Biochemical and ecotoxicological effects of 2,4,6-trinitrotoluene (TNT) and its metabolites on the soil invertebrate, *Enchytraeus albidus*

Sabine Dodard

A Thesis

In

The Department

of

Chemistry and Biochemistry

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science at

Concordia University

Montreal, Quebec, Canada

December, 2002

ABSTRACT

Biochemical and ecotoxicological effects of 2,4,6-trinitrotoluene (TNT) and its metabolites on the soil invertebrate, *Enchytraeus albidus*

Sabine G. Dodard

This M.Sc. thesis is presented as a compilation of two research articles. The toxic effects of trinitrotoluene (TNT), an explosive considered to be a recalcitrant environmental pollutant, were investigated in enchytraeids (a potworm and soil invertebrate).

The first paper describes the ecotoxicological effects of TNT on enchytraeids. TNT was shown to be transformed in the artificial soil used as an exposure matrix, and resulted in enchytraeid mortality and reductions in the number of juveniles as determined by standard tests. This study also addresses the problem of potential toxicity of TNT to juveniles, which could explain why the numbers of juveniles were decreased as judged by the reproduction endpoint test.

The toxicity of TNT and its biotransformation by enchytraeids at the organismal and biochemical levels are described in the second manuscript. Adult enchytraeids were analysed after exposure to TNT in both liquid and soil milieu. TNT-related metabolites were found in enchytraeids after exposure to TNT-spiked artificial (OECD) soil, as well as TNT in liquid. TNT was lethal to enchytraeids exposed in liquid for 20 hours. At sub-lethal concentrations, enchytraeids transformed TNT to amino derivatives (2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT) and 2,4-diamino-6-

nitrotoluene (2,4-DANT)), both *in vivo* and *in vitro*. The *in vitro* experiments showed that biotransformation of TNT in enchytraeids was dependent on the protein concentration and the period of incubation. In addition, TNT biotransformation *in vitro* was dependent on the sub-cellular fraction, with the 8,000 x g pellet containing the highest activity. Furthermore, the observed TNT-transforming activity *in vitro* was related to increased bacterial growth found in the potworm homogenate after prolonged periods of incubation. When the antibiotics, tetracycline or streptomycin-penicillin, were added to incubation mixtures, the biotransformation of TNT was inhibited and the bacterial numbers in the potworm homogenate after the exposure time period did not increase significantly. Based on the studies reported in the second manuscript, it is concluded that the transformation of TNT to selected amino-derivatives by enchytraeids is mainly due to bacteria associated with the potworms.

A literature review on enchytraeid species, and the biotransformation of TNT by bacteria, is also presented.

Acknowledgements

The completion of this study would not be done without the help and support of all my thesis committee members (from Concordia, Drs. Justin Powlowski, Paul Joyce and Judith Kornblatt, and from Biotechnology Research Institute (BRI), Dr. Geoffrey Sunahara). I would like to thank my supervisor, Dr. Sunahara, who helped me with the writing of this thesis, and provided me with the knowledge required for the interpretation of data for the manuscripts. Thank you to my research director, Dr. Powlowski, for his help and support during all phases of this Master's thesis. Thank you to Mme Carole Coutts for her help and support with the academic rules and deadlines.

Also, I would like to thank the National Research Council Canada, BRI, for the opportunity to carry out these studies in complement to my technical work at the Institute. A special thanks to Manon Sarrazin and Louise Paquet for their technical help in the maintenance of enchytraeid culture and the chemical analyses.

Thank you to Dr. Agnès Renoux for her scientific input in my thesis.

I would like to say to each person who helped me by reviewing in part or whole this Masters thesis that I really appreciated his or her help.

For reviewing the two manuscripts, I'd like to thank Drs. Jörg Römbke, Thomas Moser, Bernard Lachance, Jalal Hawari and Edmund Ziomek.

Table of Contents

List of Abbreviations

2-ADNT: 2-amino-4,6-dinitrotoluene.

4-ADNT: 4-amino-2,6-dinitrotoluene.

CL: Confidence limit corresponding to two standard deviations of the mean of a series of experimental values.

2,4-DANT: 2,4-diamino-6-nitrotoluene.

2,6-DANT: 2,6-diamino-4-nitrotoluene.

ECx: Sample concentration causing x (50%, 20% etc...) effect compared to the control. The effect could be an inhibition or a reduction of a given parameter, such as number of juveniles.

HPLC: High Performance Liquid Chromatography.

ISO: International Standards Organization

LCx: Sample concentration killing x (50%, 20% etc...) of a given population compared to a control group.

LOEC: Lowest Observed Effect Concentration; the lowest concentration tested at which a toxic effect was statistically detected compared to the control group.

NOEC: No Observed Effect Concentration; the highest concentration tested at which no significant toxic effect was observed compared to the control group.

OECD: Organization for Economic Cooperation and Development.

SD: Standard deviation of the mean.

TNT: 2,4,6-trinitrotoluene.

US-EPA: United States Environmental Protection Agency.

WL: Warning limit corresponding to three standard deviations of the mean of a series of experimental values.

List of Figures

INTRODUCTION

CHAPTER 1:

CHAPTER 2:

APPENDIX 1:

List of Tables

INTRODUCTION

CHAPTER 1:

CHAPTER 2:

Chapter 1: Introduction

1.1 The contaminant of interest

Physical and chemical properties

Explosives are typically degraded very slowly in environmental systems. Observation of mineralization in the environment is complicated by these slow rates, and the lack of accumulation of degradation products observed at explosives-contaminated sites. TNT (2,4,6-trinitrotoluene) is one of the most extensively-studied explosives because of its recalcitrant properties. This molecule is recalcitrant because it is only partially transformed in the field to related metabolites, and complete mineralization to very simple products such as methane, carbon dioxide and nitrates, has not been observed (Burrows *et al.*, 1989; Talmage *et al.*, 1999).

TNT is one of the oldest explosives, and its physical as well as chemical properties are well characterized (Table 1.1). Of the widely-used explosives, TNT is one of the most water-soluble (0.013 g/100 g). This compound reacts with light and produces a number of photodegradation products which are also water-soluble (Gordon and William, 1992). The photolytic degradation of TNT has been observed in wastewater where a number of TNT degradation products have been identified (Gordon and William, 1992).

Summary of known toxicological effects of TNT

In mammals, 2,4,6-trinitrotoluene can be absorbed by inhalation, ingestion, or skin contact. It is rapidly biotransformed in the liver, excreted in the urine, and distributed to the organs. It is metabolized primarily by reduction of the nitro group and, to a lesser

extent, by oxidation of the methyl group and ring hydroxylation (Gordon and William, 1992). Initial exposure to TNT in the atmosphere may result in mild irritation of the respiratory passages and skin. Other signs of possible intoxication include gastrointestinal disorders, nausea, anorexia and constipation. When the skin or lungs absorb sufficient amounts of TNT, liver damage as well as aplastic anemia may be observed. In fact, toxic hepatitis and aplastic anemia have been reported as the principal cause of death following TNT intoxication (Gordon and William, 1992).

Table 1.1: General Chemical and Physical Properties of 2,4,6-Trinitrotoluene (TNT)

CAS number	118-96-7
Molecular weight	227.13
Molecular formula	$C_7H_5N_3O_6$
Color	Yellow to white
Specific gravity	1.654
Liquid density	1.465 g/cm^3
Vapor pressure	0.053 mm Hg (85 °C); 0.106 mm Hg (100 °C)
Solubility in Water	0.013 g/100 g (20 °C)
Solubility in Acetone	109 g/100 g (20 °C)
Melting point	80.1 to 81.6 °C
Boiling point	210 °C (10 mm Hg) to 212 °C (12 mm Hg)
Flash point	240 °C (explodes)

Taken from Gordon and William (1992).

TNT is toxic to some species of bacteria (e.g. *Vibrio fischeri*, *Salmonella typhimurium*) and is mutagenic at low doses (Drzyzga *et al.*, 1995; Jarvis *et al.*, 1998; Sunahara *et al.*, 1999). Some other species of bacteria can tolerate the presence of nitroaromatics, reducing them and in some cases using them as sources of carbon and nitrogen (Nishino and Spain, 1993). These metabolic reactions will be discussed in more detail below.

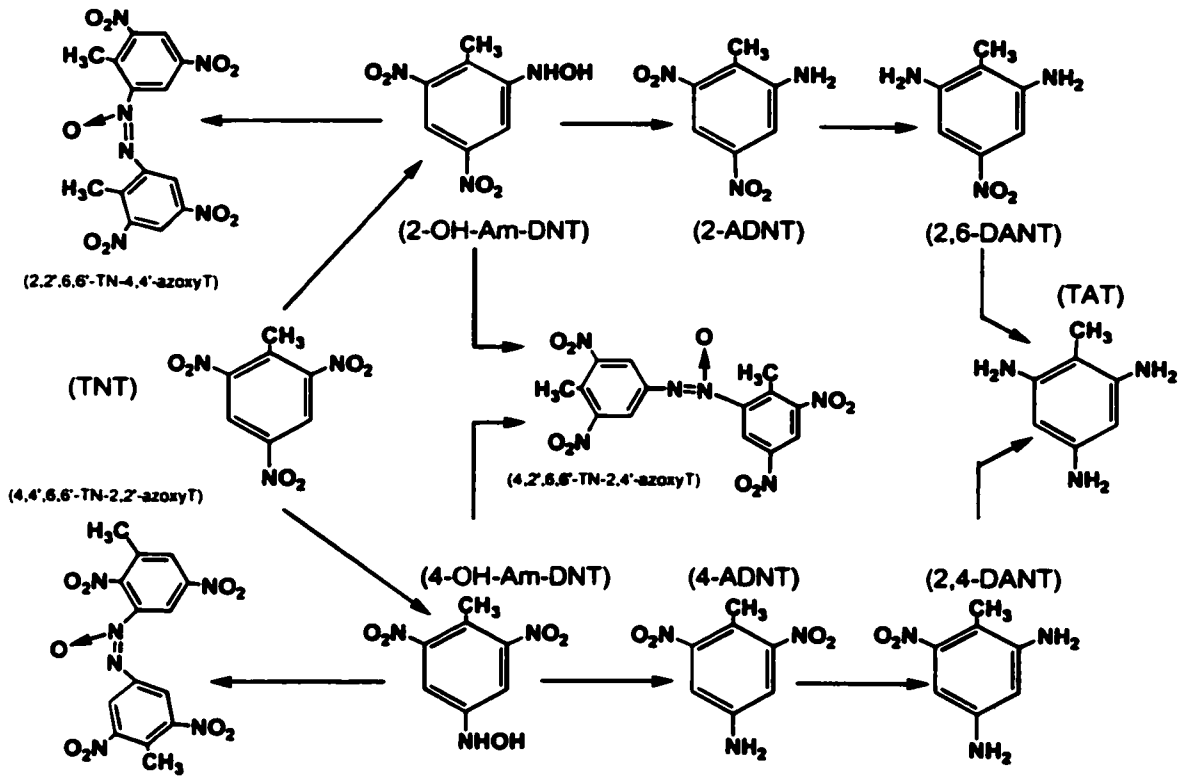
Literature on the effects of TNT on soil invertebrates is quite recent and mainly focuses on Eisenia species, Enchytraeids and Collembola. TNT caused mortality of *Eisenia andrei* and decreased its reproduction rate (Jarvis *et al.*, 1998; Robidoux *et al.*, 1999, 2000; Renoux *et al.*, 2000). Lethality, as well as decreased reproduction by TNT, was also observed in *Enchytraeus crypticus* and the collembola, *Folsomia candida* (Schäfer and Achazi, 1999).

1.2 Microbial biotransformation of TNT

The biotransformation of nitroaromatic compounds such as TNT by soil microbes (bacteria, and fungi) has been particularly well studied (Gorontzy *et al.*, 1994; Hawari *et al.*, 1999). This process can involve the formation of metabolites that are sometimes more toxic than the parent nitroaromatic compounds, and can affect other organisms that live in the immediate soil environment. The toxic effect observed depends on the sensitivity of the species (Sunahara *et al.*, 1999). In addition, the amino-containing transformation products are amenable to several attenuation mechanisms in soils. These include covalent binding to functional groups on soil organic matter, reaction at mineral surfaces, sequestration, and reversible adsorption (Pennington and Brannon, 2002).

Specific bacteria that are able to grow in nitroaromatic-enriched soil are distinguished by their ability to reduce nitroaromatic compounds (Bryant and De Luca, 1991; Nishino and Spain, 1993; Somerville *et al.*, 1995; He *et al.*, 1998). As shown in Figure 1.1, one of the initial steps in the bacterial metabolism of TNT is the reduction of nitro groups to form amino derivatives. TNT is first transformed to unstable intermediates such as hydroxyl-amino-dinitrotoluenes (2-OH-Am-DNT and 4-OH-Am-DNT, Fig. 1.1). Since these compounds are not stable they are rapidly transformed to the more stable mono-amino compounds, 2-ADNT and 4-ADNT (Fig. 1.1). Alternatively, hydroxylamino compounds may react together and form azoxytoluene polymers that can be irreversibly bound to the soil matrix (Stenersen 1992; Achtnich *et al.*, 1999). Subsequent attack on the mono-amino compounds by bacteria leads to the formation of di-amino compounds (2,6-DANT and 2,4-DANT, Fig. 1.1). The latter compounds are very reactive and can bind readily to the soil matrix (Daun *et al.*, 1998). The tertiary metabolite, triaminotoluene (TAT), is formed only under anaerobic conditions and is rapidly bound to organic material (Stenersen, 1992).

The biotransformations of TNT through amino derivatives are reactions that involve the contributions of enzymes and specific co-factors. In several studies, nitroreductases have been shown to be responsible for these biotransformation reactions (Bryant and De Luca, 1991; Nishino and Spain, 1993; Somerville *et al.*, 1995).

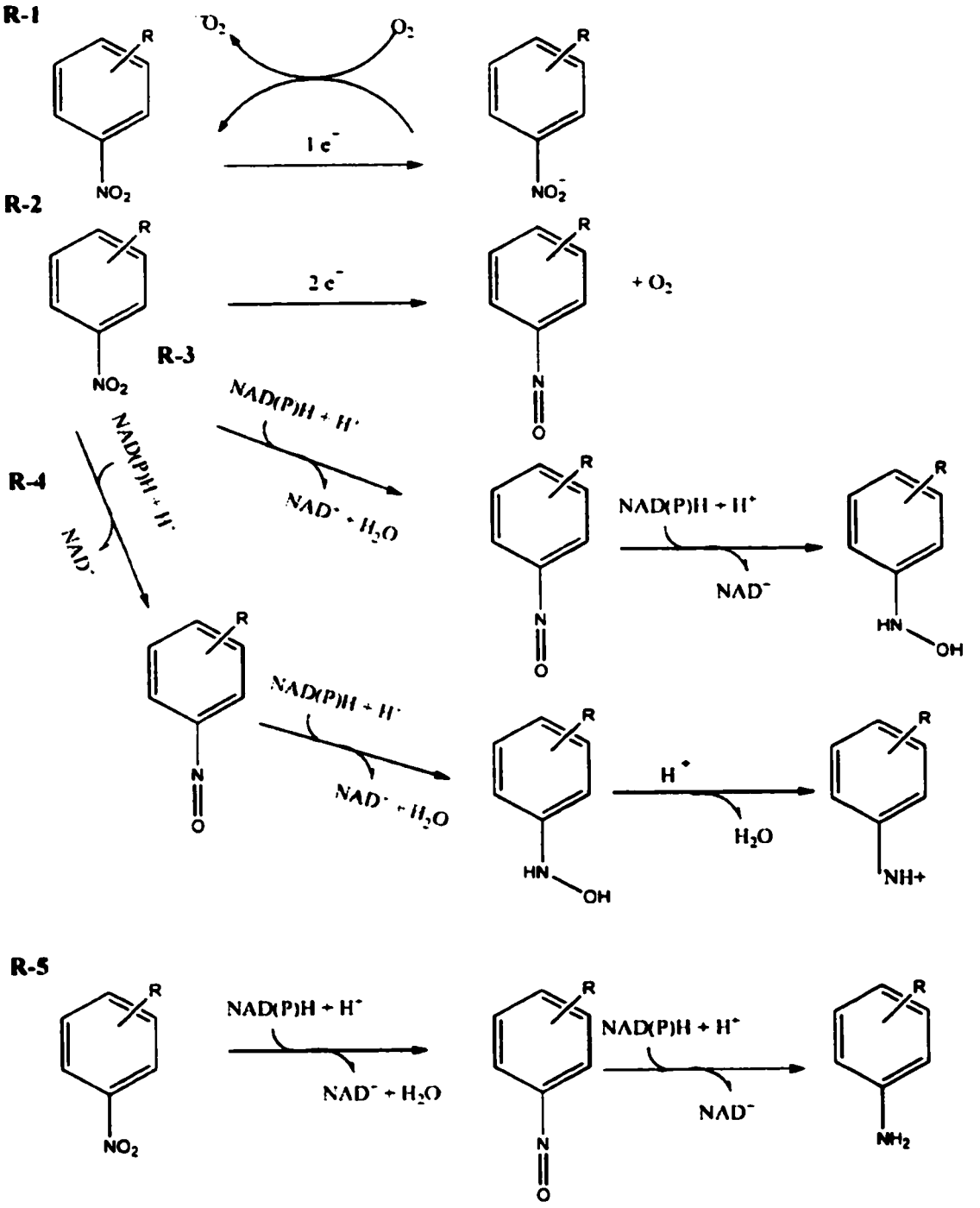


Taken from Sunahara et al., 1998

Figure 1.1: Aerobic and anaerobic reductive degradation of TNT

1.3 Properties of Nitroreductases

Nitroreductases constitute a family of enzymes that are able to reduce nitro-groups. Originally, observations of bacterial nitroreductase activity were made with cells capable of reducing chloramphenicol and *p*-nitrobenzoic acid to the corresponding aryl amines (Smith *et al.*, 1949; Saz *et al.*, 1954, both quoted by Cerniglia and Somerville, 1995). Based on these early observations, Cerniglia and Somerville (1995) proposed that nitroreductases use reduced pyridine nucleotides as electron donors, and catalyze at least one two-electron reduction reaction. Several possible pathways (Figure 1.2, R-1 to R-5) that yield different products are associated with different nitroreductase activities.



