The Mutagenic Activity of High-Energy Explosives; Contaminants of Concern at Military Training Sites

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ABSTRACT

The genotoxicity of energetic compounds (i.e., explosives) that are known to be present in contaminated soils at military training sites has not been extensively investigated. Thus, the *Salmonella* mutagenicity and Muta^{TM}Mouse assays were employed as *in vitro* assays to examine the mutagenic activity of twelve explosive compounds, as well as three soil samples from Canadian Forces Base Petawawa. *Salmonella* analyses employed strains TA98 (frameshift mutations) and TA100 (base-pair substitution mutations), as well as the metabolically-enhanced YG1041 (TA98 background) and YG1042 (TA100 background), with and without exogenous metabolic activation (S9). For *Salmonella* analyses, the results indicate that ten of the explosive compounds were mutagenic, and consistently elicited direct-acting, base-pair substitution activity. All three soil samples were also observed to be mutagenic, eliciting direct-acting, frameshift activity. Mutagenic potencies were significantly higher on the metabolically-enhanced strains for all compounds and soil samples. For Muta^{TM}Mouse analyses on FE1 cells, the results indicate that the majority of explosive compounds did not exhibit mutagenic activity. All three soil samples elicited significant positive responses (PET 1 and PET 3 without S9, and PET 2 with S9), and although there is some evidence of a concentration-related trend, the responses were weak. Correspondence of the mutagenic activity observed with the two assay systems, for both the explosive compounds and soil samples, was negligible. The differential response is likely due to differences in metabolic capacity between the two assay systems. Furthermore, it is likely that there are unidentified compounds present in these soil samples that are, at least in part, responsible for the observed mutagenic activity. Additional testing of other explosive compounds, as well as soil samples from other military training sites, using a variety of *in vitro* and *in vivo* assays, is warranted in order to reliably estimate mutagenic hazard and subsequently assess risk to human health.

RÉSUMÉ

La génotoxicité de composés énergétiques (par exemple, explosifs) qui sont connus pour être présents dans les sols contaminés sur les sites d'entraînement militaire n'a pas été étudiée de façon approfondie. Ainsi, les essais de mutagénicité in vitro Salmonella et Muta^{TM}Mouse ont été employés pour examiner l'activité mutagène de douze composés explosifs, ainsi que trois échantillons de sol provenant de la base des Forces canadiennes de Petawawa. Les analyses portant sur Salmonella ont employés les souches TA98 (mutations par décalage) et TA100 (mutations par substitution de paires de bases) ainsi que les souches métaboliques supérieures YG1041 (dérivé de TA98) et YG1042 (dérivé de TA100), avec et sans activation métabolique exogène (S9). Pour les analyses portant sur Salmonella, les résultats indiquent que dix des composés explosifs étaient mutagènes, et démontraient des mutations par substitution de paires de bases par action directe. Les trois échantillons de sol ont également été observés à être mutagènes, démontrant des mutations par décalage par action directe. L'activité mutagène était significativement plus élevée sur les souches métaboliquement supérieures pour tous les composés ainsi que les échantillons de sol. Pour les analyses sur les cellules Muta^{TM}Mouse FE1, les résultats indiquent que la majorité des composés explosifs ne présente pas d'activité mutagène. Les trois échantillons de sol ont suscité une réponse positive significative (PET 1 et 3 sans S9, et PET 2 avec S9), et bien qu'il y ait des preuves d'une tendance liée à la concentration, les réponses étaient faibles. Correspondance de l'activité mutagène observée avec les deux systèmes d'essais, à la fois pour les composés explosifs et les échantillons de sol, était négligeable. La réponse différentielle est probablement due à des différences dans la capacité métabolique entre les deux systèmes d'essais. En outre, il est probable qu'il existe des composés non identifiés présents dans ces échantillons de sol qui sont, au moins en partie, responsable de l'activité mutagène observée. Des essais supplémentaires avec d'autres composés explosifs, ainsi que des échantillons de sol provenant d'autres sites d'entraînement militaire, en utilisant une variété d'essais in vitro et in vivo, est justifiée pour une estimation fiable des risques mutagènes pour pouvoir ensuite évaluer les risques pour la santé humaine.

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Salmonella strains TA98, TA100, YG1041 and YG1042 were generous gifts from colleagues at the Environmental Carcinogenesis Division at the U.S. EPA, and I would like to express my gratitude to Dr. David Demarini, and especially Sarah Warren, who provided me with excellent guidance for troubleshooting the Ames assay. I would also like to thank Dr. Sylvie Brochu at Defence Research and Development Canada (Valcartier) for providing the soil samples that were analysed in this thesis. I would further like to thank Dr. Bernard Lachance, formerly of Biotechnology Research Institute (Montréal), for providing solutions of RDX and HMX.

I am grateful to Remi Gagné, John Gingerich and Lynda Soper for their assistance with the soil analyses and Muta^{TM}Mouse work. I would also like to thank Leonora Marro and Sandra Kuchta for their assistance with statistical and chemical analyses.

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And to my parents… I FINALLY FINISHED!

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1,3,5-TNB	1,3,5-trinitrobenzene
2,4-DANT	2,4-diamino-6-nitrotoluene
2,4-DNT	2,4-dinitrotoluene
2,6-DANT	2,6-diamino-4-nitrotoluene
2,6-DNT	2,6-dinitrotoluene
2a-DNT	2-amino-4,6-dinitrotoluene
3,5-DNA	3,5-dinitroaniline
4a-DNT	4-amino-2,6-dinitrotoluene
Å	Angstrom
ACN	Acetonitrile
Amp	Ampicillin resistance gene
ASQG	Agricultural Soil Quality Guidelines
ASE	Accelerated Solvent Extraction
atm	Atmospheres
ATSDR	Agency for Toxic Substances and Disease Registry
B[*a*]P	Benzo[*a*]pyrene
^{o}C	Temperature in degrees Celsius
CAS RN	Chemical Abstracts Service Registry Number(s)
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CFB	Canadian Forces Base
cm	Centimetre(s)
cnr	Classical nitroreductase gene
CO_2	Carbon dioxide
CRS	Congressional Research Service
CYP450	Cytochrome P450 (specific enzymes include CYP1A1 and CYP1A2)
DFT	Direct Fire Target
dH_2O	Deionized water
D-MEM/F-12	Dulbecco's modified Eagle's medium/F-12 nutrient mixture
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate buffered saline
DRDC	Defence Research and Development Canada
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FCSI	Federal Contaminated Sites Inventory
FE1	Flat Epithelial cell line derived from Muta^{TM}Mouse lung tissue
g	Gram(s)
G6P	D-glucose 6-phosphate sodium salt
GC-ECD	Gas Chromatography – Electron Capture Detector
HAA	Heterocyclic aromatic amine(s)

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMX	High Melting Explosive
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
ISQG	Industrial Soil Quality Guidelines
Kan	Kanamycin resistance gene
kg	Kilogram(s)
K_{oc}	Soil adsorption partition coefficient
K_{ow}	Octanol-water partition coefficient
λ	Lambda
L	Litre(s)
LAW	Light Anti-tank Weapon
LB	Luria-Bertani
LOQ	Limit(s) of quantification
µg	Microgram(s)
µm	Micrometre(s)
M	Molar (mol/L)
m^3	Cubic metre(s)
MF	Mutant frequency
mg	Milligram(s)
min	Minute(s)
mm	Millimetre(s)
mM	Millimolar
mm Hg	Millimetres of mercury
mol	Mole
MPa	Megapascal(s)
N/A	Not applicable
NADP	Nicotinamide adenine dinucleotide phosphate disodium salt
NC	Nitrocellulose
NG	Nitroglycerin
nm	Nanometre(s)
NO_2	Nitro moiety
NR_2	Amino group
OAT	*O*-acetyltransferase gene
PAH	Polycyclic aromatic hydrocarbon(s)
pfu	Plaque-forming unit
P-Gal	Phenyl-β-D-galactoside
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
pKM101	R-factor plamid containing *mucA/B genes*
ppm	Parts per million
psi	Pound(s) per square inch
pYG233	Plasmid containing *cnr* and *OAT* genes
RDX	Royal Demolition Explosive
rfa	Deep rough mutation
rpm	Rotations per minute

RSD	Relative standard deviation(s)
S9	Post-mitochondrial supernatant (exogenous metabolic activation system; microsomal fraction from Aroclor 1254-induced rat liver homogenate)
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
S. typhimurium	*Salmonella typhimurium*
TNT	2,4,6-trinitrotoluene
U	Units
U.S. EPA	United States Environmental Protection Agency
UV	Ultraviolet
VBME	Vogel-Bonner Medium E
v/v	Volume/volume
W	Watt
w/v	Weight/volume

1.0 INTRODUCTION

1.1 Background

Military activities often necessitate the use of energetic materials in the form of munitions. Production and subsequent testing of these munitions in training exercises at military bases are required in order to maintain combat readiness of the armed services. These actions, however, can cause dispersion of the explosive compounds employed in these munitions into the environment. The resulting exposures to explosive residues, and the concomitant risk to human health, are generally not appreciated.

The Government of Canada defines a contaminated site as an area where substances of concern are present at concentrations 1) above background levels, and pose an immediate or long term hazard to human health or the environment, or 2) exceeding the specified regulatory guidelines [1]. There is currently estimated to be up to 40 000 contaminated land sites in Canada [2], although the *Ad Hoc International Working Group on Contaminated Land* reported over 200 000 in their 2002 report [3]. The Federal Contaminated Sites Inventory (FCSI) lists nearly 20 000 contaminated sites under the custodianship of various federal departments, agencies and consolidated Crown corporations, as well as non-federal sites for which the Government of Canada has accepted financial responsibility. In total, there are 84 sites listed on the FCSI that are primarily contaminated with *energetics* (i.e., explosives and explosive residues), all of which are under the responsibility of the Department of National Defence. Contaminated soil is stated to be a medium of concern for the majority of these sites (i.e., 80 out of 84 sites list soil or surface soil as one of the contaminated media), although contaminated groundwater is also present at high frequency [1].

The existence of explosives-contaminated sites is also a problem in other countries. For example, Bhushan et al. (2006) [4] noted that the United States Departments of Defense and Energy alone, are responsible for over 21 000 contaminated sites, a large majority of which are contaminated with various explosive compounds. A Congressional Research Service (CRS) Report for Congress (2008) [5] revealed that 5356 sites, on hundreds of military installations, are slated for remediation under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA, commonly referred to as Superfund) at a cost of over $11 billion, prior to transferring these federal properties to the public domain. Internationally, numerous countries have also reported contamination at various military installations. Table 1.1 provides a list of several European countries reporting contamination, as well as the corresponding numbers of documented sites. From eighteen Western European nations that were surveyed, ten of these countries (i.e., Austria, Belgium, Denmark, Finland, France, Germany, the Netherlands, Sweden, Switzerland and Norway) have a systematic identification process for contaminated military sites. Western European nations appear to more accurately quantify these types of sites, in terms of their numbers and the extent of contamination, when compared with Eastern European countries (e.g., Estonia, Latvia, Lithuania, Poland, Slovakia, the Czech Republic, Hungary, Russia and Ukraine) [6, 7]. This is likely a result of the extremely large number of military sites belonging to the Soviet Army that were abandoned after the break-up of the former Soviet Union.

Furthermore, there are also countless numbers of explosives-contaminated sites worldwide that remain classified and unavailable for study [4].

Table 1.1: Reported numbers of contaminated military sites in several European countries [6, 7].

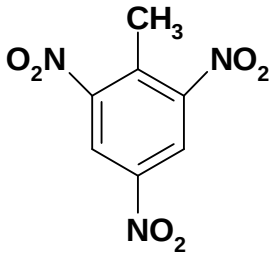
Country	Number of Contaminated Military Sites
Germany	22 513[a]
The Netherlands	2500[b]
Sweden	1244[c]
Lithuania	2743[d]
Russia	1868[e]

[a] Sites at military bases owned by the Federal Government only.
[b] Reported number represents potentially contaminated military sites.
[c] 614 identified contaminated military sites, in addition to 630 potentially contaminated military sites.
[d] Sites at military bases that are Registered Contaminated Sites only.
[e] Reported number represents military sites where pre-assessments for environmental compliance controls have been conducted. There is no estimate of the total number or the extent of contaminated military sites in this country.

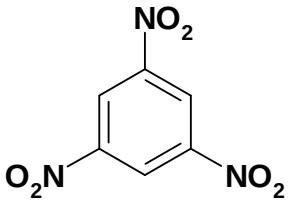
1.2 Explosive Compounds

1.2.1 Nitroaromatics and Aromatic Amines

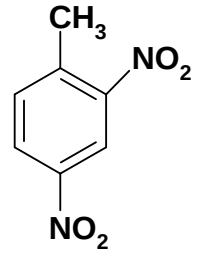
Nitroaromatic compounds are aromatic hydrocarbons that contain at least one nitro (NO_2) moiety. The majority of nitroaromatic compounds present in the environment are industrial chemicals. In addition to their use as explosives, nitroaromatics are also used as dyes, herbicides, insecticides and solvents [8]. Ten of the twelve explosive compounds examined in this thesis possess the structural properties of a nitroaromatic. Of these ten nitroaromatics, six compounds also possess the structural properties of an aromatic amine; an aromatic hydrocarbon that contains at least one amino (NR_2) group. The structures of the ten nitroaromatic and/or aromatic amine explosive compounds examined in this thesis are depicted in Figures 1.1 and 1.2.



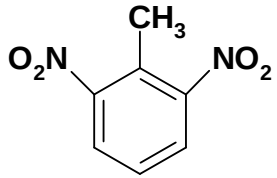
Trinitrotoluene
(TNT)



1,3,5-trinitrobenzene
(1,3,5-TNB)

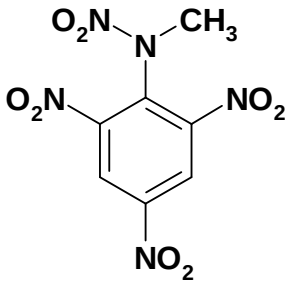


2,4-dinitrotoluene
(2,4-DNT)

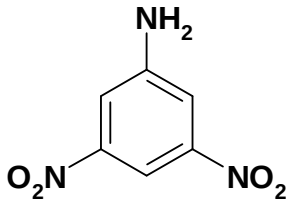


2,6-dinitrotoluene
(2,6-DNT)

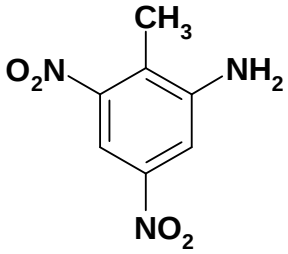
Figure 1.1: Structures of the four nitroaromatic explosive compounds examined in this thesis.



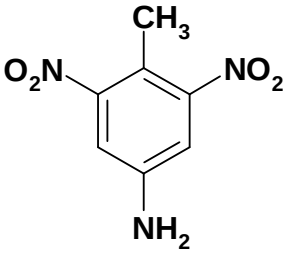
Tetryl



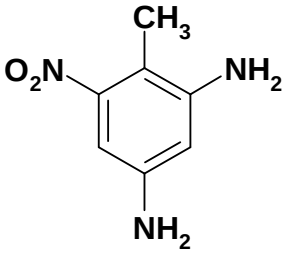
3,5-dinitroaniline
(3,5-DNA)



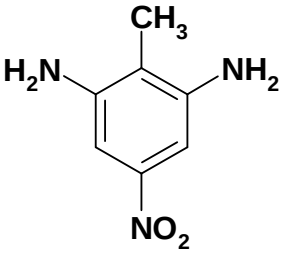
2-amino-4,6-dinitrotoluene
(2a-DNT)



4-amino-2,6-dinitrotoluene
(4a-DNT)



2,4-diamino-6-nitrotoluene
(2,4-DANT)



2,6-diamino-4-nitrotoluene
(2,6-DANT)

Figure 1.2: Structures of the six explosive compounds examined in this thesis that possess both nitroaromatic and aromatic amine properties.

TNT is not only the most commonly used nitroaromatic explosive, it is also the most common explosive compound used worldwide. Its widespread use began in World War I and has continued to the present day. TNT is used in military munitions as a booster or as a bursting charge for high-explosive shells and bombs. It is also one of the major constituents, along with RDX which will be discussed below, in nearly every munition formulation. TNT is also used in civilian applications for mining and quarrying activities [8-10]. Another nitroaromatic compound, 1,3,5-TNB, is used in specific munition formulations, and as a high-explosive for commercial mining [8, 10]. 2,4-DNT and 2,6-DNT, are used as plasticizers in single-based gun propellant [10, 11]. In addition to their use in military munitions, 1,3,5-TNB, 2,4-DNT and 2,6-DNT are also present as manufacturing impurities in the production of TNT. They are further reported to be breakdown products of TNT degradation, and thus have been observed in contaminated soils and water [8, 10-12].

