days old cultures, the rate and extent of mineralization were similar whether oleic acid (3 mM and 9 mM), or linoleic acid (3 mM) were added to 0.2% Tween 80 amended cultures (results not shown). However Fig. 3 indicates that when 6 days old cultures were amended with 0.2% Tween 80 and 3 mM oleic acid the mineralization reached 41% in 28 days, compared to 32% for the oleic acid-free cultures. This value tends to reach the 48% mineralization obtained in the presence of 1% Tween 80. If we consider that Tween 80 would be totally hydrolyzed by the fungi, a concentration of 1% and 0.2% of the surfactant would respectively provide 7.5 and 1.5 mM oleic acid. Thus the combination of 0.2% Tween and 3 mM oleic acid would provide a total concentration of 4.5 mM oleic acid. These

results are in accordance with those previously published by Kapich et al. (1999), showing that the fatty acids (directly supplied or provided as the hydrolyzed-moiety of Tween 80) might be responsible for an enhanced destruction of lignin model compound. We should mention that results obtained using higher concentrations of oleic acid (8 mM, 12 mM and 16 mM) were similar to those obtained at 3 mM. The limited water solubility of this compound may explain the absence of a dose-response effect. A study involving a simplified system composed of purified LiP or MnP and oleic acid would allow us better understand the role of such a fatty acid in helping the breakdown of the TNT molecule.

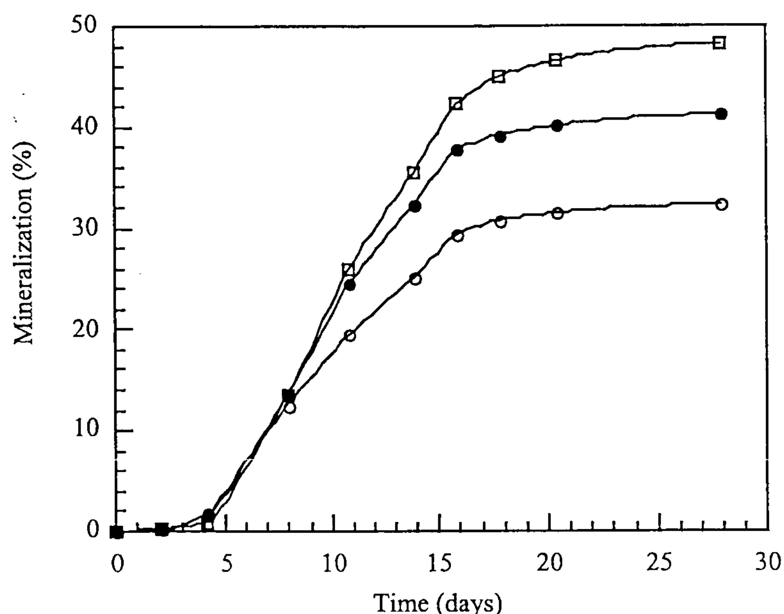

Figure 3. Effect of oleic acid on the mineralization of TNT using 6 days old *P. chrysosporium* cultures. The cultures were amended as following: 0,2% Tween 80 (○), 0,2% Tween 80 and 3 mM oleic acid (●), 1% Tween 80 (□).

3.3.3 Characterization of metabolites

While studying the degradation of TNT using *P. chrysosporium*, Hawari et al. (1999) characterized all possible transformed products. As described previously, they identified at least six transformation cycles in the degradation of TNT. Cycle 1 involved the disappearance of TNT and the formation of the prime metabolites 2-nitroso- and 4-nitroso-dinitrotoluene (NsT) and 2-hydroxylamino and 4-hydroxylaminodinitrotoluene (HADNTs). In cycles 2 to 6, subsequent biochemical and chemical reactions of HADNTs were involved. In Cycle 2 undetermined amounts of HADNTs continued their reduction

to produce the monoaminodinitrotoluenes 2-ADNT and 4-ADNT and traces of 2,4-DANT. Close to 25 % of HADNTs underwent Bamberger rearrangement to the corresponding phenolamines, PhADNTs, (Cycle 3) which disappeared with TNT disappearance (10 days). An undetermined fraction of HADNT transformed to 4-N-Ac-4-OH-2,6-dinitrotoluene and 4-N-acetoxy-2,6-dinitrotoluene in an acetylation route termed Cycle 4. A different acylation route termed Cycle 5 was also constructed which involved the formation of 2-formylamido-4,6-dinitrotoluene (and its *p*-isomer) and 2-acetylamido-4,6-dinitrotoluene (and its *p*-isomer). These latter compounds did not accumulate in the system and their disappearance was marked with retardation in the production of $^{14}CO_2$. Finally, in Cycle 6, HADNT coupled with NsT to produce the corresponding azoxy derivatives that dimerized to tetranitrohydrazotoluenes.

In the last part of this work, we performed a comparative study of the metabolites formed in the absence and the presence of 1% Tween 80. Fig. 4 shows that cultures without Tween produced transient amounts of the two prime HADNTs metabolites. LC-MS analyses confirmed this result. Using the standards and precautions described above, we were able to estimate that 2-HADNT reached a maximal value of 0,4 ppm in 5 hours of incubation and 4-HADNTattained a peak 2,3 ppm in 2 hours of incubation. One major difference between the two culture systems was that in the presence of Tween, no 2-hydroxylamino and 4-hydroxylaminodinitrotoluenes (HADNTs) were observed. The absence of HADNTs in Tween amended cultures indicates that the surfactant induces or accelerates an oxidative pathway that prevents the accumulation of HADNTs which have been shown to inhibit LiP (Bumpus and Tatarko 1994, Michels and Gottschalk 1994.). Consistently with this result a negligible concentration of 2,2'-azoxytoluene (which is a product from HADNT dimerization) was not observed in Tween 80 amended cultures while it was accumulating in the Tween-free cultures (result not shown). Thus azoxy derivatives do not lead to CO_2 formation.

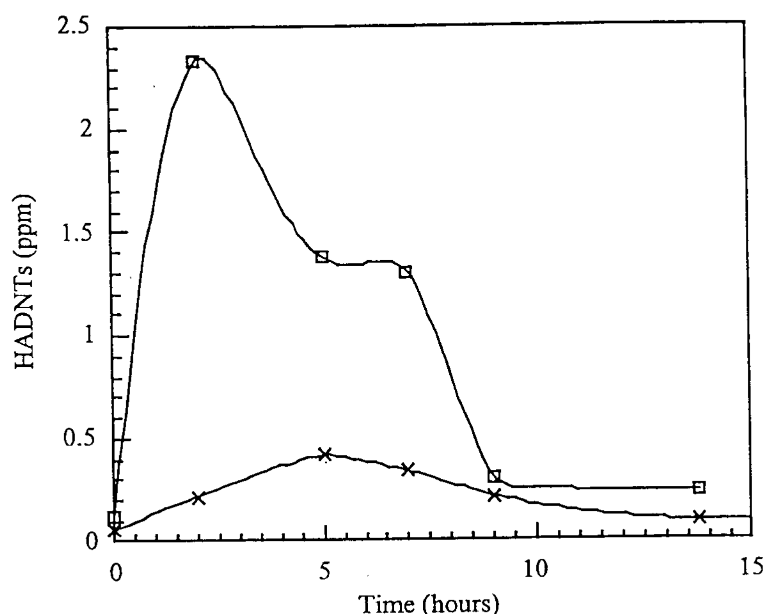

Figure 4. Time course of the formation and disapearance of 2-HADNT (X) and 4-HADNT (□) during TNT degradation using using 6 days old *P. chrysosporium* cultures in the absence of Tween 80.

The formation of the two monoaminodinitrotoluenes 2-ADNT and 4-ADNT was the same in the presence and the absence of Tween 80 (result not shown). As determined by LC-MS analyses, neither azo compounds nor hydrazo were detected in the presence of Tween while these intermediates were significantly produced in the absence of the surfactant (result not shown). As for the azoxy derivatives, hydrazo and azo derivatives do not lead to TNT mineralization.

Fig. 5 reveals another marked difference between the two systems. Only in Tween amended cultures, we observed the significant production of an unknown metabolite showing a deprotonated mass ion at 392 Da. After reaching a maximal value at 24 hours of incubation, the metabolite was degraded. The determination of the structure of this metabolite may allow us to know if Tween induces a totally distinctive degradation route that leads to an efficient ring-cleavage of TNT.

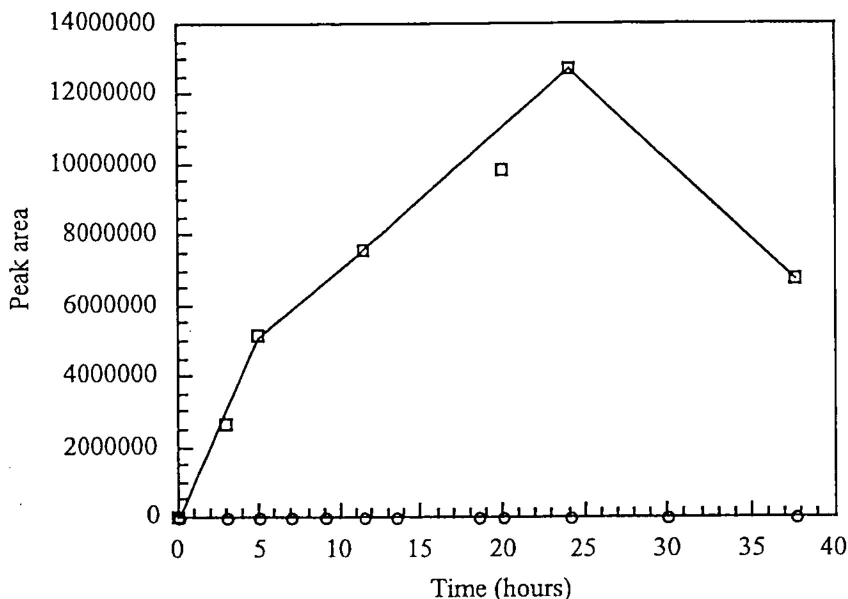

Figure 5. Time course of the production and degradation of a distinctive unknown metabolite showing a deprotonated mass ion at 392 Da produced in the presence (□) or the absence (○) of Tween 80.

3.4 Conclusion

In conclusion, this study confirms that Tween 80 plays an important role on the mineralization of TNT. It was also shown that the physiological stage of the fungi was an important parameter that potentialized the action of Tween. Oleic acid (provided by the addition of Tween 80 or directly supplied to the culture) appeared to be a crucial parameter that enhanced the degradation of TNT to CO_2. The study of the metabolites indicated that the presence of Tween modified the pattern of intermediates formed into the culture of *P. chrysosporium*. Mainly, the surfactant prevented the accumulation of toxic HADNTs as well as their dead-end derivatives. Finally, a distinctive metabolite showing a deprotonated mass ion at 392 Da never observed previously was only produced in the presence of Tween. Further investigations would allow us to establish whether or not TNT is degrading via a distinctive pathway in the presence of Tween 80.

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Chapter 4: Applied Ecotoxicology

4.1 OVERVIEW

Over the past year, the Applied Ecotoxicology Group at BRI has demonstrated how ecotoxicity data can be used towards the establishment of environmentally acceptable criteria for soil quality for energetic substances, using TNT as an example. This interest is shared by other organizations such as the US-EPA (Ecological Soil Screening Levels – EcoSSL, SERDP), and laboratories participating in the TTCP-KTA-428 collaboration. The establishment of soil quality criteria for explosives is a very important goal towards DND's Sustainable Training program.

During FY2000-2001, the Ecotoxicology module of the BRI-DREV collaboration has led to a number of recently published papers describing the ecotoxicological effects of energetic substances at the microbial and invertebrate levels. These four publications are as follows:

- Gong, P., Gasparrini, P., Rho, D., Hawari, J., Thiboutot, S., Ampleman, G. and Sunahara, G.I. (2000). An *in situ* respirometric technique to measure pollution-induced microbial tolerance in soils contaminated with 2,4,6-trinitrotoluene. *Ecotoxicology and Environmental Safety* **47**: 96-103.
- Gong, P., Hawari, J., Thiboutot, S., Ampleman, G. and Sunahara, G.I. (2001). Ecotoxicological effects of hexahydro-1,3,5-trinitro-1,3,5-triazine on soil microbial activities. *Environmental Toxicology and Chemistry* **20**: 947-951.
- Robidoux, P.Y., Svendsen, C., Caumartin, J., Hawari, J., Ampleman, G., Ampleman, S., Weeks, J.M. and Sunahara, G.I. (2000). Chronic toxicity of energetic compounds in soil determined using the earthworm (*Eisenia andrei*) reproduction test. *Environmental Toxicology and Chemistry* **19**: 1764-1773.
- Robidoux, P.Y., Hawari, J., Thiboutot, S., Ampleman, G. and Sunahara, G.I. (2001). Chronic toxicity of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in soil determined using the earthworm (*Eisenia andrei*) reproduction test. *Environmental Pollution* **111**: 283-292.

In addition, a peer-reviewed book chapter describing our integrated Ecotoxicology-Analytical Chemistry approach for the study of toxic energetic compounds, was recently accepted by the editors of the ASTM.

- Sunahara, G.I., Robidoux, P.Y., Gong, P., Lachance, B., Rocheleau, S., Dodard, S.G., Sarrazin, M., Hawari, J., Thiboutot, S., Ampleman, G., and Renoux, A.Y. (2001). Laboratory and field approaches to characterize the soil ecotoxicology of energetic substances. In: *Environmental Toxicology and Risk Assessment: Science, Policy and Standardization – Implications for Environmental Decisions: Tenth Volume STP 1403*. Greenberg, B.M., Hull, R.N., Roberts Jr., M.H., and Gensemer, R.W. (Eds). American Society for Testing and Materials, West Conshohocken, PA. (Accepted).

The first two of the following sections will describe how ecotoxicity data can be used to derive soil quality criteria for explosives using the CCME approach, and the ecotoxicological hazard and risk assessment of a TNT-contaminated site. The final two sections describe some of our recent work characterizing the soil microbial responses to

HMX, and the operational validation of the phytogenotoxicity assay (Trad-MCN assay) using the spiderwort *Tradescantia*. This bioassay is based on the measurement of the formation of micronuclei, as the indicator of chromosomal breakage. This assay will be used for future studies of toxic energetic materials.

4.2 DERIVATION OF ENVIRONMENTAL SOIL QUALITY GUIDELINES FOR 2,4,6-TRINITROTOLUENE: CCME APPROACH

4.2.1 Introduction

2,4,6-Trinitrotoluene (TNT, CAS 118-96-7) is a persistent environmental contaminant identified in soil and groundwater at sites related to military and demolition activities. Contaminated sites include those associated with the manufacture, handling, testing and disposal of these energetic materials, their by-products and degradation products. This compound represents a major international problem because of its toxicity (Yinon 1990), its persistent properties and the extent of its dispersal in the environment, particularly in North America as well as in Europe. Concentrations of these explosives in soil vary according to the use of the facility. For example, high concentrations (g/kg soil) may be found at production and handling sites (Simini *et al.* 1995) where effects include loss of vegetation and overall degeneration of soil health. On the other hand, lower levels (mg/kg soil) may be found at unexploded ordnance contaminated ranges at military training areas (Hawari, personal communications) and at open burning/open detonation (OB/OD) areas, where ecological effects are not obvious depending on the explosive. Talmage *et al.* (1999) recently reviewed the toxicological effects of TNT.

In Canada, the Department of National Defence (DND) has about 100 training sites and three OB/OD facilities, most of which are contaminated by energetic materials but at relatively low concentrations (mainly below 10 mg/kg of TNT, RDX and HMX). Some of these sites will be decommissioned and alternative land use options will be considered by DND managers. To achieve this goal, it is necessary to consider the cost and time associated with decontamination, the quality and reusability of the site (alternative land use), and the very important question of "how clean is clean?". However, no soil quality criteria are currently available in Canada for TNT.

In the Protocol for the Derivation of Environmental and Human Health Soil Quality Guidelines, the Canadian Council of Ministers of the Environment (CCME) has set out the procedures to be followed in drafting soil quality recommendations in Canada (CCME 1996). These generic recommendations are justifiable from a scientific standpoint and should help to ensure the maintenance of the ecological functions essential for activities related to the designated use of lands.

The object of this document is to provide recommended soil quality values related to TNT with the goal of protecting the environment (SQG_E). The document describes and explains the steps followed in developing environmental soil quality guidelines with respect to TNT. Furthermore, this exercise has enabled us to identify the gaps in scientific knowledge about the effects of this contaminant in soils.

4.2.2 Environmental Soil Quality Guidelines

General Principles
As the title indicates, the Protocol for the Derivation of Environmental and Human Health Soil Quality Guidelines (CCME 1996) outlines the procedure for drawing up guidelines for protecting humans and the environment. Since the present project is aimed at developing environmental soil quality guidelines, only the section on environmental guidelines in that Protocol was applied.

According to the CCME, the development of environmental soil quality guidelines (SQG_E) entails the following steps:

1) Exhaustive literature search on the contaminant. In addition to identifying key ecotoxicological properties, the goal of this research is to provide an overview of the chemical, physical, biololgical, environmental and even legal characteristics of the substance. Not all of the information collected will be used in drafting the final numerical guideline.

2) Evaluation of the quality of the available ecotoxicological data. The CCME sets minimum requirements for assessing the scientific and toxicological quality of laboratory and field data. Based on this assessment, three databases are created on direct soil contact, microbial processes and soil and food ingestion. The CCME also establishes a limit for the minimum number of data required for drafting a guideline. This step is undoubtedly the most important one in the procedure, since the quality of the resulting guideline depends on the quality of the data used.

3) Development of the "threshold effect concentration" (TEC) or the "**effect concentration low**" (ECL) for plants and invertebrates. This intermediate step involves defining an initial criterion for protecting soil fauna according to the land use designation (TEC for agricultural, residential/park lands and ECL for commercial/industrial land use) by using to the database for direct contact with the soil.

4) Comparison of the TEC or the ECL with the critical concentrations for microbial processes (nitrogen and carbon cycles). Since it is imperative to preserve ecological functions, the ecotoxicological impact of the substance on microbial processes must be assessed. By employing essentially the same approach as that used to obtain the TEC and ECL values, a critical concentration can be defined as the point beyond which microbial processes are affected. If the TEC (or the ECL) is higher than the critical value of the corresponding microbial process, the geometric mean of the two will constitute the "guideline for soil quality with respect to soil contact" (SQG_{SG}). However, if the value for the microbial process is higher than that of the TEC or the ECL, then the SQG_{SG} will be equivalent to that of the TEC or the ECL.

5) Development of the guideline for soil quality in relation to the ingestion of soil and food by mammals and birds (SQG_I); agricultural use only.Defining the SQG_I entails finding the "daily threshold effect dose" (DTED). This dose is obtained with reference to the lowest-observed-adverse-effect level (LOAEL) reported in the scientific literature for the most threatened species. The CCME is nevertheless cognizant of the lack of available information and, in the absence of acceptable data, the SQG_I will not be computed.

6) Final guideline for soil quality with the aim of protecting the environment (SQG_E). Finally, the SQG_E is obtained directly from the SQG_{SG} for residential/park, commercial and industrial land uses. In the case of agricultural use, the SQG_E will be equivalent to the lowest value of the SQG_I and the SQG_{SG} calculated for these lands.

Literature search

From the documents available within the Applied Ecotoxicology group, a review of information on the Internet and research carried out by the BRI's documentation centre, we collated some 600 documents dealing with energetic compounds. Of these, nearly 300 were identified as potentially relevant to this study. These documents cover various characteristics of TNT (toxicological, ecotoxicological, degradation, management, etc.). The databases consulted are as follows:

- Integrated Risk Information System (IRIS: http://www.epa.gov/ngispgm3/iris),
- PHYTOTOX and TERRETOX (http://www.epa.gov/earth100/records/a00132.html),
- Agency for Toxic Substances and Disease Registry (ATSDR),
- Databases accessible through SilverPlatter: INIS, BIOSYS, Current Contents, and MEDLINE.

Toxicological database for direct soil contact

The Protocol stipulates that only studies that directly involve soils will be retained. For example, the results obtained from the use of a liquid (broth, hydroponic growth, etc.) or a solid other than soil (agar) could not be retained. The review of documents made it possible to create a small ecotoxicological database specific to TNT (BRI-Ecotox TNT database available upon request) in which the information is presented according to the organisms' trophic level. The database reflects the requirements of the CCME's protocol and includes information on the organism (trophic level, common name, species name), the toxicological conditions (toxic variable measured, exposure time, temperature, standardization of the assay, field data), data (unit of measurement, NOEC, LOEC, % associated effect, effective concentration), the bibliographic reference, edaphic conditions (origin of the soil, % organic matter, sand, silt and clay, moisture, CEC, water retention capacity), statistical analysis used, and notes. The database now comprises 26 observations. Nine studies were selected during this phase. The values are divided equally among plants and animals, although the earthworm *Eisenia* represents the organism studied the most.

The two earthworm species *Eisenia foetida* and *Eisenia andrei* are represented. Although there is controversy at present over the names of these worms, the literature search did not permit a definitive determination of whether they constitute the same species or two different ones; hence, an arbitrary decision was made to consider them as separate ones. Four studies employing a standardized method reported TNT-associated decreases in weight and fertility along with mortality in the worms in two types of soil (artificial and forestry) (Phillips *et al.* 1993; Renoux *et al.* 2000; Robidoux *et al.* 1999, 2000). Various parameters such as the number of cocoons, egg hatch, juveniles and total weight for juveniles were covered in the latter study. The data selected

for the database correspond to the most sensitive parameter, i.e. the number of juveniles. Lethality and reproductive capacity were likewise studied in the invertebrate species *Enchytraeus albidus* (Dodard *et al.* 2000). The study by Parmelee *et al.* (1993) was retained, even though it did not consist of a standardized test. This author reports pertinent results and assesses the effect of TNT from an ecosystem standpoint by examining the impact on the communities of soil-dwelling microarthropods and nematodes.

With respect to plants, one high-quality study provides germination and stem growth values for four different species (watercress-*Lepidium sativum* L., turnip-*Brassica rapa* Metzg., oats-*Acena sativa* L. and wheat-*Triticum aestivum* L.) that grew in two types of soil (Gong *et al.* 1999b). Cataldo *et al.*, (1989) also derived height reduction values for plants of three different species (*Bromus mollis*, *Phaseolus vulgaris*, *Triticum aestivum*), whereas Li *et al.* (1997) studied germination and root and stem elongation in fescue. The latter two studies do not entirely satisfy the criteria for data validity, and the tests used were not standardized. Furthermore, Cataldo *et al.* (1989) did not provide statistical analyses and tested only three TNT concentrations. As a result, the EC25 values retained represent responses rather than calculated values. In the study by Li *et al.* (1997), the soils were sampled from a contaminated lot, the negative control was replaced by treatment #3 (consided as having no effect). Since the results obtained in these studies are of the same order of magnitude as those obtained by Gong *et al.* (1999b), and since the observed effects do not appear evident to us (professional assessment), they were not retained in the present databaes.

It should be noted that the studies by Dodard *et al.* (2000), Robidoux *et al.* (1999, 2000), and Renoux *et al.* (2000) were conducted in the same laboratory (BRI's Applied Ecotoxicology Group). Nonetheless, the relatively larger number of studies (nine) in the database related to direct soil contact validates this degree of redundancy. The values reported in this database appear to be fairly homogeneous overall. In fact, there is only one order of magnitude between the lowest values (24 mg/kg) and the highest values (500 mg/kg). From this database it can also be seen that plants are more sensitive to TNT contamination compared with invertebrates and that the values related to plants are more heterogeneous.

Database on microbial processes

As with the database on direct soil contact, the information in the database on microbial processes reflects the requirements of the CCME protocol and includes information on microbial processes (ecological cycle and biochemical activity), toxicological conditions (toxic variable measured, exposure time, temperature, test standardization, field data, chemical analyses), the data (unit of measure, NOEC, LOEC, % associated effect, effective concentration), the bibliographic reference, edaphic conditions (origin of the soil, % organic matter, sand, silt and clay, moisture, CEC, water retention capacity, % nitrogen (Kjeldahl)), statistical analysis used, and notes.

Only two studies were found that deal with the toxic effects of TNT on microbial processes (Fuller and Manning 1998; Gong *et al.* 1999a), although many authors had studied TNT

biodegradation by microorganisms. The research by Fuller and Manning (1998) was not retained in the database on microbial processes (available upon request). The measurements were carried out on field samples contaminated with both TNT and TNB. The very high correlation between the concentrations of these two contaminants in the soil precludes an unequivocal determination of the effect of TNT on the soil microbial communities.

In the study by Gong *et al.* (1999a), data were collected in the field and the laboratory. The microbiological cycles studied are nitrification, nitrogen fixation and overall microbial activity by measuring dehydrogenase activity. This biochemical activity was not considered pertinent for illustrating microbial ecological cycles. The values furnished are based on the TNT concentrations extracted using the acetonitrile – sonication technique (Jenkins method). TNT metabolites (mainly 2-ADNT and 4-ADNT) were also identified in the samples. With regard to the field data, the lack of uncontaminated soil (negative control) was overcome by using soils with the lowest TNT levels and biochemical activity equivalent to that found in other types of uncontaminated soil. The soil characteristics were not determined for the field samples. In contrast with the values obtained for direct soil contact (plants and invertebrates), those obtained in this study show considerable heterogeneity (0.4 - 533 mg/kg). Increased sensitivity of the 'Nitrification (PNA)' function relative to the other parameters is another aspect that should be noted.

Table 4.1 TNT toxicity data for mammalian species (route exposure: diet) (from Talmage *et al.* 1999).

Species	*Exposure period*	*NOAEL (mg/kg/d)*	*LOAEL (mg/kg/d)*	*Endpoint*	*Ref. (footnotes)*
Rat	24 mon	0.4	2	Kidney, bone, spleen	1
	13 wk	1.4	7	Anemia	2
	13 wk	5	25	Anemia (males)	3
	13 wk	5	25	Testicular atrophy	3
	13 wk	34.7	160	Testicular atrophy	2
Mouse	24 mon	10	70	Liver ; anemia	4
	13 wk	7.5 (m) 8 (f)	35.7 (m) 37.8 (f)	Anemia	2
Dog	25 wk	-	0.5	Liver	5
	13 wk	0.2	2	Liver ; anemia	2

1: Furedi *et al.* (1984) US Army Medical R&D Command, Fort Detrick, MD. Report # AD-A168 637
2: Dilley *et al.* (1982) *J Toxicol Environ Health* 9:565-585
3: Levine *et al.* (1984) *Toxicology* 82:253-265
4: Furedi *et al.* (1984) US Army Medical R&D Command, Fort Detrick, MD. Report # AD-A168 754
5: Levine *et al.* (1990). *Toxicology* 63:233-244

The literature search did not turn up any publications dealing with the effects of TNT ingestion in food and soil by browsing mammals or birds. This is confirmed by the very recent review by Talmage *et al.* (1999), which states that no studies could be found on TNT toxicity in wild mammals or birds could. PharmacoDes studies were, however, conducted on laboratory

mammals. These studies, which used rats, mice and dogs, are reported in the publication by Talmage *et al.* (1999). The toxicity effects of TNT on mammals are shown in Table 4.1. With a view to providing a preliminary value for the guideline, the data obtained for rates and mice were used in computing the SQG_I. These data were retained because, although they come from laboratory animals, other rodent species live in close contact with soil in the wild; however, this is not the case for dogs. Furthermore, in contrast with dogs, these mammals feed on plants and hence may be exposed to soil contaminants through this exposure route.

Methods for agricultural and residential/park land use

Estimation of the threshold effect concentration (TEC)

Table 4.2 gives all the values (TNT concentrations in soil with and without effect) compiled for each species from the database on direct soil contact that were used in developing the TEC. The most common values correspond to the lowest concentrations. The smallest concentrations that caused an observed effect (LOEC) have the widest range, from 30 mg/kg for decreased plant growth to 500 mg/kg for germination.

Depending on the amount of data making up the database, the CCME proposes three methods for deriving the "threshold effect concentration" (TEC; for agricultural and residential/park use): the weight-of-evidence approach, the lowest-observed-effect-concentration method (LOEC method) and the median effects method. The "effect concentration low" (ECL; for commercial and industrial land) can only be obtained by the weight-of-evidence and lowest-observed-effect-concentration methods. Uncertainty factors can be used, but this is not recommended by the CCME. Their use is prohibited in the determination of the ECL by the weight-of-evidence method.

The weight-of-evidence method, initially recommended by the CCME, applies to all the toxicological data. This procedure requires at least 10 data points collected from at least three studies. In our case, 32 data points were gathered in nine studies. The Protocol specifies that the statistical distribution must contain at least two data points on soil invertebrates and two on agricultural or other plants. Seventeen data points on soil invertebrates and 15 points related to plants were used, with most of the species consisting of agricultural plants.

A final requirement for using this method consists in having a balanced distribution of different quantiles, that is, the relative proportions of the different categories of data (LOEC, NOEC, EC and LC) must be equal. Our database comprises 40% NOEC, LOEC and 22% EC (effective concentration). These values are not indicative of the preponderance of any quantile with respect to another quantile: the basic requirements for using the weight-of-evidence method are met.

Table 4.2 TNT concentrations (mg/kg dry soil) with (LOEC and Effect Conc.) or without (NOEC) toxic effects (values compiled from the database on direct soil contact)

Trophic level	*Common name*	*Species*	*Endpoint*	*NOEC*	*LOEC*	*Effect. Conc.*
Invertebrates	Compost worm	*Eisenia andrei*	Fertility	110	220	347
Invertebrates	Compost worm	*Eisenia andrei*	Lethality	185	135	226
Invertebrates	Compost worm	*Eisenia foetida*	Lethality	200	150	325
Invertebrates	Compost worm	*Eisenia foetida*	Weight loss	110	145	
Invertebrates	Enchytraei-des	*Enchytraeus albidus*	Lethality	267		422
Invertebrates	Enchytraei-des	*Enchytraeus albidus*	Fertility	96		130
Microarthro-podes	Oribatid mites		Reduced population	100	200	
Plants	Oat	*Acena sativa*	Shoot growth	147	332	
Plants	Turnip	*Brassica rapa* Metzg.	Shoot growth	34	86	
Plants	Blando broom	*Bromus mollis*	Plant height			30
Plants	Tall fescue	*Festuca arundinacea Schreb.*	Germination	156	499	
Plants	Tall fescue	*Festuca arundinacea Schreb.*	Root-shoot growth	72	156	
Plants	Cress	*Lepidium sativum*	Shoot growth	34	86	
Plants	Bean	*Phaseolus vulgaris*	Plant height		30	
Plants	Wheat	*Triticum aestivum*	Plant height	86	222	30

In keeping with the weight-of-evidence method, the threshold effect concentration (TEC) corresponds to the value of the 25th centile, which in the case at hand means a value of 86 mg/kg. The TEC was therefore set at 86 mg/kg. With this method, three criteria could justify the use of an uncertainty factor ranging from 1 to 5:

1) Only three studies are available (minimum number of studies).

2) In spite of the existence of more than three studies, less than three taxonomic groups are represented.
3) More than 25% of the data below the 25th centile are data that show a definite effect (EC50, LC50, etc.). Such a proportion provides a criterion that does not protect ecosystem integrity sufficiently.

Since we used toxicological values for ten genera of different organisms from nine different studies (8 authors and 6 labs) and 18% of the data below the 25th centile are data with a definite effect, using an uncertainty factor greater than 1 was not justified.

Comparison with microbial processes

The following step consists in comparing the TEC with the data retained in relation to microbial processes. However, one of the minimum requirements for applying the method is that the database on microbial processes must include at least three studies. Since only the Gong *et al.* (1999a) publication was selected, this comparison cannot be made.

Derivation of Soil Quality Guideline (agricultural – residential/park land use) for soil contact (SQG_{SG})

The SQG_{SG} corresponds to the TEC in the present case since the lack of data on microbial processes precludes a comparison of this value. Therefore, the SQG_{SG} is 86 mg/kg for agricultural and residential/park land use.

Methods for commercial/industrial land use

Estimation of the effects concentration low (ECL)

Two methods are recommended for commercial and industrial land use. These are, in order of preference, the weight-of-evidence method and a method based on the geometric mean of LOEC values. The database used (database on direct soil contact), and the data that were compiled are contained in Table 4.2. However, the NOEC values were not taken into consideration, since for commercial and industrial land use, a median toxic effect is tolerated.