Adapted from Cerniglia and Somerville, 1995.

Figure 1.2: Major enzymatic reduction reactions of aromatic nitro group catalyzed by enzymes isolated from bacteria.

As shown in Figure 1.2, not all the nitroreductases yield amino derivatives as final products. The first reaction (R-1), catalyzed by enzymes extracted from *Mycobacterium species*, involves one-electron reduction of the nitro group to form a nitro anion radical, which reacts rapidly with O_2 to return to the starting compound and produces superoxide (Peterson *et al.*, 1979, quoted by Cerniglia and Somerville, 1995). The second reaction (R-2), catalyzed by cell-free extracts of *E. coli*, is a two-electron reduction which yields a stable nitroso product (Saz *et al.*, 1954 quoted by Cerniglia and Somerville, 1995). Nitroreductase enzymes of bacteria such as *Comamonas acidovorans, E. coli* and *Pseudomonas pseudoalcaligenes* catalyze the reduction of the nitroso compounds to hydroxylamino derivatives only (R-3) (Cerniglia and Somerville, 1995). Nitroreductase enzymes from *Salmonella typhimurium* may induce the formation of a nitrenium ion after acid-catalyzed decomposition of the hydroxylamino product (R-4) (Heflich *et al.*, 1985 quoted by Cerniglia and Somerville, 1995). Nitroreductases from *Rhodobacter capsulatus, Enterobacter cloacae, Bacteroides fragilis, Nocardia V.* and *Salmonella typhimurium* catalyze the reduction of aromatic nitro groups to the corresponding amino compounds (R-5) (Cerniglia and Somerville, 1995).

Purifications of various bacterial nitroreductases have been reported in the literature. Table 1.2 summarizes some characteristics of enzymes from *Pseudomonas pseudoalcaligenes, Bacteroides fragilis, Escherichia coli, Salmonella typhimurium, Enterobacter cloacae* and *Rhodobacter capsulatus* (Somerville *et al.*, 1995).

Table 1.2: Some characteristics of purified nitroreductases isolated from different bacteria.

Organism	Molecular Weight (kDa)	Electron donor	[a] -NO_2 reduced to
Bacteroides fragilis GA10624, Nrasa I	52	NADH	-NH_2
Enterobacter cloacae	27	NAD(P)H	-NHOH or -NH_2
Escherichia coli IB2	42	NAD(P)H	-NHOH
Pseudomonas pseudoalcaligenes JS45	29-33	NADPH	-NHOH
Rhodobacter capsulatus E1F1	54	NAD(P)H	-NH_2

Taken from Cerniglia and Somerville, 1995.

[a]: Possible substrate R-$C_6H_4NO_2$

All these enzymes require flavin mononucleotide (FMN) as a cofactor.

Bacterial nitroreductases fall into two general classes. Type I enzymes, which are oxygen-insensitive, use a two-electron reduction mechanism and are able to reduce nitro groups under aerobic conditions. They are FMN-requiring flavoproteins and they use either NADPH or NADH as electron donor (Table 1.2). Conversely, Type II enzymes are oxygen-sensitive, reduce nitro groups in a single electron process and form a nitro anion radical (Kitts *et al.*, 2000). Type II nitroreductases have been shown to catalyze the reaction R-1, shown in Figure 1.2.

In *Escherichia coli*, there is evidence of three different oxygen-insensitive nitroreductases (IA, IB1 and IB2) and two oxygen sensitive forms (IIa and IIb). The major

nitroreductase, IA, was simulated by NADPH in the reduction of nitrofurazone, and had a molecular weight of 50 kDa. IB1 (52 kDa) was specific for NADPH, while IB2 (42 kDa) appeared to be active with either NADH or NADPH as electron donors (Bryant *et al.*, 1981; Bryant and McElroy, 1992). The two oxygen-sensitive nitroreductases had molecular weights of 120 kDa and 760 kDa, respectively (Bryant and McElroy, 1992).

Oxygen-insensitive nitroreductase also has been partially purified from *Enterobacter cloacae* strain 96-3. This enzyme had a molecular weight of 26 kDa and showed a broad substrate specificity, with TNT as the most rapidly-reduced substrate. Either NADPH or NADH can be utilized as a source of reducing equivalents, although NADH is the preferred electron donor (Bryant and De Luca, 1991). Further purification of the enzyme from *Enterobacter cloacae* was reported by Koder and Miller (1998), who showed that the purified enzyme was stable for over 6 months in a phosphate buffer (pH 7.0) at 4°C.

Nitroreductase purified from *Pseudomonas pseudoalcaligenes* JS45 had an absorbance spectrum typical of flavoproteins, and contained tightly-bound FMN. The enzyme was active in the range of pH 5 to 9, with maximal activity at pH 8.0, and was relatively stable at temperatures below 40 °C. The molecular mass determination showed a monomer between 30-33 kDa. This enzyme was specific for NADPH and used nitrobenzene as substrate (Somerville *et al.*, 1995).

Nitroreductase purified from *Salmonella typhimurium* is a flavin-dependent oxygen-insensitive nitroreductase that uses nitrofurazone as substrate. This enzyme can use either

FMN, FAD or riboflavin as the cofactor but highest reduction rates are obtained with FMN. Both NADH and NADPH can act as electron donors (Watanabe *et al.*, 1998).

Nitroreductase also has been purified recently from *Pseudomonas aeruginosa* (Oh *et al.*, 2001). This enzyme requires NADH as a cosubstrate, and is stable between 25 to 37°C and at pH conditions between 6.5 and 8.0.

Nitroreductases also have been isolated from plants (Shah and Spain, 1996). Ferredoxin:NADP oxidoreductase (FNR) from spinach exhibits oxygen-sensitive nitroreductase activity. Under anaerobic conditions, FNR catalyzes the reduction of tetryl to N-methylpicramide with the nitroanion radical as an intermediate. This plant nitroreductase reaction resembles one found in bacteria (Figure 1.2, R-1).

To date, there is no study reported in the literature where nitroreductase activity has been identified in soil invertebrates (*e.g.* earthworms, enchytraeids or collembola). However, Renoux *et al.* (2000) showed that *Eisenia andrei* (a compost worm) was able to transform TNT into different amino-metabolites. The authors speculated that this biotransformation process may be enzymatic. TNT was transformed in *Eisenia andrei* extract to amino metabolites, 2A-DNT, 4-ADNT and 2,4-DANT, which are similar to the products formed by some bacterial nitroreductases (Figure 1.2, R5).

1.4 Enchytraeid species

The enchytraeids are a separate oligochaete family within the class clitellata (phylum Annelida). The earthworms (e.g., *Lumbricus terrestris and Eisenia andrei*) are from the family Lumbricidae. Enchytraeids are important members of the soil fauna, and have been found in many habitats including: moors, meadows, coniferous and deciduous forests, arable land, and sewage sludge (Römbke and Moser, 1999). Their abundance seems to be stimulated by factors such as low pH (5.0 to 6.5) or organic matter greater than 4.4 % (Didden and Römbke, 2001). In many areas, these potworms have a very high biomass compared to other soil-inhabitant invertebrate groups, second only to the earthworms (Rombke and Moser, 1999). They live in the uppermost centimeters of the mineral soil (litter system or detritusphere), close to or in the soil pore water. In their natural habitats, enchytaeids can thus be exposed to dissolved chemicals. Furthermore, enchytraeids have species-specific feeding preferences (fungi, bacteria, algae, protozoa, collembola excrement, and organic material such as litter) often in combination with soil particles. Despite their small individual size (from 1 to 20 mm), enchytraeids probably enhance soil structure evolution by producing excrement, active burrowing, and transportation of soil particles (Didden, 1993).

The enchytraeids are good candidate test species for ecotoxicology studies because they are sensitive to many toxicants, and to different degrees than other soil invertebrates. A study of the effects of petroleum fractions on soil invertebrates showed that fuel oil is extremely toxic to enchytraeids, but nematodes were not affected (Pirhonen and Huhta, 1984). In a herbicide toxicity study with gardoprim (a commercial herbicide in which

terbutylazine is the active ingredient), an enchytraeid, *Cognettia sphagnetorum*, was the most sensitive species compared to other soil organisms like mites and nematodes species (Salminen *et al.*, 1996). On the other hand, a study of the effects of dimethoate (a pesticide) on three soil invertebrates, earthworm (*Aporrectodea caliginosa*), collembola (*F. candida*), and enchytraeid worm (*E. crypticus*), showed that the enchytraeid was the least sensitive (Martikainen, 1996). Posthuma *et al.*, (1997) found that zinc and copper decreased juvenile production in *E. crypticus* and were lethal to adults. Sensitivity of this species to these toxicants was in the same range as the earthworm (*E. fetida*). A study comparing the sensitivity of *Enchytraeus albidus* and *Eisenia fetida* to malathion (an organophosphate pesticide) showed that the enchytraeid is more sensitive than Eisenia species. However, the reproduction rate of the species *E. albidus* was lower in a natural soil than in OECD soil (Kuperman *et al.*, 1999). Recently, the acute and chronic effects of lindane (an insecticide used for treating arable crops and seed coatings) on several soil invertebrates, *Eisenia fetida*, *Enchytraeus albidus* and *Folsomia candida*, were studied. The authors showed that Enchytraeid had the same sensitivity to lindane as Eisenia species, but the springtail (*F. candida*) was the most sensitive species (Lock *et al.*, 2002).

Enchytraeids can be used on different test substrates, including water, agar or soil, as described by Rombke and Moser (1999). However, comparison of toxicological data derived from enchytraeids tested in agar or water to that of soil may be difficult since the behaviour of a chemical within those test substrates also may be different. The artificial soil (OECD, 1984) used in the present studies was chosen because it mimics the effects observed in the field, but also because soil composition was removed as a source of

variability. This artificial soil consists of a mixture of sand (70 %) and a small quantity of organic matter (10% of peat moss).

Table 1.3 compares the life cycle of *Eisenia andrei* to that of *Enchytraeus albidus;* the latter species was chosen for this study. There are several advantages favoring the use of *E. albidus* over *Eisenia andrei* as a test organism for toxicological assessment of a chemical. Enchytraeids are true soil inhabitants, which makes them more ecologically relevant than the earthworm, *Eisenia andrei,* commonly used for toxicity tests (Jarvis *et al.*, 1998; Renoux *et al.*, 2000; Robidoux *et al.*, 2000). In fact, *Eisenia andrei* is found in compost and requires high levels of organic matter to live in soil (Rombke, 1989). In addition, enchytraeids are easy to handle and breed under laboratory conditions, and have a shorter generation time than the earthworms. Finally, they are small, and thus fewer soil samples are required to perform a toxicity test.

As shown in Table 1.3, *E. albidus* has a shorter life-cycle (juvenile development from 21 days to 56 days) than *Eisenia andrei* (from 32 days to 73 days). A large literature database exists on the acute, as well as long-term, toxicological effects of several contaminants (pesticides, metals, PAHs and PCBs) on the enchytraeid species (Rombke, 1989; Puurtinen and Martikainen, 1997; Collado *et al.*, 1999; Kuperman *et al.*, 1999). To date, no studies have been reported that describe the toxic effects of explosives (like TNT) on *Enchytraeus albidus*. However, a recent study reported by Schäfer and Achazi (1999), described the effects of TNT on *Enchytraeus crypticus* (see Chapter 2 for more details). *Enchytraeus crypticus* and *E. albidus* are in the same family, but their

sensitivities to different chemicals may vary. *E. crypticus* is found in almost all types of soil, whereas *E. albidus* prefers soil with low to medium organic matter and good humidity. Finally, *E. albidus* is larger than *E. crypticus* and is easier to manipulate.

Table 1.3: Life cycle characteristics of the species *Eisenia andrei* and *Enchytraeus albidus*.

Parameter	***E. albidus***	***E. andrei***
Optimum Temperature	12-18 °C	20 °C
Age	4-5 months	4.5 years
Length of cocoon	1-1.2 mm	5-6 mm
Eggs per cocoon	7-10	2-6
Non-developed cocoons	50-60%	21.5%
Embryonic development (days)	12-18	nf
Juvenile development (days)	21-56	32-73
Total development cycle (days)	33-74	nf
Hatching length	1-3 mm	nf
Adult length	15-41 mm	20-70 mm

Information that was not found in the literature is indicated as "nf". Refer to Edwards and Bohlen (1996), Lokke and Van Gestel (1998) and Römbke and Moser (1999).

Prior to the toxicity studies, the enchytraeids (*E. albidus*) were cultured in the laboratory for at least one year. During this acclimation period, several generations were produced. The quality of reproduction was verified using the guidelines described by Rombke and

Moser (1999). The quality of reproduction complies with those guidelines, and the relevant data are presented in the Appendix.

1.5 Study Objectives

There were three main objectives of this M. Sc. study.

The first was to determine if the enchytraeid-reproduction test could be adapted for use in our facility for assessing toxicity of contaminants to soil invertebrates. The successful implementation of this test was demonstrated by using carbendazim (a known reproductive toxicant) and by generating an enchytraeid test control chart for this compound, as described in the Appendix.

The second objective was to assess effects of TNT on the lethality and reproduction of enchytraeids. This study is presented as Chapter 2.

The third objective was to describe the metabolic pathways underlying the observed ecotoxicological responses (lethality and reproduction). This objective was partially achieved with the results presented in Chapter 3.

Contribution of Authors

I have contributed to the two manuscripts (Chapters 2 and 3) in the following manner. For each paper, I prepared the first draft. For this purpose, a complete literature search on TNT, earthworms, enchytraeids and nitro-reducing microbes was done. Then, I compiled the comments on the first draft from my research director (Dr. Powlowski) and supervisor (Dr. Sunahara), and revised the manuscripts before submission to the selected journals. Dr. Renoux contributed to the first paper by providing statistical assistance, in addition to her comments. Dr. Sunahara was the corresponding author: he completed the forms and maintained contact with the journal after submission of the research papers.

I carried out all of the experiments, except for some routine HPLC analyses that were done by Louise Paquet (BRI Analytical Chemistry Lab). I also received assistance from Manon Sarrazin for the maintenance of the enchytraeid cultures during my holidays and maternity leave.

Chapter 2: Lethal and Sub-Chronic Effects of 2,4,6-Trinitrotoluene (TNT) on *Enchytraeus albidus* in Spiked Artificial Soil

Sabine G. Dodard [1,2], Agnès Y. Renoux [2,‡], Justin Powlowski [1],

and Geoffrey I. Sunahara [2,*]

[1] Department of Chemistry and Biochemistry, Concordia University

1455 de Maisonneuve West Blvd., Montreal, Quebec, Canada, H3G 1M8

[2] Biotechnology Research Institute, National Research Council of Canada

6100 Royalmount Ave., Montreal, Quebec, Canada, H4P 2R2

Running Title: Enchytraeids and TNT Toxicity

Ecotoxicology and Environmental Safety *In Press*

*Please address all correspondence to : Geoffrey I. Sunahara, Ph.D.
Biotechnology Research Institute
National Research Council of Canada
6100 Royalmount Ave.,
Montreal, QC, Canada, H4P 2R2
Tel: (514)-496-8030
Fax: (514)-496-6265
geoffrey.sunahara@nrc.ca

‡ Present address: Sanexen, 579 Le Breton Street, Longueuil, Québec, Canada

2.1 Abstract

The effects of 2,4,6-trinitrotoluene (TNT) exposure in spiked artificial soil on the survival and reproduction rate of the white potworm *Enchytraeus albidus* were studied. Based on the initial concentrations, TNT in freshly spiked soil decreased enchytraeid survival (21-d LC50 = 422 ± 63 (SD) mg/kg, N=3) and fecundity (42-d EC50 = 111 ± 34, N=4). Data also indicated that TNT was 5 – 10 times more lethal to juveniles than adults, and lethality was less pronounced in TNT-spiked soils aged for 21 days. A time-dependent decrease in the TNT concentrations, as well as a concomitant increase in the levels of 2-aminodinitrotoluene and 4-aminodinitrotoluene was observed during the 42-d toxicity test. Taken together, TNT (or one of its metabolites) is more lethal to juvenile than adult enchytraeids. This effect may explain, at least in part, the ability of TNT to decrease fecundity as determined using the enchytraeid mortality-reproduction test.

2.2 Introduction

Soils contaminated with explosive substances such as 2,4,6-trinitrotoluene (TNT; CAS 118-96-7) remain a worldwide problem. This is mainly due to the environmental toxicity and recalcitrance of this family of pollutants (Burrows *et al.*, 1989; Gordon and William, 1992; Talmage *et al.*, 1999; Sunahara *et al.*, 1998, 1999, 2001). Little has been reported in the peer-reviewed literature about the effects of TNT on soil invertebrates for standardized soil preparations under laboratory conditions. An earlier US-Army technical report stated that TNT (150 mg/kg) is lethal to earthworms (*Eisenia sp.*) in a forest soil, whereas TNT concentrations as high as 240 mg/kg were not lethal in spiked artificial soil (Phillips *et al.*, 1993). Earthworm (*Eisenia sp.*) survival tests have been used to assess the quality of field samples of soils from a TNT-production site (Simini *et al.*, 1995). Our laboratory has recently confirmed the lethal effects of TNT in soil (Robidoux *et al.*, 1999), and also has shown that TNT causes a decrease in reproduction of the earthworm *Eisenia andrei* (Robidoux *et al.*, 2000).