One of the most well-known aromatic amine explosive compounds is tetryl. At one time, tetryl was a common component of military explosives, although it has largely been replaced by RDX in recent munition formulations. Specifically, tetryl was used as an explosive in detonators and primers and as a booster charge for other military devices. Although tetryl is no longer widely used in munitions, residues from its manufacture and use remain at contaminated sites [10, 13].

The remainder of the aromatic amine compounds examined in this thesis, including 3,5-DNA, 2a-DNT, 4a-DNT, 2,4-DANT and 2,6-DANT, are significant breakdown products of TNT. Formation of the two monoamino metabolites (i.e., 2a-DNT and 4a-DNT) is energetically favoured, and they are frequently observed in TNT-

contaminated soils and groundwater. The diamino metabolites (i.e., 2,4-DANT and 2,6-DANT) are energetically more difficult to create, and thus are observed less frequently and at lower concentrations than the monoamino compounds. 3,5-DNA is also a notable TNT biotransformation product [10-12]. Figures 1.3 and 1.4 depict the degradation pathways of TNT that generate some of these breakdown products under aerobic and anaerobic conditions, respectively.

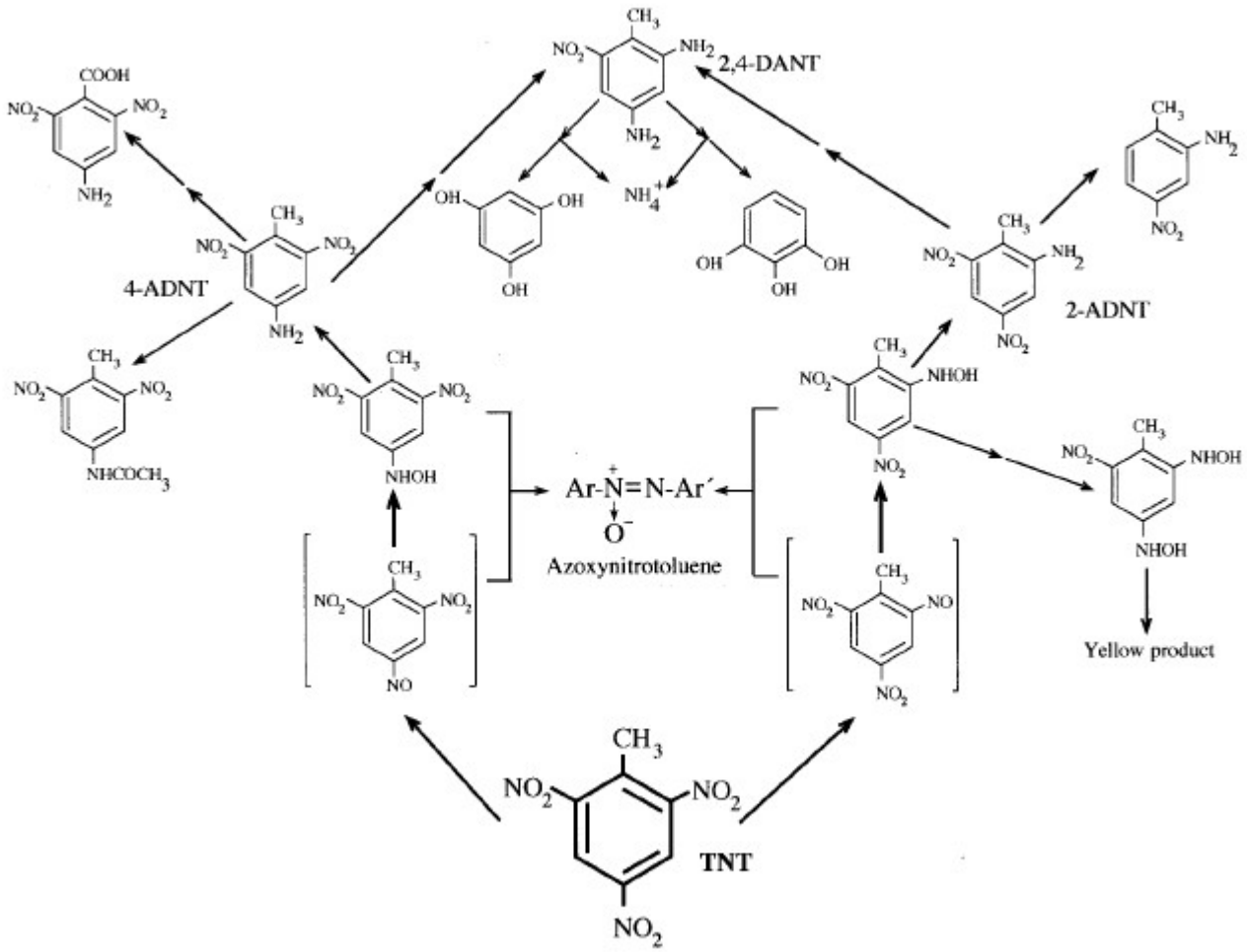


Figure 1.3: Aerobic bacterial TNT degradation pathways (adapted from Esteve-Núñez et al. 2001) [14].

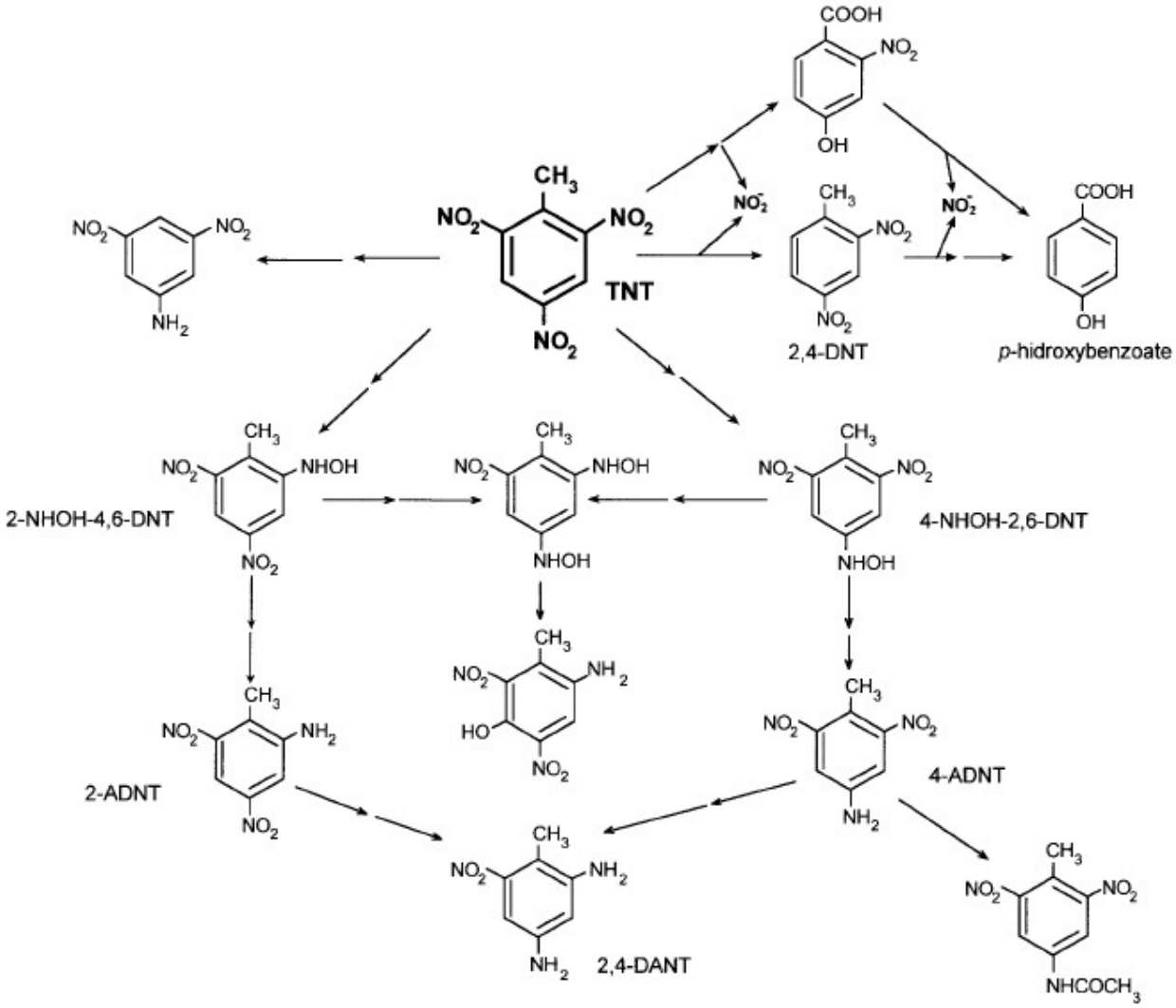
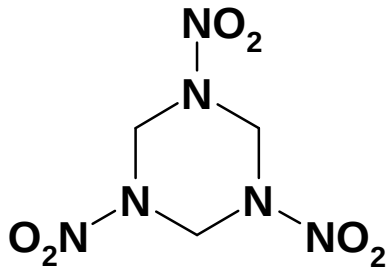


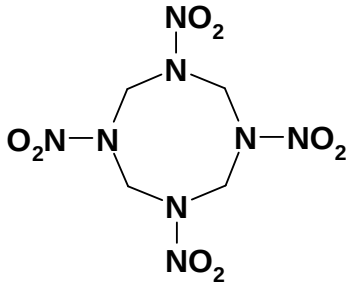
Figure 1.4: Anaerobic bacterial TNT degradation pathways (adapted from Esteve-Núñez et al. 2001) [14].

1.2.2 Heterocyclic Amines

The two remaining explosive compounds examined in this thesis are heterocyclic amines. Heterocyclic amines are cyclic compounds containing nitrogen and at least one other element within the ring structure. Heterocyclic amines should not be confused with heterocyclic aromatic amines (HAAs), where the amino group is contained within an aromatic ring. HAAs appear to have strikingly different toxicological properties than non-aromatic heterocyclic amines [15]. In addition to possessing the structural properties of a heterocyclic amine, these two explosive compounds (i.e., RDX and HMX) also possess the functional group $R_2N\text{-}NO_2$, and are therefore considered to be nitramines. Nitramines are a more recently established group of explosive compounds. These compounds are advantageous due to their higher density, and ability to produce significantly more powerful explosions than nitroaromatic and/or aromatic amine explosives [9]. The structures of the two nitramine explosive compounds examined in this thesis are depicted in Figure 1.5.



Royal Demolition Explosive
(RDX)



High Melting Explosive
(HMX)

Figure 1.5: Structures of the two nitramine explosive compounds examined in this thesis.

RDX, also known as Research Department Explosive or Hexogen, is currently the most important military high explosive. Its widespread use began in World War II and it has since become the second most widely used high explosive in the military, exceeded only by TNT. Alone, RDX can be used in military munitions as a base charge for detonators and blasting caps. It is also one of the major constituents, along with TNT, of nearly every munition. RDX is further used in civilian applications for fireworks and demolition activities [9, 10]. The other nitramine compound, HMX, also known as High Melting Explosive or Octogen, is the highest-energy solid explosive produced on a large scale. It is used exclusively for military purposes, almost always combined with other explosive compounds such as TNT, and primarily employed in maximum-performance explosive devices [9].

1.3 Explosive Compounds in Contaminated Soils at Military Training Ranges

Because terrestrial explosions are the most common, surface soil is an ideal medium for examining the types and amounts of explosive compounds released into the environment. There are often several different training ranges at military bases that are expected to contain varying concentrations of different explosive residues due to the wide variety of training activities. These include live fire training ranges for large anti-armour weapons (e.g., anti-tank rockets), as well as direct-fire training ranges for small calibre munitions (e.g., 25-mm small arms bullets) and medium to large calibre munitions (e.g., grenades and missiles). This thesis examined soils from training ranges at Canadian Forces Base (CFB) Petawawa; more specifically from sites located within Impact Area A, an area used for anti-armour weapon training, and Direct Fire Target (DFT) Area 2, an

area currently used for small arms munitions training. These types of sites, which are described in more detail below (refer to Section 2.2 in Materials and Methods), are often found to contain HMX, RDX, TNT and 2,4-DNT. For example, previous analyses of soil samples from an anti-tank target area at CFB Petawawa revealed significant levels of HMX, TNT and RDX, with mean concentrations of 750, 73 and 0.32 mg/kg, respectively. Additionally, previous analyses of soil samples from a small arms munitions firing point contained significant levels of 2,4-DNT, with a mean concentration of 1.4 mg/kg [16]. In addition to the primary explosives, several other compounds, known to be principle breakdown products of TNT degradation, are frequently identified in soils from military training ranges. Although information regarding the concentration of TNT metabolites at CFB Petawawa was not available, these compounds have been detected at other training ranges such as those located at Camp Edwards, a United States military training installation in Massachusetts. Camp Edwards encompasses a wide variety of military training areas, including several rocket/anti-tank and small arms firing ranges. Diamino TNT metabolites (i.e., 2,4-DANT and 2,6-DANT) were not detected at these sites; however, the two energetically favoured monoamino metabolites were detected, with mean concentrations of 322 and 339 μg/kg, respectively [17].

1.3.1 Other Compounds in Explosives-Contaminated Soil Samples

It is obvious that soils at military training ranges will contain significant concentrations of explosive residues. Several other classes of compounds, however, are also known to be prevalent at these types of contaminated sites, including metals and polycyclic aromatic hydrocarbons (PAHs).

A previously analysed soil sample obtained from an anti-tank firing position at CFB Petawawa was observed to have levels of chromium exceeding the Industrial Soil Quality Guidelines (ISQG), and levels of boron exceeding the Agricultural Soil Quality Guidelines (ASQG). A similar sample obtained from an anti-tank target area was determined to contain levels of copper and lead exceeding the ISQG, and levels of antimony exceeding the ASQG. A further sample obtained from a small arms munitions firing point also displayed levels of copper exceeding the ISQG [16]. Contaminated soil samples from Camp Edwards confirm the presence of these compounds at similar sites. Additionally, iron and aluminum were reported to be present in Camp Edwards soil samples at concentrations of approximately 10 322 and 8578 mg/kg, respectively. Relatively lower concentrations of arsenic and cadmium, 3.36 and 1.58 mg/kg, respectively, were also observed, in addition to several other metals [17].

Information regarding the presence of PAHs in CFB Petawawa soil samples was not available. Information regarding these compounds was available, however, for soil samples obtained from Camp Edwards. Concentrations of the sixteen PAHs classified as priority pollutants by the United States Environmental Protection Agency (U.S. EPA) are listed in Table 1.2.

Table 1.2: Concentrations of sixteen priority PAHs in contaminated soils from Camp Edwards [17].

Name	**Concentration (µg/kg)**
Acenaphthene	130
Acenaphthylene	170
Anthracene	390
Benzo[*a*]anthracene	792
Benzo[*a*]pyrene	633
Benzo[*b*]fluoranthene	1022
Benzo[*g,h,i*]perylene	404
Benzo[*k*]fluoranthene	696
Chrysene	915
Dibenz[*a,h*]anthracene	269
Fluoranthene	1713
Fluorene	425
Indeno[*1,2,3-cd*]pyrene	462
Naphthalene	206
Phenanthrene	1135
Pyrene	1528

1.4 Physical-Chemical Properties of Explosive Compounds

The physical-chemical properties of the explosive compounds examined in this thesis are important factors to consider when establishing toxicological hazard and risk. Characteristics such as water solubility, the octanol-water partition coefficient (i.e., K_{ow}), the soil adsorption partition coefficient (i.e., K_{oc}), vapour pressure, and the Henry's Law Constant will determine the extent to which these compounds are physically accessible for exposure, and their subsequent bioavailability in humans. Selected physical-chemical properties of the explosive compounds examined in this thesis are summarized in Table 1.3.