Since the use of an uncertainty factor is prohibited, the ECL corresponds directly to the lower limit of the interval of concentrations producing an effect, that is, the 25th centile in the distribution of data producing an effect. In addition to biological significance, the minimum precautions and requirements for data applying to the weight-of-evidence method of computing the TEC hold as well. As in the previous case, the procedure calls for a minimum of 10 data points collected from at least three studies (19 points from nine studies in the present case). In addition, the requirements related to the number of data points from soil invertebrates and plants are satisfied. Finally, the distribution of the different quantiles (63% of the LOEC and 37% of the EC and a fairly balanced distribution of data) does not indicate any systematic error that would prevent the use of the weight-of-evidence method.

The 25th centile lies between two values of 86 mg/kg. As a result, the ECL for commercial and industrial land use was likewise set at 86 mg/kg.

Comparison with microbial processes

As in the case of agricultural and residential/park land use, the database on microbial processes is not large enough to permit comparison with the ECL.

Derivation of Soil Quality Guideline (commercial/industrial) for soil contact (SQG_{SG})

The SQG_{SG} corresponds to the ECL in the present case, since the lack of data on microbial proceses precludes a comparison of this value. The SQG_{SG} is therefore 86 mg/kg for commercial and industrial land use.

Derivation of soil quality guidelines for soil and food ingestion

The last stage in the process of developing a soil quality guideline for agricultural land use involves computing the SQG_I. This soil quality guideline based on ingestion of soil and food requires that the daily threshold effect dose (DTED) be calculated for the most threatened species. According to the basic requirements for this calculation, three or more studies must be used and at least one of them must deal with the exposure of mammals (orally); a third study must deal with exposure in birds. With regard to the mammalian studies, one can focus on rodents, but it is necessary to also use data from studies on browsing mammals, preferably animals for which there is a high ratio between ingestion rate and body weight.

No study could be found in the scientific literature on the effects of TNT absorption by browsing mammals. The same is true for studies on oral absorption of TNT by birds. The literature search must be continued and *ad-hoc* research should be undertaken if necessary. Meanwhile, a preliminary value has been computed for the SQG_I for TNT from the data on rodents shown in Table 4.1.

Daily threshold effect dose (DTED)

The SQG_I is computed using the LOAEL derived from the toxicological data retained for rodents and presented in Table 4.1. The lowest LOAEL was that obtained by Furedi *et al.* (1984) for rats after a 24-month exposure to a TNT-enriched diet. The main chronic effects observed at 24 months were anaemia with secondary lesions in the spleen, hepatotoxicity and urogenital lesions. To compute the daily threshold effect dose (DTED), a maximum uncertainty factor (equal to 5) was used, in view of the absence of pertinent studies and the lack of representativeness of the organism chosen relative to browsing animals and birds. The DTED was calculated as follows:

DTED = LOAEL/UF

Where,

DTED = daily threshold effect dose (mg/kg bw×d)

LOAEL = lowest-observed-adverse-effect level (mg/kg bw×d)

UF = uncertainty factor; a factor of 5 was applied to take account of the limitations inherent in the available database on TNT toxicity for browsing mammals and birds

Thus,
DTED = 2/5 = 0.4 mg/kg bw×d

Parameters specific to rats

To determine the SQG_I from the DTED obtained for rats, certain parameters specific to this species had to be obtained from the literature.
Thus:

BW = 0.35 kg
Where BW = mean laboratory rat body weight (kg) given by Sample *et al.* (1996)

DMIR = 0.028 (kg/d
Where DMIR = dry matter ingestion rate (kg/d) given by Sample *et al.* (1996)

PSI = 0.083
Where PSI = geometric mean of soil ingestion proportion (dimensionless). A default value of 0.083 is recommended in the protocol (CCME 1996) for domestic species, if no information is available.

Soil ingestion rate

In order to evaluate an animal's exposure through the direct ingestion of soil, the soil ingestion rate (SIR) is calculated as follows:

$$SIR = DMIR \times PSI$$

Therefore,

$$SIR = 0.028 \times 0.083 = 0.002324 \text{ kg/d}$$

Food ingestion rate

As with the SIR, the dried food ingestion rate (FIR) for animals is expressed as a percentage of the DMIR. The FIR is the remaining percentage of the DMIR less the soil ingestion rate (SIR). The FIR therefore equals:

$$FIR = DMIR - SIR$$

Therefore,

$$FIR = 0.028 - 0.002324 = 0.025676 \text{ kg/d}$$

Bioavailability factor

Owing to the lack of precise data on the bioavailability of soil contaminants ingested by livestock and by terrestrial fauna, a value of 1 is assigned to the bioavailability factor (BF).

Bioconcentration factor

The bioconcentration factor (BCF) can be used to extrapolate the contaminant concentration that is transferred from the soil to plants which provide a food source for animals. This factor is based on bioaccumulation studies of plants. The TNT is metabolized by plants grown in TNT solutions (Palazzo and Leggett 1986; Harvey *et al.* 1990) or in soil amended with TNT (Pennington 1988; Cataldo *et al.* 1989; Thompson *et al.* 1998). This has been determined by recovering metabolites such as 2-ADNT and 4-ADNT (monoamino-dinitro-toluenes). Soil that was deliberately contaminated using TNT compounds and a ^{14}C tracer showed an abundance of polar derivatives of TNT, with the majority (>75%) being detected in the roots (Cataldo *et al.* 1989; Harvey *et al.* 1990). A portion of these derivatives were transferred to the upper part of the plants (stems, leaves, seeds). The observed concentrations depend greatly on the type of soil involved (Pennington 1988; Cataldo *et al.* 1989) and the period of time since contamination occurred (Thompson *et al.* 1998). For example, the bioconcentration of TNT and the marked derivatives decreased by a factor of 5 when the soil had been contaminated more than 270 days earlier.

Two studies explain how to compute these bioconcentration factors (Cataldo *et al.* 1989; Thompson *et al.* 1998). However, in these studies the TNT accumulated in the plants was not analysed directly, but was instead estimated from the ^{14}C. Since the measurement of radioactivity encompasses both the bioaccumulated TNT and its degradation products, the result is an overestimation of the substance in the plants.

The highest bioconcentration values were observed for derivatives that had accumulated in the roots of wheat (Cataldo *et al.* 1989), where 10 mg/kg of TNT added to the soil resulted in the accumulation of 217 µg of TNT derivatives per g of tissue (wet matter). Since water represented 90% of the tissues, the concentration of TNT derivatives in wheat was 2170 mg/kg (dry matter).

Thus:

$$\text{BCF} = \frac{\text{plant conc.}}{\text{soil conc.}} = \frac{2170}{10} = 217$$

In another study, the roots of poplar trees accumulated up to 564 mg/kg (dry matter) of TNT derivatives in soil similarly contaminated with 10.3 mg/kg of TNT (Thompson *et al.* 1998; personal communications). In this case:

$$\text{BCF} = \frac{\text{plant conc.}}{\text{soil conc.}} = \frac{564}{10.3} = 55$$

Although overestimated, the BCF value obtained for wheat by Cataldo *et al.* (1990) was used in calculating the guideline.

Calculation of the SQG_I

An animal can be exposed through more than one pathway. Total exposure results from the combined effects of contaminated food, direct soil ingestion, skin contact, contaminated drinking water and inhalation of air and dust. The combined exposure should not exceed the DTED. Assuming that drinking water, skin contact and inhalation represent 25% of total exposure (CCME 1996), 75% of exposure results from soil and food ingestion. In the case of TNT, this hypothesis may be erroneous, since skin absorption of TNT in humans and mammals represents a primary exposure route along with ingestion and inhalation (Yinon 1990; Talmage *et al.* 1999). However, no studies have so far succeeded in quantifying this absorption pathway. In view of the lack of information, the ratio 25% / 75% has been employed in evaluating exposure through direct ingestion.

Exposure through soil and food ingestion can be used to calculate the SQG_I as follows (CCME 1996):

$$SQG_I = \frac{0.75 \times DTED \times BW}{(SIR \times BF) + (FIR \times BCF)}$$

$$SQG_I = \frac{0.75 \times 0.4 \times 0.35}{(0.002324 \times 1) + (0.025676 \times 217)} = 0.0188 \text{ mg/kg of soil}$$

$SQG_I = 0.02$ mg/kg

Environmental Soil Quality Guidelines for 2,4,6-Trinitrotoluene (SQG_E)

Table 4.3 summarizes the steps involved in obtaining the numerical values for the environmental soil quality guidelines for TNT (SQG_E). The lowest value among the SQG_{SG} and SQG_I values was retained to establish the SQG_E for agricultural lands. In the present case, a preliminary value is available for the SQG_I; however, given the big difference between the two values, we cannot assume that the SQG_{SG} for soil biota is a sensitive enough measure to be used to represent the exposure pathways for contaminant ingestion in wildlife and livestock. The SQG_E for agricultural land use is therefore based on the preliminary SQG_I. However, this value is below the detection limit for TNT in soils (Rouisse 1997). In the case of residental/park, commercial and industrial land use, the Protocol stipulates that the SQG_{SG} should serve as the SQG_E.

4.2.3 Limitations of the guidelines derived and future research

In the CCME's process for developing environmental soil quality guidelines, direct contact with contaminated soil is of prime importance. In fact, the principle behind this procedure is to protect the integrity of the soil ecosystem. That is why the toxicological tests used to assess the effect of TNT on organisms cannot come from any medium other than soil. Despite the abundant literature on the toxic effects of TNT, a limited number of studies were retained to create the

present databases. However, among the different publications, the studies dealing with invertebrates and those on phytotoxicity were sufficiently relevant and complementary.

Table 4.3 Computed values that can be used to develop guidelines for TNT (mg/kg of dry soil)

	Land Use		
Guidelines	***Agriculture***	***Residential/Park***	***Commercial /industrial***
Soil contact (TEC/ECL)	86	86	86
Microbial processes	ND	ND	ND
SQG_{SG}	86	86	86
SQG_{I}	0.02* (0.3)	NA	NA
SQG_{E}	**0.02* (0.3)**	**86**	**86**

ND: not available
NA: not applicable
*preliminary values
The values in parentheses indicate the detection levels of the analytical methods, for methods that take this aspect into account

The CCME also emphasizes the importance of obtaining data from *in situ* research. During the literature search, we were unable to locate any studies reporting results obtained directly from the field. A few studies nevertheless reported values from laboratory investigations of soils that had been contaminated in the field (Cataldo *et al.* 1989; Parmelee *et al.* 1993; Phillips *et al.* 1993; Li *et al.* 1997).

The literature search also revealed a lack of quantitative information on the effects of TNT on microbial processes (nitrogen fixation, nitrogen mineralization, decomposition and respiration). According to the CCME Protocol, this information is very important for drawing up guidelines.

In the case of agricultural land use, the final environmental soil quality guideline is based on a preliminary guideline for soil and food ingestion (toxicity data obtained for laboratory animals). Values from studies on birds and browsing and livestock mammals would make it possible to provide a more realistic estimate of the ecological effects of TNT present in soils and of its transfer up the food chain. Similarly, studies providing actual TNT concentrations (versus total derivatives of TNT) bioaccumulated in plants could be used to refine the SQG_I and hence the SQG_E.

4.2.4 Conclusions

The literature search revealed a lack of scientific information on the environmental toxicity of TNT. Most of the publications examined deal with the impact of this explosive from the standpoint of humans. In addition, not only is there little information available but the quality is not uniform. In fact, the application of quality criteria for information, like the CCME criteria for including information in the database, permits the conservation of only a very small number of observations.

These restrictions related to the use of scientific research aid in identifying research directions that should be pursued to derive a final soil quality criterion for TNT with a view to protecting the environment. Four key objectives were identified: support the work done on the effects of TNT on microbial processes (development of methods and obtaining valid results), study the effects of TNT ingested in soil and food by herbivores and birds, define bioconcentration factors for plants, and make *in situ* observations of the toxic effects caused by direct contact.

Despite these gaps, the soil quality guideline for 2,4,6-trinitrotoluene (TNT) was established as SQG_E = 0.02 mg/kg for agricultural land use (preliminary value; it is below the detection level for TNT in soils), and as SQG_E = 86 mg/kg for residential/park and commercial and industrial land use.

Another aim of the present study was to assess the possibility of using the CCME's protocol for developing environmental soil quality guidelines in relation to TNT. In light of the findings for TNT, the most widely used explosive, it appears clear that the information contained in the literature about TNT metabolites and other common explosives such as octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and their metabolites is insufficient to permit the development of such criteria. These information gaps will eventually have to be overcome through *ad-hoc* research.

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4.3 ECOTOXICOLOGICAL RISK ASSESSMENT OF AN EXPLOSIVES-CONTAMINATED SITE

4.3.1 INTRODUCTION

Nitroaromatic compounds such as 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and their associated byproducts can be released into the environment at factory sites, military areas such as firing range and open burning-open detonation (OB/OD) areas, and through field use. The extent of sites contaminated by explosives worldwide represents a significant international problem. Different studies have been conducted on the impact of this type of contamination on the environment. Due to the chemical and toxicological properties of these explosives, a number of laboratory and field studies have been carried out (Talmage *et al.* 1999). The well-known explosive, TNT and some of its degradation products, are toxic and genotoxic at relatively low concentrations to a number of ecological receptors. RDX and HMX are also toxic to some organisms (Talmage *et al.* 1999; Robidoux *et al.* 2000, 2001). Because of their toxicity, these explosives may present a risk to humans and some ecological receptors. To date there is no generic criteria for explosives-contaminated soil which could be used to manage the future use of decommissioned military training sites. However, screening values have been recently calculated (Talmage *et al.* 1999). In addition, some surface water criteria have been published by different governmental organizations such as the *Ministère de l'Environnement et de la Faune du Québec* (MEFQ 1998).

Ecological risk assessment (ERA) can be used to determine if a site contaminated by explosives exceeds an environmentally acceptable level of protection and may represent a significant risk to the exposed ecological receptor. ERA may be also conducted to protect biological diversity, to determine the maximum level of contamination that permits an acceptable protection level and to compare treatment technologies or scenarios. This chapter summarizes an approach used to conduct ecological risk assessments of explosives-contaminated sites. The approach presented herein was used to evaluate an explosives production facility having TNT as the monotypic contaminant.

4.3.2 PROBLEM FORMULATION

The problem formulation provides the conceptual framework for risk assessment (US EPA 1992) and consists of the description of relevant features of the environment, the sources of contamination, and the identification of the ecological receptors at the site.

Site description

The factory site was heavily contaminated with TNT and its by-products during the second World War. This site was decommissioned in the 1970's. Vegetation on the site included

graminaceae and trees. Soil invertebrates such as earthworms were observed around the contaminated area. Mammals (*e.g.* deer and fox) and birds (*e.g.* passeriformeae) were also present on the site.

Nature and sources of stressor

Preliminary studies have shown that soils and groundwater remain contaminated by TNT. In addition, substances (*e.g.*, dinitrotoluenes) associated with TNT manufacture, as well as TNT degradation products were also detected. Heavy metals concentrations were shown to be relatively low (maximum of 2 µg Cd L^{-1}, 28 µg Cu L^{-1}, 170 µg Pb L^{-1}, 80 µg Zn L^{-1}) in the groundwater of some areas of the sites. The presence of other contaminants such as hydrocarbons was not suspected to be a significant source of contamination considering the past activities associated to the site. TNT (CAS # 118-96-7) is the most widely used military explosive because of its low melting point (80.7 °C, Yinon 1990), its stability, low sensitivity to impact friction and high temperature and its relatively safe method of manufacture.

TNT contamination in soil was heterogeneous at the study site. The contaminated areas were relatively small (ranged from 10 to 100 m^2 dispersed over approximately 5000 m^2), and corresponded to a location where TNT was synthesized (from mononitrotoluene and dinitrotoluene) and later purified (*i.e.*, removal of undesired isomers and residual dinitrated toluenes). Some areas were highly contaminated (up to 259 000 mg TNT per kg of soil). TNT contaminated-soils were generally localized with surrounding areas having low to non-detectable levels of explosive contaminants.

Physical / chemical properties and environmental behavior of stressor

Movement of TNT in the environment

Volatilization of TNT from surface and groundwater was considered to be negligible since its vapor pressure (1.99×10^{-4} mm at 20 °C; HSDB 1995) and a Henry's Law constant (4.57×10^{-7} atm-m^3 $mols^{-1}$; HSDB 1995) are low. The moderate water solubility of TNT (130 mg L^{-1} at 20 °C; HSDB 1995) favors the movement of the contaminant. Sorption to a soil matrix is low as indicated by its K_d (ranged from 4-53) and K_{oc} (1600, Spanggord *et al.* 1980). Because of its properties, TNT would not be expected to adsorb strongly to the soil.

Transformation of TNT

Environmental abiotic transformation processess of TNT in water ($t_{1/2}$ = 0.5-22 h according laboratory studies; Talmage *et al.* 1999) include photolysis. Formation of nitro compounds gives a pink color to water. TNT in water ($t_{1/2}$ < 6 months, ATSDR 1995), soil ($t_{1/2}$ < 60 days; Cataldo *et al.* 1989), sediment and sludge, can be transformed by bacterial and fungal species under both aerobic and anaerobic conditions (Spanggord *et al.* 1980). The detectable degradation products include: 2-hydroxy-aminodinitrotoluene (2-OH-ADNT), 4-hydroxy-aminodinitrotoluene (4-OH-ADNT), 2-aminodinitrotoluene (2-ADNT), 4-aminodinitrotoluene (4-ADNT), 2,4-

diaminonitrotoluene (2,4-DANT), 2,6-diaminonitrotoluene (2,6-DANT), 2,4,6-triaminotoluene (TAT) and various tetranitro-azotoluene compounds (Sunahara *et al.* 1998 ; Talmage *et al.* 1999). Mineralization of TNT is considered low (Hawari *et al.* 1998; 1999).

Bioconcentration and bioaccumulation of TNT

TNT (absorbed by inhalation, ingestion, or by dermal contact) is metabolized by different mammalian species (ATSDR 1995). Ingested TNT can be excreted within 24 h in urine (50-70 %) and feces. Distribution in tissues is less than 1 %, indicating a low potential for bioaccumulation. Metabolites recovered in urine include hydroxy-aminodinitrotoluene, diaminonitrotoluene, dinitroaminotoluene, as well as glucuronide-conjugated metabolites. Biomonitoring studies (see review by Talmage *et al.* 1999) did not reveal the presence of TNT in tissues (<0.2 mg kg^{-1}) of terrestrial wildlife (deer and small mammals).

TNT is also partially degraded by plants and earthworms (*Eisenia andrei*). Biotransformation products include 2-ADNT and 4-ADNT (Cataldo *et al.* 1989, Palazzo and Leggett 1986, Renoux *et al.* 2000). Calculated and measured log K_{ow} (1.6 – 2.7) indicate a low potential for bioconcentration in aquatic organisms (ATSDR 1995). The bioconcentration factor (BCF) calculated for fish using a calculated K_{ow} (2.03) was low (BCF = 20.5, Liu *et al.* 1983). The BCF values measured by Liu *et al.* (1983) in non-steady state conditions ranged from 202 to 453 for aquatic organisms (green alga, water flea, oligochaete and bluegill).

Ecological effects

TNT is toxic ($EC_{50} \geq 0.95$ mg L^{-1}) to aquatic organisms including bacteria (*Vibrio fisheri*; Microtox), freshwater algae (*Selenastrum capricornutum*), invertebrates (*Ceriodaphnia dubia*) and fathead minnow (*Pimephales promelas*; Smock *et al.* 1976, Dryzyga *et al.* 1995, Sunahara *et al.* 1998, 1999).

TNT-contaminated soils, composted explosives-contaminated soil and soil elutriates have also been found to be toxic ($EC_{50} \geq 1.0$ mg kg^{-1}) for organisms including bacteria (Microtox; Simini *et al.* 1995; Sunahara *et al.* 1999), subsurface soil microbial communities (Fuller and Manning 1998; Gong *et al.* 1999), plants (*Lactuca sativa*), earthworms (*Eisenia* sp.; Phillips *et al.* 1993, Simini *et al.* 1995, Peterson 1996, Jarvis *et al.* 1998, Robidoux *et al.* 1999, Robidoux *et al.* 2000, Dodard *et al.* 1999), nematodes and microarthropods (Parmelee *et al.* 1993).

Ecosystems potentially at risk

Direct contact organisms such as invertebrates, plants and micro-organisms may be affected by the presence of TNT or its (bio)transformation products. Mammals (deer and fox) and birds (passeriformeae) can also be exposed to the toxicants on the site by dermal contact and ingestion. Aquatic organisms may be potentially exposed to TNT or its products via transport by groundwater and surface runoff from contaminated soil to a nearby river.

Conceptual model
Terrestrial organisms may be exposed to TNT and its transformation products from surface soil areas. These contaminants may cause direct effects on microbial processes, terrestrial plants, soil invertebrates, mammals and birds. TNT may be transformed and/or transferred slowly to the groundwater, and then potentially to the surface water. Thus, aquatic organisms (microorganisms, microphytes, macrophytes, invertebrates and fish) may be directly affected by the presence of these contaminants. A decrease in the population of these organisms may affect other animals such as small mammals and birds. These contaminants may enter into the food chain and affect other sensitive organisms (Suter II, 1996a).

Endpoints
To evaluate if the selected receptors present on the site may be at risk, this study considered the direct effects of TNT on terrestrial and aquatic organisms. The ability of the soil and the groundwater to sustain life was assessed by considering the potential hazardous effects of the contaminated matrix on soil and aquatic organisms. The assessment endpoints (CCME 1997) for terrestrial and aquatic risks included: effects on microbial processes; effects on survival and reproduction of terrestrial plants, soil invertebrates, mammals (shrew, mouse, fox and deer) as well as birds. For aquatic organisms, the survival and reproduction microorganisms, microphytes, macrophytes, invertebrates and fish were considered. Measurement endpoints included: analysis of nitroaromatics in the surface soil (0 - 15 cm); single chemical toxicity (acute and chronic) data for soil microorganisms, terrestrial plants and invertebrates; and aquatic microorganisms, microphytes, macrophytes, invertebrates and fish. In addition, sub-lethal toxicity (earthworm reproduction) was measured on soil samples, and mesocosms were placed on site to evaluate lethal and sub-lethal (using biomarkers) effects under field conditions.

4.3.3 ASSESSMENT METHOD FOR A TNT-CONTAMINATED SITE

Methodology for Exposure assessment

Contaminated areas selected for the assessment (surface areas ranged from approximately 25 to 100 m^2) were defined according to chemical screening characterization data, and were split into two zones: one being highly contaminated by TNT (without vegetation) and another which was immediately peripheral (with low vegetation). A reference area (non-contaminated with healthy vegetation) close to the contaminated areas (approx. 30 to 150 m) was also used to define the background toxicity (laboratory and field studies) and contaminant concentrations.

Exposure values
Exposure to TNT by direct contact and ingestion is variable in space and time, and depends on the field conditions. Soil organisms (microorganisms, plants and invertebrates) are relatively immobile and can therefore be exposed to high concentrations of TNT. Considering the

distribution and the variability of the data, the 95 % upper confidence limit (UCL) of the mean TNT concentration was considered as an appropriate conservative estimate of exposure for soil organisms and other receptors used in the screening assessment. In addition, TNT-contaminated groundwater represents the worst-case exposure scenario for aquatic organisms, and the corresponding exposure values were based on measured groundwater concentrations. Thus, the UCL values were selected as the exposure values for contaminated soils and groundwater. For cases in which an adequate number of replicates was lacking and the UCL could not be calculated, then the maximal concentration was used. The UCL values were also used for a transport and transformation simulation using the *CalTOX* model (McKone 1993, McKone *et al.* 1997) in order to estimate TNT concentration in surface water. The results of this simulation were also considered in the risk assessment for aquatic organisms.

Soil and water samples

Surface soil samples were taken from each selected area using a stainless steel tool, transported and homogenized by hand at the laboratory. Groundwater samples were pumped from an observation well. Explosives were extracted from the soil using the acetonitrile sonication method (US EPA 1997). Concentrations of TNT and some of their degradation products in water or acetonitrile soil-extracts were determined by HPLC.

Methodology for Effects assessment

Single chemical toxicity data

Literature concerning the toxicity of nitroaromatic munition compounds was reviewed by Talmage *et al.* (1999). However, certain data are missing (*e.g.* DNT and metabolites of TNT) or insufficient to calculate water or soil reference values (criteria or screening benchmarks) for nitroaromatic munition compounds.

Reference values

Since standard reference values for TNT and associated substances were not available, the screening benchmarks proposed by Talmage *et al.* (1999) for aquatic and terrestrial biota were used. In some cases (*e.g.* earthworm), benchmarks were re-calculated and updated in view of new toxicological data. The lowest toxicological values (chronic) were then used as reference values.

Contaminated sites are often managed by using generic criteria or alternatively, by risk-based procedures (CCME 1996, Beaulieu 1998, CEAEQ 1998). For example, the maximal acceptable limit in Quebec (industrial site, level C) for TNT is 1.7 mg kg^{-1} (Beaulieu 1998). However, generic environmental criteria for explosive are currently in development, and some data are missing for many of these compounds. In Quebec, groundwater and surface water contamination are typically managed on a criteria-based approach (MEFQ 1998, Beaulieu 1998). Surface water criteria for TNT, 2,4-DNT and 2,6-DNT are 5.5, 910 and 930 $\mu g\ L^{-1}$, respectively.

Laboratory toxicity assessment

The lethal and sub-lethal effects of explosive contaminated-soils samples from different areas of the site were assessed using the earthworm (*Eisenia andrei*) reproduction test (ISO 1996).

Survival and growth of adult earthworms were determined after 28-d exposure to soil samples (contaminated and reference areas). Parameters associated with reproduction, including cocoon production and hatching, and juveniles' survival and growth were measured after 56 days of exposure.

Field study: Biological response and body burdens in earthworms
Earthworms (indigenous and *Eisenia andrei*) were exposed in areas of the site using field soil mesocosms (9.4 L 100-mesh Nylon bags containing soil and soil organisms exposed to field conditions). Each block of soil was carefully removed from the ground using a square stainless steel tool (30 cm x 30 cm x 15 cm depth) and was transferred as a block into the Nylon bag. Laboratory earthworms (*E. andrei*; n = 10) and ten indigenous earthworms (single species obtained close from the site and pre-acclimated for 24 h earlier in OECD artificial soil; n = 10) were then placed in each Nylon bag containing the soil block. The mesocosm (n = 3 to 6 units per area) was closed and left in place for ten days; after which the surviving earthworms were counted. Body burdens of TNT and degradation products were estimated in the surviving worms using the SW486 HPLC Method 8330 (US EPA 1997).

Field study: Biomarkers
At least three surviving earthworms were taken after exposure in mesocosms for the biomarker measurements. The determination of the Neutral Red Retention Time (NRRT) was done immediately after the exposure to the toxicant using a histochemical staining technique (Svendsen and Weeks 1996, 1997a, 1997b). Another biomarker, the Total Immune Activity (TIA) was used as described by others (Goven *et al.* 1993, Bunn *et al.* 1996).

Methodology for Risk estimation
Concentrations of contaminants in soil and groundwater were screened against toxicity data by using the quotient method (Suter II 1996b, CCME 1997). A risk index (RI) was estimated by comparing the exposure value (EV) to the reference value (RV), for each selected area and endpoint. This method permits the identification of the location and contaminants of potential ecological concern, and screening out of those chemicals that do not constitute a potential hazard to the ecological receptors. Thus, estimated risk ($RI = EV\ RV^{-1}$) values lower than 1 would indicate the absence of significant effects on the considered receptors.

4.3.4 EXPOSURE ASSESSMENT

Contaminated zones
The areas selected for the assessment were identified as sites A, B and C (TNT-contaminated areas having no vegetation and corresponding to the production sites). Peripheral contaminated areas having low levels of vegetation were also studied (identified as A', B', and C' in the text below). A non-contaminated reference zone (R) at near proximity of the contaminated areas (approx. 30 m from area A) was also used.

Direct contact

Soil

Preliminary chemical analysis indicated that the soil contained TNT, TNB, DNB, 2,4-DNT and 2,6-DNT. TNT degradation products such as 2A-DNT and 4A-DNT were also detected. The concentrations of TNT (mg kg^{-1} dry weight) in the soil samples taken from the contaminated area having no observable vegetation reached 17450 (n = 10), 24883 (n = 1) and 258 650 (n = 3) in areas A, B and C (highest contaminated area), respectively. For the peripheral contaminated areas, their concentrations (mg kg^{-1} dry weight) only reached 4425 (n = 10), 397 (n = 6) and 3.8 (n = 3) for areas A', B' and C', respectively. The exposure values (95 % UCL) for areas A, A', B, B', C and C' were 10642, 2638, 24 883, 310, 3.8 and 255 604 mg kg^{-1} of TNT, respectively. The concentrations of TNT by-products (mg kg^{-1}) in soil reached 10.6 (area B; n = 1), 6.8 (area B'; n = 6), 80 (area A; n = 10), and 210 (area A; n = 10) for TNB, DNB, 2,4-DNT and 2,6-DNT, respectively. TNT degradation products in soil reached 23.3 (B'; n = 6),and 40.5 (B; n = 1), for 2-ADNT and 4-ADNT, respectively.

Groundwater

Initial studies indicated that the concentrations of TNT in groundwater samples reached 75.5 mg L^{-1} in the highest contaminated area (A), in contrast to the reference area where TNT was not detected. The maximum concentrations (mg L^{-1}) measured in the other areas, *i.e.* A', B, B', C and C' were 0.10 (n = 1), 0.73 (n = 2), 24.8 (n = 1), 0.31 (n = 1) and 0.61 (n = 1) of TNT, respectively. The concentrations of explosive by-products (mg L^{-1}) in groundwater samples taken from area A reached 104.2 for 2,4-DNT (n=2) and 123.6 for 2,6-DNT (n=2). For area B, TNT degradation product concentrations (mg L^{-1}) in groundwater reached 1.0 (n=2), 0.87 (n=1) for 2-ADNT and 4-ADNT, respectively.

Concentrations estimated using CalTOX

A transport and transformation model, *CalTOX* (McKone 1993, McKone *et al.* 1997) was used to estimate the TNT concentrations in the different environmental compartments (soil, groundwater, plants, and surface water, CalTox values, available upon request). Results of the *CalTOX* simulation show that TNT concentrations in soil and groundwater in the contaminated area would significantly decrease over a one year period (Table 4.3).

Ingestion for wildlife

Mammals

Using a worst-case scenario (*i.e.* assuming that the animals inhabit the site all the time and consume food only in this area), the exposure to explosives in mammals by ingestion was estimated using the soil concentrations, the available bioconcentration values for plants (maximal concentrations measured in laboratory) and the earthworm tissue residues (based on the biological survey data). For plants, the bioconcentration factors (BCF) were not available.

However, Palazzo and Leggett (1986) reported 714, 614 and 2180 mg kg^{-1} tissue dry weight for TNT, 2-ADNT and 4-ADNT, respectively, in plant roots exposed to 20 mg TNT L^{-1} hydroponic solutions. These values were considered as a maximum concentration could be accumulated by plants.

It was assumed that vegetation constitutes the total diet of shrew and deer, and was potentially a large part of the diet of mouse ($\leq$ 67 %), whereas invertebrates constitute a large part of the diet of the shrew ($\geq$ 71 %) and the American robin (93 % according to Sample and Suter II 1994). The fox also consumes small mammals ($\cong$ 69 %) and birds ($\cong$ 12 %). Because areas A, B and C did not have observable vegetation, area A' was chosen as the worst case scenario for exposure. Earthworms were not found in the soil of this area, and probably may not contribute to the total exposure by ingestion by mammalian wildlife. Based on soil concentrations, the calculated maximum exposure concentrations (by ingestion) for TNT for representative mammals and birds ranged from 115 to 767 mg kg^{-1} (Table 4.4). Due to the lack of data, the exposure to other nitroaromatics was not determined.

Table 4.3 TNT concentrations (measured and estimated[a] using the CalTOX transport and transformation model) in different environmental compartments of contaminated zones.