Schäfer and Achazi (1999) recently showed that TNT could decrease the survival (7-d LC50 = 1290 mg/kg), as well as the fecundity (28-d EC50 = 480 mg/kg) of enchytraeids (*Enchytraeus crypticus*) in spiked standard LUFA 2.2 soil preparations aged for 1 month prior to the study. These exposure levels were based on nominal concentrations of TNT in soil. This approach, however, assumes that the concentrations of bioavailable TNT in soil remained constant throughout the study. This may not be the case since our laboratory has recently shown that solvent-extractable TNT concentrations decreased in soil during the 14-d earthworm toxicity test (Renoux *et al.*, 2000).

Another question that remains unresolved is the mechanism by which a toxicant such as TNT can decrease the reproduction rate of earthworms (Kula, 1998). It is not known whether the TNT-mediated decrease in fecundity (i.e., a decrease in the number of juveniles counted) as reported by Schäfer and Achazi (1999) is caused by the toxicant's effects on cocoon production and hatching, or due to its lethal effects on hatched juveniles. The present study characterizes the effects of TNT on the survival and reproduction rate of enchytraeids (*Enchytraeus albidus*) using freshly spiked artificial soil and the ISO 16387 combined enchytraeid mortality-reproduction bioassay (ISO, 2001; Didden and Römbke, 2001). Toxicity effects were related to the changes in TNT concentrations in the test soils.

2.3 Materials and methods

2.3.1 Chemicals and Reagents

TNT was obtained from ICI Explosives Canada (McMasterville, Quebec, Canada). All other chemicals were ACS reagent grade or higher, and were obtained from either BDH (Toronto, Ontario, Canada) or Aldrich Chemical (Milwaukee, WI, USA). Deionized water was obtained using a Millipore Super-Q water purification system (Nepean, Ontario, Canada) and was used throughout this study. All glassware was washed with phosphate-free detergent, rinsed with acetone, and acid-washed before a final and thorough rinse with deionized water.

2.3.2 Preparation of the Artificial Soil

The artificial soil consisted of 10% *Sphagnum sp.* peat (The Gardener, Trois Rivières, Quebec, Canada), 20% kaolin clay (Feldspar, Atlanta, GA, USA) and 70% silica sand grade 4010 (Union, New Canaan, CT, USA), according to OECD guidelines (OECD, 1984). All dry constituents were thoroughly mixed. The pH of the soil was measured using the $CaCl_2$ method, according to the ISO guideline (ISO, 1994). The soil pH was adjusted to 6.0 ± 0.5 with calcium carbonate. The moisture content and water holding capacity of the soil were also measured according to test guidelines (Römbke and Moser, 1999).

Different concentrations of TNT were prepared in acetonitrile from a 20 mg/mL stock solution. For each experiment, 5 mL of each TNT solution was added to 100 g portions of air-dried artificial soil. The final concentrations of TNT ranged from zero (negative control) to 1000 mg TNT/kg dried soil and were chosen according to earlier reports describing TNT effects on earthworms and potworms (Phillips *et al.*, 1993; Simini *et al.*, 1995; Jarvis *et al.*, 1998; Robidoux *et al.*, 1999, 2000; Schäfer and Achazi, 1999; Renoux *et al.*, 2000). These concentrations were confirmed using HPLC analysis (Sunahara *et al.*, 1999). Soil samples were left for at least 16 h in the dark under a chemical hood to permit the evaporation of the solvent. A "solvent control" group (soil samples containing acetonitrile only but no TNT added) was also included.

2.3.3 Extraction and HPLC Analysis of TNT from Spiked Artificial Soil

Two g of soil (dry weight) was taken from separate test containers (described below) and was analyzed for TNT content using the US-EPA SW846 Method 8330 (US-EPA, 1997). For this analysis, 10 mL of acetonitrile was added to each soil sample in a 50 mL screw-top glass test tube and was sonicated (Branson 3200, Haake GH) at 60 KHz for 18 h at 21°C in the dark. Five mL of an aqueous calcium chloride solution (5 mg/mL) was mixed with 5 mL soil extract in a tightly closed glass vial, and was left to precipitate in the dark. After 30 min, the suspension was filtered through a 0.45 μm membrane (Millipore) before HPLC-UV analysis. An additional 5 g of soil (dry weight) was used for the pH determinations. The remaining soil sample was stored at 4°C until further analyses. All samples were analyzed within a week.

The HPLC-UV system consisted of a Waters Model 600 pump, a Model 717 Plus sample injector, a Model 996 Photodiode-Array detector, and a temperature control module. A Supelcosil C_8 column (25 cm x 4.6 mm ID, 5 μm particles) was used, with the column temperature at 35°C. The mobile phase was 82% (v/v) water and 18% 2-propanol; the flow rate was 1 mL/min. The sample volume injected was 50 μL with a run time of 40 min. The analytes were detected at a wavelength of 254 nm. The limit of detection was approximately 25 μg/L for TNT, and at least 50 μg/L for the metabolites. The latter were obtained from Accustandard (New Haven, CT). Precision was ≥ 95% (standard deviation <2%, signal to noise ratio = 3).

2.3.4 Handling of Enchytraeids

Enchytraeus albidus Henle 1837 (donated by Dr. J. Römbke, Germany) was cultured in a mixture of garden soil and OECD artificial soil (1:1, w:w), according to Römbke and Moser (1999). The potworms were fed once a week with pre-boiled lyophilized oats. Deionized water was added to the soil culture medium to reach 60% of its water holding capacity. Mature potworms (those having eggs, seen as white spots in the clitellum region) were selected and acclimatized to artificial soil for 24 hours before the start of the experiment.

2.3.5 Enchytraeid Mortality-Reproduction Test

Briefly, 20 g of soil (dry weight) was placed into separate 200-mL glass jars and was hydrated to 60% of its water holding capacity. Each jar received 40 mg (dry weight) of lyophilized oats that were mixed into the soil. Ten acclimatized enchytraeids were then added to each test jar (except those jars used for TNT analyses in which no potworms were added). Test containers were prepared at least in triplicate. TNT concentrations in soil ranged from 50 to 1000 mg TNT/kg dry soil (nominal concentrations). Control groups included replicate jars containing all constituents but no TNT (solvent control; n=6) and some jars with no acetonitrile vehicle added (negative control; n=6). Each jar was covered with a glass Petri dish to prevent moisture loss during the exposure period (at 20°C with a 16 : 8 light-dark cycle). Each week, the moisture content was verified, 25 mg of lyophilized oats were added to the surface of the soil, and the soil was re-hydrated to 60% water holding capacity.

Lethality of the potworms was assessed on day 21 of the test. All surviving adults were removed from the soil and were counted. The soil (containing cocoons and juveniles) was then returned to the same test container, and the soil moisture was re-adjusted as described above. The container was incubated for another 21 d, under the same conditions as described above. Weekly, the soil moisture content was verified and re-adjusted accordingly; food was also added except during the fourth week of the experiment.

Fecundity of potworms was assessed by counting the number of hatched juveniles on day 42 of the test. For this, each soil sample was transferred into a separate Petri dish (150 mm x 25 mm), and 5 mL of 95% ethanol was added to the soil as a preservative. Water was added into each dish, to a depth of 1 to 2 cm. About 250 μL of an ethanolic 1% Bengal Rose solution was added to each dish as well, and the soil slurry was gently mixed and left overnight. The liquid phase was then decanted and the red-stained juveniles were counted using a fine spatula and a small forceps. This method is highly reproducible, based on the results of a round-robin international inter-laboratory study (Römbke and Moser, 1999).

A separate series of studies was carried out to test the possibility that the TNT-induced decrease in fecundity may be associated with the lethal effects of TNT upon the hatched juveniles (corresponding to days 21 to 42 of enchytraeid mortality-reproduction test). Thirteen juvenile enchytraeids (2 to 5 mm) were exposed for 21 days in triplicate to TNT (45 and 118 mg/kg) in freshly spiked and 21-d aged spiked soil samples. The latter was

included because the enchytraeid mortality-reproduction test involves the exposure of the hatched juveniles to 21-d aged spiked soil. At the end of exposure, the number of surviving juveniles was recorded.

2.3.6 Quality Control

For the enchytraeid mortality-reproduction test, the acceptability criteria for survival was >80% and the number of juveniles counted was >25 (per jar), using the solvent and negative control groups. In rare cases, some test jars were not adequately covered by their Petri dishes and were rejected from the study due to dehydration; these were not included in the analyses of data. For all control groups, the average survival (± SD) after 21 days was 89 $\pm$ 9 (n=59) and the average total number of juveniles per test unit was 49 $\pm$ 37 (n=59). These tests were judged acceptable according to test guidelines (Römbke and Moser, 1999). In addition, the performance of this test was verified using the fungicide Derosal (AgrEvo UK) as the reference toxicant (positive control group). This product contained 36% of the active ingredient carbendazim (methyl benzimidazol-2-yl carbamate; CAS 10605-21-7). Reference toxicant data (42-d EC50-fecundity = 1.03 and 2.11 mg/kg: expressed as active ingredient) from two separate studies were consistent with those reported by Römbke and Moser (1999).

2.3.7 Statistical Analyses

Maximum Likelihood-Probit analysis (Finney, 1971) was used to estimate the EC50 and EC20 values (Effective concentrations, 50 and 20%, respectively) at 95% confidence (CI). Survival data were transformed by Arcsine Square Root before analysis. The

reproduction data were used untransformed. These analyses were done using the TNT concentrations measured in soil samples collected at the beginning of the experiment (day 0). To determine the lowest-observed-effect concentration (LOEC) and the no-observed-effect concentration (NOEC), an ANOVA procedure followed by a Dunnett's Test was used. All statistical analyses were done with the ToxCalc software (Version 5.0.18, Tidepool Scientific Software, McKinleyville, CA, USA). The endpoints (EC20 and EC50) were expressed as the average ± standard deviation (SD).

2.4 Results

HPLC analyses of the acetonitrile extracts of TNT-spiked artificial soil samples taken at the beginning of the experiment showed that the measured concentrations of TNT in soil were very close to the nominal concentrations (99.1 ± 0.2 (SD) %, n=3; Table 2.1). The maximum difference between initial measured and target values was 12%, which was found in the 50 mg/kg test sample taken at day zero (data not shown). These data indicate that any variability of the toxicity, at least at the beginning of the study, would not be due to individual spiking differences at each test concentration.

On the other hand, time course studies indicated that some microbial TNT degradation might have occurred since the primary products of TNT degradation, 2-amino-dinitrotoluene (2-ADNT; CAS 35572-78-2) and 4-amino-dinitrotoluene (4-ADNT; CAS 19406-51-0), were detected in the 7-d, 21-d and 42-d spiked soil samples (Table 2.1; Fig. 2.1). In the 42-d samples, only 5.6 ± 5.5% (n=3) of the original TNT spike was detected. Preliminary studies indicated that these effects were not due to the presence of

enchytraeids, the alterations in soil pH or the presence of light. Data indicated that the pH of the soil did not change during the study (pH ranged from 6.36 to 6.49). Exposure of soil to light during the experimental procedure is not a factor since the decrease in TNT concentration and appearance of TNT metabolites occurred under both light and dark conditions (data not shown).

Table 2.1: Time Course Study Examining the Recovery of TNT and Selected Metabolites in Spiked Artificial Soil

Test day	Compounds recovered [a,b] (%)		
	TNT	2-ADNT [c]	4-ADNT [c]
0	99.1 ± 0.2 (3) [d]	0	0
1	73.8 ± 10.3 (3)	0.1 ± 0.1 (3)	0.2 ± 0.2 (3)
7	35.9 ± 29.0 (3)	2.5 ± 2.0 (3)	4.8 ± 5.3 (3)
21	20.6 ± 14.9 (3)	4.6 ± 1.8 (3)	7.3 ± 4.8 (3)
42	5.6 ± 5.5 (3)	6.4 ± 1.9 (3)	8.1 ± 2.4 (3)

Notes:

[a] Determined by HPLC analyses, from acetonitrile extracts of TNT-spiked soil samples as described in the **Materials and Methods** section.

[b] Recovery was calculated by dividing the TNT concentrations (mg TNT/kg dry soil) taken from soil samples at different periods of aging (up to 42 d) by their initial target concentrations (from 50 to 1000 mg TNT/kg).

[c] Metabolites (2-ADNT, 2-aminodinitrotoluene; 4-ADNT, 4-aminodinitrotoluene) are expressed as % of initial TNT concentrations (mg TNT/kg dry soil). Calculated by dividing the measured concentrations of metabolites from soil samples at different periods of aging (up to 42 d) by their TNT initial target concentration.

[d] Mean ± SD (n= number of samples analyzed).

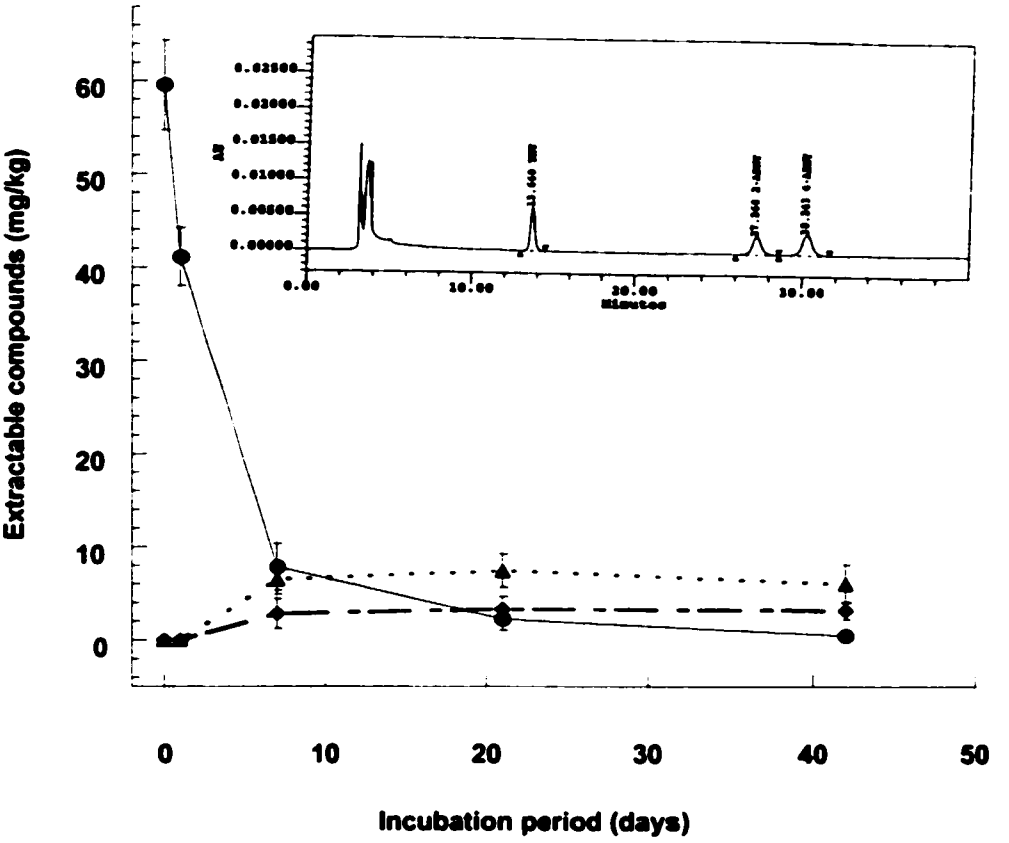


FIGURE 2.1: Compounds measured at different exposure periods in TNT-spiked (60 mg/kg dry weight) artificial (OECD) soil

Soil samples were extracted using the acetonitrile-sonication procedure, as described in the **Materials and Methods** section. Data (expressed as mean concentration ± SD, mg/kg dry soil) were taken from one experiment done in triplicate. Symbols used: ● TNT, ♦ 2-ADNT, ▲ 4-ADNT. *Inset* box shows a typical HPLC profile of a soil extract sample; the first peak is the void volume, and compounds eluted as follows: TNT (Retention time = 13.66 minutes), 2-ADNT (27.26 minutes) and 4-ADNT (30.24 minutes). Each peak is well separated.

Based on the nominal as well as the initial TNT concentrations measured in the soil samples (Table 2.1), exposure to TNT-spiked soil was lethal to *E. albidus* in a concentration-dependent manner (Fig. 2.2A and Table 2.2). A TNT concentration-dependent decrease in enchytraeid survival (pooled 21-d LC50 = 422 $\pm$ 63 mg/kg) was observed in three different experiments. The variability of enchytraeid survival at the different TNT concentrations was low. The LOEC was within the variability of the 21-d LC50 values for individual studies (Table 2.2). A fourth experiment was carried out at a lower range of concentrations (< LC20) in order to reconfirm the reproduction studies (described below).

TNT exposure in artificial soil also caused a concentration-dependent decrease in the fecundity (based on the number of juveniles counted) of *E. albidus* (Fig. 2.2B). The results of four experiments are summarized in Table 2.2. Clearly, TNT decreased potworm fecundity (42-d EC50 = 111 $\pm$ 34 mg/kg, based on TNT concentrations determined in the day 0 samples) compared to control groups. In all of the experiments carried out in this study, there was a consistent and significant decrease in fecundity (number of surviving juveniles) at sublethal concentrations of TNT in the spiked soil samples. It is possible, however, that the TNT-induced decrease in the number of surviving juveniles (days 21 to 42 of test) is due to a lethal effect on juveniles following hatching.