Table 1.3: Physical-chemical properties of explosive compounds examined in this thesis.*

Compound	Water Solubility @ 20°C (mg/L)	Log K_{ow}	Log K_{oc}	Vapour Pressure @ 20°C (Pa)	Henry's Law Constant ($atm \cdot m^3/mole$)
TNT	130	1.60	2.48 – 3.04	0.0265	4.57×10^{-7} @ 20°C
RDX	38.4	0.87	1.80	1.33×10^{-7}	1.96×10^{-11} @ 25°C
HMX	5 @ 25°C	0.06	0.54	4.40×10^{-12} @ 25°C	2.6×10^{-15} @ 25°C
Tetryl	75	2.4	3.13 – 3.47	5.33×10^{-8}	2.0×10^{-12} @ 25°C
1,3,5-TNB	350 @ 25°C	1.18	1.88	4.27×10^{-4} @ 25°C	3.08×10^{-9} @ 25°C
3,5-DNA	No data	No data	2.4 – 2.7	No data	No data
2,4-DNT	270 @ 22°C	1.98	1.65	0.6799	8.79×10^{-8}
2,6-DNT	206 @ 25°C	1.72	1.96	2.40	9.26×10^{-8}
2a-DNT	2800	1.94	No data	5.33×10^{-3}	3.39×10^{-9} @ 20°C
4a-DNT	2800	1.91	No data	2.67×10^{-3}	1.13×10^{-9}
2,4-DANT	No data	0.46	No data	No data	No data
2,6-DANT	No data	0.46	No data	No data	No data

* Table comprised of data obtained from the following references: 10-13, 18-24.

None of the explosives examined in this thesis are subject to significant volatilization due to low vapour pressures and Henry's Law Constants. TNT, tetryl, 1,3,5-TNB, 2,4-DNT and 2,6-DNT, have strong soil adsorption capacities, but also moderate water solubilities. They are also known to be environmentally unstable, and are therefore not considered to be persistent. They generally dissolve into surface water and rapidly degrade into their respective metabolites. 2a-DNT and 4a-DNT, the principle TNT metabolites, have strong soil adsorption capacities and high water solubilities. They are also known to degrade into 2,4-DANT and 2,6-DANT, but not in significant quantities. Not surprisingly, these compounds are considered to be persistent; remaining primarily in the surface soil, despite having high water solubilities. However, they have occasionally been observed in the groundwater at contaminated sites. RDX and HMX have weak soil adsorption capacities and can take decades to degrade in the environment because there are no significant degradation pathways. HMX, due to its low water solubility, will therefore remain in the surface soil. RDX, due to its moderate water solubility, can dissolve and travel to the groundwater. There is a paucity of data for 3,5-DNA, 2,4-DANT and 2,6-DANT. Tetryl, 1,3,5-TNB, 3,5-DNA, 2,4-DANT and 2,6-DANT are not present in significant quantities at most military training sites [10, 11].

Despite the fact that the log K_{ow} values for these explosives are known to be low, and thus they are not expected to bioaccumulate, the risk of repeated human exposure still exists due to the persistent nature of these compounds and/or their metabolites in surface soils and/or groundwater.

1.5 Toxicity of Explosive Compounds

The overall toxicity of explosive compounds has not been extensively investigated; however, effects that have been observed in various human studies after exposure to TNT, RDX, HMX, tetryl, 1,3,5-TNB, 2,4-DNT or 2,6-DNT include: seizures, effects of the nervous system and anaemia, as well as respiratory and skin irritation. Effects observed in animal studies are similar to those observed in humans, and further include liver and kidney damage and reproductive effects [13, 19-23].

1.5.1 Genetic Toxicity of Explosive Compounds

Genetic toxicology involves quantitative assessment of the genotoxic activity associated with a variety of environmental substances and occupational settings, as well as complex mixtures (e.g., urban air particulate matter). The ability of these substances, including explosive compounds, to induce mutations (i.e., permanent DNA sequence changes) can have serious consequences. Mutations in specific genes (i.e., tumour suppressor genes, proto-oncogenes, etc.) have been shown to be associated with the initiation of tumour formation (i.e., cancer) [25], and mutations in germ cells may contribute to heritable genetic diseases.

Given the number of substances that are currently in commerce in Canada, approximately 23 000 according to the Domestic Substances List [26], the need for reliable *in vitro* and *in vivo* assays that assess the toxicity of these substances is imperative. *In vitro* bioassays are employed because they are relatively convenient, efficient and inexpensive tools that can be used to screen for potential hazard, and justify subsequent use of more rigorous and costly *in vivo* bioassays. Several *in vitro* bioassays were designed specifically to evaluate mutagenicity and/or genotoxicity. These assays

have been developed in a variety of organisms, including bacteria and several rodent and human cell lines, and are able to measure the ability of a test article to damage DNA and/or introduce DNA sequence changes.

The genotoxic and carcinogenic effects of explosive compounds in humans or animals, determined using either *in vitro* or *in vivo* assays, have not been extensively investigated. Overall conclusions from international organizations regarding the genotoxicity and carcinogenicity of these compounds are listed in Tables 1.4 and 1.5, respectively.

Table 1.4: Genotoxicity of explosive compounds examined in this thesis.

Compound	Conclusion	Organization	Reference
TNT	- No evidence of genotoxicity *in vivo*. - Genotoxicity observed *in vitro*.	ATSDR[a] IARC[b]	19, 27
RDX	No evidence of genotoxicity *in vivo* or *in vitro*.	ATSDR	20
HMX	- Not tested for genotoxicity *in vivo*. - No evidence of genotoxicity *in vitro*.	ATSDR	21
Tetryl	- Not tested for genotoxicity *in vivo*. - Genotoxicity observed *in vitro*.	ATSDR	13
1,3,5-TNB	- Not tested for genotoxicity *in vivo*. - Genotoxicity observed *in vitro*.	ATSDR	22
3,5-DNA	Not tested for genotoxicity *in vivo* or *in vitro*.	N/A	N/A
2,4-DNT	Genotoxicity observed *in vivo* and *in vitro*.	ATSDR IARC European Commission	23, 28, 29
2,6-DNT	Genotoxicity observed *in vivo* and *in vitro*.	ATSDR IARC European Commission	23, 28, 30
2a-DNT	- Not tested for genotoxicity *in vivo*. - Genotoxicity (weak) observed *in vitro*.	ATSDR	19, 31, 32, 33
4a-DNT			19, 31, 32, 33
2,4-DANT			19, 31, 33
2,6-DANT			19, 33

[a] ATSDR = Agency for Toxic Substances and Disease Registry.
[b] IARC = International Agency for Research on Cancer.

Table 1.5: Carcinogenicity classifications of explosive compounds examined in this thesis.

Compound	Conclusion	Organization	Reference
TNT	Group C[a]	U.S. EPA	19
	Group 3[b]	IARC	27
RDX	Group C	U.S. EPA	20
HMX	Group D[c]	U.S. EPA	21
Tetryl	Group D	U.S. EPA	13
1,3,5-TNB	Group D	U.S. EPA	22
3,5-DNA	No data	N/A	N/A
2,4-DNT	Group B2[d]	U.S. EPA	23
	Group 2B[e]	IARC	28
	Category 2[f]	European Commission	29
2,6-DNT	Group B2	U.S. EPA	23
	Group 2B	IARC	28
	Category 2	European Commission	30
2a-DNT	No data	N/A	N/A
4a-DNT			
2,4-DANT			
2,6-DANT			

[a] Group C = possible human carcinogen
[b] Group 3 = not classifiable as to its carcinogenicity
[c] Group D = not classifiable as to its human carcinogenicity
[d] Group B2 = probable human carcinogen, based on animal data
[e] Group 2B = possibly carcinogenic to humans
[f] Category 2 = may cause cancer

Although several of the aforementioned explosive compounds have not been tested for genotoxicity using comprehensive *in vitro* and *in vivo* testing regimens, the experiments that have been conducted do reveal that several of these compounds exhibit mutagenic activity in various bacterial and mammalian cell assays, as well as in whole animals. Moreover, it has been determined that several of these explosives are, in fact, possible human carcinogens [13, 19-23, 27-33]. For the purposes of this thesis, two *in vitro* bioassays, namely, the Ames/*Salmonella* reverse mutation assay and the FE1 Muta^{TM}Mouse *in vitro* transgene mutation assay, were employed to assess the mutagenic activity of individual explosive compounds and extracts of contaminated soil samples. These two bioassays are described in more detail below.

1.6 Ames/*Salmonella* Reverse Mutation Assay

The *Salmonella* mutagenicity assay is an *in vitro* bacterial test employed to identify mutagenic substances. It examines the ability of the test article to induce mutations that revert the phenotype from histidine auxotrophy to wild-type. It is frequently used by government agencies and the scientific community as an initial screening tool to identify potential carcinogens. Mutagenicity observed using this assay is highly predictive of rodent carcinogenicity [34].

1.6.1 *Salmonella typhimurium* Strains

The mutagenic activity of the twelve explosive compounds and three CFB Petawawa soil extracts examined in this thesis was assessed using four *Salmonella typhimurium* (*S. typhimurium*) strains. TA98 and TA100 are well-established tester strains that have been utilised for the detection of a wide variety of frameshift and base-

pair substitution mutagens, respectively [34, 35]. YG1041 and YG1042 are metabolically-enhanced strains derived from TA98 and TA100, respectively [36, 37]. The characteristics of each tester strain are summarized in Table 2.6 (refer to Section 2.5.2 in Materials and Methods).

TA98 and YG1041 contain a mutation located in the *hisD* gene and the genotype is denoted *hisD3052*. This mutation is the result of a -1 frameshift that affects the reading frame in close proximity to a C-G dinucleotide repeat. Frameshift mutagens can target this region and induce a reversion mutation that restores the wild-type phenotype and permits the growth of revertant colonies on media lacking histidine. TA100 and YG1042 contain a mutation located in the *hisG* gene and the genotype is denoted *hisG46*. This mutation results in the substitution of leucine (GAG/CTC) for proline (GGG/CCC). Base-pair substitution mutagens can target this region and induce a reversion mutation that restores the wild-type phenotype and permits the growth of revertant colonies on media lacking histidine [34].

The four strains also contain an *rfa* mutation (deep rough) that results in partial loss of the lipopolysaccharide layer of the bacterial cell wall. This causes increased permeability to large/bulky chemicals that otherwise would not be able to penetrate the cell [34]. All four strains also contain a deletion mutation of the *uvrB-bio* genes and an addition of the R-factor plasmid, pKM101. The *uvrB* mutation removes the capacity for nucleotide excision repair. The R-factor plasmid, which contains the *mucA/B* genes, provides an enhanced capacity for error-prone DNA repair via DNA polymerase RI (i.e., by translesion synthesis) [34, 36]. Finally, YG1041 and YG1042 also harbour the plasmid pYG233 that contains the *cnr* and *OAT* genes coding for classical nitroreductase and *O*-

acetyltransferase, respectively. The enhancement of these rate-limiting metabolic enzymes has been shown to increase sensitivity to both nitroaromatics and aromatic amines [36, 37]. TA98 and TA100 also express these enzymes, but to a lesser extent.

Salmonella have a limited metabolic capacity because they do not inherently possess cytochrome P450 (CYP450) enzymes that are often important for mammalian metabolism and activation of chemical mutagens. The addition of an exogenous S9 microsomal fraction from Aroclor 1254-induced rat liver homogenate, which contains high levels of CYP450 enzymes, increases the sensitivity of the *Salmonella* strains to compounds such as aromatic amines [38, 39].

1.6.2 Metabolic Pathways in *Salmonella*

The primary metabolic activation pathways in *Salmonella* for nitroaromatic and aromatic amine compounds, with and without the addition of S9, are presented in Figure 1.6.

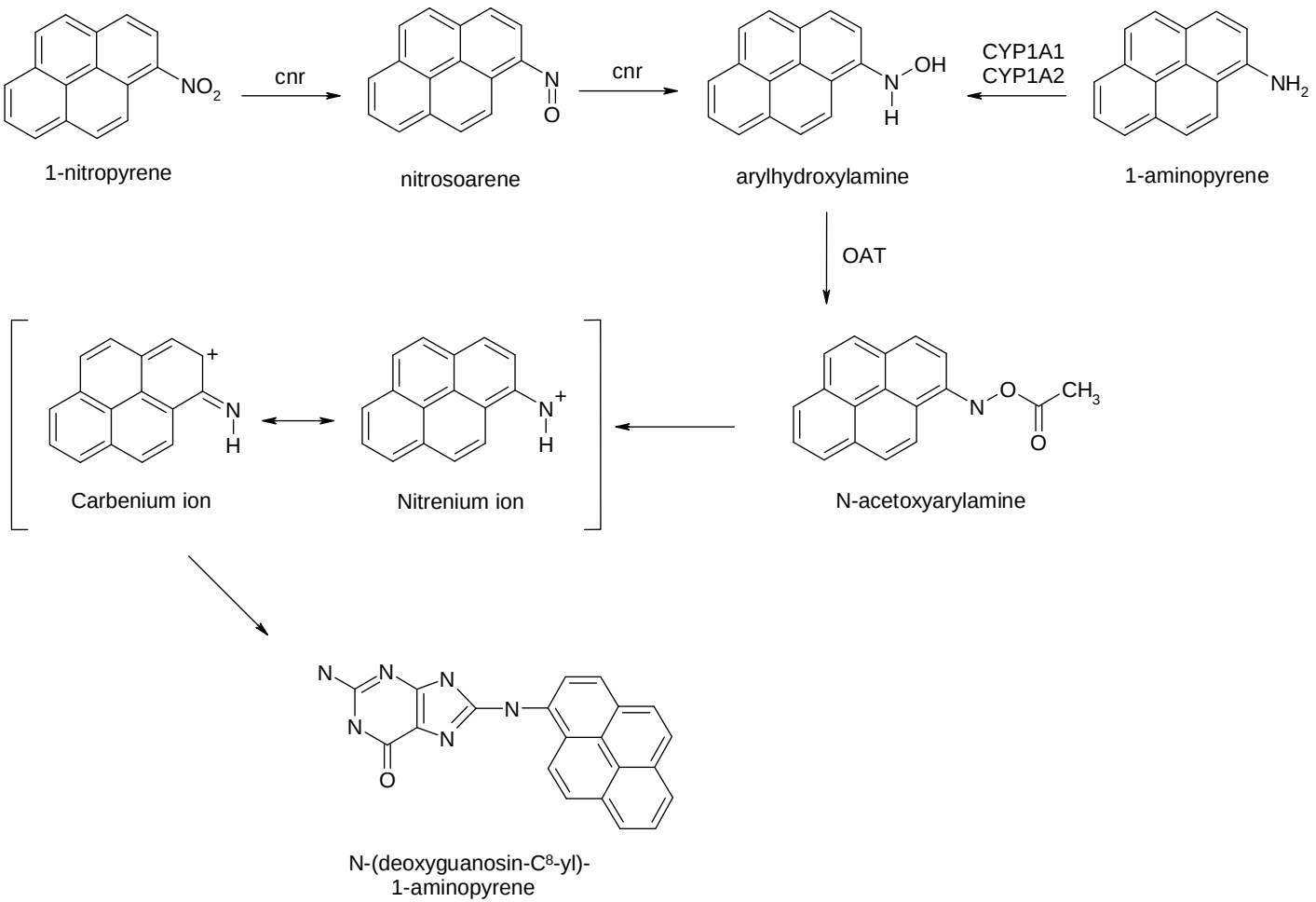


Figure 1.6: Primary metabolic activation pathways for nitroaromatic and aromatic amine compounds in *Salmonella*, with and without the addition of S9 (Lemieux 2006, reproduced with permission) [40]. CYP1A1 and CYP1A2 require the addition of exogenous S9 metabolic activation.

Using 1-nitropyrene as an example, nitroaromatics are converted to nitrosoarene and arylhydroxylamine intermediates via reduction of the nitro moiety by classical nitroreductase [37-39, 41]. Acetylation of the arylhydroxylamine intermediate to *N*-acetoxyarylamine is then catalysed by *O*-acetyltransferase [37, 39]. The acetate moiety on the *N*-acetoxyarylamine is an excellent leaving group, resulting in a highly reactive nitrenium ion that will react with DNA to form DNA adducts. Nitroaromatic mutagens are considered direct-acting because they do not require exogenous enzymes to be converted to their active form.

Using 1-aminopyrene as an example, aromatic amines induce genetic damage and mutations in a similar way to nitroaromatic compounds [37, 39]. However, they require oxidation of the amino group by CYP450 enzymes in order to be converted to an arylhydroxylamine intermediate [38, 42]. Thus, aromatic amines are considered indirect-acting because they require the addition of an exogenous metabolic activation mixture to be converted to their active form.