Environmental compartment	TNT/ area					
	A	A'	B	B'	C	C'
Measured concentrations (t = 0)[b]						
Ground-surface soil (mg kg^{-1}):	10642	2638	24 883	310.3	255 604	3.8
Groundwater (mg L^{-1}):	75.5	0.10	24.8	0.74	0.61	0.31
Estimated concentrations (t = 365 d)						
Air (mg m^3):	0.03	0.03	0.03	0.0007	0.028	0.00005
Plants (mg kg^{-1}):	13	13	12	2.9	12	0.02
Ground-surface soil (mg kg^{-1}):	43	43	43	10	40	0.06
Root-zone soil (mg kg^{-1}):	240	240	250	58	240	0.42
Vadose-zone (mg kg^{-1}):	0.28	0.28	0.53	0.18	0.28	0.0007
Groundwater (mg L^{-1}):	0.0001	0.0001	0.0001	0.00003	0.00005	0.0000001
Surface water (mg L^{-1}):	0.0051	0.0051	0.0049	0.001	0.005	0.00008
Sediments (mg kg^{-1}):	0.078	0.078	0.075	0.017	0.072	0.0001

[a] Assumptions used for the simulations are available at BRI upon request;

[b] Upper confidence limit 95 % of measured concentration (or maximum value was used if n≤2).

Table 4.4 Maximum exposure concentrations for wildlife (all areas).

Wildlife species	Diet composition (%)[b]	Maximum exposure concentrations by ingestion (mg kg^{-1})						
		TNT[a]	TNB[a]	DNB[a]	2,4-ADNT[a]	2,6-ADNT[a]	2-ADNT[a]	4-ADNT[a]
Short-tailed shrew (*Blarina brevicauda*)	Vegetation (5.4%)	38.6	ND	ND	ND	ND	33.2	117.7
	Invertebrates (≥71%)	14.0[a]	ND	ND	ND	ND	123.2[a]	153.1[a]
	Soil (13%)	342	0.64	0.86	6.4	9.2	3.0	ND
	Total	395	0.64	0.86	6.4	9.2	159	271
White-footed mouse (*Peromyscus leucopus*)	Vegetation (≤67%)	478.4	ND	ND	ND	ND	411.4	1461
	Arthropods (≥30%)	NA	NA	NA	NA	NA	NA	NA
	Soil (<2%)	52.8	0.098	0.13	0.99	1.4	0.46	ND
	Total	531	0.098	0.13	0.99	1.4	411.9	1461
Red fox (Vulpes vulpes)	Vegetation (≤10%)	41.4	NA	NA	NA	NA	61	218.0
	Small mammals (69%)	NA	NA	NA	NA	NA	NA	NA
	Birds (12%)	NA	NA	NA	NA	NA	NA	NA
	Soil (2.8%)	73.9	0.14	0.18	1.4	2.0	0.65	ND
	Total	115	0.1	0.18	1.4	2.0	61.7	218
Whitetail deer (Odocoileus virginiaus)	Vegetation (100%)	714.0	NA	NA	NA	NA	614	2180
	Soil (<2%)	52.8	0.098	0.13	0.99	1.4	0.46	ND
	Total	767	0.098	0.13	0.99	1.4	614	2180
American robin (*Turdus migratorius*)	Invertebrates (93%)	18.3[a]	ND	ND	ND	ND	161.4[a]	200.5[a]
	Fruit (7%)	NA	NA	NA	NA	NA	NA	NA
	Soil (2.1%)	55.4	0.103	0.134	1.04	1.48	0.49	NA
	Total	73.7	0.10	0.14	1.04	1.48	161.8	200.5

Note, Exposure values are based on soil concentrations, maximum BCF or concentrations accumulated in plants (Talmage *et al.* 1999) and invertebrate (this study) tissue residues; NA: Not determined (insufficient data); ND: Not detected..

[a] Based on maximum earthworm tissue concentrations measured in the biological survey (19.7, 173.5 and 215.6 mg per kg of dry tissue for, TNT, 2-ADNT, and 4-ADNT, respectively);

[b] Taken from Sample and Suter II (1994).

Birds

Exposure to TNT (and their degradation products) in birds by ingestion were estimated using maximal soil concentrations, and the earthworm tissue residues (see biological survey data). Because of lack of data, TNT exposure concentration by eating contaminated fruits (7 % of the American robin diet) were not estimated, and thus not considered. Calculated maximum TNT exposure concentrations for birds was 73.7 mg kg^{-1} of soil (Table 4.4).

4.3.5 EFFECTS ASSESSMENT

Effects of TNT in laboratory toxicity assessment

The NOAEL (no observable adverse effect level) in mammals was estimated to be 1.6 mg $kg^{-1}d^{-1}$, based on the sub-chronic LOAEL (160 mg kg^{-1} d^{-1}; testicular atrophy for rat) and an uncertainty factor of 100 (Talmage *et al.* 1999). Study of soils tested with plants (*Phaseolus vulgari*, *Triticum aestivum*) suggest a LOEC of 30 mg kg^{-1} (Cateldo *et al.* 1989). Confidence in this value is considered as moderate and additional studies are needed (Talmage *et al.* 1999). Effects data were not available for representative avian species.

Studies with the earthworms (Robidoux *et al.* 1999, 2000) showed a LOEC value of 110 mg kg^{-1}. Soil microbial processes (including nitrification, nitrogen fixation and dehydrogenase activities) were found to be decreased at 1 mg TNT kg^{-1} of soil (acetonitrile extractable concentration; Gong *et al.* 1999). Figure 4.1 shows the sensitivity distribution of different sub-lethal effects (LOECs taken from the literature) for soil organisms. Data available were linearized via the cumulative frequency distribution approach and are presented as a function of their rank of sensitivity (%; rank of the data number^{-1} of data x 100). This method permits one to examine the range of data distribution and the global proportion (percentile) of organisms that may be affected when the toxicity of a chemical is assumed to be a random variable.

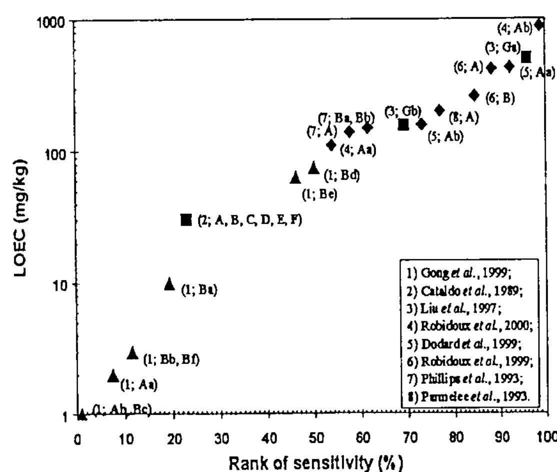

Figure 4.1.
Sensitivity distribution of sub-lethal effects for terrestrial organisms to TNT: Microorganisms (▲); plants (■); invertebrates (◆). Data correspond to different experiments or endpoints. Numbers and letters in brackets indicate: reference designated in the inset (number), experiment number (capital letter) and endpoint number (small letter). For the same citation, common capitalized letters (A, B, ...) correspond to same experimental conditions. Common small letters (a, b, ...) correspond to same endpoints. Toxicological data taken from the literature have been linearized using a cumulative frequency distribution approach, for which LOECs are presented as a function of their rank of sensitivity (%; rank of the data/ number of data x 100).

Benchmarks are usually based on this approach and are derived by rank-ordering the LOECs and then interpolating a value that approximated the 10th percentile (Efroymson *et al.* 1997a, 1997b). Thus, interpolation of the intercept of the 10th percentile of the chronic sensitivity distribution shows that 2 mg TNT kg^{-1} soil would preserve more than 90 % of the endpoints/ organisms considered (micro-organisms, plants and invertebrates). These data (Figure 4.1) indicate that microorganisms were affected from 1 to 75 mg TNT kg^{-1} in soil, whereas plants were sensitive at concentrations from 30 to 500 mg kg^{-1}, and invertebrates from 110 and 880 mg TNT kg^{-1} in soil. Clearly, more toxicological work should be carried out using plants.

Toxicity studies using aquatic organisms demonstrated acute and chronic effects of TNT for microorganisms ($EC_{50} \geq 0.95$ mg L^{-1}; LOEC: 0.03 mg L^{-1}), plants ($EC_{50} \geq 1.1$ mg L^{-1}; LOEC: 1.0 mg L^{-1}), invertebrates (LC_{50} >4.4 mg L^{-1}; LOEC: 1.03 mg L^{-1}) and fish (LC_{50} ranged from 0.8 to 3.7 mg L^{-1}; LOEC: 0.04 mg L^{-1}). Figure 4.2 shows the sensitivity distribution of sub-lethal effects (LOEC taken from the literature) for aquatic organisms. These data indicate that aquatic microorganisms were affected from 0.03 to 11 mg TNT L^{-1}, whereas microphytes and macrophytes were sensitive from 1 and 25 mg L^{-1}, invertebrates from 1 to 5 mg L^{-1}, and fish from 0.04 to 1.4 mg L^{-1} of TNT.

Reference values

The TNT screening benchmarks used for soil organisms for plants, soil invertebrates, and soil microbial process were 30 (Cataldo *et al.* 1989, Talmage *et al.* 1999), 110 (LOEC value from Robidoux *et al.* 2000) and 1 mg kg^{-1} (LOEC value from Gong *et al.* 2000), respectively. The screening benchmarks used for 2-ADNT (plants and soil microbial process) were 80 mg kg^{-1} (Pennington 1988, Talmage *et al.* 1999). Because of insufficient data, benchmarks were not determined for other nitroaromatics (TNB, DNB, 2,4-DNT, 2,6-DNT and 4-ADNT). The screening benchmarks used for mammals and birds (TNT, TNB and DNB; Table 4.5) and aquatic organisms (TNT, TNB, 2-ADNT; Table 4.6) were taken from Talmage *et al.* (1999). Because toxicity data were not available for birds, reference values were extrapolated from mammalian toxicity data.

Earthworm toxicity

The effect of soil samples from different areas (n =18) of the site were assessed. In general, soil samples from areas A and A' were lethal to earthworms. In these areas, eight out of ten samples gave 100 % mortality. TNT concentrations in these samples ranged from 1 146 to 17 063 mg kg^{-1}. A sample containing 116 mg kg^{-1} of TNT was not significantly lethal to earthworms (in agreement with Robidoux *et al.* 1999). However, one sample with low TNT concentration (25 mg kg^{-1}) in area A' was significantly lethal (33 % mortality). One out of the three samples from areas B' was lethal to earthworms (367 mg TNT kg^{-1}). Others were not lethal ($\leq$ 130 mg TNT kg^{-1}). Survival rates were 100 % for reference samples (n =5).

Table 4.5 Nitroaromatic compounds screening benchmarks for selected wildlife species.

Wildlife species		Screening benchmark		
		TNT	TNB	DNB
Short-tailed shrew (*Blarina brevicauda*)	Estimated wildlife NOAEL (mg $kg^{-1}d^{-1}$)	3.4	5.8	0.25
	Diet (mg kg^{-1} food)	5.6	9.7	0.41
	Water (mg L^{-1})[a]	15	26	1.13
White-footed mouse (*Peromyscus leucopus*)	Estimated wildlife NOAEL (mg/kg/d)	3.0	5.3	0.23
	Diet (mg/kg food)	20	34	1.46
	Water (mg L^{-1})[a]	10	18	0.75
Red fox (Vulpes vulpes)	Estimated wildlife NOAEL (mg $kg^{-1}d^{-1}$)	0.8	1.4	0.06
	Diet (mg kg^{-1} food)	8.1	14	0.60
	Water (mg L^{-1})[a]	9.6	17	0.71
Whitetail deer (Odocoileus virginianus)	Estimated wildlife NOAEL (mg $kg^{-1\ d-1}$)	0.4	0.7	0.03
	Diet (mg kg^{-1} food)	14	27	1.02
	Water (mg L^{-1})[a]	6.6	11	0.49
American robin (*Turdus migratorius*)	Estimated wildlife NOAEL (mg kg^{-1} d^{-1})	0.6[b]	ND	ND
	Diet (mg kg^{-1} food)	4.9[c]	ND	ND
	Water (mg L^{-1})[a]	4.4[d]	ND	ND

Note, Benchmarks are based on NOAEL values (Talmage *et al.* 1999); ND: Not determined (insufficient data).
[a]Water concentration that incorporates dietary exposure following both water and food consumption;
[b]Extrapolated from mammal toxicity data: Wildlife NOAEL = test NOAEL x (test organism *bw* / wildlife body weight) x uncertainty factor); *bw* = body weight (rat *bw*= 0.35 kg; mouse *bw*=0.03 kg);
[c]Concentration = Wildlife NOAEL / 0.648 $(bw)^{0.651}$ (Sample *et al.* 1996);
[d]Concentration = Wildlife NOAEL x Wildlife *bw* / 0.059 $(bw)^{0.67}$ (Sample *et al.* 1996).

Table 4.6 Nitroaromatic compounds screening benchmarks for aquatic organisms.

Criterion	Screening benchmark		
	TNT	TNB	2-ADNT
Acute water quality criterion (mg L^{-1})	0.57	ND	ND
Chronic water quality criterion (mg L^{-1})	0.09	ND	ND
Secondary acute value (mg L^{-1})	ND	0.06	0.35
Secondary chronic value (mg L^{-1})	ND	0.011	0.02
Lowest chronic value, fish (mg L^{-1})	0.04	0.12	ND
Lowest chronic value, daphnids (mg L^{-1})	1.03	0.75	ND
Lowest chronic value, plants (mg L^{-1})	1.0	0.10	>50
Sediment quality benchmark (mg kg^{-1})[a]	0.09[b]	0.002[c]	ND

Note, Water quality criterion are based on US EPA guidelines (Talmage *et al.* 1999); Secondary chronic values are based on US EPA guidelines (Talmage *et al.* 1999); Benchmarks were not determined for 2,4-DNT, 2,6-DNT and 4-ADNT (insufficient data); ND: Not determined (insufficient data).

[a]1% of organic carbon content in the sediment is assumed;

[b]9.2 mg TNT kg^{-1} of organic carbon in the sediment;

[c]0.24 mg TNB kg^{-1} of organic carbon in the sediment.

Soil samples contaminated by explosives from areas A, A' and B' caused a number of reproductive effects compared to laboratory controls (OECD soils) as evidenced by decreased number of total and hatched cocoons, the number and biomass of juveniles (in agreement with Robidoux *et al.* 1999), except for the sample having 25 mg kg^{-1} which showed only a decrease in the total number of cocoons. However, it was noted that the reference soil samples also had effects on reproduction compared to the laboratory control (artificial soil) suggesting the presence of other contaminants, or perhaps an effect of the soil matrix. Nevertheless, the explosive-contaminated samples showed higher reproductive effects compared to reference soils, except for two samples having low concentrations of TNT (25 mg kg^{-1} and 116 mg kg^{-1} respectively). Growth of surviving earthworms was generally not affected in the TNT-contaminated samples but it was increased for reference areas compared to controls.

Effects on earthworm reproduction were not correlated at low concentrations ($\leq$ 130 mg kg^{-1}) of TNT or other related substances. However, a correlation was observed (R^2=0.77, n = 4) from 116 to 367 mg TNT kg^{-1}. Soil samples having TNT concentrations from 370 to 1110 mg kg^{-1} were not available for toxicity assessment. Higher TNT concentrations ($\geq$ 1146 mg kg^{-1}) were lethal to earthworms. Soil samples from areas B, C and C' were not tested. For the earthworm toxicity study, 2-chloroacetamide, was chosen as the reference toxicant.

Biological survey data and biomarkers using the earthworm

The survival of earthworms (*E. andrei* and indigenous species) exposed to TNT-contaminated soils in mesocosms tended to decrease at increasing concentrations of TNT in soil. No survivors were found at >9033 mg TNT per kg of soil, but most were found at concentrations <1200 mg kg^{-1}. Composting worms *E. andrei* added to the mesocosms, tolerated concentrations as high as 4050 mg TNT kg^{-1}, whereas indigenous earthworms were only observed at $\leq$ 1146 mg kg^{-1}. The mortality was not significantly correlated with the soil concentrations of other nitroaromatic compounds. In addition, dried tissue concentrations of TNT and its degradation products in depurgated earthworms increased with soil levels of TNT (<1200 mg kg^{-1} dried soil; data not shown), 2-ADNT (<150 mg kg^{-1} dried soil; data not shown) and 4-ADNT (<220 mg kg^{-1} dried soil; data not shown). However, since the time of depurgation was relatively short (8 h), little interference with contaminated soil may be suspected. A maximum of 19.7 µg TNT g^{-1} dried tissue (in *E. andrei* at 116 mg kg^{-1} TNT in soil) was found in earthworm tissues; whereas a maximum of 173.5 µg 2-ADNT g^{-1} and 215.6 µg 4-ADNT g^{-1} dried tissue (in indigenous earthworms at 1146 mg kg^{-1} TNT in soil) were detected. However, TNT was not detected in earthworms exposed to soils having high concentrations of 2-ADNT and 4-ADNT, suggesting that the parent compound may have been partially degraded *in vivo*. Interestingly, the variability of the survival data was high for the reference mesocosms, ranging from 0 (*n*=3) to 90 % (*n*=2) for *E. andrei*, and 30 % to 70 % for the indigenous earthworms species suggesting the presence of other contaminants or an unfavorable effect of the experimental conditions. Effects on reproduction have also been observed in the reference areas (see *Earthworm toxicity*). Mesocosms experiments were not carried out in areas B, C and C'.

The TIA biomarker showed no significant responses in surviving earthworms exposed to the explosives-contaminated soils under field conditions. The NRRT was significantly lower for surviving earthworms (*E. andrei* and indigenous species) exposed to explosives in areas A, A' and B' (25 to 367 mg kg^{-1} of TNT) in mesocosms compared to reference soils, except for one mesocosm placed in area B' containing 116 mg kg^{-1} of TNT. In the NNRT study, copper (as $CuSO_4$) was chosen as the reference toxicant.

4.3.6 RISK CHARACTERIZATION

The potential risk to terrestrial and aquatic receptors was analyzed using a single chemical risk estimation approach (Suter II 1996b). Toxicity assessment, biological survey on earthworms and biomarkers were used to support evidence of ecological risk.

Risk estimation of single chemicals

Contaminant concentration were screened against benchmarks. Exposure values used for soil organisms were generally higher than corresponding benchmarks. This would indicate that certain ecological receptors may be at risk. For plants, the RI ranged from 0.13 (area C') to 8520 (area C). For invertebrates, the RI ranged from 0.03 (area C') to 2324 (area C). The exposure concentrations were 3.8 (area C') to 255 604 (area C) times higher than the benchmarks for microbial processes. Considering plants and microbial processes, an RI of < 0.5 was estimated for 2-ADNT separately. Because the lack of data, the risk for soil organisms was not be estimated with other explosive-associated compounds. Based on laboratory data (Figure 4.1), all soil organisms (100 %) would be affected by the concentrations of TNT in areas A, A', B and C, whereas ≥ 75 % of the organisms would be affected in area B' and ≤ 10 % in area C'.

Since exposure values for wildlife (Table 4.4) were also higher than the corresponding benchmarks (Table 4.5), RI values > 1 were found for selected species (Short-tailed shrew, White-footed mouse, Red fox, Whitetail deer, American robin). The exposure concentrations of TNT for the overall area were 14.2 (Red fox) to 70.5 (Short-tailed shrew) times higher than the toxicity benchmarks. The RI associated to DNB ranged from 0.1 (White-footed mouse) to 2.1 (Short-tailed shrew); whereas the RI values associated to TNB were ≤ 0.1. Because of the lack of data, risk for wildlife was not estimated for the other explosive-associated compounds.

Groundwater concentrations estimated using *CalTOX* (one-year period) were much lower than the measured or actual groundwater concentrations. However, considering that the concentrations in soil and groundwater were relatively stable over the last years, the concentrations measured during this study were used as a worst case scenario (Table 4.3). Thus, the concentrations of TNT, especially in areas A and B, may represent a risk for different aquatic organisms. However, TNT may be rapidly diluted and biotransformed to possibly less toxic substances in the nearby river. Figure 4.2 shows that, based on laboratory data, all aquatic organisms (100 %) would be affected by the groundwater TNT concentration from areas A and B, whereas 10 to 40 % would

be affected by those at site A', B', C and C'. Using the CalTox estimation, less than 10 % of the aquatic organisms would be affected.

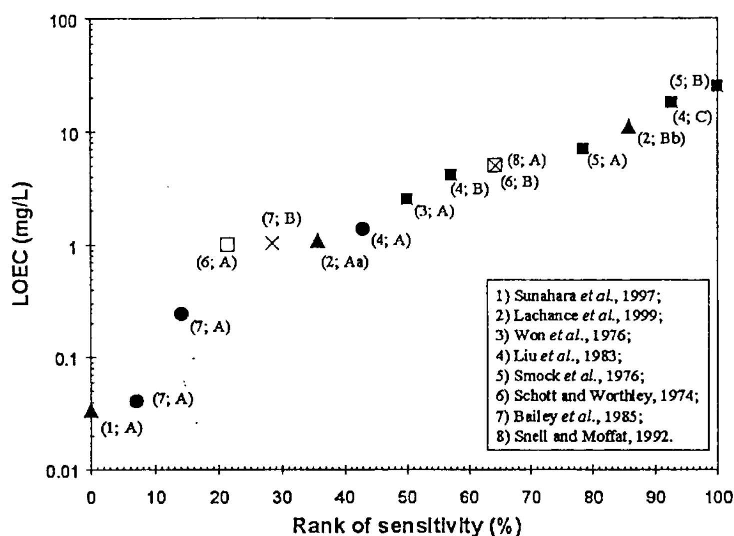

Figure 4.2
Sensitivity distribution of sub-lethal effects for aquatic organisms to TNT: Microorganisms (▲); microphytes (■); macrophytes (□); invertebrates (×); fish (λ). Numbers and letters in brackets indicate: reference designated in the inset (number), experiment number (capital letter) and endpoint number (small letter). For the same citation, common capitalized letters (A, B, ...) correspond to same experimental conditions. Common small letters (a, b, ...) correspond to same endpoints. LOEC values taken from literature (reference number in brackets) are presented as a function of their rank of sensitivity (%; rank of the data/ number of data x 100).

Toxicity assessment

Toxicity assessment using the earthworm reproduction test data showed that lethal and sub-lethal toxicities may be associated with exposure to soils from areas A, A' and B. Soils from sites having $\geq$ 367 mg TNT kg^{-1} soil may present a risk of lethality, whereas those having $\geq$ 116 mg kg^{-1} may reduce the reproduction capacity of earthworms. These results agree with earlier studies (Robidoux *et al.* 1999, Robidoux *et al.* 2000).

Biological survey

A weak trend between decreased earthworm (*E. andrei* and indigenous species) survival and increasing levels of TNT and associated nitroaromatic degradation products was observed in the mesocosm experiments. No survival of earthworms was found at concentrations > 9033mg TNT kg^{-1} of dry soil; whereas lethality was not detected at <1146 mg TNT kg^{-1}. TNT could be partially degraded (to 2-ADNT and 4-ADNT) in the tissue of surviving earthworms, suggesting that these contaminants may be transfered into the food web.

Biomarkers

Considering the NRRT responses of surviving earthworms exposed to explosives-contaminated soils under field conditions, sub-lethal effects may be associated to soils from areas A, A' and B' (25 to 367 mg kg^{-1} of TNT) compared to reference soils. These experiments support the data from single chemical and toxicity assessment.

Summary of risk characterization
Contaminants of potential concern for the ecological receptors include the nitroaromatic compounds such as TNT, their degradation (2-ADNT and 4-ADNT), and associated by-products (TNB, DNB, 2,4-DNT and 2,6-DNT). Based on the risk index, the screening risk assessment indicates that the selected contaminated areas of the heterogeneous site described herein may constitute credible hazards to soil organisms and wildlife when TNT concentrations are ≥ 1 mg kg^{-1}. However, this method does not consider the possibility of synergetic or antagonist chronic effects with the identified contaminants and other compounds. In general, earthworm data from toxicity tests, biological surveys and site-specific biomarker studies, showed lethal and sub-lethal effects and would support the preliminary risk assessment results based on single chemicals. Thus, concentrations of TNT ≥ 100 mg kg^{-1} may constitute a risk for soil organisms and /or wildlife at the study site.

Further studies, such as additional toxicity (or other measures of bioavailability) assessment of TNT degradation products and associated products, and chemical identification of other degradation products, would be necessary before concluding that TNT (over the generic criteria for industrial site, *i.e.* 1.7 mg kg^{-1}) and related contaminants at low concentrations (<100 mg kg^{-1} of TNT) constitute a risk for the soil organisms and wildlife inhabiting this site. Field studies are also required before concluding that a risk of effect exists for the aquatic receptors.

Uncertainties concerning risk

Ecological risk assessment of nitroaromatic compounds should consider uncertainty associated to different sources which may limit the conclusion of the evaluation. Sources of uncertainty (Suter II, 1993) considered for this case study include:

Ignorance
Multiple contaminant exposure. Single chemical toxicity experiments were used to calculate benchmarks. In reality, receptors are exposed to many nitroaromatic contaminants, and may be at low concentrations for other contaminants such as metals.
Bioavailability of contaminants in soil. Solvent extraction methods used in this study allowed the characterization of the total concentrations of extractable nitroaromatics, but may not accurately reflect the bioavailable quantities of these contaminants in the soil. In addition, the spiking methods used for toxicity studies may not represent the field conditions.
Transport and transformation of contaminants in water. Soils contaminated by TNT for many years probably constitute the actual source of contamination. Data from this study and others (data not shown) indicate that the groundwater TNT-concentrations have been stable for several years. This is not in agreement with the estimation given by *CalTOX* model using the theoretical proprieties of TNT (available upon request).
Uptake factors and contaminant concentrations in unanalyzed food types. Uptake factors were calculated from laboratory experiments (literature) or site-specific (mesocosms data). Because only a few data were available, maximum TNT concentrations in roots were used as the exposure

concentrations by plants for mammals. Maximum tissue residues in depurgated earthworms from the mesocosm studies were used as the exposure concentration by invertebrates for mammals. Considering that exposure conditions and food type may be different or change with respect to season, tissue burdens of contaminants are likely to differ between the laboratory and field measurements. The assumption taken the maximum concentration as the exposure value represents the worst case scenario. Thus, the level of exposure is probably overestimated.

Extrapolation from published toxicity data. Toxicity of nitroaromatic compounds on wildlife species is poorly documented in the open literature. Benchmarks (NOAEL based) were extrapolated from LOAEL for the laboratory rat giving uncertainty on the reference value.

Lack of benchmarks for some nitroaromatic compounds. Few data are available for nitroaromatic compounds other than TNT. In many cases, benchmarks for TNB, DNB, 2,4-DNT, 2,6-DNT and the TNT degradation products (2-ADNT and 4-ADNT) could not be estimated.

Toxicity data for birds. Sub-chronic or chronic studies were not available for representative avian species. Benchmarks were extrapolated from mammalian toxicity literature.

Confounding stressors. Toxicity of explosives may be affected by other biotic or abiotic factors. Toxicity of nitroaromatics may be altered by physico-chemical interaction in the soil or by field conditions leading to synergetic or antagonistic effects.

Recovery from exposure. Explosives-contaminated areas are isolated and the receptors may be exposed to the toxicant for only a short-term period without having acute effects. Benchmarks are based on laboratory toxicity data and do not consider the potential recovery if the concentrations of explosives decrease (by natural attenuation) or if the exposure period varied (*e.g.* seasonal changes in receptor activities).

Error

Chemical analysis. Technical error is relatively low when quality control procedures are applied. The TNT concentrations were generally accurate within ± 9.0 % for soil and within 15 % for groundwater (US EPA 1997).

Stochasticity

Sampling frequency. Because of their limited to low solubilities in water, the concentrations of contaminants in soil may be highly variable. The low number of samples creates uncertainty, especially when exposure concentrations are close to the benchmarks. Toxicant concentrations were, in most cases, clearly different from the benchmarks reducing the uncertainty on exposure concentrations.

Variable food and water consumption. Food and water consumption by wildlife was assumed to be similar to those reported for the same or related species (Sample and Suter II 1994). However, since weather conditions, type of food and behavior may be different in Canada and specific to the study site, food consumption by wildlife at the study site may be slightly different and should be confirmed.

Variable response to toxicants. Different species can be exposed to contaminants at different life stages. Data from which benchmarks were derived are usually not based on experiments using the same organisms found in the ecosystem assessed. Because few toxicological data are available for TNT, low to moderate confidence can be placed on the benchmarks.

4.3.7 CONCLUSION

This chapter describes a preliminary ecotoxicological risk assessment approach used for explosive-contaminated sites. This case study indicates that the selected contaminated areas of the described heterogeneous site constitute credible hazards to soil organisms, mammals and birds. The risk would be mainly associated to TNT exposure. However, cumulative risk associated to byproducts and TNT degradation products can not be estimated since scant toxicity data are available for these contaminants. Contaminated groundwater may also represent a potential risk for aquatic organisms considering a worst case scenario approach. However, the concentrations of TNT may decrease in groundwater using the transport and transformation model *CalTOX*. In addition, TNT would be diluted in the surface water. Thus, the real exposure concentration would be lower than benchmarks, resulting in a low risk to aquatic organisms. Toxicity tests, biological survey and biomarker data specific to the site using the earthworms (as indicator of exposure and effects) confirmed the potential risk to soil organisms. The uncertainties associated with this study would be reduced at low concentrations of TNT (<100 mg/kg) following further laboratory (measures of bioavailability such as tissue residues and toxicity tests of the different metabolites of TNT) and field investigations (monitoring and exposure measurement).

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4.4 MICROBIAL TOXICITY OF HMX IN SOIL

4.4.1 Introduction

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, commonly known as HMX (High Melting Explosive), is a non-aromatic heterocyclic nitramine and one of the most powerful and commonly used conventional explosive compounds. Several studies have shown that HMX exerts neither acute nor chronic adverse effects on freshwater invertebrates, fish, and algae (Bentley *et al.* 1977, 1984). Up to its limit of aqueous solubility HMX showed no toxicity to *Vibrio fischeri* (Microtox®) and green alga *Selenastrum capricornutum* (Sunahara *et al.* 1998), V79 Chinese hamster lung cells and TK6 human lymphoblastic cells (Lachance *et al.* 1999). No mutagenicity to *Salmonella typhimurium* (Ames test) was observed with or without a rat liver metabolic activation system S9 (Lachance *et al.* 1999, Tan *et al.* 1992, Whong *et al.* 1980). However, Sullivan *et al.* (1979) questioned the statistical analysis of Bentley *et al.* (1977) and concluded after reanalysis that as low as 10 mg/l of HMX significantly increased cell growth in three of the algae species and the chlorophyll A content in one species. Its subchronic toxicity to mammals (rats and mice administered with HMX in diet) was reported with the lowest observable adverse effect level being 75-270 mg/kg/d (Everett *et al.* 1985, Everett and Maddock 1985).

A large data gap exists in the terrestrial ecotoxicology for HMX. Although HMX toxicity to soil invertebrates such as earthworms (*Eisenia foetida* or *E. andrei*), enchytraeid and collembola is relatively well-documented (Philips *et al.* 1993, Robidoux *et al.* 2001, Schäfer and Achazi 1999), no studies have been found so far that tested its toxicity to avian species, terrestrial plants and soil microorganisms (Talmage *et al.* 1999). In the present study, we examined the ecotoxicological effects of HMX on indigenous soil microbial processes or activities.

4.4.2 Materials and Methods

Two uncontaminated garden soils (GS1 and GS3) were used. The GS1 soil was a sandy loam with a pH of 6.9 and total organic carbon of 11.2%, whereas the GS3 soil was a silty clay loam with a pH of 6.5 and total organic carbon of 3.5%. Immediately after collection in June 1999, both soils were sieved (2 mm) and frozen at –20°C. Before use, the soils were thawed overnight at 4°C and pre-incubated for 5-8 d at 25 ± 2°C.

HMX of technical grade (purity ≥ 99%) was obtained from the from the Defence Research Establishment Valcartier, Val-Bélair, PQ, Canada. HMX (powder) was added directly into the GS1 and GS3 soils (each ca. 1 kg of moist weight) without using any delivery solvent to achieve nominal concentrations of 0, 10, 50, 250, 1250, 6250 and 12500 mg/kg soil, followed by 5-min blending with an electric household mixer. Soils were sampled for chemical and microbiological analyses 4- and 12-wk after spiking. In a preliminary experiment, the dosage was set as 0, 10, 100, 1000 and 10000 mg/kg soil and the exposure time was one week. Each treatment was replicated four times. All soils were incubated at 25 ± 2°C in the dark. Soil moistures were adjusted every 2 wk to maintain their original levels (24.3% for GS1 and 28.6% for GS3).