Table 2.2: Lethal and Sub-Chronic Effects of TNT on *Enchytraeus albidus* in Spiked Artificial Soil

	Mortality at test day 21 (mg/kg) [a]				Fecundity at test day 42 (mg/kg) [b]			
Test	LC20 (95% CI)	LC50 (95% CI)	LOEC	NOEC	EC20 (95% CI)	EC50 (95% CI)	LOEC	NOEC
1	234 (147, 301)	362 (275, 438)	389	195	88 (38, 115)	133 (95, 173)	194	97
2	313 (231, 367)	414 (349, 478)	380	298	33 (0, 83)	107 (1, 196)	82	<82
3	403 (262, 464)	488 (392, 539)	522	309	84 (38, 114)	140 (100, 187)	197	95
4	nd [c]	nd [c]	174	147	29 (3, 49)	64 (28, 103)	51	26
	317 ± 85 (3) [d]	422 ± 63 (3) [d]			59 ± 32 (4) [d]	111 ± 34 (4) [d]		

Notes:

[a] *E. albidus* mortality was assessed on test day 21following exposure to TNT-spiked soil, as described in **Materials and Methods** section.

[b] *E. albidus* fecundity responses were assessed following 42-d exposure to TNT-spiked soil, as described in **Materials and Methods** section.

[c] nd: Lethality was not determined because this experiment was carried out at sublethal concentrations to assess exposure effects on fecundity only.

[d] Average ± SD of N experiments conducted (N).

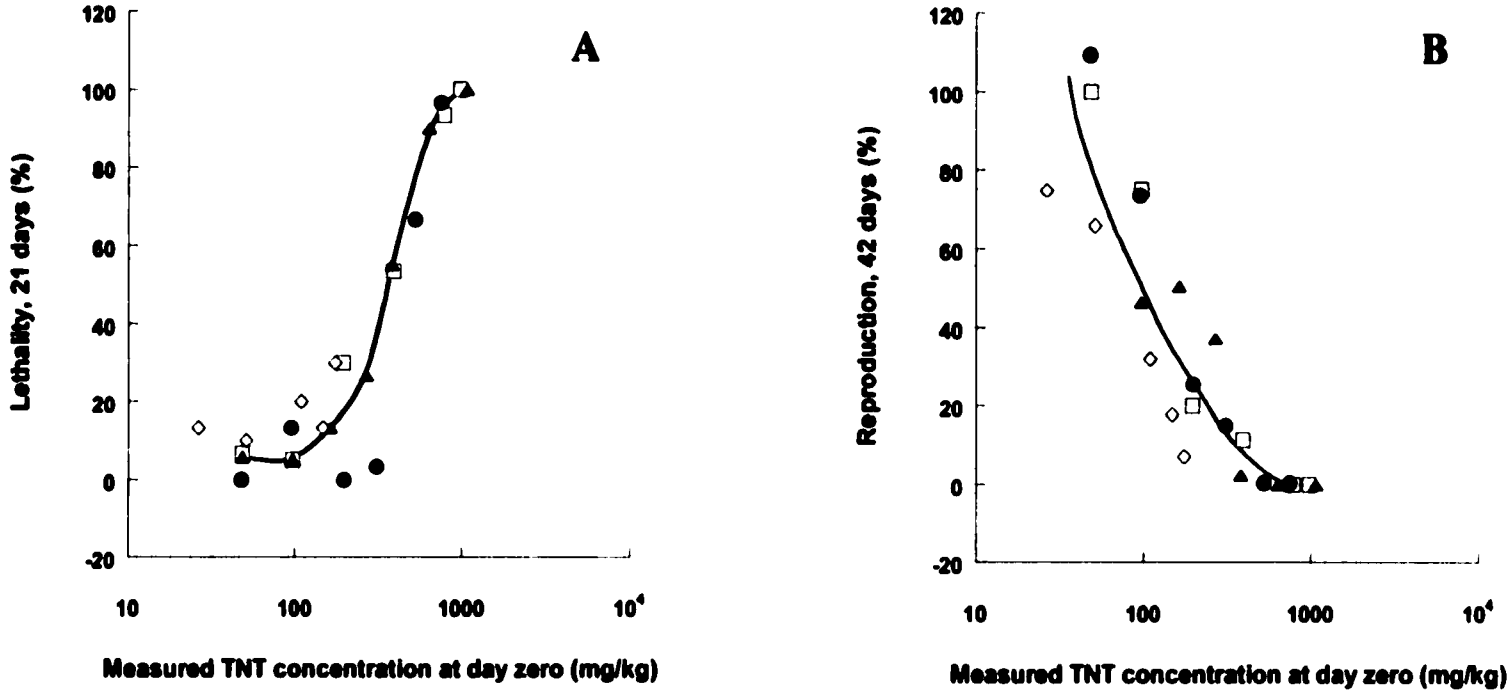


FIGURE 2.2: Effects of exposure to TNT-spiked soil on the *E. albidus* mortality at test day 21 (A) and on *E. albidus* fecundity at test day 42 (B) using the enchytraeid mortality-reproduction test

Experimental conditions are described in **Materials and Methods**. Data are plotted as a function of the TNT concentration measured in the soil samples at the start of the experiment. Data (expressed as a % of the combined negative and solvent control groups) from four separate experiments are indicated by different symbols (▲, ◇, ●, and □).

Accordingly, a series of preliminary studies was carried out using juvenile enchytraeids exposed to TNT (45 and 118 mg/kg) for 21 days in freshly spiked and 21-d aged spiked soil samples. Results indicated that freshly-spiked TNT soils were lethal to juveniles in a concentration-dependent manner (Fig. 2.3), an effect which was consistently greater than that caused by TNT-spiked soils aged for 21 days. Exposure to freshly amended TNT-contaminated soil (45 and 118 mg/kg dry weight) caused a significant decrease in the number of juveniles (Student's *t*- test, $P < 0.05$; Fig. 2.3) compared to controls (no TNT added to soil); whereas for the 21-d aged TNT contaminated soil, this effect was only observed in the high dose treatment group (118 mg/kg). The 21-d LC50 for juveniles in freshly spiked soil was 44 mg TNT/kg soil and that for the 21 day-aged soil was 89 mg/kg. Based on these results, it is also worth noting that juveniles were 5 – 10 times more sensitive to the lethal effects of TNT than adults (Table 2.2). The extractable TNT concentrations in the freshly-spiked soils at the end of the toxicity study (calculated using data from Table 2.1) were 2.27 and 6.93 mg/kg for the 45 and 181 mg/kg (nominal concentrations), respectively. For the spiked 21-d aged soils, they were 0.79 and 3.34 mg/kg for the 45 and 118 mg/kg (nominal concentrations), respectively.

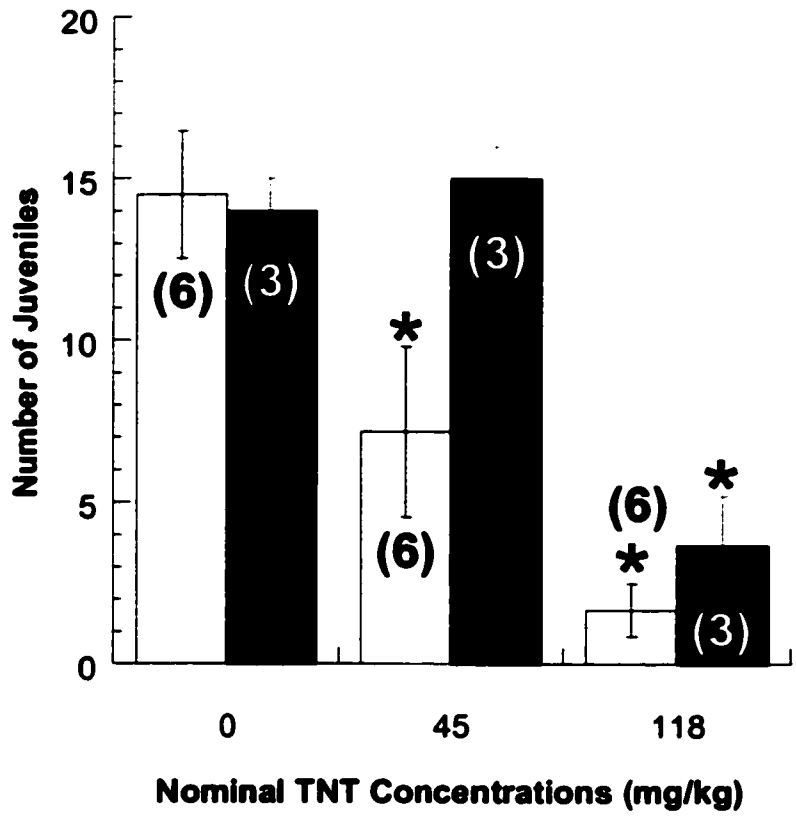


FIGURE 2.3: Effect of TNT exposure on the survival of juvenile enchytraeids in freshly-spiked (open bars) and 21-d aged artificial soils (filled bars).

Data are expressed as the mean number of juveniles per test unit ± SD (n = 3-6). Asterisk (*) denotes significant difference ($P < 0.05$ using Student's *t*-test for unpaired data) compared to their respective controls (no TNT added).

2.5 Discussion

The present study examines the lethal and sub-chronic toxic effects of TNT-spiked artificial soil on Enchytraeids (*E. albidus*) using the 42-day enchytraeid mortality-reproduction test. Lethality (number of survivors after 21-days exposure) and reproduction endpoints (number of juveniles remaining at the end of the 42-day study period) were compared to nominal, as well as measured TNT concentrations in soil. Data indicated that exposure to TNT-spiked soil was lethal to enchytraeids (21-d LC50 = 422 ± 63 mg/kg dry soil, based on nominal concentrations). These results support the conclusion of an earlier work (Schäfer and Achazi, 1999) that demonstrated similar lethal effects using another species of the genus *Enchytraeus* (*E. crypticus*) at higher soil TNT concentrations (7-d LC50 = 1290 mg/kg, nominal concentration) and a shorter exposure period. It is interesting to note that the "toxicant doses" (mg/kg multiplied by exposure period) to cause 50% mortality in enchytraeids observed in these two studies were very similar. On the other hand, discrepancies in the toxicity results reported in this article and that of Schäfer and Achazi (1999) may be due to differences related to the choice of enchytraeid test species, soil types and handling procedures.

The possibility that *Enchytraeus crypticus* (used by Schäfer and Achazi) may be less sensitive to TNT than *Enchytraeus albidus* (used in the present study) is not likely, since tests with carbendazim and 4-nitrophenol (Collado *et al.*, 1999) indicated only a two-fold sensitivity difference between *Enchytraeus albidus* and another small *Enchytraeus*-species (*Enchytraeus luxuriosus*). Future studies will be carried out to test the sensitivity of *E. albidus* and *E. crypticus* to the toxic effects of TNT.

Differences between the results of the present study and those of Schäfer and Achazi (1999) do not appear to be due to differences in the organic matter content of the soil types. These workers used the standard LUFA 2.2 soil preparation (containing 2.5% organic matter, Gong *et al.*, 1999a), whereas the OECD artificial soil (3-5% organic matter) was used in the present study. Similarities in organic matter contents between the LUFA 2.2 and OECD soils would infer similar concentrations of bioavailable (unbound) toxicant.

It should be noted that Schäfer and Achazi (1999) used a TNT-spiked LUFA 2.2 soil that was aged for one month prior to toxicity testing, whereas freshly-spiked artificial soil preparations were used in the present study. Based on HPLC analysis of the soil solvent extracts, a time-dependent decrease in TNT concentration in the artificial soil samples was observed (Table 2.1 and Fig. 2.1). This effect was not due to the presence of light (used in the light-dark cycle of the enchytraeid test). This study showed that, after 21 days, up to 79% of the TNT added to soil was not extractable using the acetonitrile-sonication technique. This decrease was even greater after 42 days when only 5.6 ± 5.5 % of the original TNT spike could be recovered. This observation would explain why Schäfer and Achazi (1999) required TNT concentrations much higher than those used in the present study, in order to estimate their LC50 values. Thus, data presented in this article strongly supports the possibility that changes in bioavailable TNT would explain the differences in the two studies, and that "soil aging" is an important variable in the conduct of the enchytraeid mortality-reproduction test.

Concomitant with the decrease in TNT levels in spiked soil samples was the appearance of two reduced TNT degradation products (2-ADNT and 4-ADNT). These results are similar to an earlier study using TNT-spiked forest soil samples (Renoux *et al.*, 2000).

It is not presently known if the observed toxic effects of TNT (lethality after 21-day exposure or decreased number of juveniles at the end of the 42-day study period) may be attributed to TNT, its reduced metabolites, or both. The relative toxicity of 2-ADNT and 4-ADNT in enchytraeids is not presently known. Studies will be conducted to characterize the relative toxicities of these TNT degradation products in adult and juvenile enchytraeids using artificial soils spiked with the pure compounds.

The present study shows for the first time that juvenile enchytraeids are more sensitive (five to ten-times; Fig. 2.3) than adults (Table 2.2) to the lethal effects of TNT, its reduced metabolites, or both (Fig. 2.1 and Table 2.1) in spiked artificial soil. The exact mechanism underlying this lethal effect is not presently known, but may reflect age-dependent differences in TNT detoxification or sensitivities at a similar locus of toxicity. Further investigations will be done to determine the identity of the active toxic compound. The process by which TNT (or one of its metabolites) exerts its inhibitory effect on potworm reproduction is not also known. It is believed that a decrease in the number of juvenile potworms counted at the end of the enchytraeid mortality-reproduction test (42d-EC50 = 111 $\pm$ 34 mg/kg) reflects a decrease in the reproductive capacity (e.g., reduced number of fertilized eggs and hatched cocoons). However, based on the present results, it is also possible that juvenile mortality may have occurred

(Fig.2.3; 21d-EC50 = 88 mg/kg) during the reproduction test, as evidenced by the exposure of juveniles to 21-d aged TNT spiked soil and may account, at least in part, for this toxicant's effect on enchytraeid fecundity.

Considering the differential sensitivity of juveniles to TNT in freshly spiked compared to 21-d aged soils (Fig. 2.3), one would expect to find more metabolites (2-ADNT and 4-ADNT) in 21-d aged soil than in freshly-spiked soil (Table 2.1). If juvenile mortality was solely due to the presence of these metabolites, then one would expect more lethal effects following exposure to the 21-d aged than freshly-spiked soil samples. This was not the case because juvenile mortality was lower following exposure to the 21-d aged soil than to the freshly-spiked soil. These results indicate that the detected TNT metabolites (2-ADNT and 4-ADNT) are not the major lethal toxicants.

If one considers the levels of extractable TNT, 2-ADNT and 4-ADNT after 0, 21 and 42 days of soil aging (Table 2.1), there was a time-dependent decrease in TNT (ranging from 100% recovery at day 0 to <6% after 42 d), with a concomitant increase in total ADNT (ranging from 0% at day 0, to about 14% after 42 d). Soil samples aged for 21 d contained intermediate levels of TNT (about 21% remaining), and total ADNT levels (<12%) comparable to those found after 42 d of aging. At the end of the juvenile survival experiment (Fig. 2.3), more juveniles were found in the test groups exposed for twenty-one days to the 21-d aged soils (representing 42 d of TNT in soil) than those exposed to the freshly-spiked soil (representing 21 d of TNT in soil). The inverse relationship

between the number of surviving juveniles and the amount of extractable TNT (and not total ADNT) strongly indicates that TNT was the causative lethal agent.

In view of the time-dependent disappearance of extractable TNT observed in the present study, the EC50 values for decreased enchytraeid fecundity described in both the present article and that of Schäfer and Achazi (1999) are probably over-estimated. TNT-soil adsorption as well as biotransformation processes may have occurred during the experiment and would effectively lead to lower bioavailable TNT concentrations to the test organism. Unfortunately, it is not known at what moment toxicity (both lethality and reproductive effects) appeared during the 42 days of exposure to TNT. For this reason, toxicity endpoints were expressed based on the initial measured concentrations. However, correcting these values for TNT recovery (Table 2.1) may be overly simplistic and misleading, since a lower 42-d EC50 value (consistent with decreased TNT concentrations in the test soil) would erroneously imply a constant exposure level.

2.6 Conclusions

In summary, TNT or one of its metabolites decreased the survival and fecundity of *E. albidus* in TNT-spiked artificial soil as measured using the standard enchytraeid mortality-reproduction test. Results indicated that TNT is more lethal to juvenile than adult enchytraeids in freshly-spiked and 21-d aged soils, and that these effects may explain, at least in part, the observed TNT-mediated decrease in enchytraeid reproduction rate.

2.7 Acknowledgements

The authors wish to express their gratitude to Drs. Sonia Thiboutot and Guy Ampleman (DREV) for their interest in this project, and to Louise Paquet and Manon Sarrazin for their technical assistance. Also, we thank Drs. Jörg Römbke and Thomas Moser (ECT Oekotoxikologie GmbH, Flörsheim am Main, Germany) for their help in the writing of this manuscript.