1.7 FE1 Muta^{TM}Mouse *in vitro* Transgene Mutation Assay

Although the *Salmonella* mutagenicity assay is useful as a screening tool to provide information regarding the potential mutagenic hazard of a variety of test substances towards mammalian receptors (e.g., humans), extrapolation of results from a bacterial assay to humans can be tenuous and complex. Despite the fact that the addition of the exogenous S9 metabolic activation system allows *Salmonella* to simulate mammalian metabolism, there remains numerous physiological differences between bacterial and mammalian cells that can affect their responses to chemical mutagens. For

example, bacterial cells contain Type I nitroreductase enzymes (i.e., oxygen-insensitive), whereas mammalian cells utilize both Type I and Type II nitroreductases (i.e., oxygen-sensitive) [43, 44]. This results in a major disparity between bacterial and mammalian metabolic capacity, particularly with respect to reduction of the nitro moiety on the explosive compounds examined in this thesis. With respect to reduction of the nitro moiety, mammalian metabolism of nitroaromatics is a highly complex, multistep process involving several enzymes and intermediates (e.g., microsomal NADPH:P450 reductase and cytosolic NAD(P)H:quinine oxidoreductase) [45]. These effects often cannot be modelled using a bacterial mutagenicity assay. Thus, the *in vitro* transgene mutation assay in Muta^{TM}Mouse FE1 cells was employed as an *in vitro* mammalian assay to verify the mutagenic activity of the twelve explosive compounds and three soil extracts.

1.7.1 Flat Epithelial (FE1) Cell Line

The Muta^{TM}Mouse assay employed in this thesis is based on the Flat Epithelial (FE1) cell line, a stable epithelial cell line derived from lung tissue of the transgenic Muta^{TM}Mouse. The cells are contact inhibited, thereby forming a flat monolayer. The cells retain several characteristics that make them useful for screening suspected mutagens *in vitro* [46]. The FE1 cell line harbours the λgt10*lacZ* shuttle vector that contains the *lacZ* transgene mutation target. This target is flanked by two lambda (λ) cohesive ends that facilitate both its retrieval from genomic DNA, and subsequent scoring of *lacZ* mutations [46-48]. The structure of the *lacZ* transgene in the λgt10*lacZ* shuttle vector is depicted in Figure 2.8 (refer to Section 2.6.1 in Materials and Methods).

Earlier studies of FE1 cells showed endogenous expression of CYP1A1, and an ability to metabolically activate PAHs such as benzo[*a*]pyrene (B[*a*]P) [46, 49]. FE1 cells

were not able to metabolize and activate heterocyclic aromatic amines such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), however, which are thought to require CYP1A2. Thus, despite the metabolic competence of FE1 cells, thorough mutagenicity assessment requires the use of an exogenous S9 metabolic activation system.

1.7.2 P-Gal Positive Selection System

Following exposure to a particular test article, the λgt10*lacZ* shuttle vector must be rescued from FE1 genomic DNA via packaging into λ bacteriophage, and subsequently scored for *lacZ* mutant frequency [50]. A *galE*$^{-}$ strain of *Escherichia coli* (*E. coli*) C bacteria is used as the host bacterium for positive selection of *lacZ* mutants [46, 51]. Phenyl-β-D-galactoside (P-Gal) is used as a selective agent to enumerate *lacZ* in packaged FE1 DNA. *E. coli* infected with FE1 λgt10*lacZ* segments harbouring the wild-type *lacZ* transgene are able to produce β-galactosidase. This enzyme will catalyze the initial step in the conversion of P-Gal into UDP-galactose. Because the strain of *E. coli* employed in this assay does not contain a functional UDP-epimerase, however, the conversion of UDP-galactose to UDP-glucose is prevented. UDP-galactose, a toxic metabolite, will therefore accumulate in cells with the wild type *lacZ*. Conversely, bacteria infected with FE1 λgt10*lacZ* segments harbouring the mutant *lacZ* transgene are unable to produce β-galactosidase, and therefore cannot initiate production of the toxic metabolite UDP-galactose [46, 47, 51]. The process thus allows for the selective identification and quantification of *lacZ* mutants. Infection of the host bacteria under non-selective conditions permits the enumeration of total plaque-forming units (i.e., λgt10*lacZ* segments isolated and packaged in phage particles). A schematic description of the P-Gal positive selection system is provided in Figure 1.7.

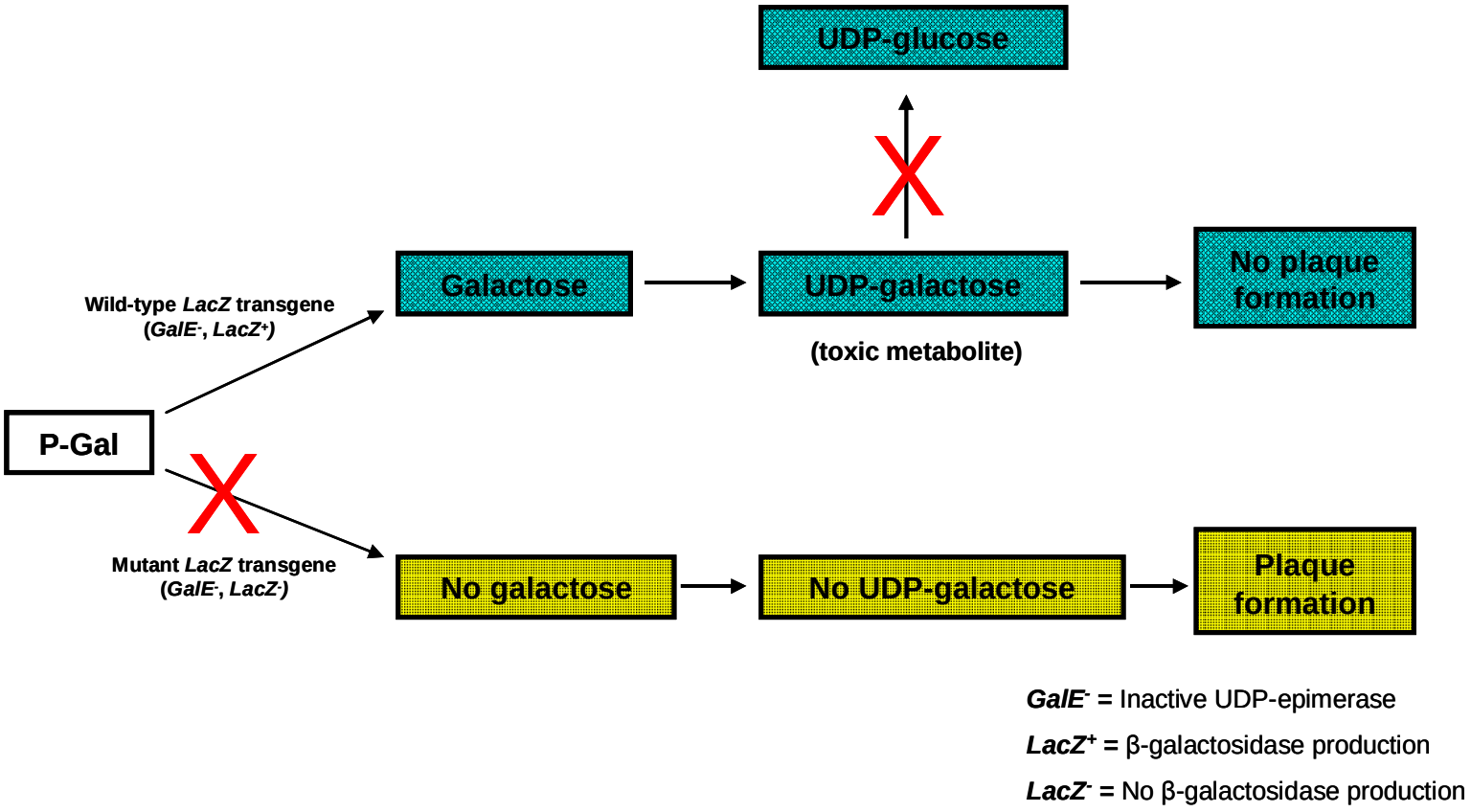


Figure 1.7: P-Gal Positive Selection System.

1.8 Risk Assessment of Explosives-Contaminated Soils at Military Training Ranges

The overall goal of a human health risk assessment for a site contaminated with potentially carcinogenic substances (i.e., explosives) is to determine the excess lifetime cancer risk associated with a specific exposure scenario. Human health risk assessment is a priority for Health Canada under the Federal Contaminated Sites Action Plan, and military training ranges contaminated with explosive residues pose a potential health risk for military personnel who engage in training exercises at these sites. Average exposure for soldiers undergoing training exercises has been estimated; however, these exposure levels are not representative of all training activities, some of which may result in higher than average exposures [52-54]. Currently, the mutagenic and carcinogenic hazards of explosive compounds at military training sites are difficult to quantify. Reasons for this include difficulties in extrapolating from the results of *in vitro* assays, a lack of information on carcinogenic potency, and difficulties in accurately quantifying exposure. This project will expand the current knowledge on this subject and, ideally, contribute to a more effective and accurate assessment of human health risk.

1.9 Objectives and Hypothesis

Objectives

1) Evaluate the mutagenic activity of individual explosive compounds known to be present in contaminated soils at military training ranges using two *in vitro* bioassays; namely, the Ames/*Salmonella* reverse mutation assay and the FE1 Muta^{TM}Mouse *in vitro* transgene mutation assay.

2) Analyse the composition of contaminated soil samples obtained from CFB Petawawa for various explosive compounds using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection.
3) Using the aforementioned bioassays, assess the mutagenic activity of organic extracts of contaminated soil samples obtained from CFB Petawawa.
4) Determine if the mutagenic activity of the aforementioned soil extracts is higher than that expected based on the concentrations and mutagenic activity of the individual explosive compounds identified.
5) Preliminary evaluation of the results of this thesis in a risk assessment context.

Hypothesis

Given that the majority of the explosives and explosive metabolites examined in this thesis possess structural properties of a nitroaromatic and/or an aromatic amine, it is likely that these compounds will induce mutations in bacterial and mammalian cells. Moreover, their presence in soil samples from military training sites will contribute to the mutagenic activity of the contaminated soils. The total mutagenic activity of contaminated soils on military training sites, however, is unlikely to be accounted for solely by known explosives and explosive residues.

2.0 MATERIALS AND METHODS

2.1 Explosive Standards

Stock solutions were custom-made by AccuStandard, Incorporated (New Haven, CT) and supplied by Chromatographic Specialties Incorporated (Brockville, ON). All solutions containing individual explosive residues were prepared at a concentration of 1 mg/ml. EPA 8330 Mixes A and B were prepared at a concentration of 0.1 mg/ml for each residue in the solutions. All solutions were prepared in acetonitrile (ACN) and stored in sealed amber vials, in the dark, at 4^{o}C until required. All solutions were prepared in order to meet the requirements of EPA Method 8330B [55]. The names and corresponding Chemical Abstracts Service Registry Numbers (CAS RNs) of each compound examined in this thesis are listed in Table 2.1.

Table 2.1: Names and CAS RNs of explosive compounds examined in this thesis.

Compound	CAS RNs
2,4,6-trinitrotoluene (TNT)	118-96-7
RDX	121-82-4
HMX	2691-41-0
Tetryl	479-45-8
2,4-dinitrotoluene (2,4-DNT)	121-14-2
2,6-dinitrotoluene (2,6-DNT)	606-20-2
2-amino-4,6-dinitrotoluene (2a-DNT)	35572-78-2
4-amino-2,6-dinitrotoluene (4a-DNT)	19406-51-0
2,4-diamino-6-nitrotoluene (2,4-DANT)	6629-29-4
2,6-diamino-4-nitrotoluene (2,6-DANT)	59229-75-3
1,3,5-trinitrobenzene (1,3,5-TNB)	99-35-4
3,5-dinitroaniline (3,5-DNA)	618-87-1
EPA 8330 Mix A[a]	N/A
EPA 8330 Mix B[b]	N/A

[a] Contains: 2-amino-4,6-dinitrotoluene, 1,3-dinitrobenzene, 2,4-dinitrotoluene, HMX, nitrobenzene, RDX, 1,3,5-trinitrobenzene and 2,4,6-trinitrotoluene. Each residue is at a concentration of 0.1 mg/ml.
[b] Contains: 4-amino-2,6-dinitrotoluene, 2,6-dinitrotoluene, 2-nitrotoluene, 3-nitrotoluene, 4-nitrotoluene and tetryl. Each residue is at a concentration of 0.1 mg/ml.

2.2 Soil Samples

The three contaminated soil samples examined in this thesis were obtained from military training ranges at CFB Petawawa, and generously donated by Dr. Sylvie Brochu (Defence Scientist, Life Cycle of Munitions Group, Energetic Materials Section, Defence Research and Development Canada (DRDC), Valcartier, Québec).

The first sample, henceforth referred to as PET 1, was collected from an anti-tank firing position (either Bay 4 or 5) located at A Range (refer to Figure 2.1). The second sample, henceforth referred to as PET 2, was collected from the T1 anti-tank target area also located at A Range (refer to Figure 2.2). A Range is located within Impact Area A (refer to Figure 2.3). The final sample, henceforth referred to as PET 3, was collected from the Delta Tower firing point located within DFT Area 2 (refer to Figures 2.4 and 2.5).

Figure 2.1: Sampling location for PET 1, an anti-tank firing position (i.e., Bay) located at A Range [16].

Figure 2.2: Sampling location for PET 2, the T1 anti-tank target area (right) located at A Range [16].

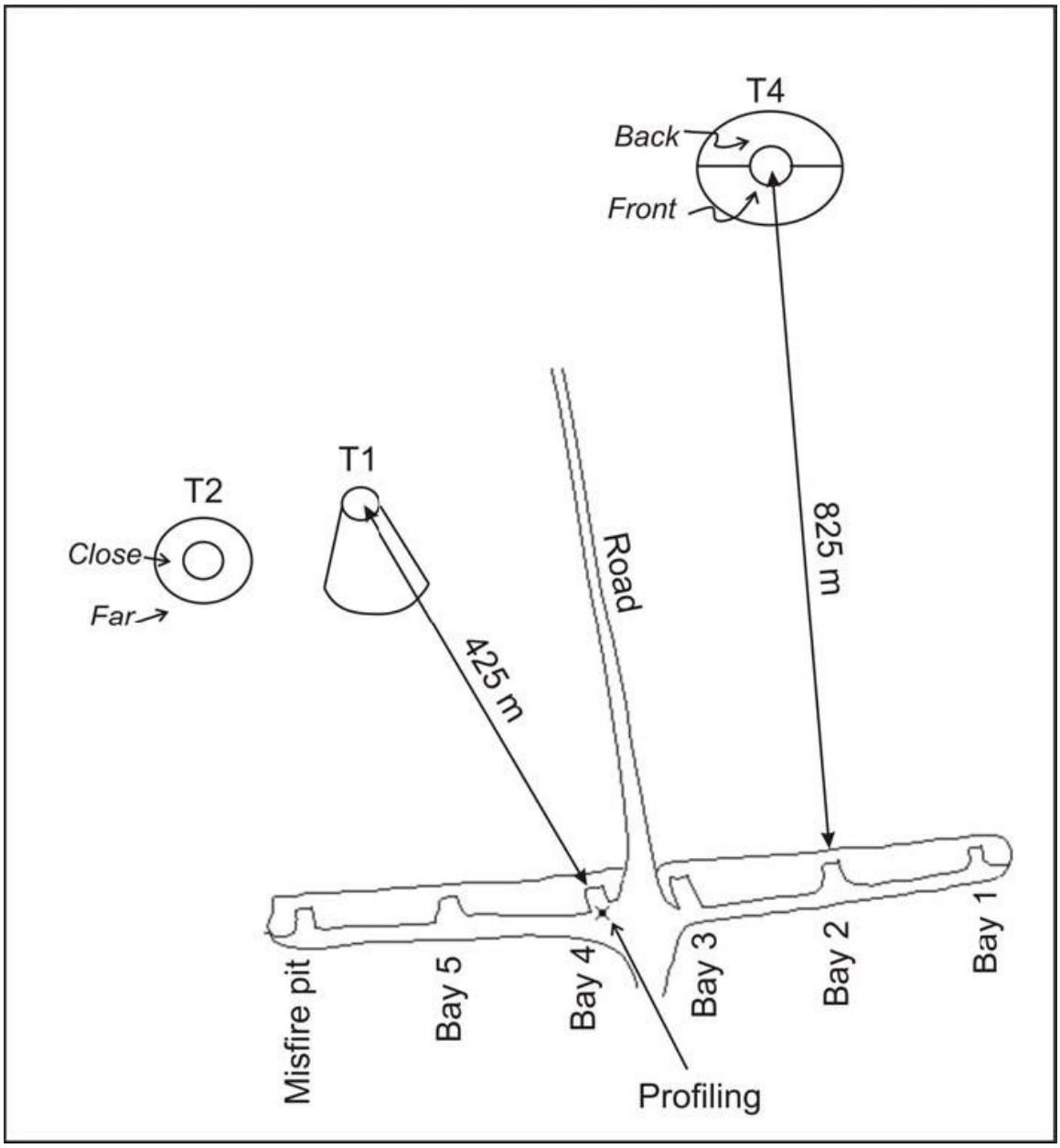


Figure 2.3: Schematic diagram of A Range [16].