Soil dehydrogenase activity (DHA), potential nitrification activity (PNA), nitrogen fixation activity (NFA), basal respiration (BR), and substrate-induced respiration (SIR), all of which are microbiologically-mediated activities or processes frequently used for assessing the health of soil microbial communities, were analyzed for each soil sample. The methods for these microbial assays were described earlier (Gong *et al.* 1999, 2001). An acetonitrile extraction-HPLC analysis method was used to extract and determine HMX and its metabolites (if any) in soil (U.S. EPA 1997). All data were expressed on an oven-dried weight basis of soil and subjected to analysis of variance (ANOVA).

4.4.3 Results and Discussion

As shown in Fig. 4.3, the recovery of spiked HMX varied tremendously at the three lowest levels, i.e., 10, 50, and 250 mg/kg. At higher levels, however, the variability in recovered HMX was low.

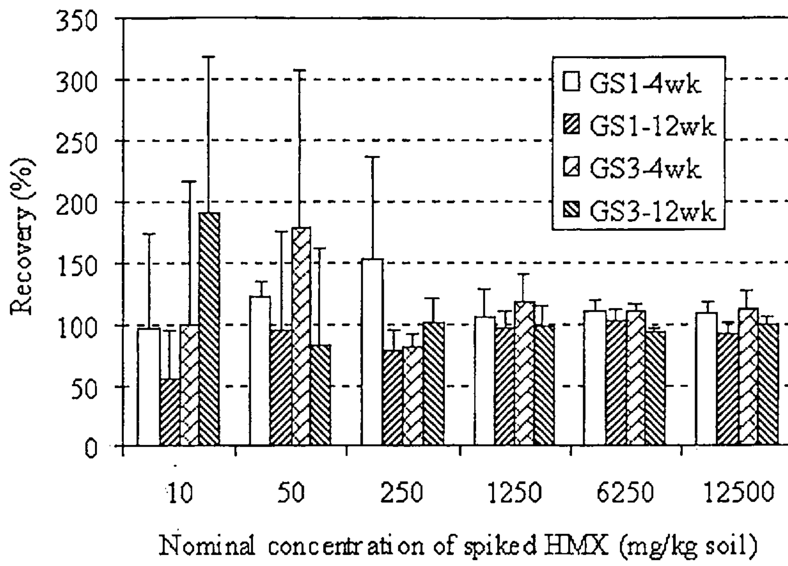

Figure 4.3
Recovery of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) 4 and 12 weeks after being spiked into the GS1 and GS3 soils. Data are expressed in mean (column) ± standard deviation (error bar) (n = 4).

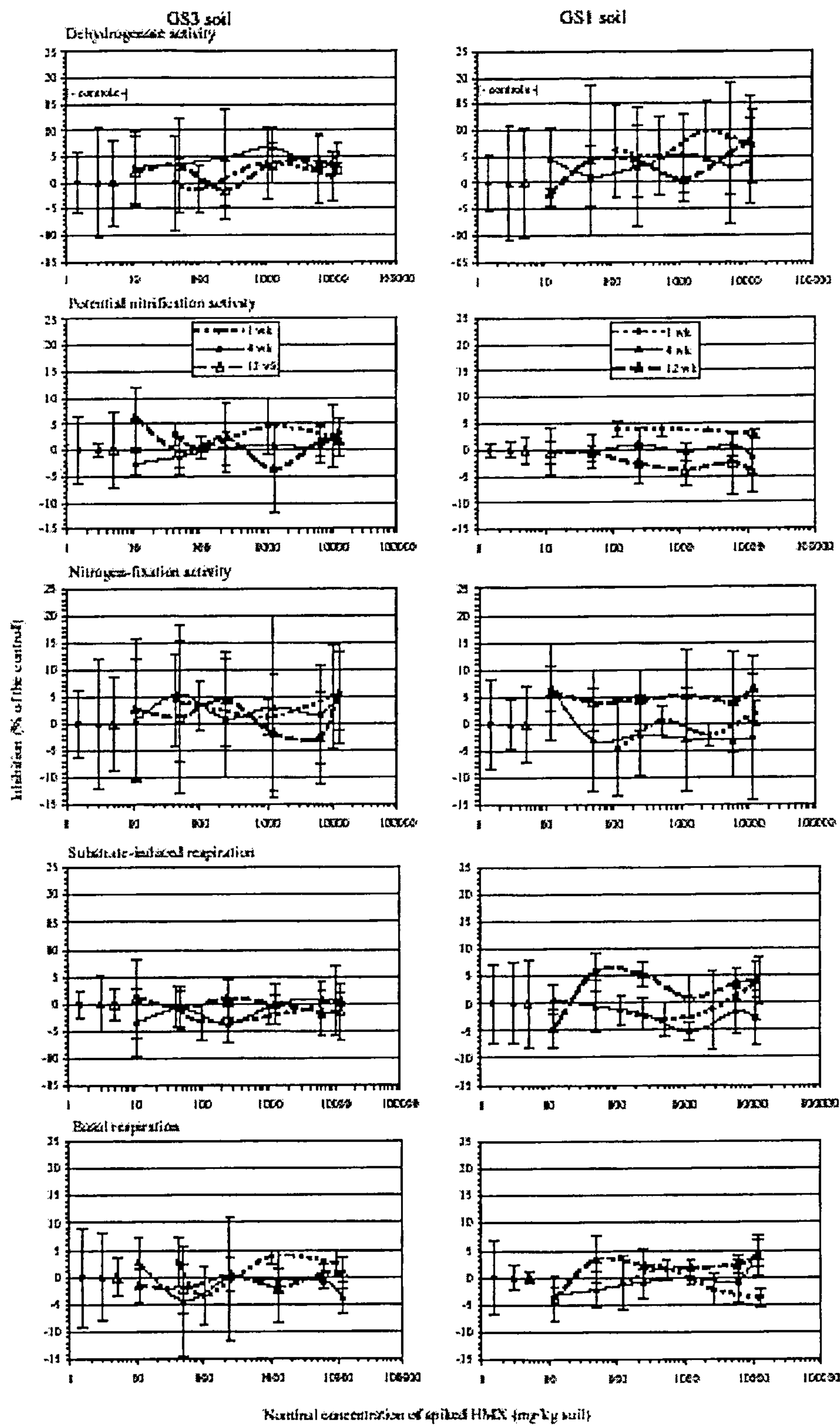

Figure 4.4 Effects of HMX on soil microbial activities in two garden soils. Data are given in mean (points) ± standard deviation (error bars) (n=4).

We believe that this was largely due to the direct amendment of powder HMX, which was done purposely to avoid the introduction of any organic solvent that might kill some soil microbes and meanwhile serve as a substrate to some others. Presumably, it is extremely difficult to achieve homogeneity if spike a tiny amount of chemical (a few mg) into a relatively large quantity of soil (a few kg). During the 12-wk incubation, no metabolites were detected. Data from the preliminary experiment were similar to those shown in Fig. 4.3.

TNT (2,4,6-trinitrotoluene) was used as a positive control and was spiked into the GS3 soil at 0, 30, 60, 125, 500, 1000 and 2000 mg/kg soil. The soil was sampled only once, i.e., one week after spiking. Results show that TNT inhibited all the microbial indicators except for basal respiration in a dose-response manner, which has been reported elsewhere (Gong *et al.* 2001). Our earlier studies also indicated that TNT adversely affected DHA, NFA and PNA in both laboratory spiked and field contaminated soils (Gong *et al.* 1999). On the contrary, HMX did not show significant effects (ANOVA, $p > 0.05$) on any of the five microbial indicators in both GS1 and GS3 soils at all three sampling times (i.e., 1-, 4- and 12-wk). Up to 12500 mg/kg soil, HMX caused less than 10% inhibition or 5% stimulation of microbial activities (Fig. 4.4), which is both statistically and biologically insignificant.

HMX is structurally similar to RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and they are expected to exhibit similar biochemical reactivities. However, HMX is less water soluble (6.6 vs. 38.4 mg/l at 20°C) (Talmage *et al.* 1999) and more chemically stable than RDX (Akhavan 1998). Therefore, under the aerobic conditions in our studies, HMX hardly influenced soil microorganisms whereas RDX only showed slightly significant effects (Gong *et al.* 2001).

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4.5 TRADESCANTIA MICRONUCLEI (TRAD-MCN) ASSAY

As an essentially important ecological receptor, higher plants have long been recognized as excellent indicators of cytogenetic and mutagenic chemicals in the environment. Since the 1970s, many higher plant species have been investigated worldwide, and the *Tradescantia* bioassays are among the few surviving higher plant systems that are frequently used for environmental genotoxin/mutagen detection. The Trad-MCN assay has been intensively reviewed under the Gene-Tox Program initiated by the US Office of Toxic Substances of the US EPA in 1980, and validated and standardized initially by the Collaborative Study on Plant Systems as part of the International Program on Chemical Safety (IPCS) from 1985 to 1992, and then by the International Program on Plant Bioassays (IPPB) that began in 1993 (Grant 1999). Both IPCS and IPPB were sponsored by the United Nations Environment Program (UNEP) and the World Health Organization (WHO). Research carried out in the past three decades has warranted the high efficiency of this bioassay for in-situ monitoring and/or detecting environmental mutagens such as heavy metals (Knasmüller *et al.* 1998).

The Trad-MCN bioassay is also referred to be as *Tradescantia* Micronuclei-in-Tetrad test because the meiotic pollen mother cells are used as the target cells and the chromosome breakage revealed in the form of micronuclei (MCN) in the tetrads as the endpoint (Ma 1983). Following the protocol described in Ma *et al.* (1994), some preliminary experiments have been carried out using $CdCl_2$., a known mutagenic agent often found in the aquatic and terrestrial environments. Young Tradescantia inflorescences bearing at least nine exposed buds were exposed to Cd^{2+} in aqueous solutions for 6 h, followed by a 24-h recovery in de-chlorined tap water. Then, the exposed buds were fixed for 24 h in a freshly prepared Carnoy's solution (ethanol : glacial acetic acid = 3 : 1). The fixed buds were stored in 70% ethanol until dissection and tetrad examination. Results given in Fig. 4.5 show a clear dose-response relationship between Cd^{2+} exposure and micronuclei frequency in *Tradescantia* pollen mother tetrad cells. The no observable effect concentration (NOEC) is 1.25 mM ($p > 0.05$, t-test) whereas the lowest observable effect concentration (LOEC) is 2.5 mM ($0.01 < p < 0.05$, t-test).

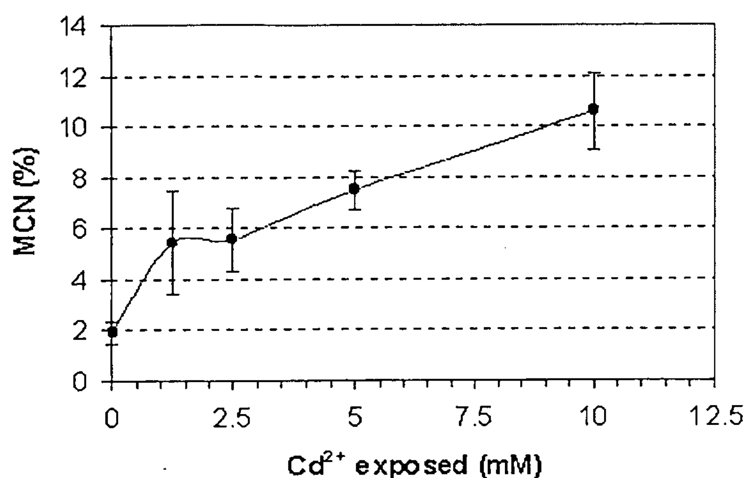

Figure 4.5
Genotoxicity of $CdCl_2$ to *Tradescantia* determined by the Trad-MCN assay. Each data point represents the average of 5-7 buds containing tetrads while the error bars the standard error.

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Chapter 5: Transformation of 2,4,6-trinitrotoluene (TNT) by immobilized *Phanerochaete chrysosporium* under fed-batch and continuous TNT feeding conditions.

Denis Rho, Jonathan Hodgson, Sonia Thiboutot, Guy Ampleman, and Jalal Hawari. 2001. Biotechnol. Bioeng. 73: 271-281.

A copy of the article is enclosed in the appendix.

5.1 Abstract

The cometabolic transformation of 2,4,6-trinitrotoluene (TNT) by an immobilized *Phanerochaete chrysosporium* culture was investigated under different TNT and/or glycerol feeding conditions in a 5-L reactor. In the fed-batch feeding mode, as a result of four spiking events at an average feeding rate of 20 mg TNT L^{-1} d^{-1} and 250 mg glycerol L^{-1} d^{-1}, the initial TNT transformation rate and the glycerol uptake rate of the 7-day-old immobilized cell culture were 2.41 mg L^{-1} h^{-1} and 16.6 mg L^{-1} h^{-1}, respectively. Thereafter, the TNT fed into the reactor depicted a negative effect on the cell physiology of *P. chrysosporium*, i.e. both rates decreased constantly. At 32 mg TNT L^{-1} d^{-1} feeding rate, also in the presence of glycerol (200 mg L^{-1} d^{-1}), this effect on the fungal cell metabolism was even more significant. When TNT was fed alone at 3.7 mg L^{-1} d^{-1}, it showed an initial 0.75 mg L^{-1} h^{-1} rate of TNT transformation, i.e. one third the initial level observed in the presence of glycerol. In contrast, in the continuous feeding mode (dilution rate, D = 0.11 d^{-1}), at 5.5 mg TNT L^{-1} d^{-1} and 220 mg glycerol L^{-1} d^{-1}, the immobilized cell culture exhibited a constant TNT transformation rate for cultivation periods of 50 day and 61 day, under uncontrolled and controlled pH conditions, respectively. Thereafter, during the latter experiment, 100 % TNT biotransformation was achieved at 1100 mg L^{-1} d^{-1} glycerol feeding rate. Immobilized cells (115-day-old), sampled from a continuous TNT feeding experiment, mineralized [^{14}C]-TNT to a level of 15.3 % following a 41-day incubation period in a microcosm.

Chapter 6. Études en microcosmes de l'activité de dégradation naturelle et stimulée du TNT et de ses composés nitroaromatiques apparentés dans les sols contaminés d'une ancienne manufacture de TNT.

C. Masson, S. Delisle, et C.W. Greer

6.1 Introduction

Une étude a été conduite afin de caractériser le potentiel de dégradation naturel et stimulé des nitro et amino aromatiques dans les sols contaminés d'une ancienne manufacture de TNT. Le site est situé à Valleyfield (Québec). Les composés cibles ont été les suivants: le TNT et deux de ses sous produits de fabrication, le 2,4-DNT et le 2,6-DNT, ainsi que deux des métabolites intermédiaires de transformation du TNT, le 2-ADNT et le 4-ADNT.

6.2 Matériel et méthodes

6.2.1 Analyses des nitroaromatiques sur HPLC

Les échantillons de sol ont été analysés pour leur contenu en nitroaromatiques en suivant la méthode 8330 de l'EPA, avec cependant les modifications suivantes: les échantillons n'ont pas été séchés à l'air, mais plutôt congelés à -80°C avant d'être lyophilisés.
Les extraits de sol ont été analysés sur un système de chromatographie de Thermoseparation Products composé d'une pompe de modèle P4000, d'un injecteur de modèle AS1000 incluant le contrôle de la température de la colonne, et un détecteur à photodiode UV 6000LP. La phase mobile était constituée de 2-propanol:eau (18:82 p/p). Les injections ont été de 50 µl dans une colonne Supelcosil C8 (particules de 5 mm) de 25 cm de long par 46 mm de diamètre interne, en phase inversée, à 35°C. Le débit était de 1 mL/ min.

6.2.2. Sols à l'étude

Deux échantillons de sol provenant d'une ancienne manufacture de TNT située à Valleyfield (Québec) ont été utilisés pour les études en microcosmes, soit le sol 1 et le sol 2. Ces deux sols ont été échantillonnés à un et deux mètres de profondeur, respectivement, sous une surface extrêment contaminée (10 000 à 20 000 ppm de TNT ainsi que plusieurs autres produits de fabrication ou de dégradation...).

6.2.3 Étude de la minéralisation de cinq nitroaromatiques

La préparation des microcosmes a été effectuée au cours de la semaine qui suivit l'échantillonnage, le temps zéro étant sept jours suivant l'échantillonnage. Des portions de 20 g de sol ont été introduites dans des bouteilles à sérum de 120 mL. Une série distincte de microcosmes en triplicatas a été préparée pour chacun des cinq composés marqués, dans le but d'évaluer le potentiel de minéralisation pour chacun de ceux-ci, et ce, pour chacun des deux sols étudiés. De plus, chacun des deux sols a été étudié à une humidité de 85 % de la capacité au champ, et sous l'effet de trois traitements: sol sans ajout, sol + acétate + Tween 80, sol + mélasse (de canne) + Tween 80. Le Tween a été utilisé à une concentration finale de 0.1%, alors que le

ratio *"carbone ajouté provenant de l'acétate ou des sucre de la mélasse: carbone déjà présent sous forme de TNT"* a été dans une proportion de 9:1 et 46:1 pour le sol 1 et 2 respectivement. Les ajouts de carbone et de Tween ainsi que l'ajustement de l'humidité ont été réalisés en ajoutant 1.5 mL de solution spécifique pour les différents traitements.
Les contrôles abiotiques ont été préparés en autoclavant deux fois 30 min à intervalle de 24 h un mélange des deux sols échantillonnés en portions égales (dans leurs bouteille à sérum). Les traitements ont été mixtes (acétate + mélasse + Tween) pour ces contrôles abiotiques préparés en triplicatas pour chacun des cinq substrats marqués. La quantité de carbone ajoutée aux contrôles stériles l'a été en considérant que les deux sols ont été mélangés à parts égales, et donc, le ratio *"carbone ajouté provenant de l'acétate ou des sucre de la mélasse:carbone déjà présent sous forme de TNT"* a été de 28:1.
Les cinq composés marqués au ^{14}C utilisés séparément ont été les suivants: ^{14}C-TNT, ^{14}C-2,4-DNT & ^{14}C-2,6-DNT (mélange 50%-50%), ^{14}C-2-ADNT, ^{14}C-4-ADNT, et finalement ^{14}C-MNT, tous préparés par Guy Ampleman au CRDV. Des aliquots de 20 µl de chacun des cinq composés radioactifs dans l'acétone ont été ajoutés à chaque portion de 20 g de sol, pour chacune des cinq séries, pour des comptes radioactifs initiaux d'environ 100 000 DPM par microcosme. La quantité de composés radioactifs ajoutée a été entre 4 et 12 ppm par microcosme de 20 g de sol (4.15, 7.20, 8.30, 11.50, et 4.05 ppm, respectivement pour chacun des composés marqués énumérés plus haut).
Une trappe à CO_2 constituée d'un tube contenant 0.5 mL de KOH 1N a été introduite dans chacun des microcosmes de sol. Chaque bouteille à sérum a été fermée avec un bouchon de butyle gris et un sceau d'aluminium. Tous les microcosmes, initialement aérobies, ont été incubés de façon statique et à l'abri de la lumière, à une température de 12°C.
La minéralisation a été régulièrement suivie en transférant le KOH des trappes dans une fiole à scintillation de 20 mL, à l'aide d'une seringue de 1 cc munie d'une longue aiguille. La seringue et l'aiguille ont été ensuite rincées de façon séquentielle avec 0.5 mL puis 1.0 mL d'eau, également transférées dans la fiole à scintillation. Du scintillant (18 mL) a été ajouté dans chaque fiole qui a été fermée d'un bouchon, agitée vigoureusement, et finalement comptée sur un compteur à scintillation Packard Tri-Carb, modèle 2100 TR.

6.2.4 Étude de la transformation des nitroaromatiques

Cette étude a été conduite sur des microcosmes non-radioactifs, qui ont été préparés et incubés de la même façon que la série de microcosmes radioactifs, à l'exception des composés marqués au ^{14}C, qui n'ont pas été pas ajoutés. Cette série de microcosmes non-radioactifs a été cependant différente de la série radioactive, afin de permettre quatre échantillonnages (T0 à T4) en sacrifiant chacun des triplicata de chaque sol et de chacun des trois traitements, pour extraction et analyse des nitroaromatiques. Les composés suivis au cours de cette étude étaient déjà présents dans le sol sur le terrain, et sont les suivants: TNT, 2,4-DNT, 2,6-DNT, et 4-ADNT. Le KOH des trappes à CO_2 a été changé à intervalles réguliers, bien que moins souvent que celles des microcosmes radioactifs. Les nitroaromatiques ont été extraits et analysés selon la méthode 8330 modifiée décrite plus haut; chaque bouteille à sérum sacrifiée étant congelée à -80°C avant la lyophilisation de son contenu.

6.2.5 Niveaux d'oxygène des microcosmes

Il a été planifié d'étudier le sol 1 en condition aérobie tout au long de l'expérience (et donc d'ajouter de l'oxygène au besoin), mais de laisser le sol 2 devenir anaérobie par lui-même. La phase gazeuse des microcosmes radioactifs et non-radioactifs a été analysée par chromatographie gazeuse. Les deux séries ne différant pas de façon significative, la série non-radioactive a été analysée la plupart du temps, sauf vers la fin de l'expérience, où toute la série non-radioactive avaient déjà été sacrifiée. Au besoin, le niveau d'oxygène a été ajusté à la hausse en injectant 60 cc d'air filtrée, tout en laissant s'échapper l'air excédant à l'aide d'une aiguille supplémentaire insérée dans le bouchon.
Au jour 264, l'activité de minéralisation était plafonnée depuis longtemps pour tous les traitements au DNT ou MNT. La phase gazeuse des triplicatas radioactifs DNT du sol 2 avec ajout de mélasse (qui était tombé rapidement anaérobie) a donc été rendue à nouveau aérobie. La phase gazeuse de tous les autres microcosmes DNT ou MNT a été changée d'aérobie à anaérobie au jour 265, avec de l'azote injectée durant une minute en laissant s'échapper l'air excédant à l'aide d'une aiguille supplémentaire insérée dans le bouchon. Ces changements des phases gazeuses ont été effectués dans le but de vérifier si l'activité de minéralisation (plafonnée dans tous les cas) reprendrait, en passant d'aérobie à anaérobie, et vice versa.

6.3 Résultats et discussion

Les résultats des dégradation et de transformation sont présentés à la Fig. 1. Une activité naturelle de dégradation (transformation) a été observée pour le TNT et le 2,4-DNT. Le TNT est pratiquement disparu du sol 1 après 150 jours. Le 2,4-DNT est complètement disparu des sols 1 et 2 en près de 25 jours. Aucune activité de transformation naturelle n'a été observée pour le 2,6-DNT. Comme observé plusieurs autres fois au cours d'études précédentes, un des deux métabolites primaires, le 4-ADNT, a augmenté puis diminué en cours de la période de transformation à l'étude.
Une stimulation de l'activité a été observée avec ajout d'acétate ou de mélasse. L'acétate a clairement accéléré la dégradation du TNT dans le sol 2, ce qui n'a pas été le cas pour le sol 1, où l'acétate a inhibé l'activité naturelle de dégradation du 2,4-DNT. La mélasse a clairement augmenté l'activité de transformation du TNT dans les deux sols à l'étude, ainsi que celle du 2,6-DNT, dans le sol 2. Ces résultats sont très encourageants, dans l'optique d'une gestion des sols contaminés aux nitroaromatiques, particulièrement au TNT.

Les activités de minéralisation sont présentés à la Fig. 2. Les potentiels naturels de minéralisation du TNT, du 2-ADNT ainsi que du 4-ADNT sont inexistants (sous 2%, ce qui est négligeable). L'ajout de mélasse ou d'acétate n'a rien changé quant à cette activité, tant pour le sol 1 que le sol 2. Par contre, une rapide activité naturelle de minéralisation totale du 2,4-DNT a été observée pour les deux sols. La minéralisation du 2,4-DNT a été considérée totale après une cinquantaine de jours, étant donné le mélange 50%-50% de ^{14}C-2,4-DNT et de ^{14}C-2,6-DNT utilisé, et également étant donné que le 2,4-DNT est complètement disparu des sols 1 et 2 en près de 25 jours, dans la série non-radioactive (Fig. 1). Le MNT a été minéralisé naturellement à près de 60% dans le sol 1, et à près de 30% dans le sol 2, tout cela en à peu près 50 jours.

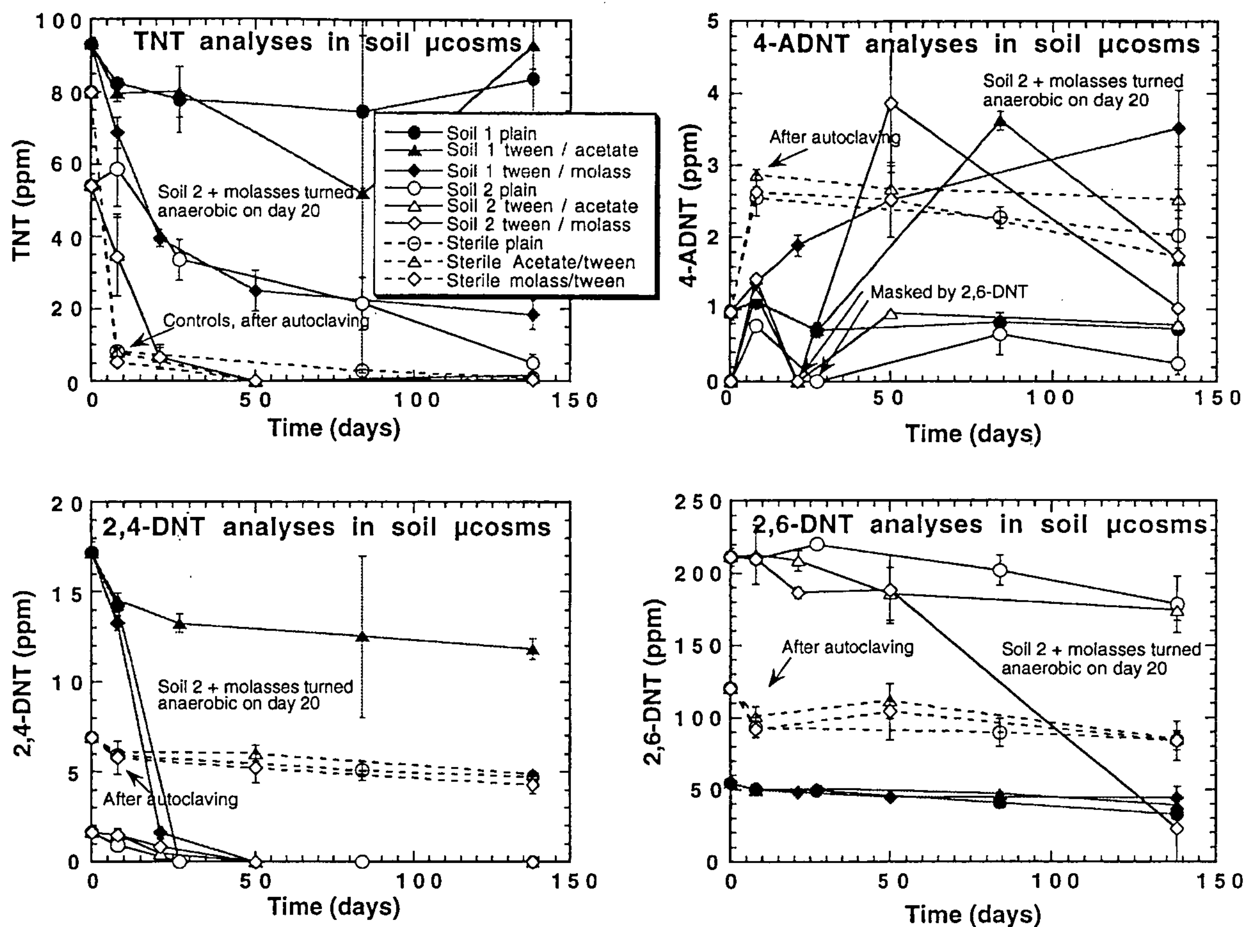
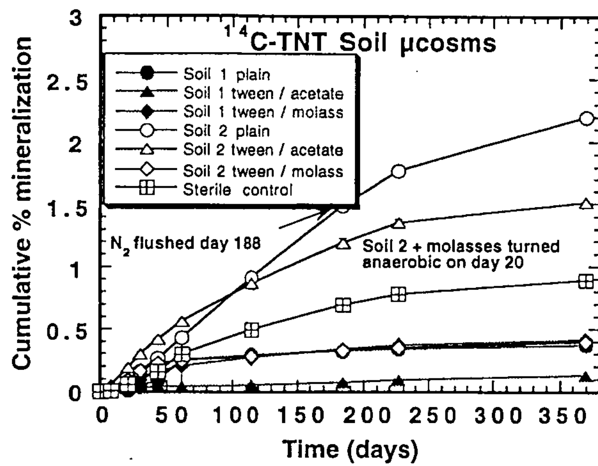
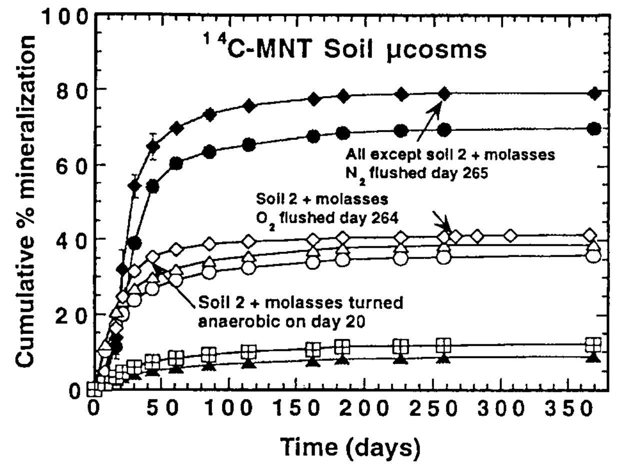
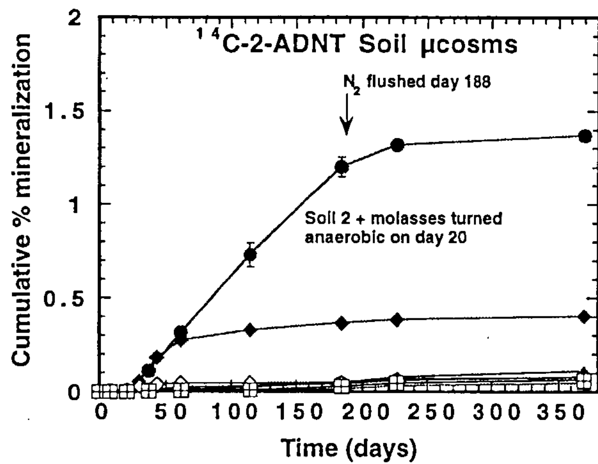


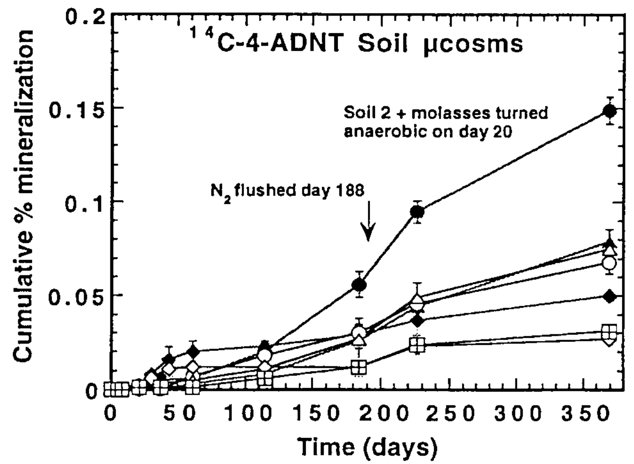
Fig. 1. Activités de dégradation (transformation) des composés nitroaromatiques étudiés (non-radioactifs).