Chapter 3: Biotransformation of TNT by Enchytraeids (*Enchytraeus albidus*)

SABINE G. DODARD[1,2], JUSTIN POWLOWSKI[1],

AND GEOFFREY I. SUNAHARA[2,*]

[1] *Department of Chemistry and Biochemistry, Concordia University*
1455 De Maisonneuve Blvd. West, Montreal, Quebec, Canada, H3G 1M8

[2] *Biotechnology Research Institute, National Research Council of Canada*
6100 Royalmount Ave., Montreal, Quebec, Canada, H4P 2R2

Submitted to : *ES&T*

*Please address all correspondence to :

Geoffrey I. Sunahara, Ph.D.
Biotechnology Research Institute
National Research Council of Canada
6100 Royalmount Ave.,
Montreal, QC, Canada, H4P 2R2
Tel: (514)-496-8030
Fax: (514)-496-6265
geoffrey.sunahara@nrc.ca

3.1 ABSTRACT

2,4,6-Trinitrotoluene (TNT) is toxic to soil invertebrates such as earthworms and enchytraeids, but little is known about its mechanism of toxicity and how it is metabolized. The biotransformation of TNT by enchytraeids (*Enchytraeus albidus*) was, therefore, examined *in vivo* and *in vitro*. Adult enchytraeids were exposed to non-lethal concentrations of TNT in amended artificial soil for 21 d, or to TNT in solution for 20 h. HPLC analyses of exposed enchytraeids indicated that TNT was transformed *in vivo* to 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene and 2,4-diaminonitrotoluene, confirming earlier studies using other soil invertebrates. Results also indicated that TNT metabolites were formed within the enchytraeid rather than being transferred from the soil into the worm. Incubation of unexposed enchytraeid homogenates with TNT led to a protein-dependent appearance of these metabolites *in vitro*, but only when incubations were carried out for extended periods (≥ 16 h). Cellular fractionation of enchytraeids indicated that most of this activity resided in the 8,000 *x g* pellet. Furthermore, this activity was completely inhibited by antibiotics (tetracycline or Pen-Strep). These results demonstrate that enchytraeids can degrade TNT to its monoamino- and diamino-metabolites, at least in part, through bacteria within the host organism.

3.2 Introduction

2,4,6-Trinitrotoluene (TNT) is an environmentally persistent explosive that has toxic effects on a number of species including humans (Talmage *et al.*, 1999; Renoux *et al.*, 2001; Sunahara *et al.*, 2001). Recent studies have shown that certain soil invertebrates (earthworms and enchytraeids) are sensitive to TNT following exposure to amended- as well as field-contaminated soils. These toxic effects include lethality and decreased reproductive capacity (Simini *et al.*, 1995; Jarvis *et al.*, 1998; Schäfer and Achazi, 1999; Robidoux *et al.*, 1999, 2000; Renoux *et al.*, 2000). The exact mechanism of these effects and their underlying metabolic pathways are not well characterized.

Earlier studies also have shown that earthworms can partially metabolize TNT *in vivo*, following exposure to TNT-contaminated soil (Renoux *et al.*, 2000; Robidoux *et al.*, 2002). The TNT metabolites detected were: 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT) and in some cases 2,6-diamino-4-nitrotoluene (2,6-DANT). Recent preliminary studies using the soil-dwelling white potworm, *Enchytraeus albidus*, exposed to TNT-amended soil have also indicated the presence of several reduced TNT degradation products (2-ADNT, 4-ADNT and 2,4-DANT) in tissue extracts. It is not known, however, whether the presence of these metabolites in the potworms is due to the transformation of TNT within the worm, or by diffusion of TNT metabolites from the soil into the worm.

The biotransformation of TNT in exposed earthworms depends on the TNT concentration in soil, as well as the duration of exposure to the toxicant (Renoux *et al.*, 2000). The same

metabolites also accumulated when homogenates of unexposed earthworms were incubated with TNT. Renoux *et al.* (2000) speculated that the metabolism of TNT in the earthworm was due to enzymes (*e.g.*, nitroreductases, cytochrome P450 oxygenases, etc.) located in the earthworm tissue or by gut-associated micro-organisms that produce these enzymes. However, it was not clear which of these two mechanisms explained the partial degradation of TNT in the earthworm.

The possibility that enzymes (*e.g.*, nitroreductase) play a role in the reductive metabolism of TNT in soil invertebrates is of considerable interest. For example, nitroreductase-type I may be involved in the initial steps in the microbial degradation of TNT, catalyzing the two-electron sequential reduction of electrophilic nitro groups to nitroso- and amino-derivatives (reviewed by Gorontzy *et al.*, 1994; Ahmad and Hughes, 2000). Nitroaromatic-metabolizing activities have been characterized in different microbial species including *Enterobacter cloacae*, *E. coli*, *S. typhimurium*, *P. pseudoalcaligenes* and *Clostridium* (Bryant and De Luca, 1991; Sommerville *et al.*, 1995, Ahmad and Hughes, 2000) as well as fungi (Hawari *et al.*, 1999; Fritsche *et al.*, 2000). To date, no studies have appeared linking the presence of microbial enzymes with the degradation of TNT in soil invertebrates. The present article describes a series of studies to characterize the metabolism of TNT in enchytraeids *in vivo* and *in vitro*, and demonstrates that this effect is due to microbes (bacteria) residing within the host organism.

3.3 Experimental Section

3.3.1 Chemical and reagents

TNT (CAS 118-96-7) was obtained from ICI Explosives Canada (McMasterville, Quebec). The reference TNT metabolites, 2-ADNT (CAS 35572-78-2), 4-ADNT (CAS 19406-51-0), 2,4-DANT (CAS 6629-29-4) and 2,6-DANT (CAS 59229-75-3) were > 99% pure and were obtained from Accustandard (New Haven, CT). All other chemicals were ACS reagent-grade or higher, and were purchased from either BDH (Toronto, Ontario) or Aldrich Chemical (Milwaukee, WI). Deionized water was obtained using a Millipore Super-Q water purification system (Nepean, Ontario) and was used throughout this study. All glassware was washed with phosphate-free detergent, rinsed with acetone, and acid-washed before a final and thorough rinse with deionized water.

3.3.2 HPLC analysis of TNT and its metabolites

The US-EPA SW-846 Method 8330 (US-EPA 1997) was used to determine the concentrations of TNT and its metabolites in solution, and in the extracts of soil and enchytraeid homogenates. Briefly, 10 mL of acetonitrile was added to each 2 g soil sample in a 50 mL screw-top glass test tube and was sonicated (Branson 3200, Haake GH) at 60 KHz for 18 h at 21°C in the dark. Five mL of an aqueous calcium chloride solution (5 mg/mL) was mixed with 5 mL soil extract in a tightly closed glass vial, and was left to precipitate in the dark. After 30 min, the suspension was filtered through a 0.45 μm membrane (Millipore) before HPLC-UV analysis.

Frozen enchytraeids (described below) were thawed on ice and homogenized in ice-cold acetonitrile (10 worms per mL) using a glass dounce (3-5 passes). The homogenate was then diluted (1:1, v/v) with ice-cold 20 mM phosphate buffer (pH 7.6). All steps were carried out on ice. Samples were vortexed for 1 min and kept at 4°C for at least 2 h to allow protein precipitation. Supernatants (from *in vivo* and *in vitro* studies, described later) were collected following centrifugation (Beckman Coulter, Allegra 21R) at 2500 *x g* for 10 min at 4 °C prior to HPLC analysis for TNT and related metabolites.

A Waters HPLC system composed of a Model 600 pump, Model 717 injector, a Model 996 Photodiode-Array detector and a temperature control module, was used to quantitate TNT and its metabolites in the test media and in enchytraeid extracts. Analytes were separated using a Supelcosil C8 column (25 cm x 4.6 mm i.d., 5 µm particles). The column temperature was maintained at 35 °C throughout each run. The mobile phase was 82 % (v/v) water and 18 % (v/v) 2-propanol. The flow rate was 1 mL/min. Sample injection volume was 50 µL and the total run time was 40 min. The analytes were detected at 254 nm. The limit of detection was 25 µg/L for TNT and at least 50 µg/L for 2-ADNT, 4-ADNT, 2,4-DANT and 2,6-DANT. Precision was ≥ 95% (standard deviation <2%, signal to noise ratio = 3).

3.3.3 Metabolism of TNT by enchytraeids *in vivo*

Enchytraeus albidus Henle 1837 (donated by Dr. J. Römbke, Germany) was cultured in a mixture of garden soil and OECD artificial soil (1:1, w/w) according to Römbke and Moser (1999). The potworms were fed weekly with pre-boiled and lyophilized oats. The

laboratory culture was renewed every four months and kept in our laboratory at least one year before use in our studies.

Initial experiments were carried out to determine if enchytraeids, like earthworms, can metabolize TNT during exposure in soil. Briefly, 20 g of OECD soil (dry weight) was placed into separate 200-mL glass jars and was hydrated to 60% of its water holding capacity. Each jar received 40 mg (dry weight) of lyophilized oats that were mixed into the soil. Ten acclimatized enchytraeids were then added to each test jar (except control jars containing TNT and no potworms added). Test containers were prepared at least in triplicate. TNT in acetonitrile was used to spike the soil samples (from 25 to 1000 mg TNT added/kg dry soil). Soil samples were then left to evaporate for at least 16 h in a darkened chemical hood. Other control groups included replicate jars containing all constituents but no TNT (solvent control; n=3). Each jar was covered with an inverted glass Petri dish to prevent moisture loss during the exposure period (at 20°C with a 16:8 light-dark cycle). Each week, the moisture content was verified, 25 mg of lyophilized oats were added to the surface of the soil, and the soil was re-hydrated to 60% of its water holding capacity. At the end of the experiment (day 21), all potworms were removed from the soil before being washed with water, depurated for 20-24 h on a Whatman filter paper (to remove gut contents) and stored at –20 °C for later workup and analysis. In this study, enchytraeids from each of the triplicate test units (each having 10 worms exposed to the same TNT concentration) were pooled in order to achieve detectable quantities of TNT and its metabolites.

Since the presence of TNT and its metabolites in potworms *in vivo* may be due to diffusion of these compounds from the soil into the tissue, exposure studies were carried out using TNT in water according to the method of Römbke and Knacker (1989). Two replicate experiments were conducted to determine whether enchytraeids can metabolize TNT in solution. In each experiment, 10 adult enchytraeids were first depurated for 24 h on a moist filter paper in separate test tubes. Worms were then exposed to different aqueous concentrations of TNT (from 0.44 to 440 nmol/mL) under ambient conditions for 20 h, in order to determine a non-lethal range of TNT concentrations. Accordingly, enchytraeids also were exposed, in triplicate test units (each containing 10 worms), to TNT (from 0 to 185 nmol/mL). TNT-exposures were carried out in the dark using tubes wrapped with aluminum foil. Control test units contained TNT only and no worms added. After this exposure period, all of the enchytraeids survived and were considered as being healthy. Worms were then rinsed with deionized water, dried on tissue paper and were weighed. The worms from triplicate test units were pooled and stored at –20°C prior to further workup. The exposure milieu was diluted with ice-cold acetonitrile (1:1, v/v, final concentration) and filtered through a Millipore filter (0.45 μm), and was kept at 4°C for no longer than 2 d before being analyzed by HPLC for TNT and its metabolites.

3.3.4 Metabolism of TNT by enchytraeid homogenates

For the time course experiments, enchytraeid homogenates (10 worms per mL) were prepared as described above, and were incubated in 20 mM phosphate buffer (pH 7.6) containing TNT (49 or 116 nmol/mL) under ambient (aerobic) conditions at room temperature (20 ± 1°C) for up to 72 h. For the protein-dependency studies, the

enchytraeid homogenates (containing up to 50 worms per mL) were diluted in phosphate buffer to obtain different protein concentrations (up to 5.5 mg/mL). All incubations were started by the addition of TNT (29 or 58 nmol/mL) to the homogenate. Reaction mixtures were vortexed and incubated in the dark at room temperature for 20 h. Control tubes contained TNT only with no homogenate added. The protein content of the homogenates and the crude cell fractions (described below) was determined using the Pierce protein assay reagent bicinchoninic acid (BCA) with bovine serum albumin (BSA) as the standard.

For the cell fractionation studies, the enchytraeid homogenates were centrifuged (Sorval Super T21) at 20 *x g* for 10 min at 4 °C, to separate the heavy soil particles from the tissue. The resulting pellet (P20) and supernatant (S20) fractions were stored on ice. The P20 pellet was re-suspended in ice-cold phosphate buffer. The S20 fraction was then centrifuged at 8,000 *x g* for 10 minutes at 4 °C. The resulting pellet (P8K) was re-suspended in the phosphate buffer. A 1-mL aliquot of each collected fraction (P20, S20, P8K and S8K) was then incubated separately for 20 h in phosphate buffer containing TNT (17.4 nmol/mL). Control mixtures lacking TNT, and those having TNT but no tissue, were also prepared. All incubations were terminated by addition of ice-cold acetonitrile (described above). The resulting suspensions were mixed and prepared for HPLC analysis as described above.

3.3.5 Effect of antibiotics on TNT metabolism *in vitro*

Antibiotics were used to investigate the possibility that transformation of TNT *in vitro* was due to the carry-over of bacteria from the enchytraeids (host organism) into the incubation mixture. Enchytraeid homogenate was prepared as described above (from 0.2 to 1.2 mg protein/mL), and split into separate aliquots. Each aliquot was incubated in the dark under ambient conditions for 20 h with TNT (19.4 nmol/mL) and different antibiotics including tetracycline (100 μg/mL) and Pen-Strep. The latter is a mixture of penicillin (100 μg/mL) and streptomycin (100 units/mL). Control studies included homogenates with no TNT added, a TNT solution lacking added homogenate, and samples incubated without added antibiotics. This experiment was also carried out using isolated cell fractions (described above). Incubations were terminated by addition of ice-cold acetonitrile. All samples were centrifuged at 2500 *x g* for 10 min at 10 °C, and supernatants were analyzed for TNT and its metabolites as described above.

3.3.6 Effect of antibiotics on bacterial growth in enchytraeid homogenates

A special soil bacteria growth medium (YTS) was used to test if the worm homogenate contained culturable bacteria (Siciliano *et al.*, 2001). This medium contained (in 500 mL distilled water): 8 g of agar (Becton Dickinson), and 125 mg each of tryptone peptone (Difco Laboratories, Detroit, MI), yeast extract (Becton Dickinson) and soluble starch (Anachemia, Montreal, Quebec, Canada). This support medium was autoclaved at 121 °C for 20 min, and was then transferred to individual Petri dishes (20 mL/dish, i.d. 125 mm) under a laminar flow hood. Dishes were cooled for at least one day and stored at 4 °C until further use.

On the day of the experiment, worm homogenates (2.7 mg protein/mL) including positive controls having no additives, and those incubated with and without tetracycline, were plated prior to, and after, 20-h incubation in the dark in the absence or presence of TNT (6.6 nmol/mL). Prior to plating, each homogenate was first diluted with 0.1 % (w/v) tetrasodium pyrophosphate up to 10^{-6} by serial dilution in sterile tubes. An aliquot of each homogenate (100 μL) was then spread onto triplicate plates that were then incubated for 2 weeks at room temperature (20 ± 1°C). Bacterial colonies were counted after 72 h and 2 weeks. Plates with between 30 to 300 colonies per dilution were chosen for the determination of the number of colony forming units (CFU).

3.4 Results and Discussion

3.4.1 TNT metabolism by enchytraeids *in vivo*

Earlier studies showed that both 4-ADNT and 2-ADNT were formed in TNT-spiked OECD soil after addition of enchytraeids (Chapter 2). In a single preliminary study, HPLC analyses of TNT-spiked soils and TNT-exposed enchytraeids revealed the presence of two major metabolites, 4-ADNT and 2-ADNT, along with a small quantity of the parent compound, TNT. The metabolite, 2,6-DANT, was not detected in these samples. Similar observations have been reported for the earthworm (*Lumbricus terrestris*) and the composting worm (*Eisenia andrei*) (Renoux *et al.*, 2000; Robidoux *et al.*, 2002). Formation of TNT metabolites in the tissue could be due to biodegradation (microbial and/or non-microbial) within the enchytraeids, or by diffusion of these metabolites (produced by soil microbes) from the soil into the potworm.

To distinguish between these possibilities, enchytraeids were exposed to sublethal concentrations of TNT in deionized water, and TNT metabolites were examined in the water and in potworm tissue.

Figure 3.1A shows that when enchytraeids were exposed to a range of TNT concentrations (0 to 185 nmol/mL) for 20 h, the major TNT metabolites in the aqueous exposure medium were 4-ADNT and 2-ADNT, with minor quantities of 2,4-DANT. The parent compound (TNT) was also detected, but rarely and at much lower concentrations than its reduced metabolites. Analyses of TNT-exposed enchytraeids (Figure 3.1B) indicated TNT concentration-dependent increases in 2-ADNT and 4-ADNT concentrations in the potworm tissue; 2,4-DANT was detected but at low quantities (up to 5.0 nmol/mL). Control test units, having TNT and no potworms added, contained only the parent compound at the end of this 20-h exposure experiment. 2,6-DANT was not detected in any of the samples tested. Preliminary mass balance calculations revealed that, of the total amount of TNT added to the aqueous exposure medium, at the end of the study from 21.8 to 58.4 % was recovered as TNT and its metabolites (data not shown). The remaining compounds (probably TNT metabolites) were not extractable and were presumably bound to the tissue (Stenersen, 1992), an effect analogous to TNT metabolites binding to organic matter in soil (Daun *et al.*, 1998; Achtnick *et al.*, 1999).