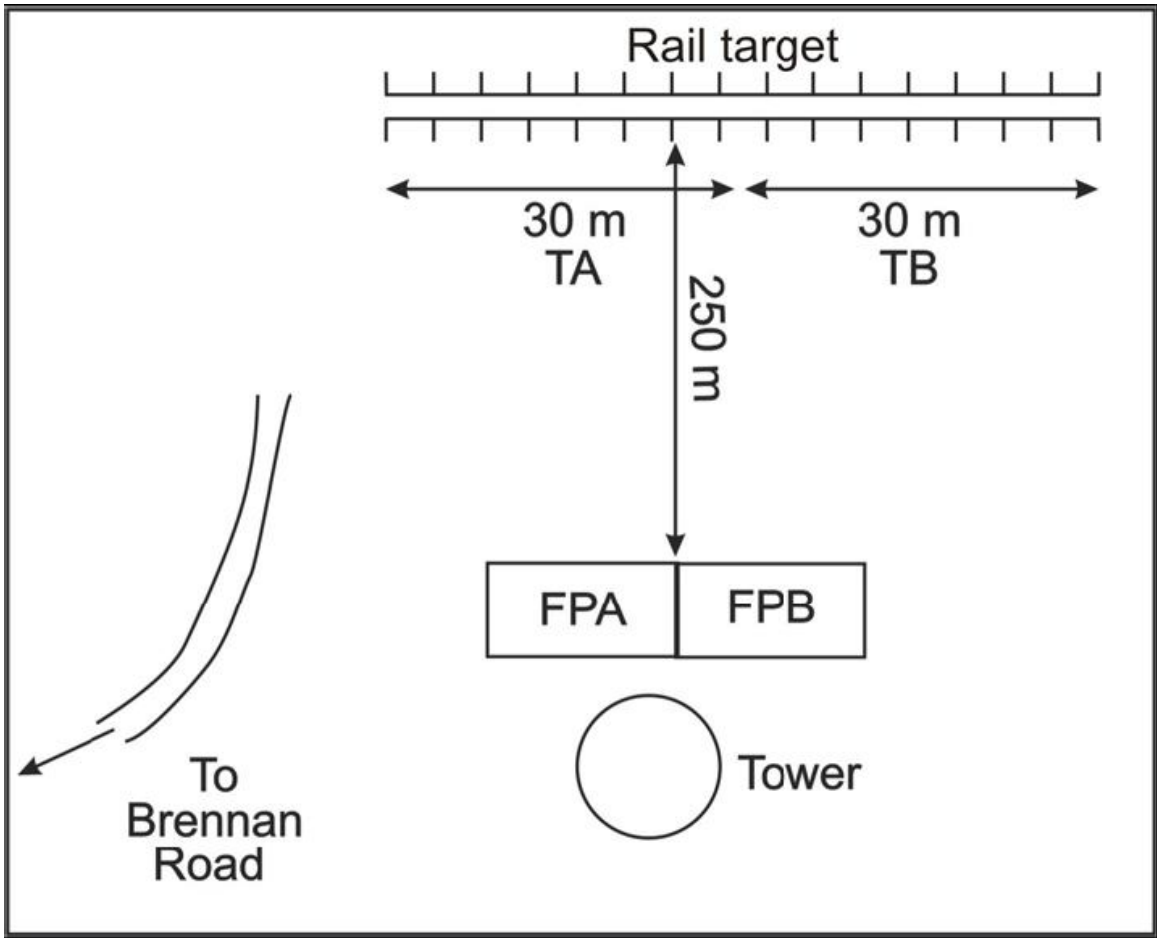


Figure 2.4: Schematic diagram of the Delta Tower firing point, located within Direct Fire Target Area 2; the sampling location for PET 3 [16].

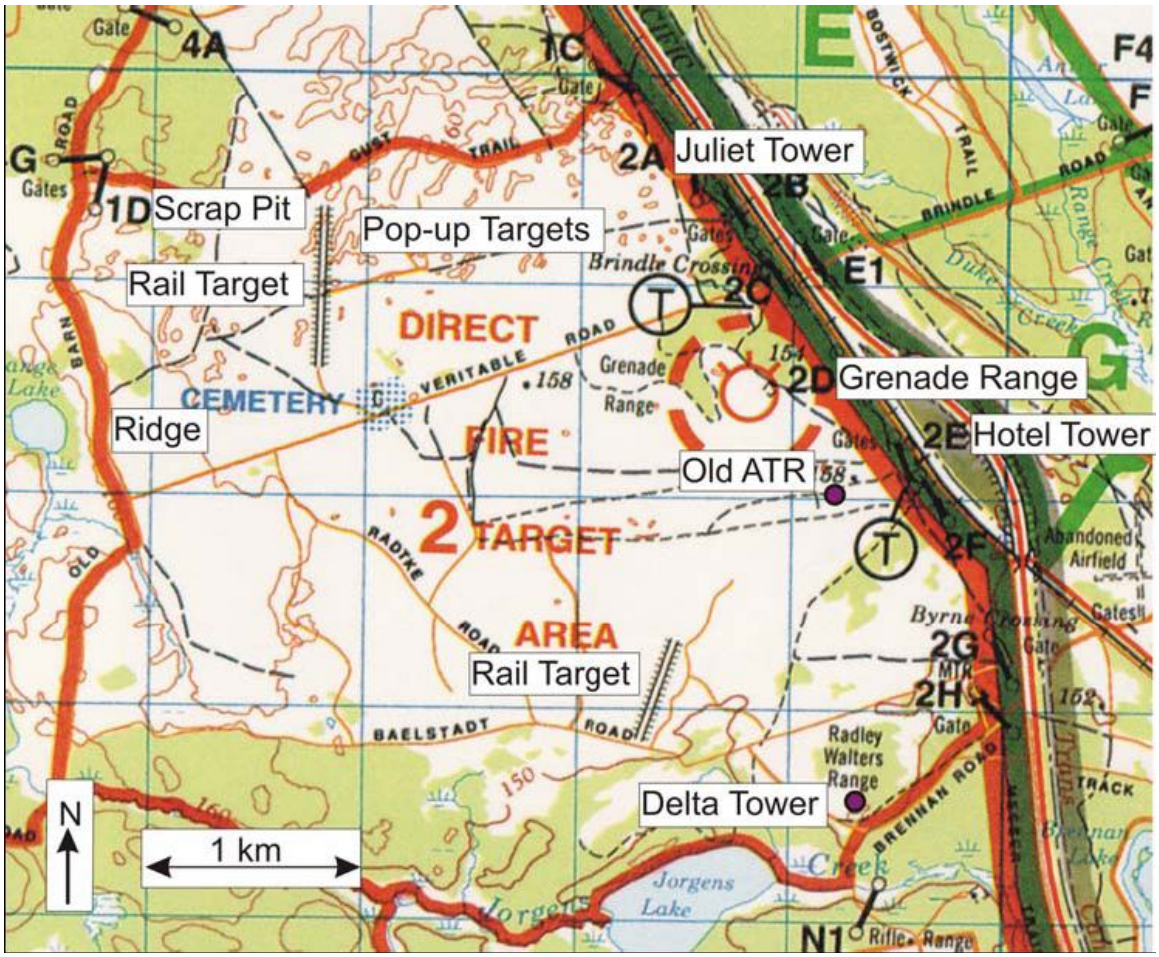


Figure 2.5: Map showing Direct Fire Target Area 2 [16].

Impact Area A is one of ten Impact/Training Areas located at CFB Petawawa. It is a live fire training range that covers an area of approximately 22 km^2. A Range (located within Impact Area A) covers an area of approximately 0.25 km^2. A-Range is used for two purposes, the one of concern being anti-armour weapon training. Since 1998, the Canadian Forces have compiled and maintained records of ammunition fired at their training ranges, including those fired at CFB Petawawa. These records indicate that approximately 5500 M72 Light Anti-tank Weapon (LAW) rockets were fired at A Range between 1998 and 2004 [16]. M72 LAW rockets contain an explosive composition known as M7 Double-Base Propellant, which is a combination of nitrocellulose (NC), nitroglycerin (NG) and potassium perchlorate in a ratio of approximately 55:36:8. Additionally, the warhead of a LAW rocket contains 0.3 kg of an explosive composition known as Octol, which is a combination of HMX and TNT in a ratio of approximately 70:30, with a tetryl or RDX booster [56]. Therefore, anti-armour weapon training ranges are expected to contain significant concentrations of HMX, TNT and RDX.

DFT Area 2 is the oldest and, until 1998, the most active training range at CFB Petawawa. It is a direct-fire range that covers an area of 12.4 km^2 and encompasses numerous distinct regions, facilitating a vast array of military training exercises. Delta Tower (located within DFT Area 2) covers an area of approximately 0.25 km^2. Until recently, Delta Tower was used primarily to fire mortars, but is no longer used for high-explosive munitions. Currently, it is used exclusively for small arms munitions training. While no records were kept specifically for the Delta Tower firing point, records do indicate that nearly 4 million rounds were fired within DFT Area 2 between 1998 and 2004. Approximately 96% were small arms bullets, 2% were 25-mm cartridges, and the

remainder was made up of approximately fifty different types of medium and large calibre munitions, including grenades, anti-tank rockets and missiles [16]. Due to its use as a small arms munitions training range, the Delta Tower firing point is certainly expected to contain gun propellant residues (e.g., NG and 2,4-DNT) [57].

Details regarding the characteristics of each soil collection site are provided in Table 2.2.

Table 2.2: Description of military training sites from which the soil samples examined in this thesis were collected [16, 57].

Site	Sample Name	Range Type (location of sample)	Explosive Residue(s) Expected (based on range type)	Soil Type	Depth of Sample (cm)
CFB Petawawa	PET 1	Direct-fire (anti-tank firing position)	NC, NG, HMX, TNT, tetryl and RDX	Sampled areas covered with sand and gravel.	≤2.5
	PET 2	Direct-fire (anti-tank target area)	NC, NG, HMX, TNT, tetryl and RDX	Sampled areas covered with gravel, sand, moss and grass.	
	PET 3	Direct-fire (firing point for small arms munitions; former firing point for mortars)	NG, 2,4-DNT	Sampled areas primarily covered with grass.	

2.2.1 Soil Collection

All soil samples were collected by DRDC Valcartier using a sampling strategy that combined twenty-five or more sub-samples from the designated area of interest. Samples were collected from the top 2.5 cm of the surface soil using acetone-rinsed, stainless steel scoops. Samples were placed in polyethylene bags that were cooled in the field, stored in the dark immediately upon collection, and subsequently refrigerated at the end of the sampling day [16].

A large sub-sample of PET 1 was later sealed inside a 10 US gallon opaque bucket. A small sub-sample of PET 2 and PET 3 were sealed inside an amber screw-top vial and a clear screw-top vial, respectively. These three sub-samples were subsequently shipped to the Mechanistic Studies Division Laboratory at Health Canada in Ottawa, Ontario. Once received, all samples were immediately stored in the dark at 4^{o}C until required for preparation and subsequent analyses.

2.2.2 Soil Preparation

Soil samples PET 2 and PET 3 were previously prepared by DRDC Valcartier before being shipped to the Mechanistic Studies Division Laboratory. These samples were air-dried, acetone-homogenized, passed through a 25-mesh (0.7 mm) sieve and split into sub-samples [16].

Soil sample PET 1 was prepared at the Mechanistic Studies Division Laboratory following EPA Method 8330B [55]. The sample was spread evenly in acetone-rinsed stainless steel trays and left to air-dry in a dark fume hood for 24 hours at room temperature. Absence of light is necessary to prevent photodegradation, particularly of TNT and its metabolites [58]. Once air-dried, large rocks and sticks were manually

removed from the sample, and the sample was passed through an acetone-rinsed, stainless steel, 10-mesh (2 mm) USA Standard Testing Sieve (VWR International, West Chester, PA). This initial sieving allowed all soil particles <2 mm to be included in the analysis, but removed any pebbles, small rocks or sticks [55]. The sample was then mechanically ground with a Polymix® Universal Mill M20 (Kinematica AG, Lucerne, Switzerland), and passed through a 200-mesh (0.075 mm) sieve. This step was performed to ensure homogeneity of the explosives-contaminated soils, as several studies have noted extremely high short-range heterogeneity. For example, Jenkins et al. (2005) [56] reported a concentration range for RDX of 0.78 – 24 mg/kg for five soil samples collected within a 10 m x 10 m area from a hand grenade range at Fort Wainwright, Alaska. Crockett et al. (1996) [59] reported a concentration range for explosive residues from below detection (<0.5 ppm) to >10 000 ppm for samples that were collected within several feet of each other. Several researchers (e.g., Walsh et al. 2002 [60]) have noted that reducing the size of the soil particles by mechanical grinding can greatly reduce the (sub)sampling error. Without grinding, the mean concentrations and corresponding relative standard deviations (RSDs) for twelve sub-samples were reported as 3.50 mg/kg (99%) for RDX, 1.72 mg/kg (143%) for TNT, and 0.69 mg/kg (61%) for HMX. After employing mechanical grinding, however, the mean concentrations and corresponding RSDs for twelve sub-samples from the same site were reported as 4.68 mg/kg (1.3%) for RDX, 1.98 mg/kg (2.6%) for TNT, and 1.15 mg/kg (1.44%) for HMX.

To further minimize the variability between sub-samples in this study, 10 g sub-samples were taken for extraction purposes, as recommended in EPA Method 8330B, instead of the 2 g sub-sample size suggested in the original EPA Method 8330 [55, 61].

2.3 Soil Extraction

2.3.1 Chemicals and Laboratory Equipment

100% ACN (EMD Chemicals Incorporated, Gibbstown, NJ) was used for extraction of all soil samples. The solvent was classified as OmniSolv® grade and is suitable for HPLC, spectrophotometry and gas chromatography.

Soil samples were extracted using Pressurized Liquid Extraction, also known as Accelerated Solvent Extraction (ASE). This study employed the ASE 200 Accelerated Solvent Extraction System (Dionex Corporation, Sunnyvale, CA). The use of ASE for the extraction of explosive residues differs from the sonication method in EPA Method 8330. A study by Dionex Corporation, however, indicates that the ASE method is equivalent or superior to sonication for extraction of explosive residues from soil [62].

2.3.2 Soil Extraction Protocol

Explosive residues were extracted from soil following a protocol recommended by Dionex Corporation [62]. A recovery study was conducted to determine the potential for recovery of these explosive compounds. Approximately 10 g of clean Ottawa Sand (Thermo Fisher Scientific Incorporated, Waltham, MA) was placed in a clean 11 ml stainless steel cell, and 10 µg of EPA 8330 Mixes A and B were spiked onto the sand and allowed to equilibrate for approximately 30 minutes. The recovery experiment was conducted in triplicate. For extraction of the military soil samples, ~10 g of collected soil was placed in a clean 11 ml stainless steel extraction cell. One of each military soil was prepared for extraction analysis. A ~10 g sample of clean Ottawa Sand was used as a "method blank" for both the recovery study and the military soil extraction runs. Extraction conditions are summarized in Table 2.3.

Table 2.3: Conditions for ASE extraction of explosive residues from spiked sand (i.e., recovery study samples) and military soil samples [62].

Condition	Description
Preheat time	0 minutes
Oven heat time	5 minutes
Oven temperature	100^{o}C
Static cycles	2
Static time	5 minutes
System Pressure	10.3 MPa (1500 psi)
Solvent	100% ACN
Flush volume	60%
Purge time	200 seconds

Two modifications were made from the aforementioned Dionex Corporation protocol. First, two static cycles were used instead of one to ensure that all explosive residues were thoroughly flushed from the soil matrix. Second, Dionex Corporation suggests the use of methanol or acetone for the extraction of explosive residues from soil matrices. However, this study employed ACN as the extraction solvent. Our initial analyses (results not shown), and information available in the literature, indicate that acetone or methanol are not ideal for the extraction of explosive residues from soil. With regards to methanol, the solubility of HMX and RDX was shown to be considerably lower in methanol as compared with ACN. Onuska et al. (2001) [63] reported that the solubilities of these two explosives are over twenty times greater in ACN compared to methanol. With regards to acetone, although the solubility of explosive residues in acetone is relatively high, acetone absorbs at 254 nm, a wavelength employed for analysis of explosives via HPLC with UV detection. While it is possible to conduct a solvent-exchange to circumvent this problem, it is not recommended because this may cause a loss of analyte and contribute to analytical uncertainty [18]. Finally, ACN is the solvent recommended for the sonication extraction in EPA Method 8330 [61].