L'activité de minéralisation du DNT ainsi que du MNT a été légèrement augmentée par l'ajout de mélasse, et ce, pour les deux sols à l'étude. Il est cependant à noter que le sol 2 stimulé avec mélasse a semblé cesser son activité de minéralisation après 25 jours, mais cela a été causé par le manque d'oxygène dans les microcosmes, dû a la consommation de cette mélasse (Fig. 3). L'activité n'a pas repris malgré l'ajout d'oxygène au jour 264 (Fig. 2). Une conclusion certaine est que le 2,4-DNT ne peut être minéralisé dans le sol 2 qu'en condition aérobie. D'autre part, pour tous les autres traitements avec DNT ou MNT, le changement provoqué de la phase gaseuse d'aérobie à anaérobie au jour 265 n'a rien changé non plus dans l'activité de minéralisation qui avait plafonnée depuis longtemps (Fig. 2).
L'acétate a très peu eu d'effet stimulant sur l'activité de minéralisation du DNT ou du MNT, dans le sol 2. Par contre, une inhibition marquée de l'activité naturelle de minéralisation du DNT ainsi que du MNT a été observée dans le sol 1 traité à l'acétate. Il









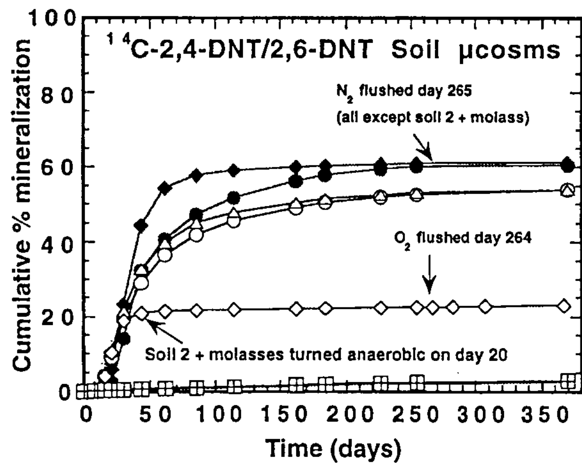


Fig. 2. Activités de minéralisation des composés nitroaromatiques marqués.

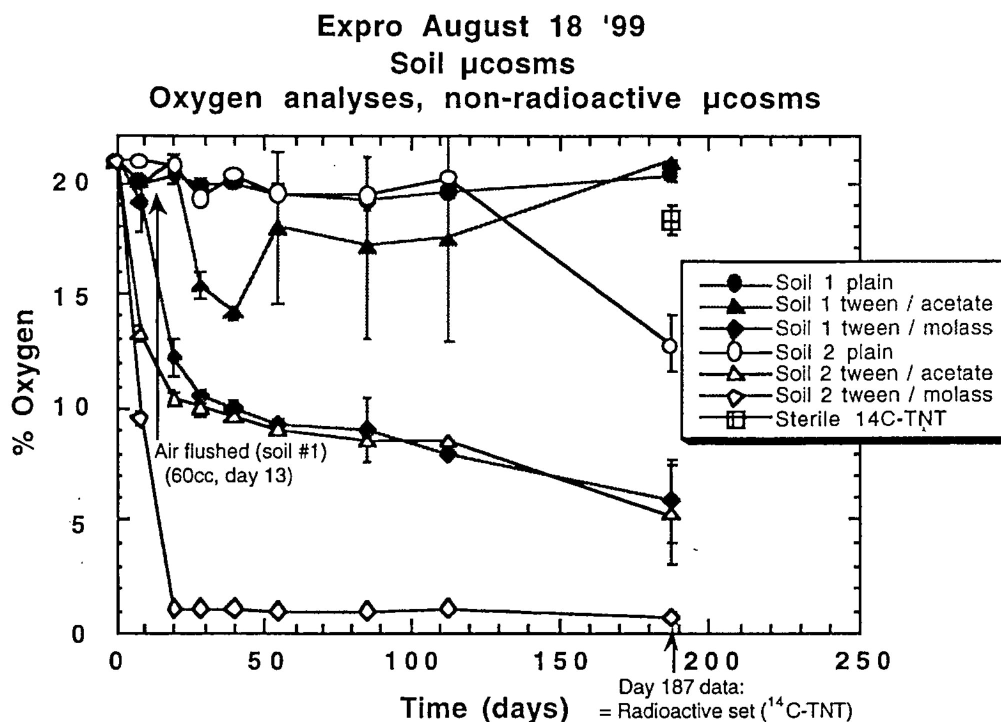


Fig. 3. Niveaux d'oxygène dans les microcosmes à l'étude.

est intéressant d'en faire un parallèle avec l'inhibition due à l'acétate, clairement observée dans la transformation du 2,4-DNT (non-radioactif), toujours dans le sol 1. Le sol 1 semble donc répondre défavorablement à l'ajout d'acétate.

L'effet du Tween n'a pas été étudiée dans la cadre de ces travaux, mais il serait intéressant de la faire ultérieurement.

Les résultats d'activités comparées (transformation et minéralisation) sont présentés au Tableau 1. Ce tableau est très utile pour conclure rapidement sur toute la présente étude, dans son ensemble. En résumé, le site à l'étude a un potentiel d'atténuation naturelle, qui semble clairement pouvoir être stimulé par l'ajout de mélasse, et possiblement par l'ajout d'acétate (avec Tween dans les deux cas).

4. Remerciements

Nous remercions grandement Louise Paquet pour les longues heures de travail effectuées lors des analyses des composés aromatiques sur HPLC incluant identification et quantification, ainsi que Danielle Ouellette pour son travail et on expertise lors de la préparation des 204 microcosmes.

Tableau 1. Activités comparées (transformation et minéralisation).

Treatment	TNT Transformation			TNT Mineralization		
	Activity	ppm	#days	Activity	%	#days
Soil #1						
Plain	-	93 → 83	138	-	non significant	250
Acetate	-	93 → 93	138	-	non significant	"
Molass	+++	93 → 25 (18)	50 (138)	-	non significant	"
Soil #2						
Plain	++	54 → 5	138	-	non significant	250
Acetate	+++	54 → 0 (7)	50 (21)	-	non significant	"
Molass	+++	54 → 0 (7)	50 (21)	-	non significant	"

Treatment	2,4-DNT Transformation			2,4-DNT (Assume 60% 2,4-DNT in ^{14}C 2,4- & 2,6-DNT mix) Mineralization		
	Activity	ppm	#days	Activity	%	#days
Soil #1						
Plain	+++	17 → 0	27	+++	100	226
Acetate	+	17 → 12	138	-	0	226
Molass	+++	17 → 0.3 (0)	21 (50)	++++	100	85
Soil #2						
Plain	+	16 → 0	27	++	87	162
Acetate	+	16 → 0 (0.4)	50 (21)	++	87	162
Molass	+ (- O_2...)	16 → 0 (0.8)	50 (21)	+++? (- O_2...)	37	43 (- O_2...)

Treatment	2,6-DNT Transformation			2,6-DNT (Assume 40% 2,6-DNT in 14C 2,4- & 2,6-DNT mix) Mineralization		
	Activity	ppm	#days	Activity	%	#days
Soil #1						
Plain	+++	55 → 32	138	-	0 (assumed)	
Acetate	++	55 → 39	138	-	0 (assumed)	
Molass	+	55 → 44	138	-	0 (assumed)	
Soil #2						
Plain	+	211 → 178	138	-	0 (assumed)	
Acetate	+	211 → 174	138	-	0 (assumed)	
Molass	++	211 → 23	138	-	0 (assumed)	

Tableau 1 (suite). Activités comparées (et minéralisation).

Treatment	MNT (2 & 4) Transformation			MNT (2 & 4) Mineralization		
	Activity	ppm	#days	Activity	%	#days
Soil #1						
Plain		nd		+	60	226
Acetate		nd		-	non significant	
Molass		nd		++	70 (60)	226 (60)
		not in soil				
Soil #2						
Plain		nd		+	35 (32)	226 (100)
Acetate		nd		++	38 (35)	226 (100)
Molass		nd		+++	40 (39)	226 (100)

Treatment	2A-DNT Transformation			2A-DNT Mineralization		
	Activity	ppm	#days	Activity	%	#days
Soil #1						
Plain		non-detectable		-	non significant	250
Acetate		(masked		-	non significant	"
Molass		by 2,6-DNT)		-	non significant	"
Soil #2						
Plain		non-detectable		-	non significant	250
Acetate		(masked		-	non significant	"
Molass		by 2,6-DNT)		-	non significant	"

Treatment	4A-DNT Appearance / Transformation			4A-DNT Mineralization		
	Activity	ppm	#days	Activity	%	#days
Soil #1						
Plain	+ / -	0.98 ↑1.09 ↓0.73	0 - 8 - 138	-	non significant	250
Acetate	+ / -	0.98 ↑3.63 ↓1.68	0 - 84 - 138	-	non significant	"
Molass	+ +	0.98 ↑2.52 ↑3.53	0 - 50 - 138	-	non significant	"
Soil #2						
Plain	+ / -	nd ↑0.77 ↓0.25	0 - 8 - 138	-	non significant	250
Acetate	+ / -	nd ↑1.21 ↓0.79	0 - 8 - 138	-	non significant	"
Molass	+ / -	nd ↑3.87 ↓0.68	0 - 50 - 138	-	non significant	"

Appendix

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The Fate of the Cyclic Nitramine Explosive RDX in Natural Soil

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The sorption–desorption behavior and long-term fate of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) was examined in sterilized and nonsterilized topsoil. Results of this study indicate that although RDX is not extensively sorbed by the topsoil (K^s_d of 0.83 L/kg), sorption is nearly irreversible. Furthermore, there was no difference in the sorption behavior for sterile and nonsterile topsoil. However, over the long-term, RDX completely disappeared within 5 weeks in nonsterile topsoil, and hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) metabolites formed in the aqueous phase. Over the same period, recovery of RDX from sterile topsoil was high (55–99%), and the nitroso metabolites were not detected. Only traces of RDX were mineralized to CO_2 and N_2O by the indigenous microorganisms in nonsterile topsoil. Of the RDX that was mineralized to N_2O, one N originated from the ring and the other from the nitro group substituent, as determined using N^{15} ring-labeled RDX. However, N_2O from RDX represented only 3% of the total N_2O that formed from the process of nitrification/denitrification.

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX, Figure 1) is an energetic compound that is commonly used as a military explosive. Various commercial and military activities that include manufacturing, waste discharge, testing and training, demilitarization, and open burning/open detonation (OB/OD) have resulted in extensive RDX contamination of soil and groundwater (*1*). The toxicity of RDX to humans and mammals is well established (*2*). Hence, remediation of contaminated soil and groundwater is necessary. Natural attenuation is an emerging remediation technology that is potentially less expensive, less intrusive, and offers a long-term solution (*3*). However, to gain acceptance by regulatory agencies, more data is required in order to accurately assess the fate of contaminants in the environment. Particularly, the fate of RDX depends on the processes of transformation, microbial degradation, and immobilization (*3*).

The metabolic pathways of polynitroaromatics, such as 2,4,6-trinitrotoluene, have been extensively characterized in liquid culture and natural soil systems (*4–7*). Two decades ago, McCormick et al. (*8*) identified nitroso metabolites of RDX. More recently, we identified several other metabolites and end products in the biodegradation of RDX with an anaerobic sludge (*9*). Particularly, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX, Figure 1) and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX, Figure 1) were formed by the stepwise reduction of $-NO_2$ in RDX. Methylenedinitramine ($O_2NNHCH_2NHNO_2$) and bis(hydroxymethyl)-nitramine ($(OHCH_2)_2NNO_2$) product formation was attributed to enzymatic hydrolytic ring cleavage of the inner C–N bonds of RDX. The four metabolites identified above disappeared to produce N-containing products (N_2O, and traces of N_2) as well as C-containing products (HCHO, CH_3OH, HCOOH, and CO_2). During the course of these experiments, 50–60% mineralization of RDX to CO_2 occurred. For aerobic biodegradation of RDX by *Phanerochaete chrysosporium*, we identified trace quantities of MNX and methanol as metabolites and N_2O and CO_2 as major mineralization products (*10*).

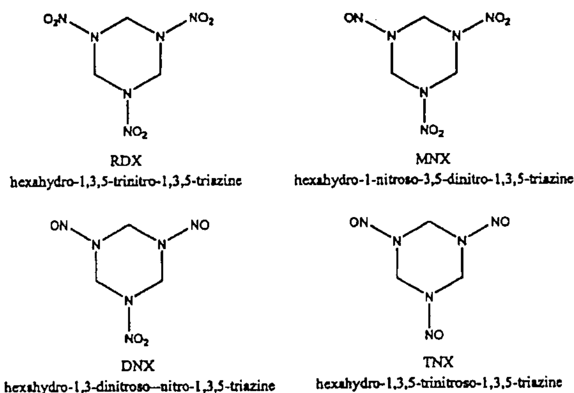

FIGURE 1. Molecular structures of RDX, MNX, DNX, and TNX.

Knowledge of the products and transformation pathways from the anaerobic (*9, 11*) and aerobic (*10*) biodegradation of RDX in liquid culture have provided us with information that is necessary in the design of effective remediation technologies. However, it is also necessary to assess the transformation, microbial degradation, and immobilization behavior of RDX in natural soil to assess the effectiveness of natural attenuation. Therefore, the objective of the present study was to determine the sorption–desorption behavior and the long-term fate of RDX in sterile and nonsterile topsoil.

Experimental Section

Chemicals. RDX (>99% purity) was provided by Defense Research Establishment Valcartier (Valcartier, PQ, Canada). Uniformly labeled [UL-^{14}C]RDX was synthesized and recrystallized to achieve chemical and radioactive purity of 99% and 97%, respectively (*12*). The specific activity of the radioactive compound was 28.7 μCi/mmol. The ring-labeled [^{15}N]RDX (>98% purity) was synthesized similarly (*13*). The MNX and TNX were synthesized according to the methods of Brockman et al. (*14*). All other chemicals used were reagent grade.

Soil. An agricultural topsoil was obtained from Varennes, Quebec. Properties of the topsoil are summarized in Table 1. Qualitative mineralogical analysis was determined by X-ray diffraction (Geochemical Laboratories, McGill University, Montreal, Quebec).

Soil Sterilization. The topsoil was sterilized by gamma irradiation from a cobalt-60 source at the Canadian Irradia-

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TABLE 1. Properties of the Topsoil Used in Experiments with RDX

particle size distribution			%		CEC,[a]
% clay (<2 μm)	% silt (2–53 μm)	% sand (>53 μm)	organic matter	pH	mequiv/100 g
4	12	83	8.4	5.6	14.6
major elements		minor elements		trace elements (<0.05%)	
quartz (SiO_2)		albite-ordered ($Na{\cdot}Al{\cdot}Si_3O_8$)		crossite ($Na_2(Fe,Mg)_5$-$(Si,Al)_8O_{22}(OH)_2$)	

[a] Cation exchange capacity.

tion Centre (Laval, Quebec) with minimum and maximum doses of 35.4 and 40.4 kGy, respectively. Gamma irradiation was examined for sterilization since it has minimal impact on the sorption of other contaminants by soil (*15–17*). Irradiated topsoil was combined with a solution that contained 0.1% (w/w) sodium pyrophosphate in water. Three dilutions were spread plated onto tryptone-yeast extract agar (*18*). The absence of colony forming units (CFUs) after 20 days incubation at 35 °C is indicative that gamma irradiation was effective.

Sorption–Desorption. Sorption was conducted in batch reactors at 25 °C. Aqueous RDX solutions were prepared from stock solutions in acetonitrile (10.27 mg/mL). A specific volume from the stock solution was added to deionized water to give the following initial RDX concentrations 5, 10, 15, 20, and 30 mg/L. In 16 mL borosilicate centrifuge tubes, fitted with Teflon coated screw caps, 15 mL of the aqueous RDX solutions was combined with 2 g of nonsterile topsoil.

Centrifuge tubes were wrapped in aluminum foil, sealed with Teflon coated caps without degassing, and agitated on a Wrist Action shaker (Burrell Corp., Pittsburgh, PA) for 22 h. The tubes were centrifuged for 30 min at 3500 rpm, and the supernatant was filtered using a Millex-HV 0.45 μm filter unit (Millipore Corp., Bedford, MA). Sorption of the RDX by the filter unit was negligible. The mass of RDX sorbed by topsoil was calculated by difference. All experiments were conducted in triplicate.

Desorption was conducted by adding 15 mL of distilled water to the soil pellets for all five initial concentrations following sorption. The initial desorption step was 24 h, as this time frame was determined to be sufficient to achieve equilibrium (results not shown). The interstitial solution volume that remained with the soil pellet was estimated gravimetrically. Using the same methods just described, a second desorption step that lasted 88 h was also employed.

Sorption Kinetics (Sterile and Nonsterile Soils). To verify that the results for 22 h sorption were not coupled with biotic losses of RDX, sorption was studied with nonsterile and sterile topsoil. These results were also used to verify the time required to achieve equilibrium with respect to sorption. The initial RDX concentration was 10 mg/L, and samples were analyzed in triplicate following 1, 19, 24, 44, and 72 h of sorption.

Long-Term Fate. The long-term fate of RDX in sterile and nonsterile topsoil was monitored over 7 weeks. Particularly, 15 mL of 10 mg/L RDX was combined with 2 g of topsoil, as was done for the sorption–desorption experiments. Sufficient vials were prepared to allow for sacrificial sampling. The flasks were sealed with aluminum coated capes with degassing. Each week, the aqueous phase and the remaining soil pellet were subjected to acetonitrile extraction (see next section), in triplicate. The aqueous phases and acetonitrile extracts were analyzed for RDX (described subsequently). These experiments were static and were conducted at 25 °C.

Acetonitrile Extraction. RDX was extracted from sterile and nonsterile topsoil using the EPA SW-846 Method 8330 acetonitrile extraction procedure (*19*). Briefly, the soil was combined with 10.0 mL of acetonitrile, vortexed, and placed in a sonicator bath (20 kHz) cooled to 22 °C (Blackstone Ultrasonics, Jamestown, NY) for 18 h. After sedimentation, 5.0 mL of the supernatant was combined with 5.0 mL of a 5 g/L $CaCl_2$ solution. The solutions were agitated and settled for 15 min prior to sample preparation for HPLC/LC-MS analysis. Based on a material balance, the percent recovery of RDX was calculated as follows

$$\%\ \text{recovery} = ((RDX_{solid} + RDX_{aqueous})/RDX_{total}) \times 100\% \quad (1)$$

where RDX_{solid} is the RDX recovered by acetonitrile extraction of soil, $RDX_{aqueous}$ is the RDX present in the aqueous phase, and RDX_{total} is the total amount of RDX present. All terms in eq 1 are expressed in units of moles.

Mineralization. Mineralization experiments were prepared in 120 mL serum bottles with 3 g of topsoil and 15 mL of distilled water. For RDX mineralization to CO_2, 0.051 μCi of [UL-^{14}C]RDX was combined with unlabeled RDX for a total RDX amount of 1.27 mg in the serum bottle. Samples were prepared in triplicate with sterile and nonsterile topsoil and incubated statically at 35 °C. The headspace was not augmented with air or oxygen. It was determined that the RDX was completely soluble at this temperature. Each serum bottle was fitted with a small test tube containing 1 mL of 0.5 M KOH to trap liberated $^{14}CO_2$. Microcosms with [UL^{14}C]RDX were routinely sampled (every 2–3 days) for determination of $^{14}CO_2$ in the KOH trap using a Tri-Carb 4530 liquid scintillation counter (model 2100 TR; Packard Instrument Company, Meriden, CT). At the end of the experiments, the unfiltered and filtered aqueous phase (Millex-HV 0.45 μm filter unit) was also analyzed by liquid scintillation counting to determine the residual radioactivity remaining in the respective phases.

For RDX mineralization to N_2O, microcosms were prepared as just described for CO_2 mineralization, except that only unlabeled RDX (1.27 mg) was used (the headspace was sampled every 2–3 days and analyzed by GC, as described subsequently). Microcosms were similarly prepared with ring-labeled [^{15}N]RDX, and the headspace was analyzed by gas chromatography–mass spectroscopy (GC-MS), as described subsequently.

Analytical Methods. RDX concentrations were determined by reversed-phase high-pressure liquid chromatography (HPLC) with a photodiode array (PDA) detector. The Waters (Waters Associates, Milford, MA) HPLC system consisted of a model 600 pump, 717 Plus autosampler, and a 996 PDA detector ($\lambda = 254$ nm). A Supelcosil LC-CN column (25 cm × 4.6 mm, particle size 5 μm) was coupled with a Temperature Control Module held at 35 °C. The methanol/water gradient was at a flow rate of 1.5 mL/min. The initial solvent composition was 30% methanol and 70% water that was held for 8 min. Following this, a linear gradient was run from 30 to 65% methanol over 12 min. The solvent ratio was changed to the initial conditions over 5 min and held for another 5 min. The system was outfitted with Millenium data acquisition software.

Liquid chromatography/mass spectrometry (LC/MS) was used to verify the presence of RDX, MNX, DNX, and TNX. Analyte ionization was achieved in a negative electrospray ionization ES (−) mode. This system consisted of a Micromass Platform II benchtop single quadrupole mass detector fronted by a Hewlett-Packard 1100 series HPLC system equipped with a photodiode array detector. Samples (50 μL) from the microcosms were injected into a Supelcosil LC–CN column (25 cm × 4.6 mm; 5 μm particle size) at 35 °C. The instrument

TABLE 2. Freundlich Sorption and Desorption Parameters for RDX and Nonsterile Topsoil[a]

operation	K_d	n	r^2
sorption (K^s_d)	0.83	1.1	0.96
desorption # 1 ($K^{d}_{d}{}^{1}$)	4.13	1.1	0.96
desorption # 2 ($K^{d}_{d}{}^{2}$)	16.05	1.0	0.88

[a] 95% confidence intervals are shown for values of K_d and n determined from 18 data points.

conditions used are reported elsewhere (*9*). Confirmation of the identity of targeted metabolites (MNX and TNX) was accomplished by comparison with reference compounds simulated according to the methods of Brockman et al. (*14*).

A Perkin-Elmer Sigma 2000 GC connected to a Chromsorb 102 (60–80 mesh, 12′ × 1/8″) stainless steel column (Supelco, ON) coupled with an electron capture detector (ECD) (350 °C) was used for N_2O detection. Gas samples from the headspace of the serum bottles were sampled using a gastight syringe for subsequent injection to the GC using helium as a carrier gas (30 mL/min) at 50 °C. Identification was confirmed by comparison with a reference compound. The presence of N_2O as a product of RDX was confirmed by analyzing the headspace of microcosms containing ring-labeled [^{15}N]RDX by GC-MS to monitor the masses at 45 Da ($^{15}N^{14}NO$). A Hewlett-Packard 6890 GC (Mississauga ON), coupled with a 5973 quadrupole mass spectrometer, was used for this analysis. A GS-Gas Pro (30 m × 0.32 mm) capillary column (J & W Scientific, Folsom CA) was used under splitless condition. The column was maintained at 150 °C and 280 °C, respectively. The injection volume was 50 μL.

Results and Discussion

Sorption–Desorption. The following Freundlich isotherm has been found to be adequate to describe equilibrium sorption and desorption of explosives by and from soil (*5, 20–22*)

$$\frac{x}{m} = K_d C^n \quad (2)$$

where x/m is the mass of solute sorbed per unit mass of soil at equilibrium (μg/g), K_d is the capacity constant (L/kg), C is the aqueous equilibrium phase solute concentration (mg/L), and n is a constant. The capacity constant for sorption is denoted as K^s_d, and for desorption it is denoted as K^d_d. When $K^s_d < K^d_d$, sorption–desorption hysteresis exists.

The sorption capacity constant for RDX is considerably less than (K^s_d of 0.83 L/kg, Table 2) those of 2,4,6-trinitrotoluene and its two amino metabolites for the same topsoil (6.38–11.96 L/kg (*5*)). For other surface soils, values of K^s_d that range from 0.58 to 11 and 0.21–0.33 L/kg have been reported for TNT and RDX, respectively (*23*). However, since the desorption isotherms lie above the sorption isotherm (Figure 2), and the capacity constants for desorption are 10 to 100 times greater than for sorption (Table 2), there is considerable sorption–desorption hysteresis.

Long-Term Fate. Results of kinetic experiments indicate that equilibrium was achieved within 1 h and that there were no differences between the sterile and nonsterile systems over 72 h (data not shown). Therefore, the fate of RDX in sterile and nonsterile topsoil systems was studied over 7 weeks. The results indicate that following the first week, similar quantities of RDX were recovered from both sterile and nonsterile systems (Figure 3). Therefore, the sorption–desorption hysteresis described in the previous section (Figure 2) is attributable to abiotic processes such as sorption.

Between the second and seventh weeks, the recovery of RDX (eq 1) from sterile systems remained between 55 and

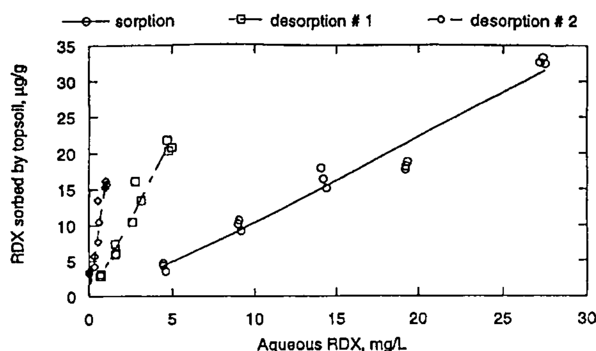

FIGURE 2. Sorption–desorption isotherms for RDX and topsoil.

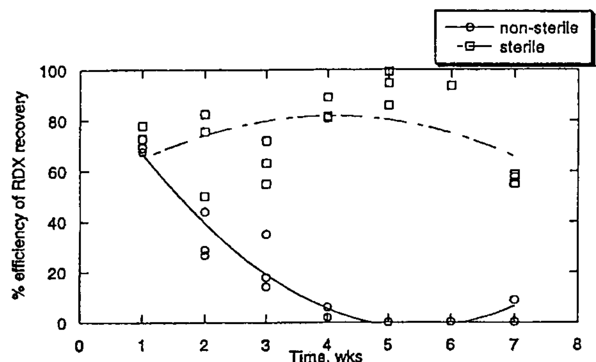

FIGURE 3. Efficiency of total RDX recovered from the aqueous and solid-sorbed phases of sterile and nonsterile topsoil.

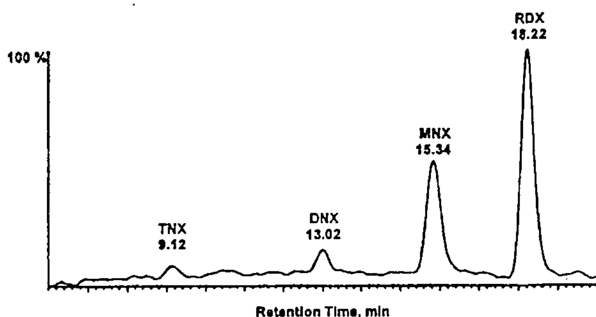

FIGURE 4. LC-MS chromatogram for aqueous phase following 4 weeks of contacting RDX (10 mg/L) with nonsterile topsoil.

99%. In contrast, the recovery of RDX from nonsterile topsoil fell from 43 to less than 1% during the same period. Furthermore, the three nitroso metabolites were evident (mostly in the aqueous phase) for the nonsterile topsoil between two and seven weeks. The nitroso metabolites were absent in sterile topsoil over the entire experiment. The presence of these three metabolites in the aqueous phase in nonsterile topsoil (4 weeks contact) was confirmed by LC-MS, and a representative chromatogram is shown in Figure 4. Anaerobic conditions were evidenced by the stench when vials were sampled, and since sampling was sacrificial, air was not introduced to the vials during the course of the experiment.

The fact that the three nitroso metabolites were mainly observed in the aqueous phase, and not in the solid sorbed phase (i.e. in the acetonitrile soil-extracts), is not surprising since the sorption capacity constant of the topsoil for RDX is quite low (K^s_d of 0.83 L/kg, Table 1). Hence, the nitroso metabolites form in the aqueous phase, and they are not subsequently sorbed by topsoil. However, since we did not have access to pure forms of the nitroso metabolites (they are not commercially available), it was not possible to further characterize their behavior in soil.

Mineralization of RDX. In our previous work, we identified CO_2 and N_2O as products from the mineralization of RDX by *Phanerochaete chrysosporium* (*10*) and an anaerobic consortium from food processing sludge (*9*, *11*). By using ring labeled [^{15}N]RDX, both studies confirmed that one nitrogen in N_2O originates from RDX. Previous to these studies, it was not known that N_2O could form from RDX by ring cleavage. In the present study, we sought to determine whether N_2O formed from RDX by microbial activity of the indigenous microorganisms in the topsoil. Results of this study indicate that only traces of the N_2O that formed originated from RDX. Particularly, one N originated from the ring, and the other from the nitro substituent. This was concluded by analyzing the N_2O formed in experiments using ring labeled [^{15}N]RDX. In particular, the headspace was analyzed by GC-MS, and the mass spectra showed a mass of 45 Da (indicative of $N^{15}N^{14}O$ (*9*)). The mass of 45 Da represented a maximum of 3% of the total N_2O that formed. However, N_2O formation by indigenous microorganisms, presumably by nitrification and denitrification, was far greater than what was formed by ring cleavage of RDX. In particular, nonsterile controls (i.e. topsoil with no RDX) showed N_2O evolution with time. Coupled with the fact that there was little conversion of RDX to CO_2, it can be concluded that RDX was not extensively mineralized in the topsoil.

Attempts to identify other ring cleavage products or metabolites by LC-MS were not successful. McCormick et al. (*8*) postulated that the biodegradation of RDX proceeds by successive reduction of the nitro groups to a point where destabilization and fragmentation of the ring occurs in liquid culture. In two later studies, we showed that N_2O and CO_2 were the final end products in both anaerobic and aerobic liquid cultures (*9*, *10*). The absence of extensive mineralization in the present system may be due to the adsorption of ring cleavage products by soil or binding with dissolved organic matter. The topsoil contained 8.4% organic carbon (Table 1), and the presence of dissolved organic matter was visible. Binding of organic contaminants by such solid soil particles and possibly by dissolved organic matter are known to reduce the availability of organic contaminants to microorganisms for biodegradation in soil (*24*–*27*). To further investigate this possibility, the radioactivity in the aqueous phase was measured at the end of the [^{14}C]RDX mineralization experiments. Results indicate that although RDX had disappeared, and the nitroso metabolites were no longer present, 1.93% ($\pm$ 0.59) of radioactivity was recovered as $^{14}CO_2$, and 53.06% ($\pm$ 2.47) of the radioactivity was recovered from the aqueous phase. Therefore, by difference, approximately 45% of the radioactivity partitioned with the solid-sorbed phase. Such partitioning may have rendered the sorbed phase as unavailable to the indigenous microorganisms for mineralization. However, at this point, the chemical structures of the breakdown products in the aqueous and solid-sorbed phases remain unknown. Based on the results of the present study, in contrast to what we observed in liquid culture (*9*, *10*), N_2O formation in topsoil is slow and the yield is small; hence, measurement of N_2O for monitored natural attenuation does not appear to be a useful option.

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Biotransformation Routes of Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by Municipal Anaerobic Sludge

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Recently we demonstrated that hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a trimer of methylene nitramine (CH_2=N—NO_2) undergoes spontaneous decomposition following an initial microbial attack using a mixed microbial culture at pH 7 in the presence of glucose as carbon source. The present study describes whether the second cyclic nitramine octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), a more strained tetramer of CH_2=N—NO_2, degrades similarly using sludge of the same source. Part of HMX biotransformed to give products that are tentatively identified as the nitroso derivatives octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine (mNs-HMX) and octahydro-1,3-dinitroso-5,7-dinitro-1,3,5,7-tetrazocine and its isomer octahydro-1,5-dinitroso-3,7-dinitro-1,3,5,7-tetrazocine (dNs-HMX). Another fraction of HMX biotransformed, apparently via ring cleavage, to produce products that are tentatively identified as methylenedinitramine ($O_2NNHCH_2NHNO_2$) and bis(hydroxymethyl)nitramine ($(HOCH_2)_2NNO_2$). None of the above intermediates accumulated indefinitely; they disappeared to predominantly form nitrous oxide (N_2O) and formaldehyde (HCHO). Formaldehyde biotransformed further to eventually produce carbon dioxide ($^{14}CO_2$). Nitrous oxide persisted in HMX microcosms containing glucose but denitrified rapidly to nitrogen in the absence of glucose. The presence of nitrous oxide was accompanied by the presence of appreciable amounts of hydrogen sulfide, a known inhibitor of denitrification.

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are powerful highly energetic chemicals that are widely used in various commercial and military activities (*1*, *2*). Both cyclic nitramines are toxic (*3*, *4*) and have adverse effects on the central nervous system of mammals (*5*). The widespread contamination by these explosives necessitates that contaminated soil and groundwater be remediated. Physicochemical techniques such as incineration (*2*) and alkaline hydrolysis (*6*, *7*) are neither cost-effective nor environmentally safe. Several studies on the biodegradation of RDX and HMX have been reported (*8*–*15*), but in most cases very little information is available on the type of products and the degradation pathways of these chemicals.