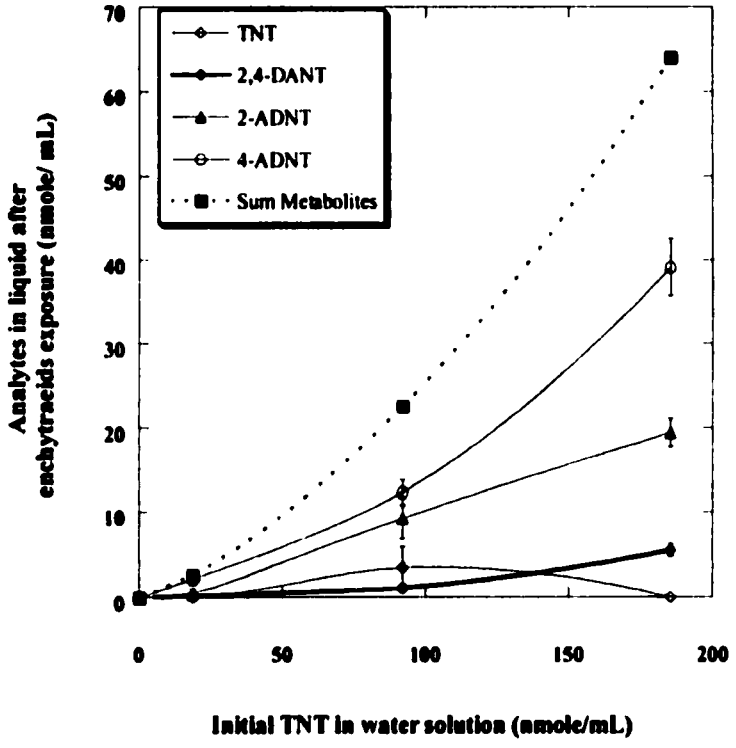
Hydroxyamino-dinitrotoluene, a rapidly-formed precursor to amino-dinitrotoluene during microbial reduction of TNT, can be irreversibly bound to soil components (Daun *et al.*, 1998), an effect that may be also occurring in the enchytraeid. More extensive mass-

balance studies will be conducted using worms exposed to radioactive TNT and its metabolites to better determine the fate of TNT under the experimental conditions described above. Nevertheless, the present results demonstrate that enchytraeids are capable of partially metabolizing TNT in spiked soil and aqueous solutions.

3.4.2 Metabolism of TNT by enchytraeids *in vitro*

To further characterize the reductive metabolism of TNT by enchytraeids, protein concentration dependence and time course studies were carried out *in vitro* using whole homogenates as well as crude cell fractions. Figure 3.2A shows the results of an experiment in which homogenates were incubated in the dark for 20 h after addition of TNT (29 nmol/mL) at several different protein concentrations (0 to 5.5 mg/mL). These data indicate a protein-dependent increase in the formation of 4-ADNT (major metabolite) and 2-ADNT (minor metabolite), along with a concomitant decrease in TNT concentration. The amount of 4-ADNT formed was linear up to 2.0 mg/mL protein, with a maximum total formation of reduced metabolites at higher protein concentrations (4.0 and 5.5 mg/mL). This experiment was repeated at a higher concentration of TNT (58 nmol/mL) and showed a similar effect although the plateau appeared at 2.0 mg protein/mL (Figure 3.2B). Taken together, a substrate concentration-dependency is also evident. Based on the linear portions of these curves, the TNT metabolizing activity (Σ metabolites formed) nearly doubled with respect to the TNT concentrations added (Figures 3.2A and 3.2B).

A



B

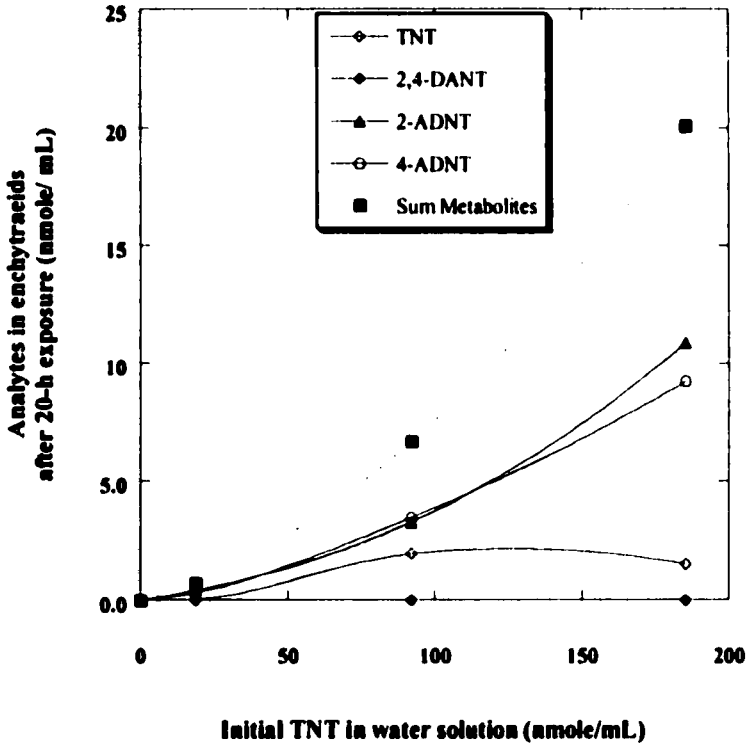


FIGURE 3.1: Formation of TNT metabolites in the exposure milieu (1A) and in tissue extracts (1B) following exposure of enchytraeids for 20 h to different aqueous concentrations of TNT.

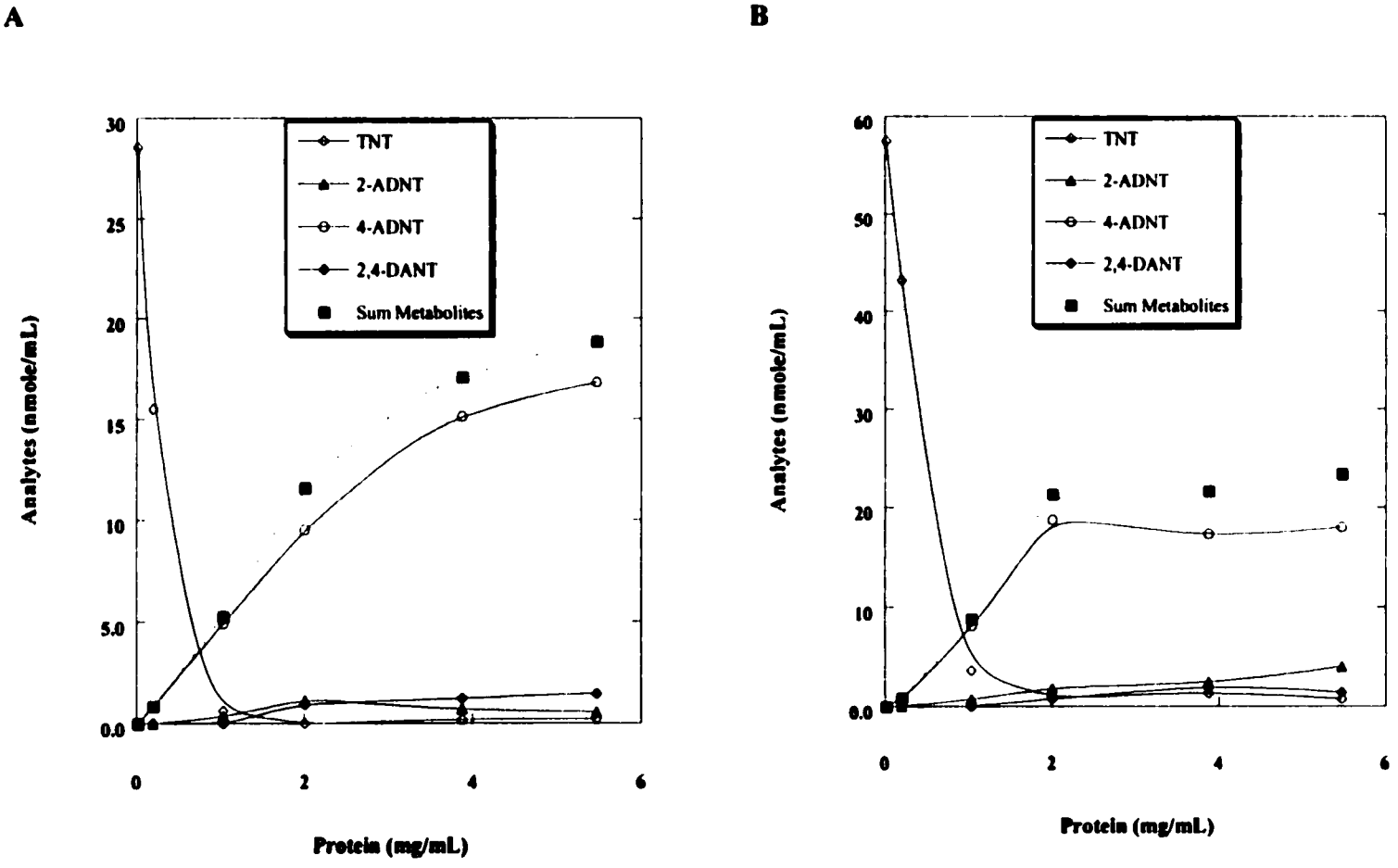


FIGURE 3.2: Formation of reduced TNT metabolites at different protein concentrations of enchytraeid homogenate. Enchytraeid homogenates were incubated with 29 nmol TNT/mL (A) and 58 nmol TNT /mL (B).

Figure 3.3 shows the results of an experiment in which unfractionated enchytraeid homogenates (2 mg protein/mL) were incubated in the dark at room temperature for up to 72 h after addition of TNT (48 nmol/mL). There is a time-dependent decrease in TNT concentrations in the homogenate, with virtually no TNT being detectable after 48 h. During this time, control tubes (containing TNT only and no homogenate) gave constant levels of the parent compound (data not shown). However, in the presence of the homogenate, the formation of reduced TNT metabolites (4-ADNT and 2-ADNT) was detected only after 16 h of incubation, with maximal levels being achieved after 48 h. This experiment was also carried out using 164.8 nmol TNT/mL, and similar effects were observed (data not shown). These data are consistent with our earlier studies showing the appearance of these reduced metabolites of TNT *in vitro* using *Eisenia andrei* homogenates (Renoux *et al.*, 2000). It is interesting that, during the first 16 h of the experiment, the disappearance of TNT preceeded the appearance of 4-ADNT (major product detected), 2-ADNT and 2,4-DANT (minor products). It is plausible that intermediate metabolites, such as the hydroxyamino- products, that are produced prior to the reductive formation of 4-ADNT and 2-ANDT, may have reacted rapidly with the enchytraeid tissue constituents and were not detected using the extraction procedure described in this article.

To test the hypothesis that the TNT metabolizing activity originated from intact microbes residing in the enchytraeid homogenate, the following experiments were carried out using isolated cell fractions and antibiotics.

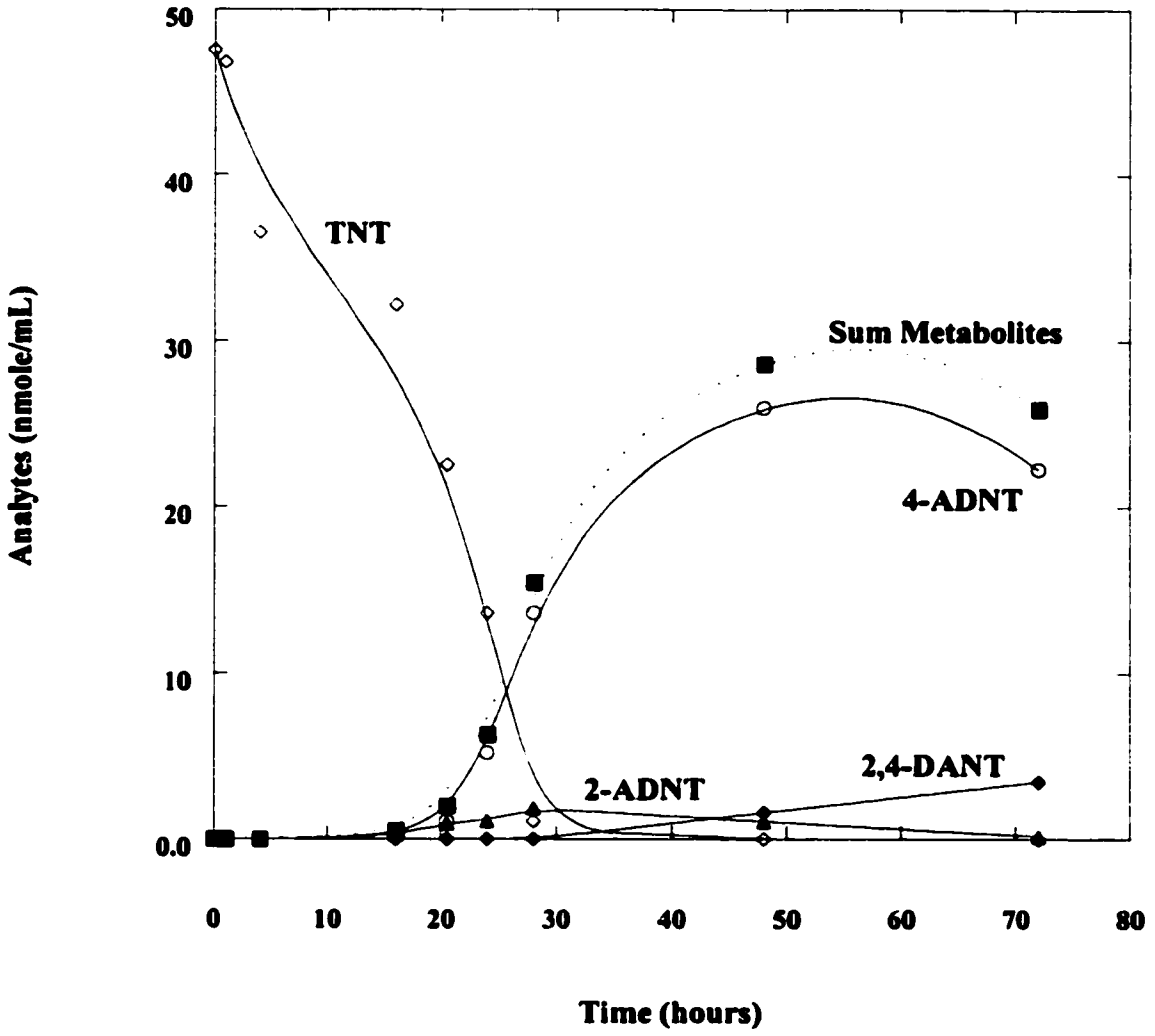


FIGURE 3.3: Time-dependent disappearance of TNT and formation of reduced TNT metabolites in enchytraeid homogenates. Concentrations used: 48 nmol TNT/mL and 2 mg protein/mL.

Sequential differential centrifugation was used to identify which crude cellular fraction of the enchytraeid whole homogenate was responsible for the reductive metabolism of TNT *in vitro*. This study involved two repeat experiments, each involving triplicate determinations for each fraction. Table 3.1 summarizes the results of one of these experiments and shows that in the whole homogenate incubated with TNT (19.4 nmol/mL) for 20 h, 4-ADNT was the major metabolite formed (3.18 nmol/mL) followed by 2-ADNT (0.64 nmol/mL). The S20 fraction had a higher TNT metabolizing activity (Σ metabolites = 9.0 μmol/mg protein) than the P20 fraction (Σ metabolites = 3.7 μmol/mg protein). Further fractionation of the S20 preparation into 8,000 *x g* pellet (P8K) and supernatant (S8K) fractions was carried out, and metabolites formed by these fractions after incubation with TNT for 20 h were examined.

Table 3.1 shows that in the P8K fraction, like the S20 fraction, 4-ADNT was the principal metabolite (2.03 nmol/mL) compared to 2-ADNT (0.24 nmol/mL). In contrast, the major analyte detected in the 8,000 *x g* supernatant fraction (S8K sample) was the unmetabolized TNT (9.74 nmol/mL) with only a small amount of the metabolite 4-ADNT (1.28 nmol/mL) detected. The poor recovery of analytes from all fractions tested suggested that these and other metabolites were not extracted by acetonitrile and may have been bound to cellular debris within these fractions (as discussed above). Table 3.1 also shows that the 8,000 *x g* pellet (P8K sample) had more TNT-metabolizing-type activity (Σ metabolites = 8.1 μmol/mg protein) than the S8K fraction (Σ metabolites = 2.0 μmol/mg protein) and accounted for most of the activity present in the S20 fraction. This

experiment was repeated and confirmed that the 8,000 *x g* pellet contained most of the TNT metabolic activity (data not shown).

These data are consistent with the hypothesis that microbial TNT-metabolizing activity present in the enchytraeids was carried over from the whole homogenate into the P8K fraction. Soil microbes within the host enchytraeid could be fungi (Hawari *et al.*, 1999; Fritsche *et al.*, 2000) or bacteria (Gorontzy *et al.*, 1994; Ahmad and Hughes, 2000). However, in addition to intact microbes, the P8K fraction also contains enchytraeid constituents such as mitochrondria and cell membranes, that could harbor TNT-metabolizing activity. Therefore, to test the possibility that the observed TNT metabolism *in vitro* was bacterial in origin, enchytraeid homogenates and crude cell fractions were incubated with TNT in the presence or absence of different broad-spectrum antibiotics: tetracycline, which inhibits protein synthesis, or Pen-Strep, which inhibits bacterial cell wall formation.

3.4.3 Inhibition of TNT metabolism and bacterial growth by co-incubation with antibiotics

Figure 3.4 shows that the reductive metabolism of TNT in enchytraeid homogenate (1.2 mg protein/mL) was completely inhibited by addition of tetracycline (100 μg/mL) or Pen-Strep (100 μg penicillin/mL and 100 units streptomycin/mL), antibiotics that are directed toward bacteria. Since almost all the TNT added was recovered after 20 h in the presence of both antibiotics, TNT did not react with the antibiotics used.