2.4 Chemical Analysis

2.4.1 Chemicals and Laboratory Equipment

Methanol was OmniSolv® grade (EMD Chemicals). Laboratory-grade water was prepared using the Milli-Q Ultrapure Water Purification System (Millipore Corporation, Billerica, MA). Anhydrous calcium chloride was obtained from Sigma-Aldrich Corporation (St. Louis, MO).

Soil extracts were chemically analysed using HPLC/UV. The Alliance® HPLC System (Waters Corporation, Milford, MA), was employed with an autosampler to inject each sample into an e2695 Separations Module. The sample flow rate was maintained using the Waters 515 HPLC pump. Compounds of interest were detected using the 2475 Multi-Wavelength Fluorescence Detector.

Although Gas Chromatography coupled with an Electron Capture Detector (GC-ECD) is also used to analyse explosives-contaminated soil extracts, and is known to have detection limits that are 2-3 magnitudes of order lower than those for HPLC/UV (refer to Table 2.4), there is one notable problem. GC requires relatively high temperatures to vaporize compounds during the analysis. Therefore, all GC methods must contend with the thermal instability of several explosive compounds. GC-ECD was shown to be successful in analysing nitroaromatics, but caused thermal degradation of nitramines, particularly RDX, HMX and tetryl, which have lower boiling points [18]. HPLC methods avoid this issue because HPLC/UV is conducted at 30^{o}C.

Table 2.4: Detection limits for explosive compounds in soil (mg/kg) [10].

Compound	HPLC	GC-ECD
TNT	0.3	0.0013
RDX	1	0.0034
HMX	1	0.025
Tetryl	0.7	0.020
2,4-DNT	Not provided	Not provided
2,6-DNT	Not provided	Not provided
2a-DNT	0.3	0.002
4a-DNT	0.3	0.0015
2,4-DANT	0.3	0.00068
2,6-DANT	0.3	0.00069
1,3,5-TNB	0.3	0.0016
3,5-DNA	Not provided	Not provided

2.4.2 Calibration Standards

New calibration standards were prepared prior to each experiment. EPA 8330 Mixes A and B were combined and subsequently diluted in a 50:50 methanol:aqueous calcium chloride solution (5 g/L) to yield concentrations of 50 µg/ml, 5 µg/ml, 0.5 µg/ml and 0.1 µg/ml. Methanol was used as a negative control for the analysis. These calibration standards, along with the control, were used for two purposes. First, to determine the elution time of each explosive compound, and employ these values to subsequently identify explosive residues present in the soil samples. Second, to establish a standard concentration-detector response curve for each explosive compound. The slope and intercept values obtained from these curves were subsequently utilized to determine both the detection limit and the concentration of each explosive compound present in the soil samples.

2.4.3 HPLC/UV Protocol

Following extraction, a ~1 g aliquot of each recovery or military soil extract was removed for HPLC/UV analysis. For the recovery experiment, each aliquot was brought to a volume of 2 mls in 50:50 methanol:aqueous calcium chloride. The Ottawa Sand method blank was treated in the same manner. For the military soil experiment, PET 1 and PET 2 extract aliquots were brought to a volume of 10 mls in 50:50 methanol:aqueous calcium chloride. The PET 3 extract aliquot was brought to a volume of 1 ml.

The remainder of the military soil extracts were concentrated to a volume of 10 mls under ultra-pure nitrogen gas, using the TurboVap® II concentration system (Caliper

Life Sciences, Hopkinton, MA). This portion of the extract was used for mutagenicity analysis with the *Salmonella* mutagenicity and Muta™Mouse assays.

Explosive residues from both the recovery and military soil extracts were analysed following a protocol provided by Dionex Corporation [64] using Acclaim® Explosives (E1 and E2) columns. These columns are reversed-phase columns that are designed specifically for the separation of the fourteen explosive compounds listed in EPA Method 8330. The E1 column replaces the C18 reversed-phase column recommended in EPA Method 8330. The E2 column acts as a confirmatory column by providing complimentary selectivity for the same fourteen compounds. These columns are silica-based (ultrapure) and have a particle size of 5 µm. The pore volume is 0.9 ml/g and the average pore diameter is 120Å.

The mobile phase for the E1 column was 43:57 methanol:water, and 48:52 methanol:water for the E2 column. Each sample was allowed to run for 42 minutes, followed by cleaning of the columns with methanol from 42-50 minutes, and re-equilibration of the columns (43:57 methanol:water for the E1 column and 48:52 methanol:water for the E2 column) from 50-65 minutes. The flow rate was maintained at 1.23 ml/min, column temperature was maintained at 30^{o}C, the injection volume was 10 µl, and analysis occurred at a wavelength of 254 nm.

2.4.4 Chromatogram Analysis

Chromatograms were visually inspected prior to any quantitative analysis to ensure proper peak formation. For the recovery and military soil samples, the recovered quantity of each explosive residue was determined according to the following two-step calculation:

1) $$x = \frac{(y - b)}{m}$$

Where: x = concentration of the explosive compound of interest.
y = area of the peak representing the explosive compound of interest.
b = intercept of the calibration curve for the explosive compound of interest.
m = slope of the calibration curve for the explosive compound of interest.

2) $$e = x \text{ x dilution volume x } \frac{\text{weight of total ASE extract}}{\text{weight of aliquot taken for HPLC analysis}}$$

Where: e = recovered quantity of the explosive residue spiked onto clean sand or present in the soil sample.
x = concentration of the explosive compound of interest (obtained in Step 1).

An explosive residue was termed "identifiable" if the peak was three times the background noise, and "quantifiable" if the peak was ten times the background noise.

2.5 Ames/*Salmonella* Reverse Mutation Assay

Protocols employed for the preparation of solutions, media and *Salmonella* cultures, as well as *Salmonella* strain checks, adhered to the Standard Operating Procedures of the Environmental Carcinogenesis Division of the U.S. EPA (Research Triangle Park, NC). These protocols were adapted from Mortelmans and Zeiger (2000) and Maron and Ames (1983) [34, 35]. All *Salmonella* mutagenicity assay-related work was conducted under sterile conditions in a laminar flow hood (Model No. BM6-2B-49, Microzone Corporation, Nepean, ON). Unless otherwise stated, all solutions and media, as well as their components, were autoclaved for sterilization at 121^{o}C for 20 minutes using an Amsco® Century® Small Steam Sterilizer (Steris Corporation, Mentor, OH), and

all incubations were carried out at 37^{o}C in a GCA/Precision Scientific incubator (Model No. 6M).

2.5.1 Solutions and Media

Table 2.5 provides a detailed description of the *Salmonella* mutagenicity assay solution/media components. Preparation of the solutions and media are described in the text below. Milli-Q Ultrapure water was used to prepare all solutions and media, as well as their components.

Table 2.5: *Salmonella* mutagenicity assay solution/media components.

Component	Reagents	Amount	Concentration	Source
30% Dextrose	D-Glucose	300 g	1.67 M	Sigma-Aldrich Canada, Limited (Oakville, ON)
	dH_2O	1 L	-	-
50X Vogel-Bonner Medium E (VBME)	Magnesium sulfate	10 g	0.04 M	Sigma-Aldrich Canada
	Citric acid monohydrate	100 g	0.48 M	
	Potassium phosphate dibasic, anhydrous	500 g	2.87 M	
	Sodium ammonium phosphate	175 g	0.84 M	
	dH_2O	topped up to 1 L	-	-
0.5 mM Histidine/Biotin	L-Histidine-HCl	52.5 mg	0.25 mM	Sigma-Aldrich Canada
	d-Biotin	97.6 mg	0.4 mM	
	dH_2O	1 L	-	-
0.2 M Phosphate Buffer (pH 7.4)	Sodium dihydrogen phosphate	3.31 g	0.02 M	Sigma-Aldrich Canada
	Disodium hydrogen phosphate	25 g	0.18 M	
	dH_2O	1 L	-	-
Microsomal Salt Solution	Magnesium chloride hexahydrate	81.4 g	0.4 M	Sigma-Aldrich Canada
	Potassium chloride	123 g	1.65 M	
	dH_2O	topped up to 1 L	-	-

Stock solutions of 30% dextrose and microsomal salt solution were prepared according to Table 2.5, autoclaved, and stored at 4^{o}C until required. Stock solutions of 50X VBME were prepared according to Table 2.5, filtered using a 0.45 µm Nalgene MF75 Series sterile disposable bottle-top filter (Thermo Fisher Scientific), autoclaved, and stored at room temperature until required. Stock solutions of 0.5 mM histidine/biotin were prepared according to Table 2.5, filtered for sterilization using a 0.2 µm Nalgene MF75 Series disposable bottle-top filter, and stored at 4^{o}C until required. Stock solutions of 0.2 M phosphate buffer were prepared according to Table 2.5, autoclaved, and stored at room temperature until required.

Glucose Minimal Agar Plates

Glucose minimal agar media was used as the bottom agar for the *Salmonella* mutagenicity assay. Glucose minimal agar plates were prepared using a MediaClave™ (Integra Biosciences AG, Chur, Switzerland). For each batch of plates, 5310 mls of water and 90 g of Difco granulated agar (1.5% w/v in final solution) (Thermo Fisher Scientific) were autoclaved at 121^{o}C for 25 minutes. Following sterilization, the contents were cooled to 50^{o}C and 400 mls of 30% dextrose (6.8% v/v), 120 mls of 50X VBME (2% v/v) and 60 mls of 0.5 mM histidine/biotin solution (1% v/v) were added. 26 mls of media was then dispensed onto sterile 100 mm Petri dishes (VWR International) using a Tecnomat Line for automatic plate dispensing (Integra Biosciences AG). Plates were sterilized using UV light, allowed to solidify on a level surface, and subsequently stored at room temperature until required.

Top Agar Tubes

Top agar consisted of 6 g of Difco granulated agar (0.6% w/v) and 5 g of sodium chloride (0.09 M) (Sigma-Aldrich Canada) per litre of water. 2.2 mls of top agar was dispensed into 13 mm x 100 mm disposable glass culture tubes (Thermo Fisher Scientific) using a Socorex Calibrex 520 bottle-top dispenser (VWR International). Tubes were capped, and stored at room temperature until required. Prior to each experiment, top agar tubes were autoclaved and maintained at approximately 50^{o}C in a DB-3 Dri-Block® heater (Bibby Scientific Limited, Staffordshire, UK).

Nutrient Agar Plates

Nutrient agar consisted of 25 g of Oxoid Nutrient Broth No. 2 (2.5% w/v) (Oxoid Limited, Basingstoke, UK) and 15 g of Difco granulated agar (1.5% w/v) per litre of water. This solution was autoclaved and, following sterilization, the contents were cooled slightly. 25 mls of media was then dispensed onto sterile 100 mm Petri dishes. Plates were allowed to solidify on a level surface and subsequently stored at 4^{o}C until required.

Nutrient Broth

Nutrient broth consisted of 25 g of Oxoid Nutrient Broth No. 2 (2.5% w/v) per litre of water. This solution was autoclaved and subsequently stored at 4^{o}C until required.

S9 Metabolic Activation Mixture

An exogenous metabolic activation system was used in an effort to simulate mammalian hepatic metabolism. S9 metabolic activation mixture was prepared without the addition of rat liver S9, which was added fresh on the day of experimentation. Each 1 L batch consisted of 430 mls of sterile dH_2O, 500 mls of 0.2 M phosphate buffer (50% v/v), 20 mls of microsomal salt solution (2% v/v), 1410 mg of D-Glucose 6-Phosphate Sodium Salt (G6P) (5 mM) (Sigma-Aldrich Canada) and 3060 mg of Nicotinamide

Adenine Dinucleotide Phosphate Disodium Salt (NADP) (3.89 mM) (Roche Applied Science, Laval, QC). 38 ml aliquots of the mixture were dispensed into 50 ml sterile polypropylene centrifuge tubes (DiaMed Lab Supplies Incorporated, Mississauga, ON), and stored at -30^{o}C until required. Prior to each experiment, a 38 ml aliquot was thawed and 2 mls of Aroclor-1254 induced rat liver S9 (Moltox Incorporated, Boone, NC) was added to provide a 5% v/v concentration. 1 ml of S9 contains microsomes from ~250 mg of wet liver and has a CYP450 protein content of ~40 mg/ml [35]. The S9 metabolic activation mixture was maintained on ice for the duration of the experiment.

2.5.2 *Salmonella typhimurium* Strains

Four *S. typhimurium* strains were used to detect the mutagenic activity of the individual explosive compounds and military soil extracts. TA98, TA100, YG1041 and YG1042 were generously donated by Dr. David DeMarini (Environmental Carcinogenesis Division, U.S. EPA). The characteristics of each tester strain are summarized in Table 2.6.

Table 2.6: Properties of *S. typhimurium* strains used in the *Salmonella* mutagenicity assay.

***S. typhimurium* Strain**	**Mutation**	**Target DNA Sequence**	**Plasmid**	**Additional Mutations**		**Use**	**Reference**
				LPS	**Repair**		
TA98	*hisD3052*	C-G dinucleotide repeat	pKM101 – *mucA/B, Amp*	rfa	uvrB	Detects frameshift mutagens.	34-36
YG1041	*hisD3052*	C-G dinucleotide repeat	pKM101 – *mucA/B, Amp* pYG233 – *cnr, OAT, Kan*	rfa	uvrB	- Derived from TA98. - Classical nitroreductase / *O*-acetyltransferase-overproducing strain. - Sensitive to nitroaromatics and aromatic amines.	36, 37
TA100	*hisG46*	GGG	pKM101 – *mucA/B, Amp*	rfa	uvrB	Detects base-pair mutagens.	34-36
YG1042	*hisG46*	GGG	pKM101 – *mucA/B, Amp* pYG233 – *cnr, OAT, Kan*	rfa	uvrB	- Derived from TA100. - Classical nitroreductase / *O*-acetyltransferase-overproducing strain. - Sensitive to nitroaromatics and aromatic amines.	36, 37

2.5.3 Preparation of *Salmonella* Master Stock Plates, Overnight Cultures and Frozen Permanent Cultures

Master stock plates were prepared by streaking a loopful of the bacterial culture from a bacterial agar stab onto a nutrient agar plate in order to obtain isolated colonies. These plates were inverted and incubated for 72 hours. TA98 and TA100 master stock plates were supplemented with 25 μg/ml of ampicillin. YG1041 and YG1042 master stock plates were supplemented with 25 μg/ml of both ampicillin and kanamycin. New master stock plates were prepared approximately every 4 weeks and stored at 4^{o}C until required.

Overnight cultures were prepared by inoculating 20 mls of nutrient broth with one isolated colony from the master stock plate in a 50 ml Erlenmeyer flask. In order to maintain the selective pressure, TA98 and TA100 overnight cultures were supplemented with 25 μg/ml of ampicillin, and YG1041 and YG1042 overnight cultures were supplemented with 25 μg/ml of both ampicillin and kanamycin. The cultures were incubated in a Max^{Q} Mini 4450 Shaker (Thermo Fisher Scientific) at 37^{o}C and 200 rpm, for 16 hours or until an optical density of ~1.0-1.2 at 660 nm was achieved.

Frozen permanent cultures were prepared by adding 90 μl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich Canada) to 1 ml of the overnight culture in a 1.5 ml Eppendorf tube. Frozen permanent cultures were stored at -80^{o}C until required.

2.5.4 *Salmonella* Strain Checks

Strain checks were performed prior to the commencement of experimentation to ensure the utility of the *Salmonella* tester strains.

Histidine dependence was confirmed by adding 100 μl of the bacterial culture to a glucose minimal agar plate supplemented with 0.4 mM d-biotin (no histidine), and

additionally by adding 100 µl of the bacterial culture to a glucose minimal agar plate supplemented with 0.5 mM d-biotin and 0.024 M L-histidine. Plates were inverted and incubated for 72 hours. No growth was observed for any strain on the biotin-only supplemented plate, while confluent growth was observed on the histidine/biotin supplemented plate.

Deletion of the *uvrB* gene was confirmed by demonstrating that the strains maintained sensitivity to UV light. This was accomplished by adding 50 µl of the bacterial culture to a nutrient agar plate and subsequently exposing half of the plate to a 15-W germicidal UV light, for 8 seconds, at a distance of 33 cm. Plates were inverted and incubated for 24 hours. No growth was observed for any strain on the irradiated side of the plate, while confluent growth was observed on the non-irradiated side.