Earlier McCormick et al. (*8*) proposed a degradation pathway for RDX based on the sequential reduction of the energetic chemical to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine, and hexahydro-1,3,5-trinitroso-1,3,5-triazine. The nitroso metabolites are suggested to undergo further reduction prior to ring cleavage to yield HCHO, CH_3OH, NH_2NH_2, and $(H_3C)_2NNH_2$. Most recently Hawari et al. (*16*) reported the dominant formation of CO_2 and N_2O as end products from the biodegradation of RDX with a domestic anaerobic sludge and identified at least two pathways for its degradation. One route involved reduction of the nitro groups in RDX to the nitroso derivatives, and another novel route involved a direct ring cleavage to produce methylenedinitramine ($O_2NNHCH_2NHNO_2$) and bis(hydroxymethyl)nitramine ($(HOCH_2)_2NNO_2$) (*16*). The nitramines undergo spontaneous chemical decomposition in water to nitrous oxide and formaldehyde.

It is our objective in the present study to apply a combination of analytical techniques (LC/MS, SPME/GC–MS, capillary electrophoresis/UV, and other GC methods) to identify most possible intermediate products involved in the (bio)transformation of HMX with anaerobic sludge taken from the same source used to treat RDX (*16*). A comprehensive knowledge of the degradation products of this family of cyclic nitramine explosives would help to understand their degradation pathways and, consequently, would improve our understanding of the metabolic routes to their mineralization.

Experimental Section

Materials and Methods. Commercial grade HMX (with a purity >99%) was provided by Defense Research Establishment Valcartier, Quebec, Canada. Uniformly labeled [UL-^{14}C]HMX was synthesized according to the procedure described in ref *17*. The chemical and radiochemical purity as measured in our laboratory reached 94% and 91%, respectively. The specific activity of the radioactive compound was 93.4 μCi/mmol. All other chemicals were reagent grade. The municipal sludge, which in the past proved to be an excellent source of microorganisms, particularly methanogens (*18*), was obtained from a food factory (Cornwall, ON, Canada) and was used as the exogenous source of microorganisms. The sludge was always obtained fresh and stored at 4 °C when not in use. The viability of the sludge was measured using a glucose activity test (*19*). On average, the biomass concentration of the sludge was 8 g of VSS/L (volatile suspended solid) with a 0-mV reduction potential (E_h) before incubation that dropped down to a range of −250 to −300 mV during the biodegradation of HMX. The drop in E_h is possibly related to several fermentative processes such as those leading to the production of hydrogen from other cosubstrates. The sludge was also found to contain several heavy metals (mg/kg dry weight) including iron (8300), copper (44), nickel (36), and manganese (11). A BBL dry anaerobic indicator (VWR, Canlab, ON, Canada) was placed inside the microcosm to detect air leaks to ensure anaerobic conditions.

Microcosms Description for the Degradation of HMX. In a typical setup, a serum bottle (100 mL) was charged with anaerobic sludge (5 mL) and a mineral salt medium (10 mL) composed of 0.15 g/L NaH_2PO_4, 0.45 g/L K_2HPO_4, 0.02 g/L $MgSO_4$, and 0.24 g/L Na_2SO_4. Glucose (2.1 g/L) was used to serve as a carbon source and HMX (100 mg/L) as the only

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extraneous N-source for the degrading microorganisms using a final volume of 50 mL. However, we expect the sludge to contain other organic nitrogenous compounds that could also serve as a nitrogen source to the degrading microorganisms. We used high concentrations of HMX in an attempt to generate sufficient amounts of metabolites for detection. To account for an insoluble or suspended portion of HMX [water solublity is ca. 5 mg/L (4)], all the content of the microcosm was extracted in acetonitrile. Some microcosms were supplemented with [UL-^{14}C]HMX (100 000 dpm) and then fitted with a small test tube containing 1.0 mL of 0.5 M KOH to trap liberated carbon dioxide ($^{14}CO_2$). The headspace in each microcosm was flushed with nitrogen-free argon gas to maintain anaerobic conditions and then sealed with butyl rubber septa and aluminum crimp seals to prevent the loss of CO_2 and other volatile metabolites. For the analysis of N_2 and N_2O, sampling of the gaseous products from the headspace was performed using a gas-tight syringe. Two control microcosms were prepared: one contained the sludge without HMX, and the second contained HMX and an autoclaved sludge. Each microcosm was wrapped with aluminum foil to protect the mixture against photolysis. Microcosms with [UL-^{14}C]HMX were routinely sampled (on hourly and/or daily basis) for the determination of $^{14}CO_2$ in the KOH trap using a Packard, Tri-Carb 4530 liquid scintillation counter (model 2100 TR, Packard Instrument Company, Meriden, CT).

Analysis of HMX and Its Intermediate Products. The analytical part was worked out as described in ref *16*. Briefly, acetonitrile (50 mL) was added to the HMX-treated culture medium and mixed for few minutes at room temperature. Aliquots (1 mL) from the above treated culture medium were filtered through a 0.45-μm pore-size Millex-HV filter. The filtered culture medium (50 μL) was analyzed for the remaining HMX using a Waters HPLC system (Waters Chromatography Division) composed of a model 600 pump, an auto-injector (model 717 plus), and a Model 996 photodiode array detector. Detection and quantification was accomplished at λ 254 nm (*16*).

The nitroso derivatives and ring cleavage products of HMX were analyzed using a Micromass Plattform benchtop single quadrupole mass detector fronted by a Hewlett-Packard 1100 series HPLC system connected to a Supelcosil LC−CN column (25 cm × 4.6 mm; 5 μm particle size). The solvent system consisted of a methanol/water gradient at a flow rate of 1 mL/min. Analyte ionization was done in a negative electrospray ES(−) ionization mode producing mainly $[M-H]^-$. Further details on the method can be found in ref *16*.

Formaldehyde was detected as its oxime derivative using an SPME fiber coated with poly(dimethylsiloxane)/divinylbenzene (Supelco) and the derivatizing agent *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine as described by Martos and Pawliszyn (*20*) and Hawari et al. (*16*). Formic acid, HCOOH, was analyzed by capillary electrophoresis (CE) and UV detection using a Hewlett-Packard ^{3D}HPCE system consisting of a photodiode array detector following the procedure that was described by Chen et al. (*21*) and developed latter for the analysis of RDX metabolites (*16*). Formic acid was initially detected and quantified at 340 nm (210 nm reference) with a limit of detection of 200 μg/L. The identity of HCOOH as a degradation product of HMX was confirmed using [UL-^{14}C]HMX and by collecting the product by HPLC fractionation for subsequent radioactivity measurement.

Measurement of HMX Gaseous End Products N_2O, N_2, and $^{14}CH_4$. A SRI 8610 GC (INSUS Systems Inc.) connected to a Supelco Porapack Q column (2 m) and coupled with either a electron capture detector (ECD) (330 °C) for the detection of N_2O or a radioactivity detector (RAM) for the detection of $^{14}CH_4$ were used. The gaseous products from the headspace of the culture medium were sampled using a gas-tight syringe for subsequent injection inside the GC using helium as a carrier gas (21 mL/min) at 60 °C. Gas identification was confirmed by comparison with reference materials. The detection limit for RAM and ECD was 150 dpm and 12 ppm, respectively. Gaseous nitrogen was analyzed with an HP GC connected to Supelco Chomosorb 102 column coupled with a thermal conductivity detector (TCD).

Attempted Analysis of Hydrazine, Dimethyl Hydrazine, and Other Nitrogen Species Such as NH_4^+ and NO_2^-. Ion chromatography (Dionex model DX-500 ion chromatograph system) consisting of a GP40 gradient pump and coupled with electrochemical detector (pulsed detection mode) was used to analyze hydrazines as described by Larson and Strong (*22*). Samples (25 μL) from the culture medium were injected into a Hamilton PRP-X200 (250 mm × 4.1 mm × 10 μm) analytical cation-exchange column using 30% methanol in 4 mM nitric acid at a flow rate of 1 mL/min. Standards of hydrazine and dimethylhydrazine were employed for confirmation. A more sensitive technique (SPME/GC−MS) with a picogram detection limit was also employed to confirm the absence of hydrazines as HMX metabolites. Ammonium cation was analyzed for in the aqueous phase of the culture medium using an SP 8100 HPLC system equipped with a Waters 431 conductivity detector and a Hamilton PRP-X200 (250 mm × 4.1 mm × 10 μm) analytical cation-exchange column using 30% methanol in 4 mM nitric acid at a flow rate of 0.75 mL/min. The NO_2^- was analyzed using the CE as described by Okemgbo et al. (*23*) using sodium borate (25 mM) and hexamethonium bromide (25 mM) as an electrolyte at pH 9.2.

Results and Discussion

HMX Nitroso Metabolites. The LC/MS ES(−) of HMX after treatment with domestic anaerobic sludge over a period of 6 days (Figure 1) showed several peaks with deprotonated molecular mass ions [M − H] appearing at *m/z* 279, 263, and 263 Da, matching molecular mass formulas of $C_4H_8N_8O_7$, $C_4H_8N_8O_6$, and $C_4H_8N_8O_6$, respectively. Using the above obtained deprotonated molecular mass ions [M − H], the peaks are tentatively identified as mono nitroso, octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine [279 Da] (mNs-HMX), and two isomers of the dinitroso derivative, (octahydro-1,3-dinitroso-5,7-dinitro-1,3,5,7-tetrazocine) [263 Da] and (octahydro-1,5-dinitroso-3,7-dinitro-1,3,5,7-tetrazocine) [263 Da] (dNs-HMX). We did not detect any of the trinitroso derivative, although this compound was formed as a major product during biodegradation of HMX in soil slurry (30% w/v) using the same type of sludge (*24*).

None of the above nitroso derivatives accumulated in the system indefinitely. They biotransformed with no clear indication of their actual fate. We did not observe any hydroxylamino derivatives (HOHN−HMX) described earlier during biodegradation of RDX with anaerobic sludge by McCormick et al. (*8*). Other studies reported the formation of nitroso derivatives during biodegradation of RDX and/or HMX with either soil isolates (*Providencia rettgeri, Citrobacter freundii, Morganella morganii*) of the *Enterobacteriaceae* family (*11*) or consortia of a horse manure under O_2-depleting conditions (*12, 13*).

Ring Cleavage Intermediates. The LC/MS chromatogram of the sludge-treated HMX also showed the initial presence of several other peaks and presumed to be ring cleavage products of the original HMX molecule (Figure 1). For instance, one LC/MS peak showed a deprotonated molecular mass ion [M − H] at 135 Da, matching a molecular mass formula of $CH_4N_4O_4$ (MW 136 Da). Another characteristic mass ion was detected at *m/z* 61 Da, representing the fragment mass ion -$HNNO_2$. Whereas another LC/MS peak

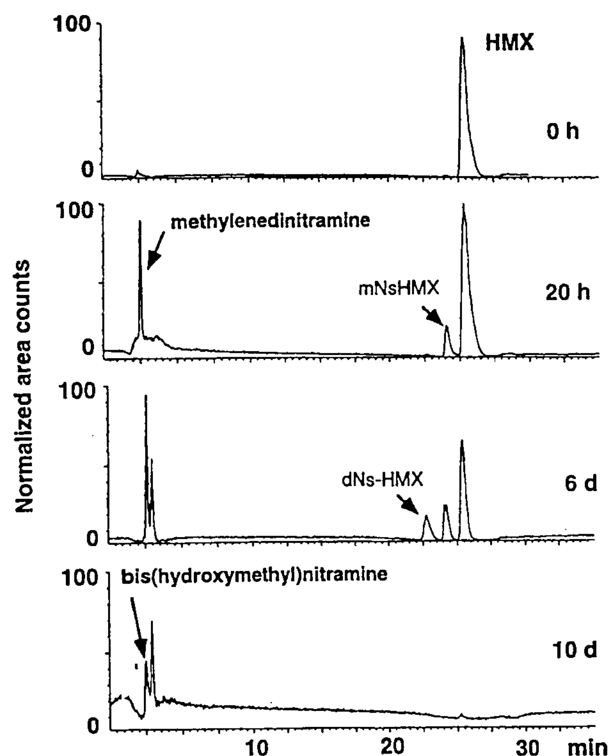

FIGURE 1. LC/MS (ES−) time course of biotransformation of HMX (100 mg/L) with a domestic anaerobic sludge in the presence of glucose as a carbon source at pH 7.0. The *y*-axis represents normalized area counts.

showed a deprotonated molecular mass ion [M − H] at 121 Da matching a molecular mass formula of $C_2H_6N_2O_4$ (MW 122 Da). Other relevant mass ions included one at *m/z* 139 Da representing ([M − H] + H_2O). We work out the identities of both peaks by comparing their chromatographic and mass data (retention times and mass ions) with those obtained earlier from the degradation of RDX with sludge taken from the same source (*16*). Both RDX and HMX gave the two products at the same retention time (3.09 and 3.88 min, respectively). In the case of RDX, the two peaks were tentatively identified using ring-labeled [^{15}N]RDX as the ring cleavage products methylenedinitramine ($O_2NNHCH_2NHNO_2$) and bis(hydroxymethyl)nitramine [$(HOCH_2)_2NNO_2$], respectively. Likewise, we tentatively identified the two LC/MS peaks observed during treatment of HMX with the sludge as the ring cleavage products methylenedinitramine and bis-(hydroxymethyl)nitramine, respectively. This is not surprising in view of the fact that the two cyclic nitramines RDX and HMX are structurally similar. Both compounds are cyclic oligomers (trimer and tetramer, respectively) of the same chemical unit methylenedinitramine, $CH_2{=}N{-}NO_2$ [$(CH_2NNO_2)_3$ for RDX and $(CH_2NNO_2)_4$ for HMX].

Furthermore, we detected several other LC/MS peaks including one that showed a [M − H] at *m/z* 121 Da, matching a molecular mass formula of $CH_6N_4O_3$ (MW 122). Other relevant mass ions in this peak included one at *m/z* 139 Da representing the solvent adduct ([M − H] + H_2O) and another at *m/z* 46 Da, representing the fragment mass ion -NO_2 (*m/z* 46 Da). The peak was tentatively identified as *N*-nitramino-*N'*-hydroxylaminomethylamine ($O_2NHNCH_2NHNHOH$). We are not sure at the present time whether the product is a reduced form of the previously formed methylenedinitramine ($O_2NHNCH_2NHNO_2$) or is a ring cleavage product of the hydroxylamine derivative of HMX (HOHN−HMX). McCormick et al. (*8*) postulated the presence of HOHN−RDX during biotreatment of RDX with anaerobic sludge but did not observe them.

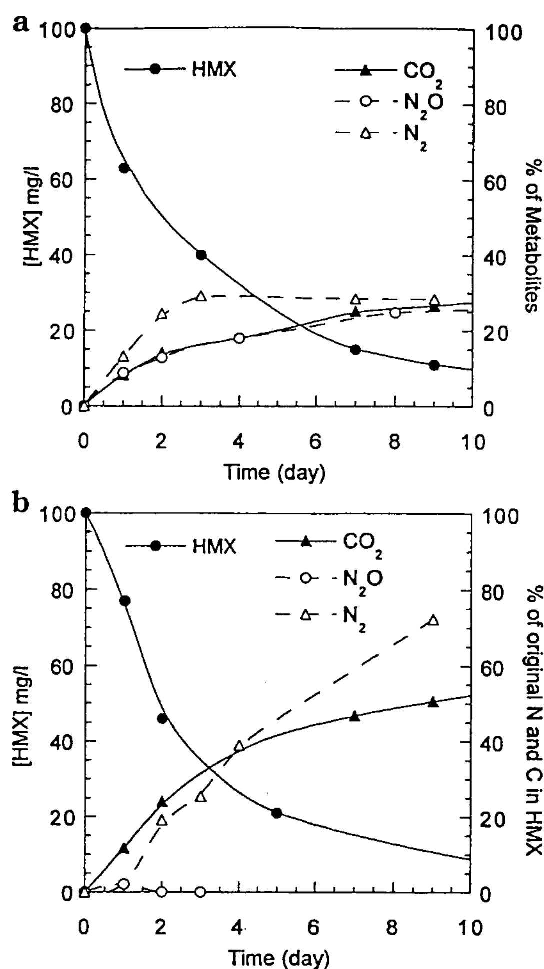

FIGURE 2. Time course study for the biotransformation of HMX with the domestic anaerobic sludge (a) in the presence of glucose and (b) in the absence of glucose.

None of the above HMX ring cleavage products accumulated indefinitely. They all transformed and produced predominantly formaldehyde (HCHO) and nitrous oxide (N_2O). The presence of formaldehyde as HMX degradation product was confirmed by the detection of $H^{14}CHO$ using [UL-^{14}C]HMX. We were also able to detect formic acid and confirmed its presence by the detection of $H^{14}COOH$. Eventually HCHO (bio)transformed to carbon dioxide (detected as $^{14}CO_2$).

Neither N_2O nor $^{14}CO_2$ was observed in controls containing either the buffer and HMX or HMX and dead biomass. Roughly 40% of total HMX initial concentration (100 mg/L) was attributed to mineralization ($^{14}CO_2$) after 40 d of incubation. Furthermore, methane ($^{14}CH_4$) was detected and confirmed by the use of uniformly labeled [UL-^{14}C]HMX. Neither hydrazine nor dimethylhydrazine [$(CH_3)_2NNH_2$] reported earlier by McCormick et al. (*8*) was observed in either the present study or the previously described RDX study (*16*).

On the other hand, we detected nitrogen when HMX was treated with the sludge under a blanket of argon. Earlier we confirmed that RDX biodegradation with the same sludge gave N_2O and N_2 as end products. The formation of the two gases was confirmed by using ring-labeled [^{15}N]RDX and the detection of $^{15}N^{14}NO$ (*m/z* 45 Da) and $^{15}N^{14}N$ (*m/z* 29 Da) (*16, 25*). No ^{15}N-labeled HMX was available at the time of the present experiment, but the presence of N_2O and N_2 was confirmed by comparing their GC retention times with reference standards and also by their absence in the controls (described above).

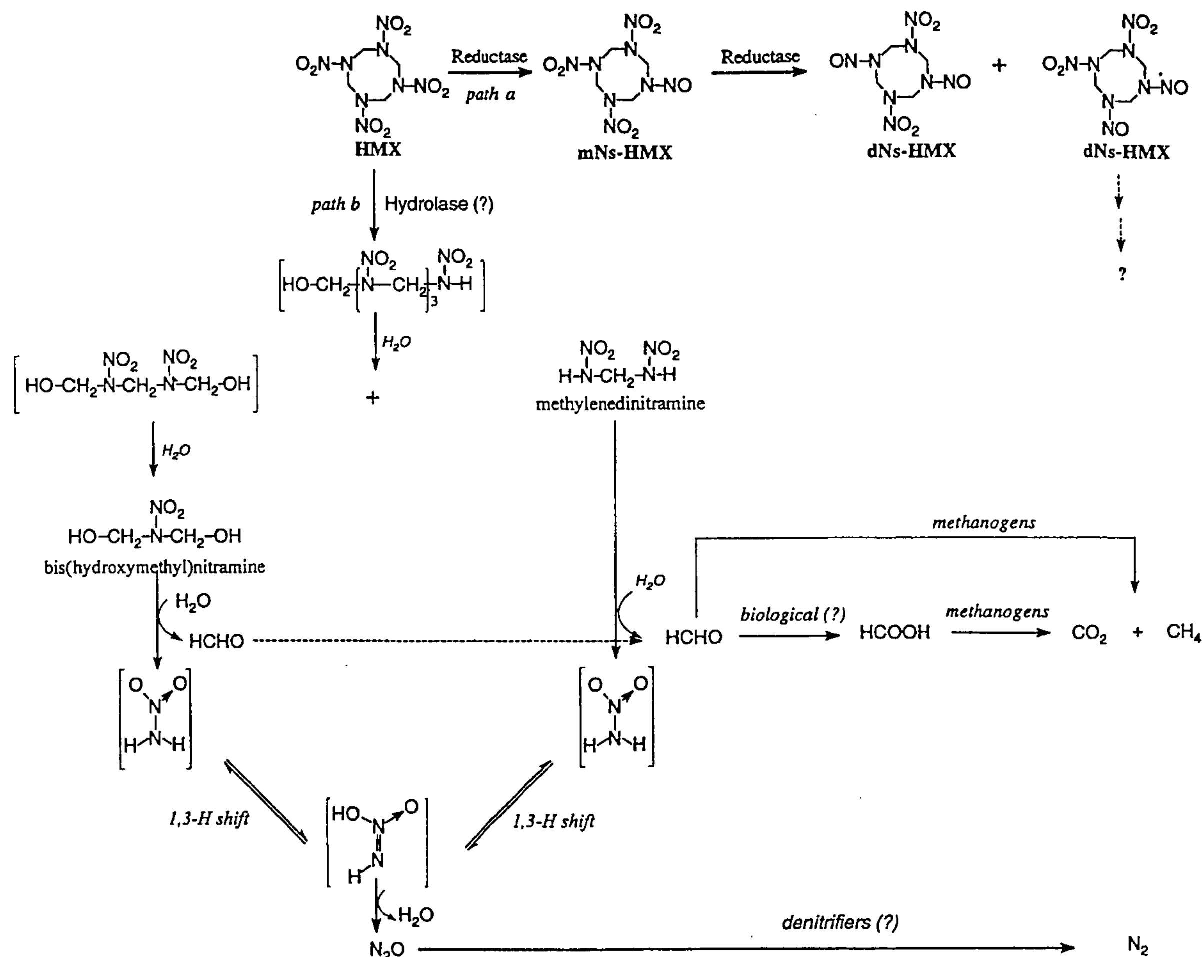

FIGURE 3. Potential biodegradation routes of HMX during treatment with anaerobic sludge. Path a: Reduction via nitroso route who were later removed from the system without identifying their products (?). Path b: Ring cleavage followed by competing chemical and biochemical transformations. A square bracket indicates unidentified product whereas a question mark indicates potential presence that requires further experimental verification. In the case of RDX, McCormick et al. (*8*) reported the formation of hydrazines via this route.

Time Course Study and the Degradation Pathway. Figure 2, panels a and b, represents time course studies for the disappearance of HMX and the appearance of N_2O, N_2, and CO_2 in the presence and absence of glucose, respectively. Roughly 50% of the total nitrogen content of reacted HMX (90%) was detected as gaseous nitrogen and nitrous oxide. Using solubility data, another 4% of N_2O could be accounted for as a dissolved fraction in the aqueous phase (*26*). Formaldehyde did not accumulate, and its disappearance was accompanied by the formation of formic acid, HCOOH, methane (CH_4), and carbon dioxide (CO_2). The concentrations of HCHO and HCOOH were not high enough and thus are excluded from Figure 2. This is in contrast to RDX where we successfully measured HCHO and the ring cleavage product methylendinitramine with time (*16*). This is possibly caused by a lower biological reactivity of HMX as compared to RDX. It is known that HMX is less water soluble (5 mg/L) than RDX (40 mg/L) (*4*) and is chemically more stable (*27*).

Furthermore, controls containing either HMX in a phosphate buffer (pH 7) in the absence of the sludge or HMX in a killed biomass did not give any of the previously described nitroso or ring cleavage products, indicating the essential role of enzymatic reactions on HMX. We expect the domestic sludge used in the present study to contain a microbial community capable of producing several enzymes including nitroreductases and hydrolases. Recently Kitts et al. (*28*) reported the degradation of RDX by the enteric bacteria *Morganella morganii* via the oxygen-insensitive type I nitroreductase. Reduction of the $-NO_2$ group in HMX by the same type of nitroreductase would produce the corresponding nitroso derivative. Subsequent reduction of the resulting nitroso group might produce the HOHN–HMX derivative, which was earlier implicated as a prerequisite entity prior to ring cleavage in the RDX pathway described by McCormick et al. (*8*) (Figure 3, path a). Neither the present HMX study nor the earlier study on RDX revealed the presence of these hydroxylamino derivatives (*16*).

On the other hand, we speculate that the presence of a hydrolase enzyme in the sludge might cleave a C–N bond in HMX to give the primary nitramine product $HOCH_2\text{-}[CH_2\text{-}NNO_2]_3\text{-}NHNO_2$ containing two reactive terminal functional groups, $-CH_2OH$ and $-NHNO_2$ (Figure 3, path b). Such compounds are not stable in water, and their continued spontaneous decomposition (enzymatic or chemical) would eventually produce nitramide (NH_2NO_2) and HCHO (Figure 3, path b). Hydroxyalkylnitramines are known to be unstable in water and exist as equilibrated mixtures with their dissociated products HCHO and NH_2NO_2 (*29–31*). The two metabolites methylenedinitramine and bis(hydroxymethyl)-nitramine do not represent the only HMX ring cleavage products in our study. There are possibly other undetected products that were produced in trace amounts. However, the detection of the above two products are particularly important because, as we described above, we were able to identify them using the chromatographic and mass data obtained in our earlier study with RDX (*16*).

Once formed, the nitramide molecule can undergo spontaneous hydrolytic decomposition to produce nitrous

oxide, N_2O (*6, 29–31*) (Figure 3, path b). The chemical decomposition of nitramide to nitrous oxide is well established and is suggested to initially involve a 1,3-H shift from nitrogen to oxygen followed by hydrolysis as shown in Figure 3 (*32*). Using ring-labeled [^{15}N]RDX, we previously found that one nitrogen atom in N_2O originated from the -NO_2 group and the second one originated from the ring (*16*).

Whereas the formation of nitrogen during degradation of HMX was considered to be biological because the gas only appeared in microcosms that contained HMX and live sludge (incubated under an atmosphere of argon), possibly unidentified denitrifiers in the sludge caused the transformation of nitrous oxide to nitrogen gas. St. John and Hollocher (*33*) and Garber and Hollocher (*34*) reported the formation of nitrogen as a metabolite from N_2O under anaerobic conditions using *Pseudomonas aeruginosa*. Also it has been reported that facultative and obligate anaerobic bacteria can reduce N_2O to nitrogen (*35*). Also we found that microcosms that contained glucose as a carbon substrate during HMX treatment with the anaerobic sludge showed both nitrous oxide and nitrogen gas as major products (Figure 2a). Unlike microcosms that did not receive glucose, nitrogen concentrations increased drastically and nitrous oxide was beyond the detection limit (Figure 2b). As one of our reviewers suggested, we expected to observe more reduction of N_2O to N_2 by denitrifies in the presence of excess e-donors such as glucose. Interestingly we found relatively high amounts of hydrogen sulfide in microcosms that received glucose. In contrast, only a trace amount of the sulfide was detected in the absence of glucose. It has been reported that sulfides are inhibitory to denitrification processes, and thus their presence might have contributed to the inhibition of denitrification, leading to the accumulation of N_2O (*36, 37*) (Figure 2a). Furthermore, VSS and the heavy metal cationic species (Fe, Mn, Ni, and Cu) in the sludge, depending on their oxidation states, might have also served as source of electron donors for the denitrification observed in the absence of glucose (Figure 2b).

Formic acid (detected as $H^{14}COOH$) was detected only in trace amounts that we were unable to measure at accurate concentration with time. We are not sure at the present time how HCOOH is produced in our system, although it has been reported that the acid can be formed as a ring cleavage product following the alkaline hydrolysis of cyclic nitramines. Formaldehyde, another detected HMX product, can also produce HCOOH under alkaline conditions (Canizarro reaction) (*6, 7*), but the pH during our study stayed near neutral. On the other hand, we speculate that methanogens in the sludge biotransformed formic acid into methane and carbon dioxide (Figure 2). Previously, a sludge of the same origin to the one used here proved to contain several consortia including methanogens (*18*).

Presently we are unable to determine the extent of the nitroso route (Figure 3, path a) relative to the ring cleavage one (Figure 3, path b) for HMX degradation. Also, we did not have any direct evidence of other degradative mechanisms such as reductive denitration. In the case of RDX, nonbiological reductive denitration via a bimolecular elimination (E_2) of HNO_2 yields the unstable intermediate 3,5-dinitro-1,3,5-triazacyclohex-1-ene, which undergoes spontaneous decomposition with a rate constant that is 10^5 times higher than that of RDX itself (*38*). On the other hand, the initial cleavage of an external N–NO_2 or C–H bonds of HMX would destabilize inner C–N chemical bonds (<5 kcal/mol), forcing the molecule to undergo rapid autodecomposition to N_2, N_2O, HCHO, and HCOOH (*32*). The above analysis might help explain why most reported biodegradation studies of cyclic nitramines (*25*) did not describe more than the removal of the explosive and in more specific cases the initial denitration processes as diagnostic tools to describe biodegradation (*39*). Often poor knowledge of intermediate metabolites are described, and without such knowledge the (bio)degradation pathways of RDX and HMX will stay unknown.

In summary, the present study shows that unidentified anaerobic microorganisms from a domestic sludge can convert HMX via at least two independent mechanisms: one involved the familiar reduction of -NO_2 to form the corresponding nitroso derivatives, and the other involved a direct ring cleavage. The present observation confirms the earlier results obtained with RDX using sludge of the same source (*16*). Since both explosives are frequently found together at contaminated sites, the observed similarity in their degradation patterns might help future efforts for their joint successful remediation. Certainly further investigation is needed to learn more about intermediate products of both RDX and HMX and the enzymes that produce them.

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An *in Situ* Respirometric Technique to Measure Pollution-Induced Microbial Community Tolerance in Soils Contaminated with 2,4,6-Trinitrotoluene[1]

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Long-term exposure to 2,4,6-trinitrotoluene (TNT) can induce changes in the structure and activities of soil microbial communities. Such changes may be associated with an elevated microbial tolerance. An *in situ* respirometry technique based on the analysis of the substrate-induced respiration response to freshly added TNT was used to examine soil microbial tolerance to TNT at the community level. The specific growth rate derived by fitting an exponential equation to respiration data was taken as the measurement endpoint. Microbial tolerance was evaluated using a tolerance index defined as the ratio of the specific growth rate at a spiking dose of 2000 μg TNT/g soil to that of the control with no spiked TNT. Three soils with long-term exposure histories (TNT level in soil: 1.5, 32, and 620 μg TNT/g, respectively) exhibited significantly higher microbial community tolerance to TNT than two uncontaminated control soils. A soil containing 29,000 μg TNT/g exhibited the highest tolerance. Findings from this study support the hypothesis that pollution-induced community tolerance can be used as a means of identifying those compounds that have exerted selective pressure on the community. © 2000 Academic Press

Key Words: **soil microbial community; 2,4,6-trinitrotoluene; pollution-induced community tolerance; substrate-induced respiration; specific growth rate.**

INTRODUCTION

Pollution-induced community tolerance (PICT) has recently been used as an ecotoxicological tool for assessing the toxic effects of a pollutant on an ecosystem at the community level (Blanck *et al.*, 1988; Dahl and Blanck, 1996; Rutgers *et al.*, 1998; Siciliano *et al.*, 2000). The PICT theory is founded on the principle that communities become tolerant due to individual acclimation, genetic or physiological adaptation, and loss of sensitive species as a result of long-term exposure to a toxicant or a mixture of toxicants (Posthuma, 1997). An elevated PICT can be used as an evidence that a toxicant has previously had contact with the community and may have caused structural changes.

A variety of approaches to examine the tolerance or resistance of soil microorganisms to heavy metals and recal-citrant organic compounds have been developed. A conven-tional approach is to isolate and culture the tolerant microorganisms from soil samples (Klausmeier *et al.*, 1974; Jordan and Lechevalier, 1975; Duxbury and Bicknell, 1983). However, an intrinsic shortcoming of the plate count tech-nique is that more than 99% of soil bacteria cannot be isolated on agar plates (Torsvik, 1995). Therefore, only a small proportion of soil microorganisms are available for further sensitivity testing. More recently, a thymidine or leucine incorporation technique (Bååth, 1992; Díaz-Raviña *et al.*, 1994; Díaz-Raviña and Bååth, 1996; Pennanen *et al.*, 1996) and Biolog microtiter plates (Rutgers *et al.*, 1998; Siciliano *et al.*, 2000) have been used to assess the tolerance of bacterial communities in soil. However, these two tech-niques also result in an incomplete evaluation of the entire microbial community because of insufficient extraction of bacteria from soil (Rutgers *et al.*, 1998; Siciliano *et al.*, 2000; Witter *et al.*, 2000).