Table 3.1 : TNT metabolism in different crude cellular fractions of enchytraeid homogenate in the presence and absence of tetracycline

Test Fractions	Tetracycline	TNT (nmol/mL)	4-ADNT (nmol/mL)	2-ADNT (nmol/mL)	Σ Metabolites (nmol/mL)	Recovery (%)	Protein (mg/mL)	Σ Metabolites (µmol/mg protein)
Whole Homogenate	-	0.38	3.18	0.64	3.82	21.7	1.2	3.3
	+	17.29	ND	ND		89.2		ND
(S20) 20 *x g* Supernatant	-	0.35	8.52	0.61	9.31	49.0	1.0	9.0
	+	17.23	ND	ND		88.9		ND
20 *x g* Pellet (P20)	-	11.74	0.64	0.10	0.64	64.4	0.2	3.7
	+	17.38	ND	ND		89.7		ND
(S8K)	-	9.74	1.28	ND	1.28	56.9	0.6	2.0
8,000 *x g* Supernatant	+	17.82	ND	ND		92.0		ND
8,000 *x g* Pellet (P8K)	-	8.98	2.03	0.24	2.27	58.1	0.3	8.1
	+	17.02	ND	ND		87.0		ND

Samples were prepared and incubated for 20 h at room temperature following addition of 19.4 nmol/mL TNT (± 100 mg tetracycline per mL). Incubations were terminated with cold acetonitrile, prior to HPLC analysis. ND denotes not detected (limits of detection: 25 µg/L for TNT and 50 µg/L for the TNT metabolites).

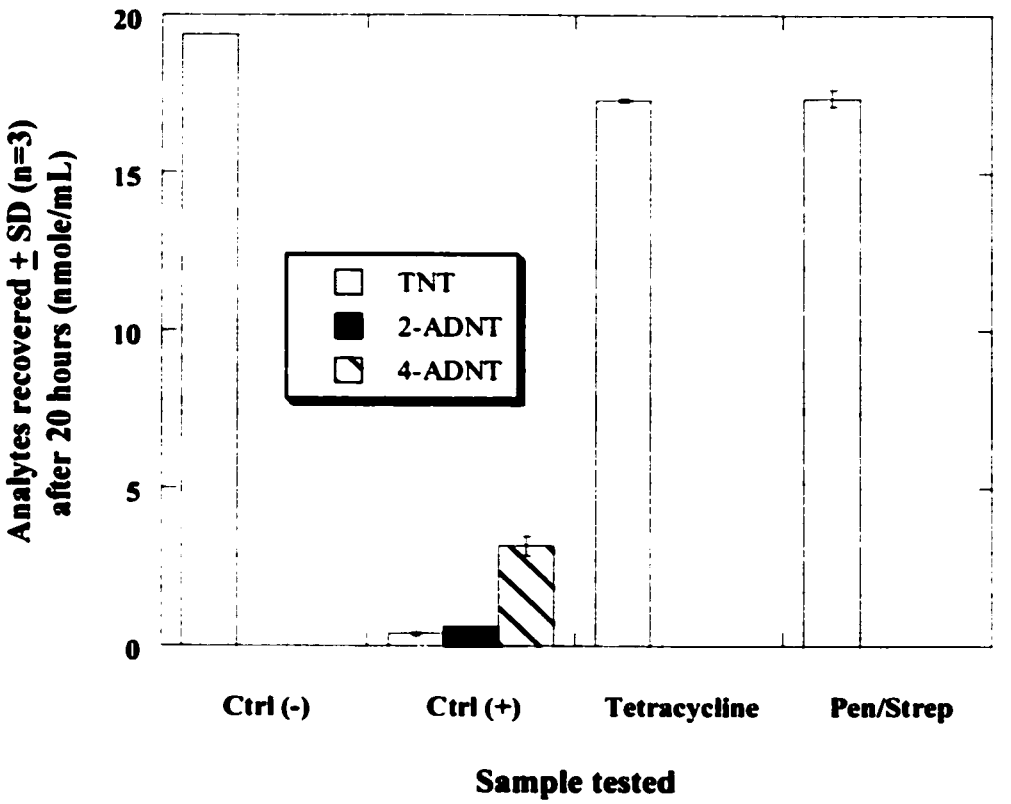


FIGURE 3.4: Effects of tetracycline and penicillin–streptomycin (Pen/Strep) on the biotransformation of TNT by enchytraeids *in vitro*

Ctrl (-) represents the negative controls (no homogenate added) incubated with only TNT (19.4 nmol/mL) at room temperature in the dark for 20 h. Ctrl (+) denotes enchytraeid homogenates incubated with TNT. No metabolites were detected in the samples incubated with TNT and 100 µg tetracycline/mL or combined penicillin (100 units/mL)–streptomycin (100 µg/mL).

When enchytraeid homogenates were incubated for 20 h without the antibiotics (positive control group), only 3.5 nmoles/mL of TNT were recovered from the 19.4 nmoles/mL TNT originally added. Both 2-ADNT and 4-ADNT were also present, consistent with the results obtained using homogenates (Figure 3.4). Thus, enchytraeid-associated bacteria are required for the disappearance of TNT and formation of reduced TNT metabolites. Furthermore, the non-extractable fraction of TNT metabolites bound to enchytraeid constituents also may require bacterial activity. A preliminary literature search failed to show any evidence that the antibiotics used in the present study are capable of directly inhibiting enzymes responsible for TNT metabolism.

To determine whether the TNT-metabolizing activity within the P8K fraction was also associated with the presence of bacteria, the effects of tetracycline on the accumulation of reduced metabolites by crude cellular fractions were examined. Tetracycline (100 μg/mL) inhibited the biotransformation of TNT in all cell fractions tested, including in the S20 and P8K samples (Table 3.1), which would be likely to contain any bacteria originally present in the homogenate. In the absence of the antibiotic, the total recovery of analytes remained low (≤ 64.4 %) in all fractions, whereas in the presence of tetracycline, this recovery increased up to 92 %. These data indicate that the partial reductive degradation of TNT *in vitro* (as reflected by the formation of 2-ADNT and 4-ADNT) was due to the presence of tetracycline-sensitive bacteria, in both the whole homogenates and the enchytraeid crude cell fractions. Our data are consistent with the notion that bacteria are responsible for the TNT-metabolizing activity observed in enchytraeids. The presence of bacteria in earthworms is well known (for reviews see Edwards and Bohlen, 1996, Brown

et al., 2000). Bacteria serve as a food source, and as primary degraders of soil organic matter, and can provide nutritive carbon sources to host organisms such as earthworms and enchytraeids.

In return, the earthworm provides the microbes with a protective and nutritive environment (humidity and mucus) in their gut, as well as a means for microbial propagation throughout the soil (so-called soil microbial-earthworm mutualism). It is intriguing to speculate about the possibility that by enhancing this ecological relationship, one may optimize the TNT degrading activity of microbes used for the bioremediation of soils contaminated with TNT.

To determine if the observed TNT-metabolizing activity was associated with increased culturable bacterial growth, worm homogenates were incubated with TNT (6.6 nmol/mL) for 20 h in the absence or presence of tetracycline (100 μg /mL). Aliquots of homogenates were then plated on YTS bacterial growth media for up to 2 weeks to assess the presence of soil bacteria in the homogenates. As shown in Table 3.2, no bacterial colonies were present on plates containing the control growth media (no homogenate or tetracycline added). Culturable microbes were found in all worm homogenates, particularly those incubated for 20 h at room temperature without the antibiotic added. Tetracycline decreased the number of CFU on all of the plates tested, confirming that the enchytraeid whole homogenates used in the present study contain soil bacteria. TNT-spiked homogenates (6.6 nmol TNT/mL) that were not incubated for 20 h prior to the 2-week bacterial growth test, showed >2-times lower numbers of bacterial colonies (0.065

x 10^6 CFU) than the controls (no TNT added, 0.147 x 10^6 CFU). These results indicate that addition of TNT to the homogenate inhibited bacterial colony formation, which is consistent with TNT's toxic effects on certain bacteria (Klausmeier *et al.*, 1973; Drzyzga *et al.*, 1995; Sunahara *et al.*, 1998, 1999; Gong *et al.*, 1999b; Frische, 2002).

Results provided in this article show that, in the absence of tetracycline, the growth of bacteria in the enchytraeid homogenates incubated for 20 h in the presence of TNT (Table 3.2) correlates well with the time-dependent disappearance of TNT and formation of reduced TNT metabolites in the enchytraeid homogenates (Figure 3.3). On the other hand, the inhibition of TNT-metabolizing activity by tetracycline in homogenates incubated for 20 h (Figure 3.4 and Table 3.1) is explained by the inhibition of bacterial growth compared to controls where no tetracycline was added (Table 3.2).

It is tempting to speculate that the appearance of 4-ADNT and 2-ADNT in TNT-exposed enchytraeids is *solely* due to microbial action within the host organism. If true, then the metabolic profiles observed *in vitro* and *in vivo* should be similar. Data indicate that this was not the case, since concentrations of 4-ADNT were > 10-times that of 2-ADNT in the TNT-incubated homogenates (an effect associated with microbial degradation of TNT, Table 3.1), whereas equimolar concentrations 4-ADNT and 2-ADNT were present in TNT-exposed enchytraeids (Figure 3.1B). Clearly, other TNT degradative mechanisms must be involved *in vivo*.

Table 3.2: Effects of tetracycline and TNT on microbial colony formation in enchytraeid homogenates incubated for 20 h

Plate Identification	CFU / mL	
	No incubation (10^{+6})	20 h-incubation (10^{+6})
Control growth medium (no additives)	0	0
Homogenate only	0.147	2.000
Homogenate + Tetracycline	0.063	0.033
Homogenate + TNT	0.065	2.480
Homogenate + TNT + Tetracycline	0.067	0.061

Enchytraeid homogenates (2.7 mg protein/mL) were prepared as described in the Experimental section. Samples were then incubated for 20 h at room temperature following addition of 6.6 nmol/mL TNT (± addition of 100 µg tetracycline/mL). An aliquot of each sample was then plated (in triplicate) on the YTS agar growth medium for up to 2 weeks, prior to the counting of microbial colonies.

Using two different antibiotics (tetracycline or Pen-Strep) in whole homogenates and in crude cell fractions, we have demonstrated, for the first time, that the appearance of reduced TNT metabolites *in vitro* is due to the presence of bacteria present in the host organism. Further studies are being conducted to characterize the bacterial enzyme(s) responsible for this activity, and to determine if this biotransformation represents a detoxification process in enchytraeids and other soil invertebrate species.

3.5 Acknowledgements

The authors wish to express their gratitude to Drs. Sonia Thiboutot and Guy Ampleman (DND-DREV) for their interest in this project. Also, we thank Louise Paquet and Manon Sarrazin for the HPLC analysis and enchytraeid culture maintenance, and Drs. Jalal Hawari and Edmund Ziomek, for their review of an earlier version of this manuscript. This article has been assigned NRCC publication # XXXX (to be added later).

Conclusions and Summary

Studies presented in this thesis demonstrate that soil contaminated with TNT is toxic to enchytraeids. During the lethality and reproduction test periods, this explosive was partially degraded in the artificial soil to form amino derivatives. Lethality and a decrease in reproductive capacity of enchytraeids were dependent on the concentration of TNT measured at the beginning of the experiment. There is no data to explain which of the metabolites formed in the soil during the exposure period play a role in the observed toxicity effects. Therefore, additional experiments should be conducted to compare the sensitivity of both adults and juveniles to these metabolites. Nevertheless, data provided by the fresh/aged soil experiment demonstrate for the first time that juvenile enchytraeids are more sensitive to TNT than adult enchytraeids are. Consequently, the toxicity observed is not associated with the transformation of TNT to low concentrations of metabolites within the incubation test period.

In addition to these ecotoxicity responses *in vivo*, the formation of TNT metabolites in enchytraeid tissue and test media (soil and water) was observed. Since these metabolites are dependent (*in vitro*) on the exposure time period and the protein concentration, the presence of these metabolites appeared to be related to the presence of bacteria. This was confirmed with the antibiotic studies in which the formation of TNT metabolites was inhibited by the antibiotics, tetracycline and a mixture of penicillin-streptomycin. Numerous reports in the literature have shown that bacteria are able to transform TNT to amino metabolites via nitroreductase enzymes. Data provided in this thesis showed that bacteria present in enchytraeids are responsible for the *in vitro* biotransformation of TNT.

Since there are many bacteria in soil that are able of catalyzing the biotransformation of TNT, it is not evident which bacterial enzyme is responsible for the partial degradation of TNT in the enchytraeid. Additional experiments would be required to determine this.

Bibliography

Achtnick, C., Sieglen, U., Knackmuss, H-J., and Lenke, H. 1999. Irreversible binding of biologically reduced 2,4,6-trinitrotoluene to soil. *Environ. Toxicol. Chem.* **18**, 2416-2423.

Ahmad, F., and Hughes, J.B. 2000. Chapter 8. Anaerobic transformation of TNT by *Clostridium*. In: *Nitroaromatic Compounds and Explosives* (J.C. Spain, J.B. Hughes and H-J. Knackmuss, Eds). Lewis Pub., pp. 185-211.

Brown, G.G., Barois, I., and Lavelle, P. 2000. Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. *Eur. J. Soil Biol.* **36**, 177-198.

Bryant, C., and De Luca, M. 1991. Purification and characterization of an oxygen-insensitive NAD(P)H nitroreductase from *Enterobacter cloacae*. *J. Biol. Chem.* **266**, 4119-4125.

Bryant, C. and McElroy, W.D. 1992. Nitroreductases *in* Chemistry of flavoenzymes vol. II, chapter 9, 291-304, CRC Press, Boca Raton FL.

Bryant, D.W., McCalla, D.R., Leeksma, M. and Laneuville, P. 1981. Type I nitroreductases of *Escherichia coli*. *Can. J. Microbiol.* **27**, 81-86.

Burrows, E. P., Rosenblatt, D. H., Mitchell, W. R., and Parmer, D. L. 1989. Organic Explosives and Related Compounds: Environmental and Health Considerations. Technical Report 8901, US Army Biomedical Research and Development Laboratory, Fort Detrick, Frederick, MD.

Cerniglia, C. E. and Somerville, C. C. 1995. Reductive metabolism of nitroaromatic and nitropolycyclic aromatic hydrocarbons. From the book *Biodegradation of nitroaromatic compounds*. Edited by J.C. Spain, Plenum Press, New York, pp. 99-115.

Collado, R., Schmelz, R.M., Moser, T., and Römbke, J. 1999. Sublethal responses of two *Enchytraeus* species (Oligochaeta) to toxic chemicals. *Pedobiologia* **43**, 625-629.

Daun, G., Lenke, H., Reuss, M. and Knackmuss, H-J. 1998. Biological treatment of TNT-contaminated soil. 1. Anaerobic cometabolic reduction and interaction of TNT and metabolites with soil components. *Environ. Sci. Technol.* **32**, 1956-1963.

Didden, W.A.M. 1993. Ecology of terrestrial enchytraeidae. *Pedologia* **37**, 2-29.

Didden, W., and Römbke, J. 2001. Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. *Ecotoxicol. Environ. Safety* **50**, 25-43.

Drzyzga, O., Gorontzy, T., Schmidt, A., and Blotevogel, K.H. 1995. Toxicity of explosives and related compounds to the luminescent bacterium *Vibrio fischeri* NRRL-B-11177. *Arch. Environ. Contam. Toxicol.* **28**, 222-235.

Edwards, C.A. and Bohlen, P.J. 1996. *Biology and Ecology of Earthworms* (3rd edition). Chapman and Hill, London, UK.

Finney, D. J. 1971. *Statistical Methods in Biological Assays*. Griffin and Company, London.

Fritsche, W., Scheibner, K., Herre, A., and Hofrichter, M. 2000. Chapter 9. Fungal degradation of explosives: TNT and related nitroaromatic compounds. In: *Nitroaromatic Compounds and Explosives* (J.C. Spain, J.B. Hughes and H-J. Knackmuss, Eds). Lewis Pub., pp. 213-237.

Frische, T. 2002. Screening for soil toxicity and mutagenicity using luminescent bacteria – A case study of the explosive 2,4,6-trinitrotoluene (TNT). *Ecotoxicol. Environ. Saf.* **51**, 133-144.

Gong, P., Wilke, B.-M., and Fleischmann, S. 1999a. Soil-based phytotoxicity of 2,4,6-trinitrotoluene (TNT) to terrestrial higher plants. *Arch. Environ. Contam. Toxicol.* **36**, 152-157.

Gong, P., Siciliano, S.D., Greer, C.W., Paquet, L., Hawari, J. and Sunahara, G.I. 1999b. Effects of bioavailability of 2,4,6-trinitrotoluene in spiked and field-contaminated soils to indigenous microorganisms. *Environ. Toxicol. Chem.* **18**, 2681-2688.

Gordon, L., and William, R. H. 1992. 2,4,6-Trinitrotoluene (TNT). In "*Drinking Water Health Advisory: Munitions*" (W. C. Roberts, Ed.), pp. 327-398. Lewis Publishers, Boca Raton, FL.

Gorontzy, T., Drzyga, O., Kahl, M.W., Bruns-Nigel, D., Breitung, J., von Loew, E. and Blotevogel, K-H. 1994. Microbial degradation of explosives and related compounds. *Crit. Rev. Microbiol.* **20**, 265-284.

Hawari, J., Halasz, A., Beaudet, S., Paquet, L., Ampleman, G. and Thiboutot, S. 1999. Biotransformation of 2,4,6-trinitrotoluene with *Phanerochaete chrysosporium* in agitated cultures at pH 4.5. *Appl. Environ. Microbiol.* **65**, 2977-2986.

He, J., Davis, JK., Spain, JC. 1998. Purification, characterization, and sequence analysis of 2-aminomuconic 6-semialdehyde dehydrogenase from *Pseudomonas pseudoalcaligenes* JS45. *J. Bacteriol.* **180**, 4591-4595.

International Organization for Standardization (ISO) 1994. *Soil Quality – Determination of pH.* ISO 10390: 1994 (E). Geneva, Switzerland.

International Organization for Standardization (ISO) 2001. Soil Quality – Effects of Pollutants on Enchytraeidae (*Enchytraeus sp.*) - Determination of Effects on Reproduction and Survival. ISO DIS 16387: 2001. Geneva, Switzerland.