The *rfa* mutation was confirmed by demonstrating that the strains maintained sensitivity to crystal violet. This was accomplished by adding 100 µl of the bacterial culture to a nutrient agar plate and subsequently placing a disc soaked in 0.1% v/v crystal violet (Sigma-Aldrich Canada) into the middle of the plate. Plates were inverted and incubated for 24 hours. A clear zone of inhibition around the crystal violet disc was observed for all strains.

The presence of the pKM101 plasmid was confirmed by demonstrating that the bacterial strains maintained resistance to ampicillin. This was accomplished by streaking a loopful of the bacterial culture onto a nutrient agar plate supplemented with 25 µg/ml of ampicillin (Sigma-Aldrich Canada). Plates were inverted and incubated for 24 hours. Confluent growth was observed for all strains. The presence of the pYG233 plasmid in strains YG1041 and YG1042 was confirmed by demonstrating that these strains

maintained resistance to kanamycin. This was accomplished by streaking a loopful of the bacterial culture onto a glucose minimal agar plate supplemented with 25 μg/ml of kanamycin (Sigma-Aldrich Canada). Plates were inverted and incubated for 24 hours. Confluent growth was observed for both strains.

2.5.5 *Salmonella* Mutagenicity Assay Protocol

The standard plate incorporation version of the *Salmonella* mutagenicity assay, as described in Mortelmans and Zeiger (2000) [34], was used to determine the mutagenic activity of the individual explosive compounds and military soil extracts. A basic description of this protocol is provided in Figure 2.6.

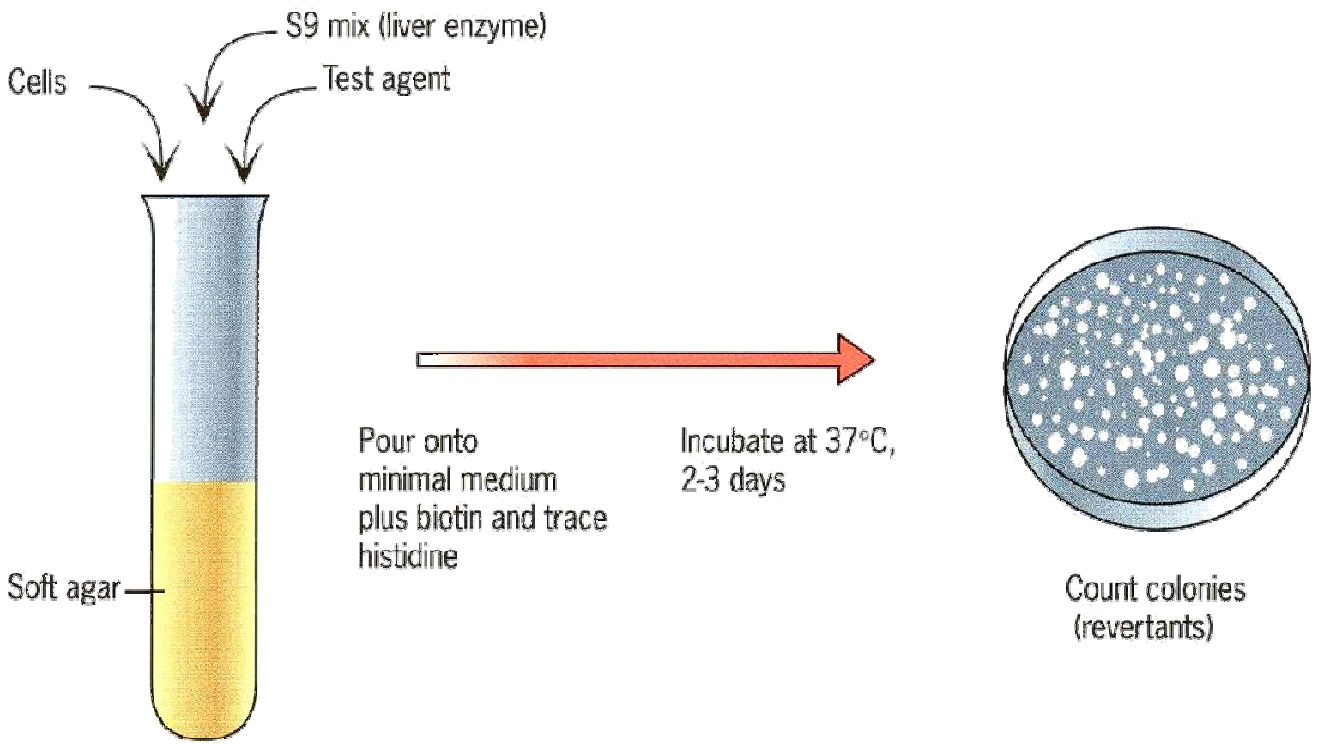


Figure 2.6: Basic schematic diagram of the *Salmonella* mutagenicity assay (Möller 2000, reproduced with permission) [65].

Overnight cultures of all four *Salmonella* strains were prepared as described above and placed on ice for the duration of the experiment. Top agar tubes were prepared as described above, and used to contain all assay ingredients. 100 µl of overnight culture, the appropriate volume of test article (not exceeding 100 µl), and 500 µl of S9 metabolic activation mixture (where required), were added to the top agar tube. The solution was vortexed briefly to ensure homogeneity, distributed evenly onto a glucose minimal agar plate and allowed to set on a level surface. Once solidified, the plates were inverted and incubated for 72 hours. Revertant colonies on each plate were scored using a ProtoCOL SR automated colony counter (Synbiosis Limited, Frederick, MD). Each plate was counted twice, the second count occurring after the plate was rotated 180^{o}, and the mean number of revertant colonies per plate was calculated.

Each concentration of the test substance, as well as each positive and negative control, was tested in triplicate using all four *Salmonella* strains, with and without S9. All explosive compounds were tested on three separate occasions, using a minimum of six concentrations up to 100 µg compound/plate. All soil extracts were tested once, using ten concentrations up to 100 mg dry soil equivalents/plate.

Positive and negative controls were tested for each experiment to ensure that the appropriate number of spontaneous and chemically-induced revertant colonies was produced for each strain and S9 combination (i.e., that the test system was functioning properly). The positive controls and corresponding concentrations used for each strain, with and without S9, are listed in Table 2.7.

Table 2.7: Positive controls and corresponding concentrations used in the *Salmonella* mutagenicity assay.

Strain	Exogenous S9 Metabolic Activation	Positive Control	Concentration (µg/plate)	Source
TA98	Yes	2-aminoanthracene	0.5	Premeasured aliquots purchased from Moltox
	No	2-nitrofluorene	3.5	
TA100	Yes	2-aminoanthracene	0.5	
	No	Methyl methane sulfonate	0.5*	
YG1041	Yes	2-aminoanthracene	0.1	
	No	2-nitrofluorene	1	
YG1042	Yes	2-aminoanthracene	0.05	
	No	2-nitrofluorene	0.1	

* Concentration in µl/plate

The *Salmonella* mutagenicity assay is generally conducted using DMSO as a carrier solvent for the test substance. The explosive compounds and soil extracts tested in these experiments, however, were prepared using ACN as the carrier solvent. Thus, 100 µl of DMSO and 100 µl of ACN were both tested as negative controls. DMSO was tested to ensure that the appropriate number of spontaneous revertant colonies was produced for each strain and S9 combination. ACN was tested to ensure that the number of spontaneous revertant colonies produced was comparable to that of DMSO (i.e., no mutagenic effect of ACN), and thus that the number of spontaneous revertant colonies was appropriate for each strain and S9 combination. A study conducted by Maron *et al.* (1981) [66], found that ACN produced similar numbers of revertant colonies to DMSO when tested up to 200 µl/plate, and that ACN is toxic at 500 µl/plate. Thus, due to the fact that the volume of test substance in ACN was never to exceed 100 µl, it was expected that ACN could be used as a solvent without any detrimental consequences.

2.5.6 Statistical Analysis

For each test substance, strain and S9 combination, a concentration-response curve was constructed by plotting the number of revertant colonies per plate against concentration. SAS Version 9.2 for Windows (SAS Institute, Cary, NC) was used for the analysis of all *Salmonella* mutagenicity results. Responses were considered positive if a concentration-dependent increase in the number of revertant colonies was observed, and a minimum two-fold increase over the number of background revertant colonies was maintained for two consecutive concentrations. When a concentration-dependent increase in the number of revertant colonies was observed but a two-fold increase over background for two consecutive concentrations was not observed, the response was

considered to be marginally positive [67]. For each positive or marginally positive response, a mutagenic potency (revertants/μg compound or revertants/mg dry soil equivalents) was calculated. Mutagenic potency, defined as the slope of the linear portion of the concentration-response curve, was calculated using ordinary least squares regression analysis [67].

Furthermore, the predicted mutagenic potencies for each soil sample, strain and S9 combination, were determined, using an assumption of additivity, by calculating and summing together the relative contribution of each explosive compound to the total mutagenic activity of the soil. This was achieved using the following formula:

Predicted Mutagenic Potency =

$\sum_{i=1}^{n}$ (Observed mutagenic activity of the explosive compound x Explosive compound concentration in the soil) for explosives 1 through *n*.

2.6 FE1 Muta^{TM}Mouse *in vitro* Transgene Mutation Assay

The Muta^{TM}Mouse assay, as described in White et al. (2003) and Vijg and Douglas (1996) [46, 47], was used to expose FE1 cells to the individual explosive compounds and military soil extracts, rescue the transgenic *lacZ* from FE1 genomic DNA, and enumerate the frequency of mutations in exposed FE1 cells. A schematic description of the basic Muta^{TM}Mouse assay protocol using the P-Gal positive selection system is provided in Figure 2.7.

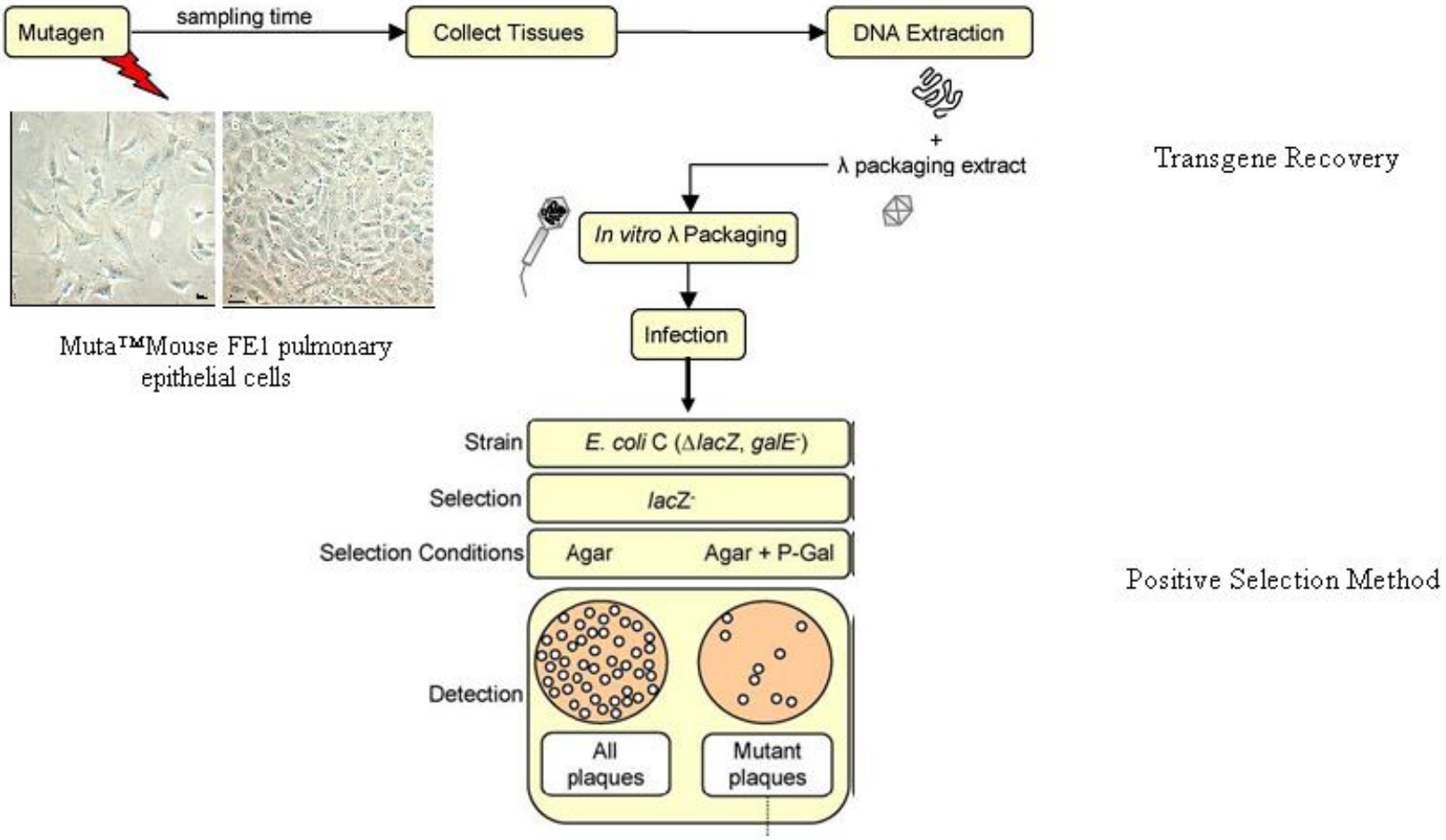


Figure 2.7: The Muta[TM]Mouse assay showing the P-Gal positive selection system for scoring *lacZ* mutations (adapted from Lambert et al. 2005) [68].

2.6.1 Flat Epithelial (FE1) Cell Line

The FE1 cell line is a stable epithelial cell line derived from lung tissue of the transgenic Muta^{TM}Mouse. The cells are contact inhibited, thereby forming a flat monolayer. The cells retain several epithelial and pulmonary characteristics that make them ideal for screening suspected mutagens *in vitro*. The FE1 cell line harbours the λgt10*lacZ* shuttle vector that contains the *lacZ* transgene mutation target [46]. The structure of the *lacZ* transgene in the λgt10*lacZ* shuttle vector is depicted in Figure 2.8.

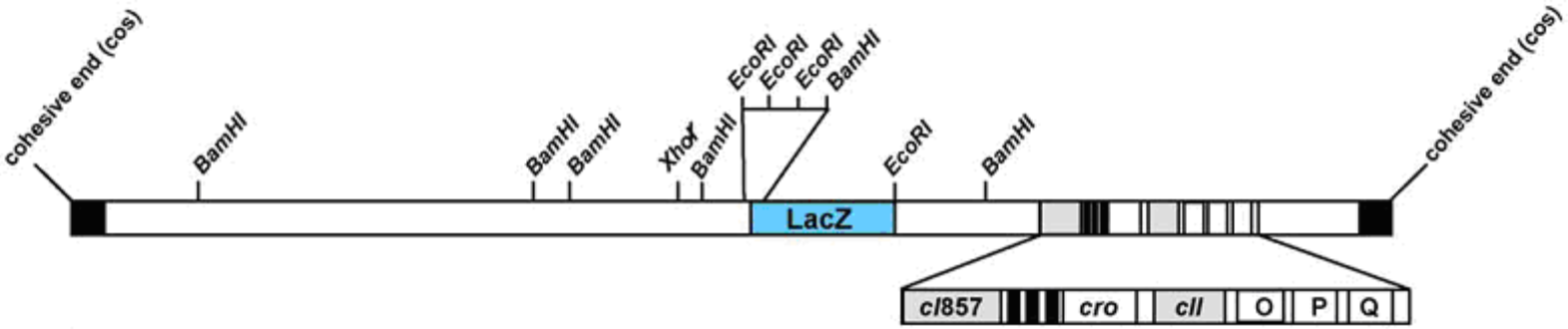


Figure 2.8: *λgt10lacZ* shuttle vector containing the *lacZ* transgene (adapted from Lambert et al. 2005) [68].

2.6.2 Muta^{TM}Mouse Assay Protocol

All Muta^{TM}Mouse assay-related work was conducted under sterile conditions in a laminar flow hood (Model No. BM6-2B, Canadian Cabinets Company Limited, Ottawa, ON; Model No. BK-2-4, Microzone Corporation, Ottawa, ON), including the preparation of all solutions and media. Unless otherwise stated, all incubations were carried out at 37^{o}C, 95% humidity and 5% CO_2, in a CO_2 Cell Culture Incubator with SafeCell^{TM} UV (Model No. MCO-20AIC, Sanyo Biomedical, Wood Dale, IL). Cell cultures and mutagen exposures were conducted in 100 mm non-pyrogenic, sterile, polystyrene dishes (Corning Life Sciences, Lowell, MA). Milli-Q Ultrapure water was used to prepare all solutions and media.