In a previous study, Witter *et al.* (2000) developed an *in situ* respirometric technique based on the analysis of the substrate-induced respiration (SIR) response to test the microbial tolerance to heavy metals at the community level in soil ecosystems. These workers demonstrated that microbial metal tolerance increased as soil metal concentrations increased (Witter *et al.*, 2000). In the present study, this newly developed technique was used on soils contaminated with 2,4,6-trinitrotoluene (TNT) for several decades, since TNT is a proven toxicant to soil microorganisms and is very recalcitrant in soil (Klausmeier *et al.*, 1974; Fuller and

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ECOTOXICOLOGICAL EFFECTS OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE ON SOIL MICROBIAL ACTIVITIES

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Abstract—Although hexahydro-1,3,5-trinitro-1,3,5-triazine (also called RDX or hexogen) is a potentially toxic explosive compound that persists in soil, its ecotoxicological effects on soil organisms have rarely been assessed. In this study, two uncontaminated garden soils were spiked with 10 to 12,500 mg RDX/kg dry soil. Soil microbial activities, i.e., potential nitrification, nitrogen fixation, dehydrogenase, basal respiration, and substrate-induced respiration were chosen as bioindicators and were determined after 1-, 4-, and 12-weeks of exposure. Experimental results indicate that RDX showed significant inhibition (up to 36% of control) on indigenous soil microbial communities over the period of this study. All five bioindicators responded similarly to the RDX challenge. The length of exposure also affected the microbial toxicity of RDX, with 12-week exposure exerting more significant effects than the shorter exposure periods, suggesting that soil microorganisms might become more vulnerable to RDX when exposure is extended. The estimated lowest observable adverse effect concentration of RDX was 1,235 mg/kg. No biodegradation products of RDX were detected at all three sampling times. Compared with 2,4,6-trinitrotoluene (TNT), RDX is less toxic to microbes, probably because of its resistance to biodegradation under aerobic conditions, which precludes metabolic activation of nitro groups.

Keywords—Soil Hexahydro-1,3,5-trinitro-1,3,5-triazine Microbial activities Toxicity

INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine, commonly known as cyclonite, hexogen, or RDX (British code name for Research Department Explosive or Royal Demolition Explosive), is a crystalline high explosive [1]. As one of the most important military explosives, its ecotoxicity has been assessed, but primarily in aquatic systems. In acute studies, RDX is moderately toxic to fish with the 96-h LC50s ranging from 3.6 to >100 mg/L [2–4]. The RDX can significantly inhibit the growth of algae [5] even though no EC50 was derivable [2]. Recently, Burton et al. [6] and Sunahara et al. [7] reevaluated the acute 96-h toxicity of RDX to a green algae, *Selenastrum capricornutum,* both showing that nominal RDX concentrations up to 40 mg/L reduced about 40% of cell density. When a bioluminescent bacterium *Vibrio fischeri* was exposed to RDX, significant toxic effects were observed [7,8] with an EC50 above water saturation point [8]. However, at concentrations near its aqueous solubility limit (42.3 mg/L at 20°C [1]), RDX is neither mutagenic to *Salmonella typhimurium* strains TA98 and TA100 with or without S9 [9,10] nor cytotoxic to Chinese hamster lung V79 cells and TK-6 human lymphoblastic cells [10] and elicits little or no toxicity to the survival, growth, development, and reproduction of freshwater invertebrates [2,11]. Furthermore, concentrations of RDX up to 10 mg/L do not affect the growth of bush beans (*Phaseolus vulgaris*) when grown for 7 d under hydroponic conditions [12].

In the literature, there is very little information on RDX toxicity to terrestrial organisms. Simini et al. [13] reported that RDX concentrations (≥100 mg/kg in soil) caused significant biomass reduction in cucumber seedlings. Winfield et al. [14] found that exposure to RDX (up to 4,000 mg /kg soil) during the early life stage resulted in adverse responses in sensitive terrestrial plants such as sunflower and sanfroin. Significant sublethal effects of RDX were observed on the reproduction of earthworm *Eisenia andrei* at concentrations as low as 95 mg/kg soil [15]. However, no effects were found on the mortality and reproduction of two terrestrial invertebrates, *Enchytraeid* and *Folsomia,* in soils spiked with up to 1,000 mg RDX/kg soil [16].

To the best of our knowledge, no studies have been documented with regard to the effects of RDX on soil microorganisms whose functions are important for the health of the terrestrial ecosystem [17]. In the present study, soil microbial communities were exposed to RDX for up to 12 weeks. Soil dehydrogenase activity (DHA), potential nitrification activity (PNA), nitrogen fixation activity (NFA), basal respiration, and substrate-induced respiration, all of which are microbiologically mediated activities or processes frequently used for assessing the health of soil microbial communities [18], were chosen as bioindicators in order to evaluate the ecotoxicological impact of RDX on indigenous soil microorganisms.

MATERIALS AND METHODS

Experimental design

Two uncontaminated garden soils (GS1 and GS3) were used in this study. The GS1 soil was a sandy loam with a pH of 6.9 and total organic carbon of 11.2%, whereas the GS3 soil was a silty clay loam with a pH of 6.5 and total organic carbon of 3.5% (see [18] for their detailed physical and chemical characteristics). The soils used in the present study were collected in June 1999 from the same garden as in our previous studies [18]. The soils were sieved (2 mm) and frozen at −20°C for long-term storage [19]. Before use, the soils were thawed

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CHRONIC TOXICITY OF ENERGETIC COMPOUNDS IN SOIL DETERMINED USING THE EARTHWORM (*EISENIA ANDREI*) REPRODUCTION TEST

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Abstract—Earthworm survival tests are commonly used in terrestrial ecotoxicology to assess the toxicity of compounds in soil. Earthworm (*Eisenia andrei*) reproduction tests were used to assess the sublethal and chronic effects of 2,4,6-trinitrotoluene (TNT) and 1,3,5-trinitro-1,3,5,7-triazacyclohexane (RDX). Effects on reproduction parameters (total number of cocoons, number of hatched cocoons, number of juveniles, juvenile biomass, and hatchability) were measured in TNT- and RDX-spiked artificial soil. For TNT, the lowest-observed-effect concentration (LOEC) was 110 mg/kg dry soil, and the no-observed-effect concentration (NOEC) was 55 mg/kg. For the RDX-spiked soil, the LOEC was 95 mg/kg dry soil, and the NOEC was <95 mg/kg. The growth of adult worms was also reduced when exposed to TNT-spiked soil at the highest tested concentration (881 mg/kg dry soil). Taken together, data analysis showed that the number of juveniles was strongly correlated with the number of cocoons but poorly correlated with the growth of adults. This information could permit one to optimize the application of the *Eisenia* sp. reproduction assay when used as a sublethal effect assessment tool for TNT- or RDX-contaminated soils.

Keywords—Explosives Contaminated soil 2,4,6-Trinitrotoluene 1,3,5-Trinitro-1,3,5,7-triazacyclohexane 2-Chloroacetamide

INTRODUCTION

Nitroaromatic compounds such as 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitro-1,3,5,7-triazacyclohexane (RDX), and 1,3,5,7-tetranitro-1,3,5,7-tetrazacyclo-octane (HMX), as well as their metabolites, can be found in explosive-contaminated soil and groundwater [1,2]. These chemicals can be toxic to a number of aquatic organisms, including bioluminescent bacteria *Vibrio fisheri* (Microtox®, AZUR Environmental, Carlsbad, CA, USA), freshwater algae (*Selenastrum capricornutum*), invertebrates (*Ceriodaphnia dubia*), and fathead minnow (*Pimephales promelas*; [3–6]). Cytotoxic and mutagenic effects in bacterial cells have also been found using aqueous solutions of TNT [7–11] and TNT-contaminated soil [12]. In addition, TNT and other nitroaromatic compounds, such as 1,3,5-trinitrobenzene (TNB) and 2,6-dinitrotoluene (2,6-DNT), were cytotoxic using the rat liver hepatoma H4IIE [13]. Furthermore, TNT metabolites, such as 2-amino-dinitrotoluene (2A-DNT) and 4-amino-dinitrotoluene (4A-DNT), when in solution, showed bacterial mutagenicity [9–11].

The acute toxicity of nitroaromatic compounds in contaminated soils, composted explosive-contaminated soil, and elutriates has also been assessed using different test systems, including bacteria (Microtox; [1,5]) and terrestrial species such as plants (*Lactuca sativa*), earthworms (*Eisenia* sp.; [1,14–17]), nematodes, and microarthropods [18]. Effects on subsurface soil microbial communities have also been demonstrated [19,20]. Only a few studies have been reported in the peer-reviewed literature describing the sublethal and chronic effects of explosives and their metabolites on soil oligochaetes.

Lethal and sublethal effects (weight change) on earthworms (*Eisenia* sp.) by different nitroaromatic compounds (TNT, RDX, HMX) spiked into soils have been reported earlier [14,21]. In a U.S. Army Technical Report, the lethal effects of TNT on earthworms were found at a concentration of 150 mg/kg in a forest soil but not at 240 mg/kg of TNT in an artificial soil [14]. However, the lethal effects on earthworms using an artificial soil were measured at a much higher concentration (420 mg/kg; [21]). Phillips et al. [14] have also observed a sublethal effect (weight loss) at 140 mg/kg of TNT in artificial soil and at 150 mg/kg in a forest soil. These effects were not seen by Robidoux et al. [21]. No significant lethal effects were measured by Phillips et al. [14] at RDX and HMX concentrations under 500 mg/kg using a toxicity range-finding study; however, a concentration-dependent weight loss with both explosives was detected using the earthworm and would indicate the possibility of sublethal effects. The acute toxicity of TNT using the Organization for Economic Cooperation and Development (OECD) filter paper method [22] has also been reported [21].

Lethal and sublethal effects of nitroaromatic substances on *Eisenia* were also observed in mixed explosives-contaminated soil [14]. Soil containing TNT (60 mg/kg), TNB (100 mg/kg), 2,4-DNT (125 mg/kg), and 2,6-DNT (40 mg/kg) was lethal to earthworms. However, soils contaminated in part by HMX (50.7 mg/kg), RDX (55.9 mg/kg) and some TNT (0.8 mg/kg), 2,4-DNT (50.0 mg/kg), and 2,6-DNT (20.0 mg/kg) did not produce acute effects on earthworms. In addition, toxicity test-

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Chronic toxicity of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in soil determined using the earthworm (*Eisenia andrei*) reproduction test[☆]

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"Capsule": *Fecundity of the earthworm* Eisenia andrei *was reduced by the explosive HMX in soil.*

Abstract

The sublethal and chronic effects of the environmental contaminant and explosive octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in artificial soil were assessed using the earthworm (*Eisenia andrei*). Based on various reproduction parameters (total and hatched number of cocoons, number of juveniles and their biomass), fecundity was reduced at the different concentrations of HMX tested (from 280.0 ± 12.3 to 2502.9 ± 230.0 mg kg^{-1} dry soil) in spiked artificial soil (LOEC: 280.0 ± 12.3 mg kg^{-1} dry soil). The growth of adult *E. andrei* was also reduced at the different concentrations tested, though no mortality occurred, even at the highest tested concentrations. The number of juveniles produced was correlated with the number of total and hatched cocoons, and the biomass of juveniles was correlated with the number of cocoons. Pooled results of these and earlier studies on explosives (TNT, RDX) using the *E. andrei* reproduction test confirm that effects of HMX on cocoon production are indicative of some reproductive consequences (number of juvenile and their biomass), whereas adult growth, in general, does not correlate strongly with change in reproduction capacity. Crown Copyright © 2000 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Explosives; Contaminated soil; Toxicity test; TNT; RDX; HMX; 2-Chloroacetamide; Solvent effects; Acetonitrile

1. Introduction

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; high melting explosive) is commonly present in explosive-contaminated soil and groundwater (Griest et al., 1993; Simini et al., 1995; Hovatter et al., 1997; reviewed by Talmage et al., 1999). The environmental concentrations of HMX in soil on contaminated sites can range from 0.7 to 5700 mg kg^{-1}, whereas the groundwater and surface water ranged from 1.3 to 4200 and 1.9 to 67 μg l^{-1}, respectively (reviewed by Talmage et al., 1999). This explosive is toxic to some aquatic organisms, including fathead minnows (*Pimephales promelas*) and the freshwater microcrustacean *Daphnia magna* (reviewed by Talmage et al., 1999). HMX was not toxic to the bioluminescent bacteria *Vibrio fisheri* (liquid phase Microtox; Sunahara et al., 1998) and was not cytotoxic or mutagenic to bacterial and mammalian cells in aqueous solutions of HMX (Lachance et al., 1999).

HMX is also toxic to some terrestrial organisms including mice and rats (see review by Talmage et al., 1999). Lethal and sublethal effects (weight change) on earthworms (*Eisenia fetida* sp.) tested in soil containing different energetic substances (TNT, RDX, HMX) spiked soils have been reported earlier (Phillips et al., 1993; Robidoux et al., 1999a). An HMX concentration-dependent weight loss (14-day test) was detected in the earthworms and would indicate the possibility of sublethal effects, but no lethality was observed at concentrations of HMX up to 500 mg kg^{-1} (Phillips et al., 1993). However, little or no information is available on the

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Transformation of 2,4,6-Trinitrotoluene (TNT) by Immobilized *Phanerochaete chrysosporium* Under Fed-Batch and Continuous TNT Feeding Conditions

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Abstract: The cometabolic transformation of 2,4,6-trinitrotoluene (TNT) by an immobilized *Phanerochaete chrysosporium* culture was investigated under different TNT and/or glycerol feeding conditions in a 5-L reactor. In the fed-batch feeding mode, as a result of four spiking events at an average feeding rate of 20 mg TNT L^{-1} d^{-1} and 250 mg glycerol L^{-1} d^{-1}, the initial TNT transformation rate and the glycerol uptake rate of the 7-day-old immobilized cell culture were 2.41 mg L^{-1} h^{-1} and 16.6 mg L^{-1} h^{-1}, respectively. Thereafter, the TNT fed into the reactor depicted a negative effect on the cell physiology of *P. chrysosporium*, i.e., both rates decreased constantly. At 32 mg TNT L^{-1} d^{-1} feeding rate, also in the presence of glycerol (200 mg L^{-1} d^{-1}), this effect on the fungal cell metabolism was even more significant. When TNT was fed alone at 3.7 mg L^{-1} d^{-1}, it showed an initial 0.75 mg L^{-1} h^{-1} rate of TNT transformation, i.e., one-third the initial level observed in the presence of glycerol. In contrast, in the continuous feeding mode (dilution rate, D = 0.11 d^{-1}), at 5.5 mg TNT L^{-1} d^{-1} and 220 mg glycerol L^{-1} d^{-1}, the immobilized cell culture exhibited a constant TNT transformation rate for cultivation periods of 50 and 61 days, under uncontrolled and controlled pH conditions, respectively. Thereafter, during the latter experiment, 100% TNT biotransformation was achieved at 1,100 mg L^{-1} d^{-1} glycerol feeding rate. Immobilized cells (115-day-old), sampled from a continuous TNT feeding experiment, mineralized [^{14}C]-TNT to a level of 15.3% following a 41-day incubation period in a microcosm. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **73**: 271–281, 2001.

Keywords: *Phanerochaete chrysosporium*; 2,4,6-trinitrotoluene; TNT; cell immobilization; biodegradation; explosive; fungi

INTRODUCTION

Over the last century, human and industrial activities have contributed to the disruption and pollution of many aquatic and terrestrial ecosystems. The manufacturing of 2,4,6-trinitrotoluene (TNT) is one such activity. For instance, as reported by Fritsche et al. (2000), Lenke et al. (1998), and Tharakan and Gordon (1999), in Germany and the US, its current use and past disposal practices have resulted in extensive contamination of soils, sediments, surface waters, and groundwater. In Canada, the exact extent of TNT contamination is not known; however, two major munition producers have generated significant TNT wastes impacting soil and groundwater (Ampleman, pers. comm.). Moreover, TNT and its metabolites are xenobiotic compounds of known toxicity (Lachance et al., 1999; Talmage et al., 1999; Yinon, 1990) whose presence in the environment is a problem of public concern which needs to be addressed.

Fritsche (2000) provides an excellent overview of the degradation of TNT by fungal microorganisms, whereas Hawari et al. (1999) report a detailed mechanism of the TNT biotransformation by *Phanerochaete chrysosporium*. Over the past 10 years, various biological means have been developed for treating TNT-contaminated soils and waters. TNT reduction to its metabolites has been extensively demonstrated using various bioreactor configurations and under aerobic (Fernando et al., 1990; Fuller and Manning, 1997; Kalafut et al., 1998; Pucik et al., 1998; Rieger and Knackmuss, 1995), anaerobic (Boopathy et al., 1998; Ederer et al., 1997; Funk et al., 1993; Hawari et al., 1998, 1999; Lenke et al., 1998; Rieger and Knackmuss, 1995), and microaerophilic (Gunnison et al., 1997) conditions, but also under abiotic conditions (Brannon et al., 1998). Mineralization of TNT, however, has only been demonstrated by certain wood- and litter-decaying basidiomycetes (Scheibner et al., 1997). One such basidiomycete is *P. chrysosporium*, a white-rot fungi known to degrade xenobiotic compounds (Bumpus and Aust, 1986; Paszczynski and Crawford, 1995), and which has shown promise for its use in the bioremediation of TNT-contaminated soils and waters (Fernando et al., 1990; Hodgson et al., 2000; Sublette et al., 1992). Interestingly, and of relevance to this study, the physiological capability of *P. chrysosporium* with respect to

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TNT mineralization was demonstrated to be superior under static growth conditions (Hodgson et al., 2000) and consequently has inspired the development of an immobilized process for the biotreatment of TNT-contaminated liquids using a continuous feeding mode.

Immobilization is a general term that describes many different forms of cell attachment or entrapment in terms of flocculation, adsorption on surfaces, covalent bonding to carriers, cross-linking of cells, encapsulation in a gel, and entrapment in a matrix (Cassidy et al., 1996). Bailey and Ollis (1986), Fitch et al. (1998), and Cassidy et al. (1996) provide an excellent overview of the importance and the role of biological fixed-film systems related to some environmental applications. Incentives for operating continuous-flow bioreactors with immobilized cells include attainment of high cell densities, absence of cell washout, and extended biochemical or biotransformation reaction time. The use of *P. chrysosporium* as an immobilized cell technology for the treatment of TNT-contaminated liquids has been previously reported by Fernando et al. (1990), Sublette et al. (1992), and Bumpus and Tatarko (1994), but little information was provided by these authors on the fluxes (i.e., rates) of TNT and its metabolites.

The experimental results presented herein are part of a comprehensive project aimed at studying the biodegradation of highly energetic compounds (Ampleman et al., 1995; Hawari et al., 1998, 1999; Sunahara et al., 1999). In addition to the development of a biological process for treatment of TNT-contaminated liquids, the objective of this study was to determine the cometabolic TNT transformation rate of an immobilized *P. chrysosporium* culture. In order to assess the physiological activity (i.e., viability) of the fungal biofilm, this study focused also on the determination of glycerol (the cosubstrate) uptake rate throughout different fed-batch and continuous TNT feeding experiments.

MATERIALS AND METHODS

Fungal Strain, Inoculum, and Culture Media

Phanerochaete chrysosporium BKM-F-1767 (ATCC 24725) was maintained on malt agar slants (20 g agar, 20 g malt extract, and 1 g yeast extract in 1 L distilled water). As described by Hodgson et al. (2000), a suspension of conidia was obtained from a 7-day-old culture of *P. chrysosporium* grown on malt agar, in 1-L Roux culture bottle, and used as a seed culture for the reactor inoculum. One mL of this suspension (10^7 conidia mL^{-1}) was added to 75 mL sterile growth medium [10 g glycerol, 2.3 g disodium tartrate, 2 g KH_2PO_4, 1.07 g NH_4Cl, 1 g yeast extract, 700 mg $MgSO_4 \cdot 7H_2O$, 500 mg NAT 89 (commercial soybean phospholipids, Natterman Phospholipid GmbH, Köln, Germany), 140 mg $CaCl_2 \cdot 2H_2O$, 70 mg $FeSO_4 \cdot 7H_2O$, 67 mg veratryl alcohol, 46 mg $ZnSO_4 \cdot 7H_2O$, 35 mg $MnSO_4 \cdot H_2O$, 7 mg $CuSO_4 \cdot 5H_2O$, and 2.5 mg thiamin-HCl per L distilled water, final pH 5.4] into 1-L sterile Roux culture bottle. Three bottles were laid flat and incubated static for 7 days at 37°C. The cohesive mycelial mats of three Roux bottles and 100 mL of the spent growth medium were aseptically homogenized for 5 sec using an Omni-mixer blade homogenizer (OCI Instruments, Kansas City, MO) and then used to inoculate 4 L or 3.5 L of sterile cultivation medium, i.e., the liquid working volumes for the fed-batch and continuous feeding experiments, respectively. This medium contained the same nutrients as for the growth medium, except that 2 g glycerol and 214 mg NH_4Cl were added and NAT 89 was omitted.

5-L Air-Lift Reactor Setup

The reactor setup was modified from an existing NB Celligen reactor (Fig. 1), which consisted of a 5-L round bottom glass vessel equipped with a stainless steel (s.s.) apparatus (#1) located 4 cm from the base of the reactor. It was designed for promoting liquid recirculation and holding a strip of textile (61 × 14 cm) fixed in a spiral configuration (top view, #3). The textile was made of nonwoven polyester

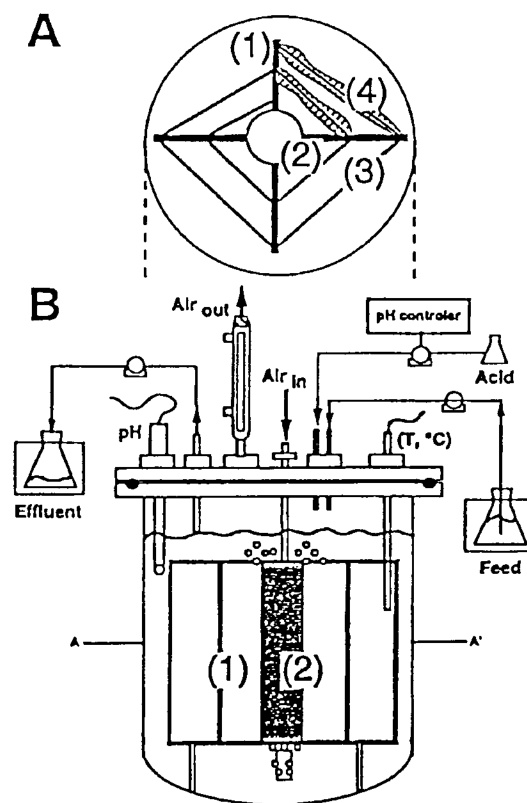

Figure 1. Schematic representation of the 5-L air-lift reactor setup. A: Cross-section view A-A'. B: Front view. Note: 1–4 refer to the cage, the central tube, the strip of textile, and the biofilm developed on the textile, respectively. See Materials and Methods for more details regarding the peripherals used (or not) to perform these experiments.

short fibers (Texel, St-Elzéar de Beauce, QC, Canada) and was used for the purpose of immobilizing the fungal cells (#4). An air sparger (5 μm sintered s.s.) was located in the lower end of the central tube (internal diameter, 3 cm) (#2) to ensure oxygenation and recirculation of the liquid medium with minimal shear stress forces for the immobilized cells. The incoming air (600 mL min^{-1}) was filtered through a hydrophobic 0.45 μm filter. The off-gas line was equipped with a West condenser and maintained at 6°C to minimize water losses due to evaporation. Once assembled, the reactor filled with cultivation medium was sterilized by autoclaving at 120°C for 30 min and ready to use for the fed-batch experiments. For the continuous feeding experiments, however, in addition to the above-mentioned reactor setup, a Teflon line was used to feed sterile cultivation medium with 50 mg L^{-1} TNT, whereas the spent cultivation medium was withdrawn through an effluent line into a reservoir. The feed tank and the reservoir were both maintained at 6°C and covered with aluminum foil to minimize exposure to light. The reactor was also covered with aluminum foil. A Metler pH probe, a Cole Parmer pH controller, and an acid reservoir filled with 0.25 M HCl completed the setup for operating in the continuous mode.

A total of three fed-batch and two continuous experiments (runs FB_1, FB_2, FB_3, C_4, and C_5) were carried out under different TNT and/or glycerol feeding conditions. Over the course of all three fed-batch experiments, initiated 7 days after inoculation, a volume of 3.2 mL (FB_1), 6.4 mL (FB_2), and 0.4 mL (FB_3) of a 50-mg TNT mL^{-1} acetone solution was injected manually through an injection port into the corresponding fungal culture. In runs FB_1 and FB_2, the four spiking events occurred on days 7, 9, 13, and 15, and on days 7, 11, 13, and 17, respectively. Whereas, in run FB_3, 20 mg TNT was fed daily, 5 days a week, over a 50-day period, for a total of 36 injections. Thus, the immobilized fungal culture was subjected upon each spiking event to an initial TNT concentration of 40, 80, and 5 mg L^{-1} in runs FB_1, 2, and 3, respectively. Moreover, in both FB_1 and FB_2 experiments, following each TNT spiking event, 20 mL of a 100 g L^{-1} sterile glycerol solution was also injected, whereas in the FB_3 experiment no C-source was added. The concentrations of glycerol, TNT, and TNT metabolites (see Analytical Methods) were determined 0, 2, 7, and 24 h following each spiking event in runs FB_1 and FB_2 or, on average, every second spiking event in run FB_3. Using these results, glycerol uptake and TNT transformation rates were then calculated and expressed as mg L^{-1} h^{-1}. The continuous mode, however, was initiated upon onset of feeding, which commenced also 7 days after inoculation. Here, the feed was introduced at a rate of 16 mL h^{-1} and an amount of 192 mL was removed every 12 h, ensuring a hydraulic residence time (HRT) of 9.1 days. The temperature of the reactor was maintained constant at 37°C.

Radio-Respirometry Test

At the end of the C_5 run, a fungal biomass sample (93 mg dry weight (d.w.)) was recovered nonaseptically and resuspended in 10 mL sterile cultivation medium with 50 mg L^{-1} TNT and 500 mg L^{-1} ampicillin. The use of this antibiotic limited the bacterial growth to a level barely detectable by a light microscope. Immediately after, the cell suspension was spiked with [U-^{14}C] TNT (120,000 dpm; Ampleman et al., 1995) and incubated under static condition at 37°C. Measurement of radiolabeled $^{14}CO_2$ was performed in triplicate in sealed 120-mL serum bottle as described by Hodgson et al. (2000).

Analytical Methods

All liquid samples collected throughout each experiment were immediately filtered on 0.45 μm sterile membranes and stored at –20°C until the day of analysis. First, determination of 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT), 2,6-diamino-4-nitrotoluene (2,6-DANT), thereafter referred to as the TNT metabolites, TNT, and trinitrobenzene (TNB) concentrations was done according to the following method. One volume of liquid sample was mixed with an equal volume of acetonitrile in a glass vial, maintained for 1 h at 4°C and centrifuged 4 min at 5,000 rpm using a model 5415C Eppendorf centrifuge, and then injected on a Supelcosil LC-8 column (250 × 4.6 mm, 5-μm particles; Supelco, Oakville, ON, Canada) maintained at 35°C. The flow of the isocratic mobile phase (18% 2-propanol and 82% water) was 1 mL min^{-1}. A Waters liquid chromatograph (LC) system (i.e., a model 600 pump, a model 717 plus autosampler, and a model 996 photo diode array detector set at 254 nm) was used to detect these nitroaromatic compounds. Second, ammonium ions were detected using a model SP8800 LC system (Spectra-Physics, San Jose, CA) equipped with a PRP-X200 column (250 × 4.1 mm, 10 μm poly-(styrene-divinylbenzene) particles) (Hamilton, Reno, NV) and a Waters 431 conductivity detector (Waters Chromatography Division, Milford, MA). The mobile phase consisted of 35% methanol and 65% HNO_3 6 mM (v v^{-1}) and its flow was 2 mL min^{-1}. Third, glycerol was analyzed using a model 590 LC system (Waters) equipped with an ION-300 organic acid column of 300 × 7.8 mm (Interaction Chromatography, San Jose, CA) and a model 410 differential refractometer (Waters). The mobile phase was 17 mM H_2SO_4 and the flow rate was 0.4 mL min^{-1}.

Commercial standard solutions of the above-mentioned nitroaromatic compounds were purchased from AccuStandard (New Haven, CT). TNT used throughout this work was supplied by the Defence Research Establishment Valcartier (Val Bélair, Canada) and its chemical purity was 99.5% (LC analysis).

RESULTS

Fungal Colonization of the Textile

The following is a very short description of the growth phase and fungal colonization process observed during the

7-day period upon inoculation of each run reported herein. One day after inoculation, the cultivation medium recirculating in the reactor became limpid and a fungal biofilm was visible on some areas of the textile. Glycerol (2 g L^{-1}) was used as the primary C-source (Fig. 2). Following a 7-day growth period, a fungal mat had completely covered the textile. During the time course of these experiments the liquid recirculation was not hindered, although the biofilm thickness (visual observation) increased with incubation time, i.e., the volume between two textile layers was not completely filled with fungal biomass (Fig. 1A, 4).

Fed-Batch TNT Feeding in the Presence of Glycerol

To overcome the toxic effects of TNT, the fed-batch cultivation mode was used to feed minute amounts of TNT together with glycerol to a 7-day-old immobilized *P. chrysosporium* culture. Two TNT and glycerol fed-batch feeding rates were investigated: 20 mg TNT L^{-1} d^{-1} and 250 mg glycerol L^{-1} d^{-1} for 8 days (run FB_1) and 32 mg TNT L^{-1} d^{-1} and 200 mg glycerol L^{-1} d^{-1} for 10 days (run FB_2). After the first spiking event, glycerol and TNT as well as 2-ADNT, 4-ADNT, 2,4-DANT, and 2,6-DANT (i.e., the TNT metabolites) were followed over a 24-h period; glycerol uptake and TNT transformation occurred concomitantly with the accumulation of 2-ADNT and 4-ADNT in the spent cultivation medium (results not shown). Thus, consequent to this first injection, TNT transformation rates were 2.41 and 2.38 mg L^{-1} h^{-1} and glycerol uptake rates were 16.6 and 16.2 mg L^{-1} h^{-1}, respectively, in runs FB_1 and FB_2 (Fig. 2). Thereafter, as the process of feeding TNT and glycerol continued, both rates decreased significantly and proportionally to the TNT feeding rate (Fig. 2). Following the third or fourth injection, at 20 mg TNT L^{-1} d^{-1} (run FB_1), rate of TNT transformation and glycerol uptake was, respectively, 2.13 mg L^{-1} h^{-1} and 12.3 mg L^{-1} h^{-1}, which represents a 12% and 26% decrease as compared to the initial values. On day 15, the actual TNT transformation rate was (inexplicably) high. On the other hand, at 32 mg TNT L^{-1} d^{-1} (run FB_2), these rates were 1.73 and 2.90 mg L^{-1} h^{-1}, respectively, which represents a 27% and 82% decrease as compared to the initial values.

Under both TNT feeding rates, 2-ADNT and 4-ADNT were the predominant TNT metabolites to accumulate in the spent cultivation medium throughout the TNT fed-batch process (Fig. 3B). Final concentrations of these two TNT metabolites were, respectively, 3.8 and 13.0 mg L^{-1} on day 16, at 20 mg TNT L^{-1} d^{-1}, and 7.6 and 21.9 mg L^{-1} on day 19, at 32 mg TNT L^{-1} d^{-1}. 2,4-DANT and 2,6-DANT concentrations were below 0.5 mg L^{-1} and below detection limit, respectively. Interestingly, throughout both fed-batch

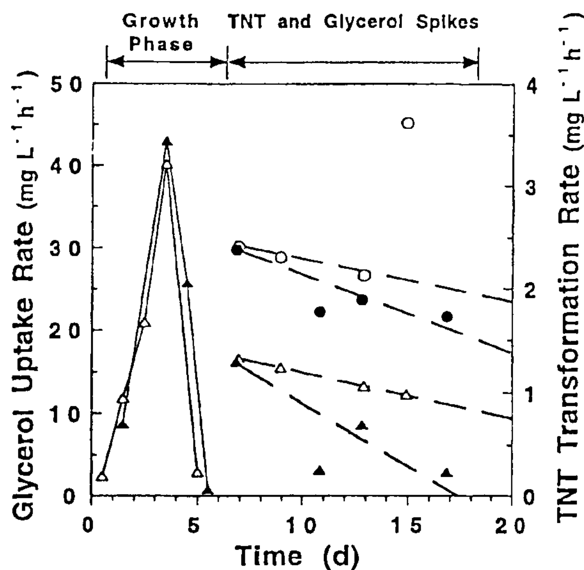

Figure 2. Fed-batch TNT feeding experiment in the presence of glycerol. Glycerol uptake (△,▲) and TNT transformation (○,●) rates of immobilized *P. chrysosporium* culture fed with 20 mg TNT L^{-1} d^{-1} and 250 mg glycerol L^{-1} d^{-1} for 8 days (run FB_1, open symbols) or with 32 mg TNT L^{-1} d^{-1} and 200 mg glycerol L^{-1} d^{-1} for 10 days (run FB_2, closed symbols). The first spiking event was on day 7, whereas the time of the other three events is reported in the materials and methods. Glycerol uptake rates relative to the growth phase were calculated from the glycerol consumption curve, whereas all other rates were calculated from residual TNT and glycerol concentration profiles (results not shown) and plotted at the time corresponding to the spiking event. Regression lines (---) through these rates are also shown.