Jarvis, S. A., McFarland, V. A., and Honeycutt, M. E. 1998. Assessment of the effectiveness of composting for the reduction of toxicity and mutagenicity of explosive-contaminated soil. *Ecotoxicol. Environ. Safety* **39**, 131-135.

Kitts, C.L., Green, C.E., Otley, R.A., Alvarez, M.A. and Unkefer, P.J. 2000. Type I nitroreductases in soil enterobacteria reduce TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). *Can. J. Microbiol.* **46**, 278-282.

Klausmeier, R.E., Osmon, J.L. and Walls, D.R. 1973. The effect of trinitrotoluene on microorganisms. *Dev. Ind. Microbiol.* **15**, 309-317.

Koder, R.L., Miller, A.-F. 1998. Overexpression, isotopic labeling and spectral characterization of *Enterobacter cloacae* nitroreductase. *Protein expression and purification* **13**, 53-60.

Kula, C. 1998. Endpoints in laboratory testing with earthworms: experience with regard to regulatory decisions for plant protection products. In "*Advances in Earthworm Ecotoxicology*" (S. Sheppard, J. Bembridge, M. Holmstrup and L. Posthuma, Eds), pp 3-14. SETAC Press, Pensacola, FL.

Kuperman, R.G., Simini, M., Phillips, C.T. and Checkai, R.T. 1999. Comparison of malathion toxicity using enchytraeid reproduction test and earthworm toxicity test in different soil types. *Pedologia* **43**, 630-634.

Lock, K., De Schamphelaere, K.A.C. and Janssen, C.R. 2002. The effect of lindane on terrestrial invertebrates. *Arch. Environ. Contam. Toxicol.* **42**, 217-221

Lokke, H. and Van Gestel, C.A.M. 1998. *Handbook of soil invertebrate toxicity tests*. John Wiley & Sons ltd. West Sussex, England.

Martikainen, E. 1996. Toxicity of dimethoate to some soil animal species in different soil types. *Ecotoxicology and Environmental Safety* **33**, 128-136.

Nishino, S.F. and Spain, J.C. 1993. Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes*. *Appl. Environ. Microbiol.* **59**, 2520-2525.

Oh, B.-T., Sarath, G. and Shea, P.J. 2001. TNT nitroreductase from *Pseudomonas aeruginosa* strain isolated from TNT-contaminated soil. *Soil Biology and Biochemistry* **33**, 875-881.

Organization for Economic Cooperation and Development (OECD) 1984. *Guidelines for Testing of Chemicals: Earthworm Acute Toxicity Test*. OECD Guideline No. 207, Paris, France.

Pennington, J.C. and Brannon, J.M. 2002. Environmental fate of explosives. *Thermochimica Acta* **384**, 163-172.

Phillips, C. T., Checkai, R. T., and Wentsel, R. S. 1993. *Toxicity of Selected Munitions and Munition-contaminated Soil on the Earthworm (Eisenia foetida).* Technical Report, US Army Chemical and Biological Defense Agency, Aberdeen Proving Ground, MD.

Pirhonen, R. and Huhta, V. 1984. Petroleum fractions in soil: Effects on populations of nematoda, enchytraeidae and microarthropoda. *Soil Biol. Biochem.* **16(4)**, 347-350.

Posthuma, L., Baerselman, R., Van Veen, R.P.M. and Dirven-Van Breemen, E.M. 1997. Single and joint toxic effects of copper and zinc on reproduction of *Enchytraeus crypticus* in relation to sorption of metals in soils. *Ecotoxicology and environmental safety* **38**, 108-121.

Puurtinen, H.M. and Martikainen, E.A.T. 1997. Effect of soil moisture on pesticide toxicity to an enchytraeid worm *Enchytraeus* sp. *Arch. Environ. Contam. Toxicol.* **33**, 34-41.

Renoux, A.Y., Sarrazin, M., Hawari, J., and Sunahara, G.I. 2000. Transformation of 2,4,6-trinitrotoluene in soil in the presence of the earthworm *Eisenia andrei*. *Environ. Toxicol. Chem.* **19**, 1473-1480.

Renoux, A.Y., Caumartin, J., Thiboutot, S., Ampleman, G., and Sunahara, G.I. 2001. Derivation of environmental soil quality guidelines for 2,4,6-trinitrotoluene using the CCME approach. *Human and Ecological Risk Assessment*. **7**, 1715-1735.

Robidoux, P.Y., Hawari, J., Thiboutot, S., Ampleman, G., and Sunahara, G. I. 1999. Acute toxicity of 2,4,6-trinitrotoluene in earthworm (*Eisenia andrei*). *Ecotoxicol. Environ. Saf.* **44**, 311-321.

Robidoux, P.Y., Svendsen, C., Coumartin, J., Hawari, J., Ampleman, G., Thiboutot, S., Weeks, J., and Sunahara, G.I. 2000. Chronic toxicity of energetic compounds in soil determined using the earthworm (*Eisenia andrei*) reproduction test. *Environ. Toxicol. Chem.* **19**, 1764-1773.

Robidoux, P.Y., Svendsen, C., Sarrazin, M., Hawari, J., Thiboutot, S., Ampleman, G., Weeks, J.M., and Sunahara, G.I. 2002. Evaluation of tissue and cellular biomarkers to assess 2,4,6-trinitrotoluene (TNT) exposure in earthworms: Effects-based assessment in laboratory studies using *Eisenia andrei*. *Biomarkers* (Accepted for publication).

Rombke, J. 1989. *Enchytraeus albidus* (Enchytraeidae, Oligochaeta) as test organism in terrestrial laboratory systems. *Arch. Toxicol.* **13**, 402-405.

Römbke, J., and Knacker, T. 1989. Aquatic toxicity test for enchytraeids. *Hydrobiologia* **180**, 235-242.

Römbke, J., and Moser T. 1999. Organization and Performance of an International Ringtest for the Validation of the Enchytraeid Reproduction Test. Research Report 296 64 906 (UBA-FB 98-104). Environmental Research of the Federal Ministry of the Environment, Nature Conservation and Nuclear Safety. German Federal Environment Agency, Berlin.

Salminen, J., Eriksson, I. And Haimi, J. 1996. Effects of terbutylazine on soil fauna and decomposition processes. *Ecotoxicology and Environmental Safety* **34**, 184-189.

Schäfer, R., and Achazi, R.K. 1999. The toxicity of soil samples containing TNT and other ammunition derived compounds in the Enchytraeid and collembola-biotest. *Environ. Sci. Pollut. Res.* **6**, 213-219.

Shah, M.M. and Spain, J.C. 1996. Elimination of nitrite from the explosive 2,4,6-trinitrophenylmethylnitramine (tetryl) catalyzed by ferredoxin NADP oxidoreductase from spinach. *Biochemical and Biophysical Research Communications* **220**, 563-568.

Siciliano, S.D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumier, D., Ouellette, D., Roy, R., Whyte, L.G., Banks, M.K., Schwab, P., Lee, K., and Greer, C.W., 2001. Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Appl. Environ. Microbiol.* **67**, 2469-2475.

Simini, M., Wentsel, R. S., Checkai, R. T., Phillips, C. T., Chester, N. A., Major, M. A., and Amos, J. C. 1995. Evaluation of soil toxicity at Joliet Army Ammunition plant. *Environ. Toxicol. Chem.* **14**, 623-630.

Somerville, C.C., Nishino, S.F., and Spain, J.C. 1995. Purification and characterization of nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* JS45. *J. Bacteriol.* **177**, 3837-3842.

Stenersen, J., 1992. Uptake and metabolism of xenobiotics by earthworms. In: *Ecotoxicology of Earthworms*. (P.W. Greig-Smith, H. Becker, P.J. Edwards and F. Heimbach, Eds). Intercept Ltd., Andover, Hants, UK., pp. 129-138.

Sunahara, G. I., Dodard, S., Sarrazin, M., Paquet, L., Ampleman, G., Thiboutot, S., Hawari, J., and Renoux, A. Y. 1998. Development of a soil extraction procedure for ecotoxicity characterization of energetic compounds. *Ecotoxicol. Environ. Saf.* **39**, 185-194.

Sunahara, G. I., Dodard, S., Sarrazin, M., Paquet, L., Hawari, J., Greer, C. W., Ampleman, G., Thiboutot, S., and Renoux, A. Y. 1999. Ecotoxicological characterization of energetic substances using a soil extraction procedure. *Ecotoxicol. Environ. Saf.* **43**, 138-148.

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 polynitro explosives. In "*Environmental Toxicology and Risk Assessment: Science, Policy and Standardization – Implications for Environmental Decisions: Tenth Volume, ASTM STP 1403*" (B.M. Greenberg, R.N. Hull, M.H. Roberts, Jr., and R.W. Gensemer, Eds), pp 293-312. American Society for Testing and Materials, West Conshohocken, PA.

Talmage, S. S., Opresko, D. M., Maxwell, C. J., Welsh, C. J. E., Cretella, F. M., Reno, P. H., and Daniel, F. B. 1999. Nitroaromatic munition compounds: Environmental effects and screening values. *Rev. Environ. Contam. Toxicol.* **161**, 1-156.

U.S. Environmental Protection Agency (US-EPA) 1997. Method 8330. Nitroaromatics and nitramines by high performance liquid chromatography (HPLC). In "*Test Methods for Evaluating Solid Waste*" EPA, SW-846 Update III; Part 4: 1 (B), Washington, DC.

Watanabe, M., Nishino, T., Takio, K., Sofuni, T., Nohmi, T. 1998. Purification and characterization of wild-type and mutant "classical" nitroreductases of *Salmonella typhimurium*. *The Journal of Biological Chemistry* **273** (11), 23922-23928.

APPENDIX 1: Quality control for the enchytraeid reproduction test

Prior to the toxicity studies, the enchytraeids (*E. albidus*) were cultured in the laboratory for at least one year. During this acclimation period, several generations were produced. The quality of reproduction was verified using the guidelines described by Rombke and Moser (1999). The guidelines state that the adult survival should be at least 80%. As seen in Figure A-1, this criterion was always met. The number of juveniles per lot of ten adults should be at least 25 individuals, and this criterion was also met.

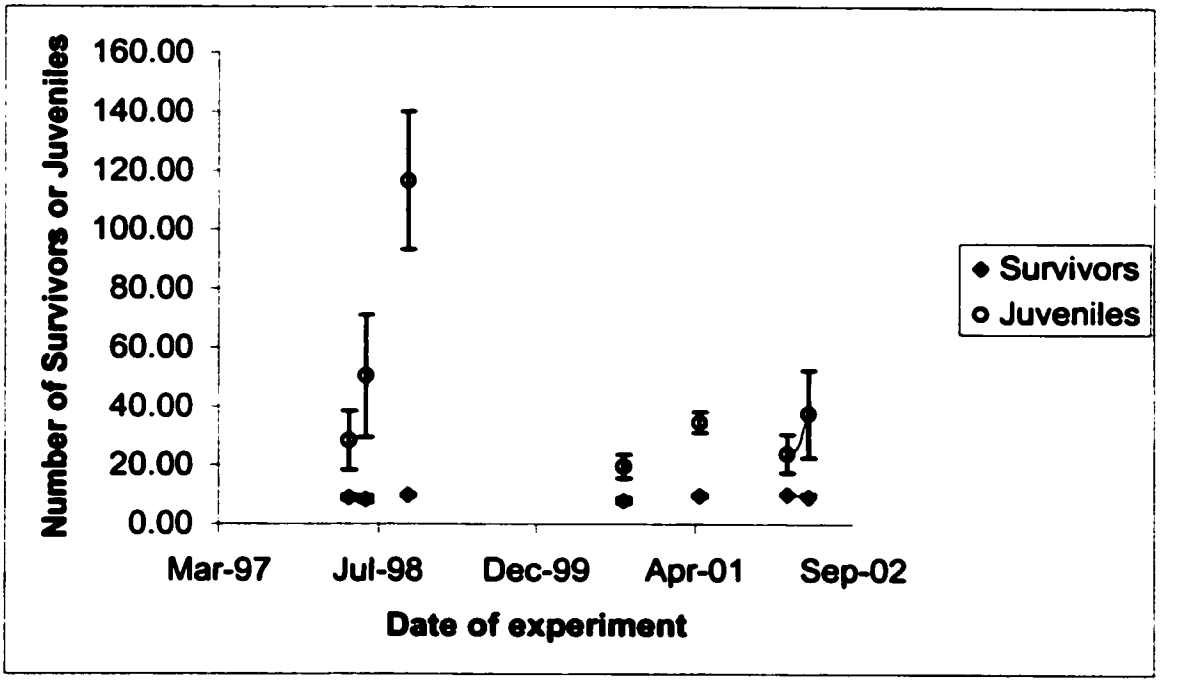


Figure A-1: Enumeration of survivors after lethality period (21 days), and juveniles counted after the reproduction period (42 days) in control jars. Experiments were carried out with at least three replicates (10 enchytraeids per replicate).

Since the enchytraeid reproduction test is a new test in our facility, validation of data is required. For this purpose, the consistent sensitivity of the species was evaluated using the reference toxicant, carbendazim. A control chart (Figure A-2) was established from 1998 to 2002. In a control chart, two limits are established with mean data obtained from different experiments performed during a certain period of time. The first limit is a control limit (CL) that corresponds mathematically to two standard deviation of the mean. For a consistent response, all data recorded should be within this limit. The second limit is the warning limit (WL) and corresponds mathematically to three standard deviations of the mean. When a value is outside of this limit, the test performed is not consistent with the others. All EC50 values were in the acceptable control limit (denoted CL= 1 to 2.5) for the five experiments for which the observed values were within 2 standard deviations of the mean. Therefore data presented in Figure A-2 show consistent reproduction response.

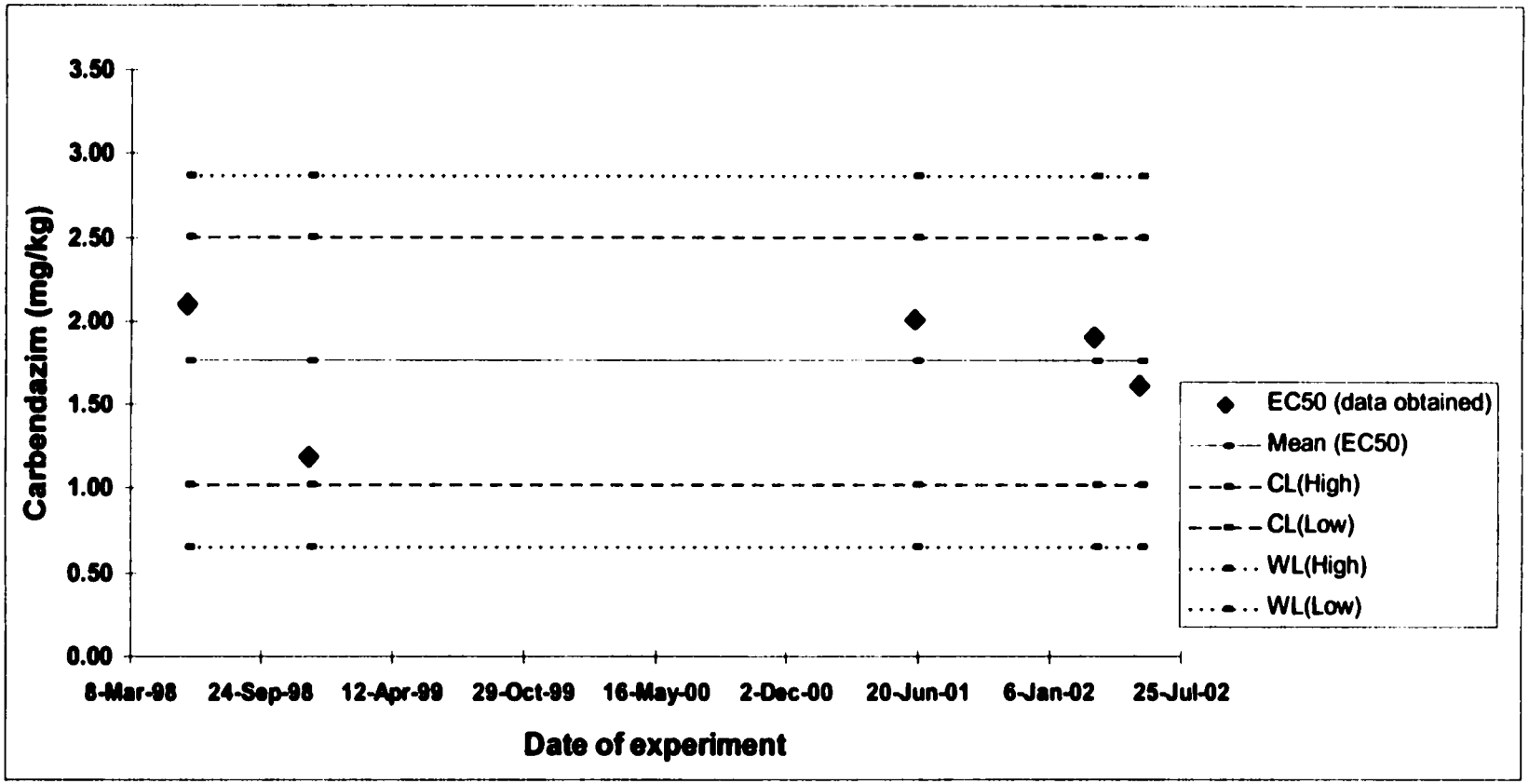


Figure A-2: Control chart for carbendazim showing EC50 values as well as control limit (CL) and warning limit (WL). Experiments were carried out with OECD soil contaminated with carbendazim (concentrations ranged from 0 to 10 mg/kg in three replicates). EC50 values were estimated with probit or linear interpolation for each experiment and plotted. The mean value of all EC50 values was calculated. The control limits (CL) correspond to two standard deviations of the mean. The warning limits (WL) correspond to three standard deviations of the mean.