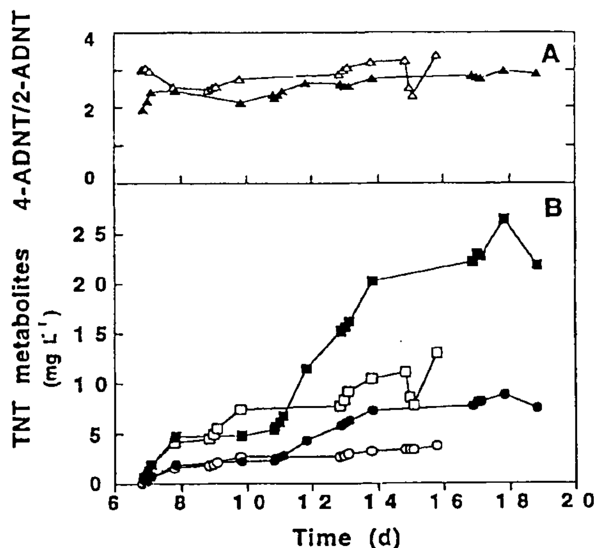

Figure 3. As a result of four spiking events, (A) 4-ADNT:2-ADNT concentration ratios (dimensionless) (△,▲) and (B) cumulative (of the two predominant) TNT metabolites (□,■, 4-ADNT; ○,● 2-ADNT) concentration profiles of the two immobilized *P. chrysosporium* cultures described in Figure 2.

runs 4-ADNT/2-ADNT concentration ratio was constant at ~2.7 (Fig. 3A).

Fed-Batch TNT Feeding in the Absence of Glycerol

The fed-batch process was used once more to evaluate the TNT transformation rate of a 7-day-old immobilized fungal culture, but at a low 3.7 mg L^{-1} d^{-1} TNT feeding rate and under C-source starvation (run FB_3, Fig. 4). Figure 4A shows the evolution of TNT metabolites concentrations in the cell-free spent cultivation medium. The 4-ADNT was the predominant metabolite throughout the entire fed-batch experiment; its concentration increased steadily to a maximum of 23 mg L^{-1}. The 2-ADNT and 2,4-DANT were detected in the spent cultivation medium, but only at concentrations not higher than 2.7 mg L^{-1} and 0.7 mg L^{-1}, respectively. Throughout this experiment, 2,6-DANT was not detected in the spent cultivation medium. Until day 21, TNT was not detected in the spent cultivation medium, but thereafter TNT concentration increased at 1.84 mg L^{-1} d^{-1} and reached a maximum concentration of 38 mg L^{-1} on day 41 (Fig. 4B). Noticeably, TNB was first detected on day 30, and its concentration increased constantly and reached 1.14 mg L^{-1} on day 49 (Fig. 4B). In the first 10 days of this fed-batch experiment, ammonium concentration gradually increased and peaked at 170 mg L^{-1} (Fig. 4B). From day 16 onwards, ammonium concentration began to decrease and leveled at 26 mg L^{-1}. Initial TNT transformation rate of the immobilized fungal culture was 0.75 mg L^{-1} h^{-1}, which dropped to 50% of that value after 23 days and even lower between day 26 and day 41 (Fig. 4C). In an attempt to stimulate the biotransformation of TNT, but also to test if the immobilized fungal culture was still alive, on day 44, glycerol (5 g L^{-1}) was injected into the spent cultivation medium. Consequently, 5 days later (day 49), the rates of glycerol uptake and TNT transformation were 2.0 mg L^{-1} h^{-1} (results not shown) and 0.09 mg L^{-1} h^{-1} (Fig. 4C), respectively. The pH of the spent cultivation medium increased from pH 5.4 to pH 8.5 (results not shown). At the end of this fed-batch experiment (day 49), 8.7 g biomass d.w. was found immobilized on the textile.

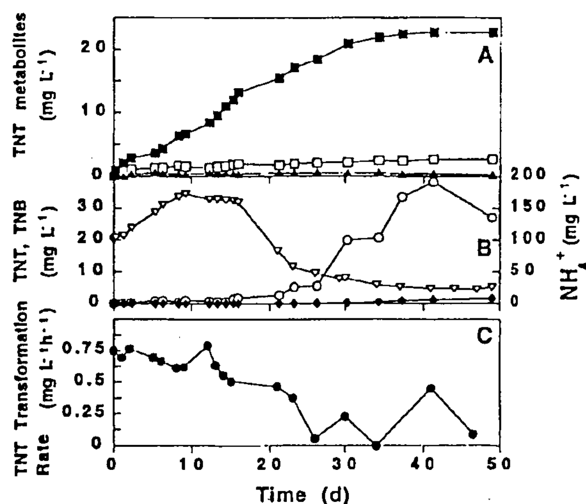

Figure 4. Fed-batch TNT feeding (3.7 mg L^{-1} d^{-1}) experiment in the absence of glycerol. A–B: TNT metabolites (■, 4-ADNT; □, 2-ADNT; ▲, 2,4-DANT), TNT (○), TNB (♦), and ammonium (▽) concentration profiles as a result of 36 injections of 20 mg TNT (the concentration reported herein are those measured 24 h following each spiking event, with few exceptions). C: TNT transformation rate (●); as determined every second injection. The reactor was inoculated 7 days prior to t_0.

Continuous TNT Feeding in the Presence of Glycerol (Uncontrolled pH)

To study the interrelations between *P. chrysosporium* and TNT, the continuous culture technique was used but under uncontrolled pH (from 5.5–7.8). Results of continuous TNT feeding at 5.5 mg L^{-1} d^{-1} are presented in Figure 5 (run C_4). The dilution rate (D) was 0.11 d^{-1}, which corresponds to an HRT of 9.1 days. First, between day 8 and day 22,

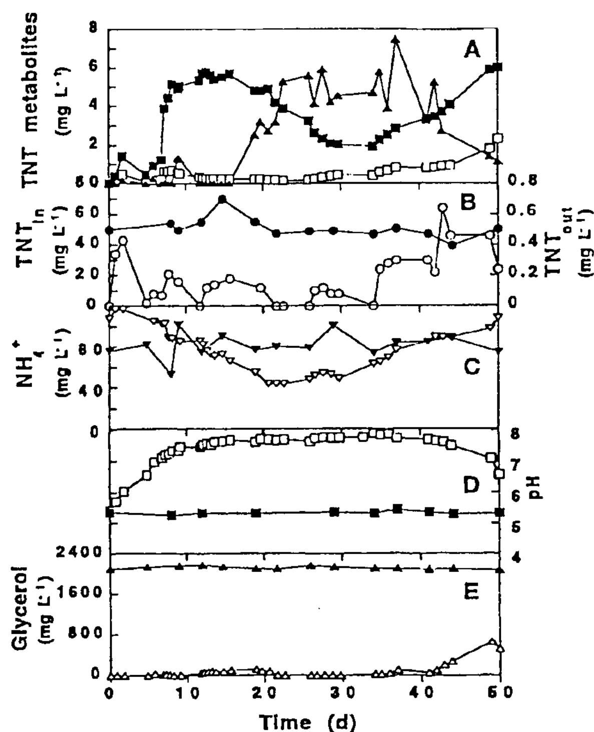

Figure 5. Continuous TNT feeding (5.5 mg L^{-1} d^{-1}) experiment in the presence of glycerol (no pH control). A: TNT metabolites (■, 4-ADNT; □, 2-ADNT; ▲, 2,4-DANT) concentration profiles of the effluent. B–E: TNT_{in} (●) and TNT_{out} (○), respectively, ammonium (▼,▽), and glycerol (▲,△) concentration profiles and pH (■,□) of the influent (closed symbols) and the effluent (open symbols). The reactor was inoculated 7 days prior to t_0.

4-ADNT concentration reached 5.1 ± 0.6 mg L^{-1} (n = 8, number of data points), whereas 2-ADNT and 2,4-DANT concentrations were 0.4 ± 0.2 mg L^{-1} (n = 12) and below detection limit, respectively. Second, 2,4-DANT was produced by the immobilized cell culture, and thus between day 23 and day 42 its concentration in the effluent was 5.0 ± 1.1 mg L^{-1} (n = 12). However, between day 28 and day 37, the concentration of 4-ADNT decreased from its previous level to 2.3 ± 0.3 mg L^{-1} (n = 7). Finally, between day 40 and day 50, the concentration of 4-ADNT started to increase, whereas the 2,4-DANT concentration decreased to a low level of 1.0 mg L^{-1}. In the effluent, the concentration of 2-ADNT increased only during the last 25 days without exceeding 2.5 mg L^{-1}, on day 50, whereas the 2,6-DANT was not detected in the spent cultivation medium throughout the entire cultivation period. TNT concentration in the feed was constant throughout this experiment (TNT_{in}, Fig. 5B). TNT was detected in the effluent but always at concentrations below 1 mg L^{-1} (TNT_{out}, Fig. 5B). Ammonium concentration remained constant at ~80 mg L^{-1} in the feed. In the effluent it decreased constantly during the first 20 days and then increased constantly to reach 110 mg L^{-1} on day 50 (Fig. 5C). Throughout this continuous feeding experiment, the pH of the effluent increased from 5.4–7.8 within the first 10 days, then leveled-off at 7.8 for a 30-day period and decreased to 6.5 between day 42 and day 50 (Fig. 5D). Until day 41, glycerol was not detected in the effluent, after which time the glycerol concentration increased and reached 650 mg L^{-1} on day 50 (Fig. 5E). On the trial day of this experiment, up to 29.5 g biomass d.w. was found immobilized on the support.

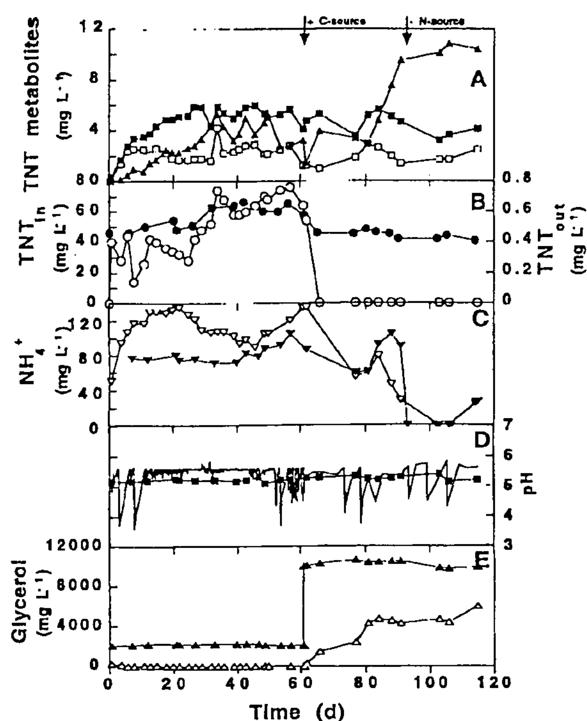

Figure 6. Continuous TNT feeding (5.5 mg L^{-1} d^{-1}) experiment in the presence of glycerol (at pH 5.5). A: TNT metabolites (■, 4-ADNT; □, 2-ADNT; ▲, 2,4-DANT) concentration profiles of the effluent. B–E: TNT_{in} (●) and TNT_{out} (○), ammonium (▼,▽) and glycerol (▲,△) concentrations profiles and pH (■,—) of the influent (closed symbols) and the effluent (open symbols). The continuous line (D) represents the actual pH of the cultivation medium in the reactor. On day 61, the glycerol concentration in the feed was increased to 10 g L^{-1} (+C-source) and on day 93 the N-source was omitted from the feed (N-source). pH was controlled at 5.5 by addition of 0.25 M HCl. The reactor was inoculated 7 days prior to t_0.

Continuous TNT Feeding in the Presence of Glycerol (at pH 5.5)

The continuous culture technique was used once more to investigate the effect of different C- and N-sources feeding conditions on the biotransformation of TNT, but at pH 5.5. Results of continuous TNT feeding at 5.5 mg L^{-1} d^{-1} (run C_5) are presented in Figure 6. The HRT was once again kept at 9.1 days. Three different operational conditions were tested throughout this experiment: 1) from day 0–61, 220 mg glycerol L^{-1} d^{-1} and 7.9 mg NH_4 L^{-1} d^{-1}; 2) from day 61–93, 1,100 mg glycerol L^{-1} d^{-1} and 7.9 mg NH_4 L^{-1} d^{-1}; and 3) from day 93–115, 1,100 mg glycerol L^{-1} d^{-1} and 0 mg NH_4 L^{-1} d^{-1}. In the first case, concentration of 4-ADNT, 2-ADNT, and 2,4-DANT increased gradually from 0 mg L^{-1} to a constant level of 5.3 ± 0.6 mg L^{-1} (n = 15; between day 27 and day 62), 2.1 ± 0.5 mg L^{-1} (n = 33; between day 5 and day 115), and 4.0 ± 0.9 mg L^{-1} (n = 13; between day 29 and day 61), respectively (Fig. 6A). Once again, 2,6-DANT was not detected during this experiment. Between day 1 and day 61, TNT concentration in the effluent increased from 0.4 mg L^{-1} to 0.8 mg L^{-1} (Fig. 6B), the ammonium concentration reached an average value of 116 ± 14 mg L^{-1} (n = 22) (Fig. 6C). To maintain pH constant at 5.5 (Fig. 6D), from day 0 to day 61, the HCl feeding rate was 2.27 mmol L^{-1} d^{-1} (results not shown). The glycerol fed to the immobilized culture was entirely metabolized, as no glycerol was detected in the effluent (Fig. 6E).

This experiment was continued for an additional 65 days. In the second case, glycerol concentration in the feed was increased from 2 g L^{-1} to 10 g L^{-1} (Fig. 6E); as a result, the TNT concentration in the effluent rapidly dropped to zero (Fig. 6B). TNT metabolites concentration profiles remained (on average) as before, from day 81, 2,4-DANT concentration started to increase significantly (Fig. 6A). Ammonium concentration kept on decreasing during that time period (Fig. 6C). From day 93 onwards (the third case), the N-source was eliminated from the feed (Fig. 6C). TNT concentration in the effluent was still 0 mg L^{-1} by day 115, whereas 2,4-DANT concentration leveled at 11 mg L^{-1}, and 4-ADNT and 2-ADNT concentrations were nearly as they were before the N-source was eliminated from the feed (Fig. 6A). To maintain pH at 5.5 (Fig. 6D), from day 61 to day 115, the HCl feeding rate was 0.45 mmol L^{-1} d^{-1} (results not shown). On day 115, the immobilized biomass present

in the reactor amounted to 28.1 g d.w. Following the dismantling of the reactor, a sample of these immobilized cells was tested for its TNT mineralization activity (i.e., production of $^{14}CO_2$) in a microcosm. After a 41-day incubation period, the 115-day-old cells mineralized TNT to a level of 15.3% ± 2.1% (n = 3).

DISCUSSION

In an attempt to overcome the toxic effect of TNT and TNT metabolites, two different approaches to feed TNT were tested: the fed-batch and continuous feeding modes.

First, the fed-batch mode was used to feed minute amounts of TNT (Figs. 2–4, Table I). The cultivation of this fungal strain under similar TNT concentrations have been shown not to interfere with its mineralization level (Hodgson et al., 2000). Without a cosubstrate (run FB_3), the initial TNT transformation rate (0.75 mg L^{-1} h^{-1}) was approximately one-third the rate observed following the first TNT and glycerol injection in FB_1 and FB_2 runs (2.4 mg L^{-1} h^{-1}), which supports the idea that optimal TNT transformation requires a C-source such as glycerol. Similarly, Boopathy et al. (1994) reported that the TNT transformation rate in molasses-fed culture was 10 times higher than in the cultures fed with other C-sources (i.e., succinate, citrate, malic acid, acetate, sucrose, and glucose), whereas only 2% TNT was transformed by the culture that received TNT as the sole C-source. Although most immobilized processes offer significant advantages over the processes based on cell suspension alone, the immobilized cells must stay alive and maintain their activity toward the target compound for prolonged periods of time. Thus, not only glycerol was essential to the transformation of TNT, it served as a C-source for the growth and maintenance of *P. chrysosporium*. Beyond the first injection of TNT, as more TNT was fed to the immobilized culture, it displayed a negative effect on cell physiology (see regression lines in Fig. 2) and, moreover, the higher the TNT feeding rate, the more significant was the effect on cellular metabolism. The slope of these regression lines (m_{TNT} and $m_{glycerol}$) quantify this effect (Table I). In other words, throughout each fed-batch experiment, the metabolic activity of the fungal biofilm (i.e., glycerol uptake and TNT transformation rates) decrease constantly with incubation time, which allow the calculation of the $t_{50\%}$ factor, i.e., the contact time required to observe a 50% decrease of the initial TNT transformation rate and glycerol uptake rate or a (pseudo) half-life constant (Table I). As a result of a 1.6× increase in the TNT feeding rate, the $t_{50\%}$ of the corresponding TNT transformation and glycerol uptake rates decreased by 1.7× and 2.9×. This observation suggests that TNT more adversely affects the viability of the fungal biofilm as measured by the uptake of glycerol than the TNT transformation activity. Although the products of fungal reduction of TNT by *P. chrysosporium* are well known (Hawari et al., 1999), the enzymatic mechanisms of TNT transformation are still under discussion (Fritsche, 2000; van Aken et al., 2000).

Second, the continuous cultivation technique was used to feed the immobilized culture with 5.5 mg TNT L^{-1} d^{-1} (i.e., 4–6 times less than in fed-batch mode), but under a high HRT of 9.1 days. In contrast to the fed-batch mode of feeding, when immobilized *P. chrysosporium* cells were continuously fed with both TNT and glycerol, the TNT fed did not display a negative effect on the cell metabolic activity. Glycerol uptake was unhindered for 50 days (run c_4) or up to 115 days (run C_5), which is far beyond the $t_{50\%}$ estimated during the fed-batch experiments (see above). Although these fed-batch and continuous feeding experiments were not performed under the same TNT feeding rate (Table I), the residual TNT concentration was in all experiments below 1 mg L^{-1} (4.4 μM) either 24 h following each TNT spiking event (fed-batch experiments) or throughout the continuous feeding experiments. Thus, the concentration level of the TNT metabolites in the spent cultivation medium may be one of the reasons that explains the discrepancy between the responses of the fungal biofilm. Throughout the continuous feeding experiments (runs C_4 and C_5), the TNT metabolites concentration was ~7 mg L^{-1} (~35 μM) except during the last 22 days of the C_5 run, whereas on the last sampling day in run FB_2, the TNT metabolites concentration increased up to 24 mg L^{-1} (i.e., 121 μM, run FB_2). TNT and TNT metabolites at μM concentration proved to be cytotoxic to *Salmonella typhimurium* reference strains (Lachance et al., 1999). They proposed that these energetic compounds could induce both basepair substitutions and/or frameshift type mutations. The mechanism by which TNT and TNT metabolites affect the fungal cell activity and consequently the TNT biotransformation activity is still under discussion (Stahl and Aust, 1995). Alternatively, the sensitivity of *P. chrysosporium* cells to TNT was first described by Spiker et al. (1992), but as shown by Stahl and Aust (1993), the extracellular detoxification of TNT can be maximized by starting with significant amounts of mycelium. This is the main reason why TNT was fed to the immobilized culture only after a 7-day growth period. It also suggests that the accumulation of immobilized biomass circumvents the potentially toxic effect of TNT and TNT metabolites. Consequently, contrary to the fed-batch feeding mode, the continuous feeding mode provides not only the necessary C-source, but maintained the concentration of TNT metabolites below a threshold value. Another reason to explain this discrepancy could be simply due to the molar ratio of TNT fed to glycerol consumed, i.e., the cometabolic yield ($Y_{TNT/glycerol}$) (see below). Actually, there is a difference of one order of magnitude between the respective ratios of the continuous feeding experiments and the fed-batch feeding experiments (Table II).

In cometabolic biotransformation, microorganisms grow on a primary C-source (the cosubstrate) and metabolize the contaminant without any gain in energy. Others have added cosubstrates like glycerol, glucose (Boopathy et al., 1994; Daun et al., 1998; Stahl and Aust, 1994; Sublette et al., 1992; von Fahnestock and Forney, 1995), acetate (Zappi et

Table I. Summary of operating conditions and results of TNT (glycerol) fed-batch and continuous feeding experiments following the 7-day growth period.

Feeding mode	Run (#)	TNT feeding rate (mg L^{-1} d^{-1})	Glycerol feeding rate (mg L^{-1} d^{-1})	Time[1] (d)	TNT fed (mg)	TNT transformed[2] (%)	TNT metabolites accumulated[3] (%)	X_{final} (g)	m_{TNT}[4] (mg L^{-1} h^{-1}) d^{-1}	$t_{50\%}$[5] (d)	$m_{glycerol}$[4] (mg L^{-1} h^{-1}) d^{-1}	$t_{50\%}$[5] (d)
Fed-batch[6]	FB_3	3.7	0	49	720	87.4	11	8.7	−0.015 (r^2 = 0.920)	25.5	n/a[7]	n/a
	FB_1	20	250	8	640	99.5	12	n.d.[8]	−0.048 (r^2 = 0.998)	25.4	−0.54 (r^2 = 0.998)	15.3
	FB_2	32	200	10	1,280	99.8	14	n.d.	−0.078 (r^2 = 0.623)	15.3	−1.52 (r^2 = 0.537)	5.3
Continuous[9]	C_4	5.5	220	50	971	98.5	18	29.5	0[10]	—	0	—
(D = 0.110 d^{-1})	C_5 (t_{0-61})	5.5	220	61	1,314	99.1	21	—	0	—	0	—
	C_5 (t_{62-115})	5.5	1100	54	932	99.9	35	28.1	0	—	0	—

[1]Time period of TNT feeding either in fed-batch or continuous mode.

[2]Based on TNT and TNT metabolites detected in the effluent and in the spent cultivation medium, biomass, and textile at the end of the experiment.

[3]Over the TNT transformed.

[4]Slopes of the four regression lines (forced through the first data point) shown in Figure 2 (runs FB_1 and FB_2) and slope of a linear fit of the TNT transformation rates obtained between day 0 to day 23 (Fig. 4, run FB_3).

[5]Contact time upon injection of TNT required to observe a 50% decrease in the TNT transformation rate or glycerol uptake rate.

[6]Liquid working volume, 4 L.

[7]Not applicable.

[8]Not determined.

[9]Liquid working volume, 3.5 L.

[10]Inferred to be 0 as the TNT transformation and glycerol uptake rates were constant throughout the cultivation period.

Table II. Rates of TNT feeding (TNT_{in}) and TNT-metabolites production throughout each fed-batch and continuous TNT feeding experiments.

	Feeding and production rates (μmol L^{-1} d^{-1}) and glycerol uptake rate (mmol L^{-1} d^{-1})						
	a[1] TNT_{in}	b[2] TNT	c 4-ADNT	d 2-ADNT	e 2,4-DANT	Glycerol	$Y_{TNT\ in/glycerol}$ (μmol $mmol^{-1}$)
FB_1 (t_{7-16})	88	0.44	7.51	2.18	0.36	2.7	33
FB_2 (t_{7-19})	141	0.28	9.44	3.25	0.24	2.2	64
FB_3 (t_{0-21})	16.3	0.00	3.55	0.51	0.00	0	—
(t_{21-41})[3]	16.3	8.11	2.03	0.20	0.00	0	—
C_4 (t_{8-22})	24.2	0.36	2.84	0.20	0.00	2.4	10
(t_{23-42})	24.2	0.36	1.27	0.41	3.29	2.4	10
C_5 (t_{27-92})	24.2	0.22	2.94	1.17	2.63	2.4	10
($t_{100-115}$)	24.3	0.02	2.08	1.17	6.89	7.2	3

The 2,6-DANT production rate was below detection limit throughout all these experiments.
[1]The identification letters (a–e) refer to Figure 7.
[2]TNT not biotransformed.
[3]TNB accumulation rate of 0.33 μmol L^{-1} d^{-1}.

al., 1995), and toluene (Tharakan and Gordon, 1999) to stimulate the biotransformation of TNT. To carry out efficient reductive TNT transformation, microorganisms need a cosubstrate, but little is known about the efficiency of this process. Daun et al. (1998) used the fed-batch feeding mode to supply the cosubstrate (but not TNT) for the transformation of TNT into TAT under anaerobic conditions; thus, they obtained a $Y_{TNT/glycerol}$ of 9.8 μmol TNT $mmol^{-1}$ glucose. Similar $Y_{TNT/glycerol}$ has been also obtained with a soil bacterial consortium cultivated under aerobic conditions (Boopathy et al., 1994). The cometabolic yields of the different *P. chrysosporium* immobilized cultures varied between 3 and 64 μmol TNT $mmol^{-1}$ glycerol depending on the cultivation mode (Table II). Actually, the highest and cometabolic yields were observed when the glycerol uptake rates were at a minimum and a maximum, respectively. As mentioned by Daun et al. (1998) and Boopathy et al. (1994), it is of practical importance to determine the conditions under which a high cometabolic yield could be obtained, as it allows the remediation of TNT contaminated soils and waters using a minimum amount of cosubstrate. It is also important to determine the optimal C-source feeding rate as the cell viability depends on the cosubstrate.

The present study highlights the dynamics of the TNT transformation process by *P. chrysosporium* immobilized culture, although the carbon mass balance was not complete. Based on the TNT biotransformation pathway proposed by Hawari et al. (1999), Figure 7 illustrates the fluxes (i.e., production rates) of those TNT metabolites detectable by the LC technique used herein. The presence of the nitroso- and hydroxylamino-intermediates and the acetylated intermediates was confirmed in liquid samples from the two continuous cultures using the same analytical procedure as described in Hawari et al. (1999), but only in traces (results not shown). In this figure and in Table II, the letters a–e refer to the rate of production for each TNT metabolite at pseudosteady-state, for one or two significant time periods

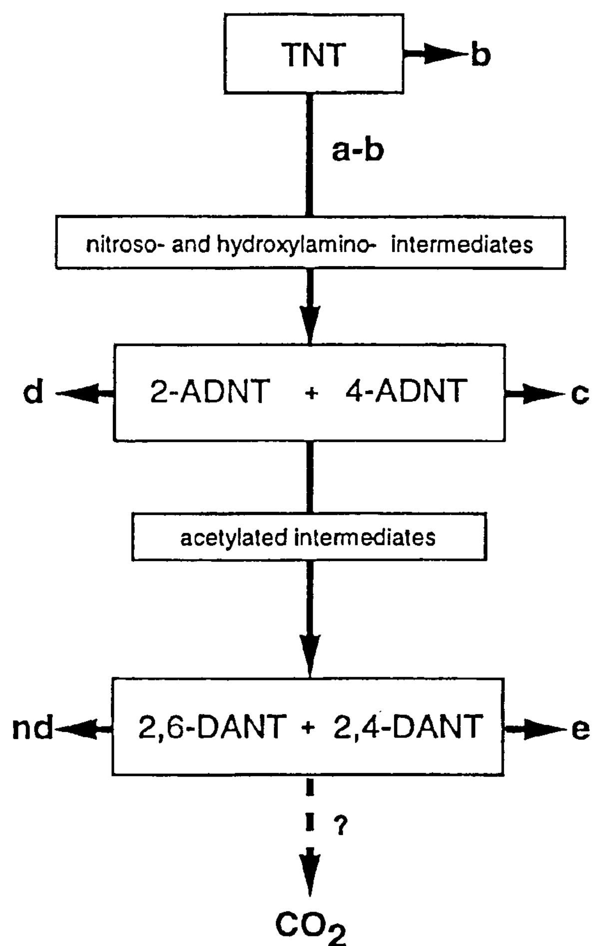

Figure 7. Simplified schematic representation of the transformation of TNT into 2-ADNT, 4-ADNT, 2,4-DANT, and 2,6-DANT, but with a reference to their respective production flow rate; see letters a–e in Table II. Note: Not detected (nd); not determined (?).

during each run. In the fed-batch cultivation mode, the dynamics shows that 4-ADNT and 2-ADNT accumulate with little production of 2,4-DANT (0.4% of TNT_{in} flux), as if the pathway was halted at that level. The preferential reduction of the TNT *para* nitro-group has been attributed to regioselectivity of the TNT molecule, but the reasons why the ratio of 4-ADNT/2-ADNT was either constant or variable under specific feeding conditions have yet to found. Interestingly, when TNT was fed without a cosubstrate (run FB_3), the biotransformation activity of the 7-day-old fungal biofilm declined to a level below the TNT feeding rate, thus, TNT accumulates in the spent cultivation medium. Coincidentally, traces of TNB were detected under these cultivation conditions; the flux of TNB represents 4% of the net TNT flux (Table II). The oxidation of the methyl function of TNT and a subsequent decarboxylation has been proposed as a source of TNB (as reported by Bruns-Nagel et al., 2000), but photodecomposition of TNT could not be ruled out. In contrast, under a continuous TNT feeding mode the dynamics of the fungal biofilm was significantly different, as the rate of production of each TNT metabolite undergoes several changes throughout the respective cultivation period of the C_4 and C_5 runs. The most significant one is the modification of the 2,4-DANT flux, which increases from 0–3.29 μmol L^{-1} d^{-1} in run C_4 (Table II). This modification was observed after a cultivation period of 20 days, i.e., as the biofilm thickness increase. In other words, while TNT reduction to monoaminodinitrotoluenes (e.g., 2-ADNT and 4-ADNT) occurs under oxic (and anoxic) conditions, further reduction to diaminonitrotoluenes (e.g., 2,4-DANT) requires a lower redox potential (Spain, 1995). Although the dissolved oxygen level within the biofilm was not measured herein, this condition was presumably found within the biofilm formed on both surfaces of the textile. It was 1–2 cm thick the day the reactor was dismantled (results not shown). Similarly, in run C_5 the 2,4-DANT flux was nearly as high as in run C_4 (Table II); moreover, it increased 2.6 times (from 2.63–6.89 μmol L^{-1} d^{-1}) as a result of a higher glycerol feeding rate and, to a certain extent, because of the N-limiting condition prevailing in the reactor at that time.

Finally, a direct demonstration of the mineralization of TNT (production of CO_2) by the immobilized cells was not done throughout these experiments. However, a batch test using 115-day-old immobilized *P. chrysosporium* cells (run C_5) incubated with 50 mg TNT L^{-1} showed a 15.3% mineralization level after an additional 41-day incubation period. This respiration activity is similar to the 12.3% mineralization level observed on day 57 of a *P. chrysosporium* batch culture (Hodgson et al., 2000). This shows that immobilized cells have the ability to mineralize TNT, and we have yet to determine the cultivation conditions promoting a high TNT mineralization rate. Hodgson et al. (2000) showed that addition of 1% Tween 80 into the cultivation medium enhanced the mineralization of TNT by a factor of two, but it has not been used with the present cell immobilization process. The present data points to the importance of continuing our investigations on the cellular metabolic functions of *P. chrysosporium* involved in the degradation of TNT towards the optimization of its mineralization, as this may constitute the basis for the development of a TNT remediation technology.

CONCLUSION

Immobilized *P. chrysosporium* culture maintained its viability and its cometabolic transformation activity for a prolonged period of time. Without a primary C-source, the biotransformation of TNT prove to be limited. The present study clearly demonstrates the negative effect of high TNT feeding rates (20 and 32 mg L^{-1} d^{-1}) in the fed-batch mode on the glycerol uptake rate and TNT transformation rate of this immobilized fungal culture. In the continuous feeding mode, at a low TNT feeding rate (5 mg L^{-1} d^{-1}) and a high HRT 9.1 days, it alleviates this negative effect.

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