2.6.2.1 Culturing FE1 Cells

Approximately 300 000 FE1 cells were seeded onto a dish containing 10 mls of culture medium. Culture medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium:F-12 nutrient mixture (D-MEM/F-12) supplemented with 2% v/v fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 1 ng/ml murine epidermal growth factor (EGF). D-MEM/F-12, FBS, L-glutamine, penicillin G and streptomycin sulfate were Gibco®-brand and were obtained from Invitrogen Canada Incorporated (Burlington, ON). EGF was obtained from Roche Applied Science. The cells were incubated for 3-4 days, which allowed them to grow to ~100% confluence (~2-5 million cells). The medium was discarded and the cells were washed with Gibco®-brand Dulbecco's phosphate-buffered saline (D-PBS). 1 ml of Gibco®-brand 0.25% Trypsin (1X) was added to the cells, gently swirled around the dish to ensure contact with all cells, and immediately discarded. The cells were incubated for

5 minutes to allow detachment, and resuspended in 10 mls of culture medium. Cell concentration was determined using a Coulter Particle Counter (Model No. Z1 D, Beckman Coulter, Incorporated, Brea, CA).

2.6.2.2 FE1 Cell Exposures

Approximately 300 000 cultured FE1 cells were seeded onto a dish containing 10 mls of culture medium. The cells were incubated overnight, the medium discarded, and replaced with 5 mls of treatment medium. Treatment medium consisted of the aforementioned culture medium (without FBS supplementation), the test article, and an S9 metabolic activation cofactor mixture (where required). Tables 2.8 and 2.9 list the concentrations tested for the explosive compounds and soil extracts, respectively. For exposures requiring an exogenous metabolic activation system, an S9 cofactor mixture without Aroclor-1254 induced rat liver S9 was prepared fresh on the day of experimentation, maintained on ice for the duration of the experiment, and finally added to the treatment medium. The mixture consisted of 0.5 mls of 20 mM HEPES sodium salt solution (pH 7.2) (Sigma-Aldrich Canada), 25.2 µl of 0.5 M magnesium chloride hexahydrate solution, 25.1 µl of 3.3 M potassium chloride solution, 252 µl of 50 mM G6P solution and 250 µl of 40 mM NADP solution. This provided final concentrations of 1 mM HEPES, 1.26 mM magnesium chloride, 8.30 mM potassium chloride, 1.26 mM G6P and 1 mM NADP. Aroclor-1254 induced rat liver S9 was added directly to the culture dish to provide a 0.5% v/v final concentration. The cells were incubated for 6 hours, the treatment medium discarded, and the cells washed with D-PBS. 10 mls of culture medium (with FBS supplementation) was then added and the cells incubated for 72 hours to allow mutation fixation.

Table 2.8: Concentrations of explosive compounds tested in the Muta^{TM}Mouse assay.

Explosive Compound	Concentrations Tested (µg compound/ml)
TNT	0.1, 1, 10, 20, 40
RDX	0.1, 1, 10, 20, 40, 250, 500, 1000
HMX	0.1, 1, 10, 20, 40, 150, 300, 600
Tetryl	0.1, 1, 2, 3, 10
2,4-DNT	0.1, 1, 10
2,6-DNT	0.1, 1, 10
2a-DNT	0.1, 1, 10
4a-DNT	0.1, 1, 10
2,4-DANT	0.1, 1, 10
2,6-DANT	0.1, 1, 10
1,3,5-TNB	0.1, 1, 10
3,5-DNA	0.1, 1, 10

Table 2.9: Concentrations of soil extracts tested in the Muta^{TM}Mouse assay.

Soil Sample	Concentrations Tested (mg dry soil equivalents/ml)
PET 1	0.2114, 2.114, 21.14
PET 2	0.1673, 1.673, 16.73
PET 3	0.194, 1.94, 19.4

Each concentration of the test substance, as well as each positive and negative control, was tested in duplicate, with and without S9. Positive and negative controls were tested in each experiment to ensure that the appropriate number of spontaneous and chemically-induced *lacZ* mutants was produced (i.e., that the test system was functioning properly). To ensure that the assay was working effectively both with and without S9, B[*a*]P (Sigma-Aldrich Canada) and PhIP (Toronto Research Chemicals Incorporated, Toronto, ON) were used as positive controls. B[*a*]P can be metabolized and activated by FE1 cells and was used as a positive control in the absence of S9. PhIP requires exogenous metabolic activation, presumably via CYP1A2, and was used as a positive control in the presence of S9. B[*a*]P was tested at a concentration of 0.1 μg/ml, and PhIP was tested at a concentration of 0.7 μg/ml.

The Muta^{TM}Mouse assay is generally conducted using DMSO as a carrier solvent for the test substance. The explosive compounds and soil extracts tested in these experiments, however, were prepared using ACN as the carrier solvent. Thus, 0.1 mls of ACN was tested as the negative control. Additionally, a vehicle control, containing no test substance, was run concurrently.

2.6.2.3 Cell Lysis, Extraction, Purification and Precipitation of Genomic DNA

Following the 72-hour mutation fixation period, the culture medium was discarded and replaced with 3.5 mls of lysis buffer. Lysis buffer consisted of 10 mM Tris (pH 7.6), 10 mM ethylenediaminetetraacetic acid (EDTA), 100 mM sodium chloride, 1 mg/ml Proteinase K, and 1% w/v sodium dodecyl sulfate (SDS). Tris was obtained from Caledon Laboratories Limited (Georgetown, ON), EDTA from Sigma-Aldrich Canada, and Proteinase K and SDS were Gibco®-brand from Invitrogen Canada. The cells were

incubated overnight. The cell digest was collected in a 15 ml sterile polypropylene centrifuge tube (DiaMed Lab Supplies Incorporated, Mississauga, ON), and the DNA extracted and purified in a two-step process. The first step employed a mixture of phenol/chloroform, and the second step used chloroform alone. This two-step process effectively removes proteins from the aqueous phase of the cell digest. Approximately 3 mls of 1:1 phenol:chloroform was added to the cell digest and the tube rotated end-on-end for 20 minutes, at 20 rpm, using a Caframo rotator (Model No. REAX 2, Caframo Limited, Wiarton, ON). The tube was subsequently centrifuged at 2500 rpm for 10 minutes at room temperature to separate the aqueous and organic phases (Sorvall® Legend® RT Centrifuge, Thermo Fisher Scientific). A portion of the top layer (~2.5 mls) was carefully removed with a glass pipette and transferred to a new 15 ml sterile polypropylene centrifuge tube. An additional 3 mls of chloroform was added to the cell digest, and the tube rotated and centrifuged as described. A portion of the top layer (~2 mls) was carefully removed as described, and transferred to a clean 15 ml sterile polypropylene centrifuge tube. Approximately 40 µl of 5 M sodium chloride was then added to each tube to bring the final concentration of sodium chloride to 200 mM.

The DNA was precipitated by adding two volumes (~4 mls) of ethanol to each tube and rolling the tubes horizontally to promote the formation of a DNA bead. The precipitated DNA was then spooled onto a sealed Pasteur pipette, washed in 70% ethanol, allowed to air-dry for a few minutes, and finally dissolved in 15-75 µl of Tris-EDTA (10 mM Tris (pH 7.6) and 1 mM EDTA). Extracted DNA was stored at 4^{o}C until required.

2.6.2.4 Packaging of Extracted DNA into λ Phage

λgt10*lacZ* transgenic segments were rescued from FE1 genomic DNA and packaged into λ phage using the Transpack™ Lambda Packaging System (Stratagene, La Jolla, CA). 4 µl of extracted FE1 DNA was placed in a 1.5 ml Eppendorf tube using a wide-bore pipette. 4.8 µl of Reagent 1 of the Transpack™ Lambda Packaging System was added to the Eppendorf tube, and homogenized via repeated pipetting. The tube was centrifuged for 5 seconds at 10 000 rpm in an Eppendorf 5417C Centrifuge (Eppendorf Canada, Mississauga, ON). The tube was then incubated at 30°C in a Lo-Boy Tissue Float Bath (Lab-Line Instruments, Mumbai, India) for 90 minutes. Following this initial incubation, 4.8 µl of Reagent 2 of the Transpack™ Lambda Packaging System was added to the Eppendorf tube. The resulting solution was treated as described, and following this final incubation, 500 µl of SM buffer (100 mM sodium chloride, 16 mM magnesium sulfate, 50 mM Tris (pH 7.6), 0.01% w/v gelatin) was added to the Eppendorf tube. Gelatin was obtained from J.T. Baker Chemical Company (Phillipsburg, NJ). The resulting solution was rotated end-on-end for 30 minutes, at 20 rpm, using a Caframo rotator (Model No. REAX 2). The solution was then vortexed and centrifuged briefly.

2.6.3 P-Gal Positive Selection System

The P-Gal positive selection system was used to score *lacZ* mutations in the rescued λgt10*lacZ* segments of FE1 genomic DNA. Refer to Figure 2.7 (above) for a basic illustration of the protocol. All P-Gal positive selection system-related work was conducted under sterile conditions in a laminar flow hood (Model No. BK-2-4; Model No. BM6-2B). Unless otherwise stated, all media were autoclaved for sterilization at 121°C for 20 minutes using an Amsco® Century® Small Steam Sterilizer, and all incubations

were carried out at 37°C in a Binder incubator (Model No. BD240-UL, Binder Incorporated, Bohemia, NY). Milli-Q Ultrapure water was used to prepare all media.

2.6.3.1 Media

Luria-Bertani (LB) broth consisted of 20 g of dehydrated LB mixture (2% w/v) (Thermo Fisher Scientific) per litre of water. LB broth was autoclaved and stored at 4°C until required.

Minimal agar media was used as bottom agar for the P-Gal assay, and consisted of 7.5 g of Difco granulated agar (0.75% w/v), 5 g of dehydrated LB mixture (0.5% w/v) and 6.4 g of sodium chloride (0.11 M) per litre of water. Minimal agar plates were prepared using a MediaClave™ and each batch was autoclaved at 121°C for 35 minutes. Following sterilization, the contents were cooled to 50°C and 8 mls of minimal agar was then dispensed onto sterile 100 mm Petri dishes. This was accomplished using a Tecnomat Line for automatic plate dispensing. Plates were sterilized using UV light, allowed to solidify on a level surface, and subsequently stored at room temperature until required.

Top agar media consisted of 7.5 g of Difco granulated agar (0.75% w/v), 5 g of dehydrated LB mixture (0.5% w/v), 6.4 g of sodium chloride (0.11 M) and 2.46 g of magnesium sulfate (10 mM) per litre of water. Top agar media was prepared on the day of the experiment, autoclaved, and maintained at approximately 50°C in a Tissue Mat Water Bath (Thermo Fisher Scientific).

2.6.3.2 Description of *Escherichia coli* Strain, Preparation of Overnight Cultures and Frozen Permanent Cultures

An *E. coli* C bacterial strain was used as the host bacterium for λ phage particles. The genotype of the strain employed is: Δ*lacZ*$^-$, *galE*$^-$, *recA*$^-$, pAA119 with *galT* and *galK* [46, 51].

Overnight cultures were prepared by inoculating 10 mls of LB broth supplemented with 0.2% w/v maltose (Thermo Fisher Scientific), 50 μg/ml of ampicillin and 20 μg/ml of kanamycin, with a scraping of frozen permanent culture in a 50 ml sterile polypropylene centrifuge tube. The cultures were incubated in a Max^Q Mini 4450 Shaker at 37^{o}C and 220 rpm, for 16 hours or until an optical density of ~0.1 at 600 nm was achieved. Frozen permanent cultures were prepared by adding 15% v/v of glycerol to the overnight culture. Frozen permanent cultures were stored at -80^{o}C until required.

2.6.3.3 P-Gal Positive Selection System Protocol

Briefly, an overnight culture of the *E. coli* strain was prepared as described above, and an aliquot of this culture was diluted in LB broth (1:100) and incubated in the Max^Q Mini 4450 Shaker at 37^{o}C and 220 rpm, for a further 3.5 hours or until an optical density of ~0.1 at 600 nm was achieved. This culture was centrifuged at 15^{o}C and 2400 rpm for 10 minutes using a Sorvall® RC5CPlus centrifuge (Mandel Scientific Company Incorporated, Guelph, ON). The bacterial pellet was then diluted approximately 100-fold in cold LB broth supplemented with 10 mM magnesium sulfate. The culture was placed on ice for the duration of the experiment.

For each concentration of test substance, as well as each positive and negative control, 2 mls of *E. coli* culture was used for mutant selection (selective culture) and 2 mls was used for titre measurement (titre culture). The 2 mls of selective culture and the 2

mls of titre culture were added to two separate 50 ml sterile polypropylene centrifuge tubes. 500 µl of assembled λ phage was added to the selective culture tube, and the solution was left to stand for 30 minutes to allow for phage adsorbtion. After 30 minutes, a 15 µl aliquot of the selective culture was transferred to the titre culture. 32 mls of top agar was subsequently added to the titre culture, and 32 mls of top agar supplemented with 0.3% w/v of P-Gal (Sigma-Aldrich Canada) was added to the selective culture. The resulting solutions for both the selective and titre cultures were distributed evenly onto four minimal agar plates and allowed to set on a level surface. Once solidified, the plates were inverted and incubated for 24 hours. Mutant plaque-forming units (pfu) on the selective plates, and total pfu on the titre plates were scored manually.

2.6.4 Statistical Analysis

For each concentration of test substance, a mutant frequency (MF) was calculated using the following formula:

$$\text{Mean MF} = \frac{\text{Mutant pfu}}{\text{Total pfu}}$$

SAS Version 9.2 for Windows was used for the analysis of all Muta^{TM}Mouse assay results. Analysis was completed using Poisson regression and the data were fit to the model:

$$\log(E(Yi)) = \log t_i + \beta x_i$$

Where: E(Y*i*) = the expected value for the i^{th} observation
β = the vector of regressions coefficients
x*i* = a vector of covariates for the i^{th} observation
t*i* = the offset variable used to account for differences in observation count period (i.e., total plaque counts). The offset (i.e., natural log of total plaque count) was given a constant coefficient of 1.0 for each observation.

Log-linear relationships between mutant count and test substance concentration were specified by a natural log link function. Type 1, or sequential analysis, was employed to examine the statistical significance of the chemical treatment, and custom contrasts were employed to evaluate the statistical significance of responses at selected concentrations. Custom contrasts were accomplished by specifying an L matrix and computing statistics for pair-wise comparisons based on the asymptotic chi-square distribution of the likelihood ratio.

3.0 RESULTS

3.1 Ames/*Salmonella* Reverse Mutation Assay

The standard plate incorporation version of the *Salmonella* mutagenicity assay was employed to assess the mutagenic activity of the individual explosive standards and military soil extracts. The study employed *S. typhimurium* strains TA98, TA100, YG1041 and YG1042, both with and without exogenous S9 metabolic activation.

3.1.1 Positive and Negative Controls

Positive and negative controls were tested for each experiment to ensure that the appropriate number of spontaneous or chemically-induced revertant colonies was produced for each strain. Furthermore, because the explosive standards and soil extracts examined in this thesis were prepared in ACN rather than DMSO, which is the typical solvent used for the *Salmonella* mutagenicity assay, both ACN and DMSO were used as negative controls. The number of spontaneous revertants observed for each strain is presented in Tables 3.1 and 3.2, and the number of chemically-induced revertants observed for each strain is presented in Table 3.3.

Table 3.1: Mean number of spontaneous revertants for the ACN negative control.

Strain	S9	Mean revertants per plate[a] ± SEM[b]	n[c]
TA98	N	18.3 ± 0.6	117
	Y	28.1± 0.8	
TA100	N	125.2 ± 1.9	
	Y	151.9 ± 2.2	
YG1041	N	21.3 ± 0.7	
	Y	49.6 ± 1.2	
YG1042	N	104.2 ± 1.9	
	Y	307.2 ± 13.9	

[a] Test volume = 100µl ACN.
[b] SEM = Standard error of the mean.
[c] n = Number of observations for each strain and S9 combination.

Table 3.2: Mean number of spontaneous revertants for the DMSO negative control.

Strain	S9	Mean revertants per plate[a] ± SEM[b]	n[c]
TA98	N	20.9 ± 0.9	36
	Y	31.1 ± 2.0	
TA100	N	126.6 ± 3.9	
	Y	162.9 ± 6.5	
YG1041	N	24.6 ± 1.0	
	Y	46.3 ± 1.9	
YG1042	N	101.9 ± 3.3	
	Y	244.8 ± 15.2	35

[a] Test volume = 100µl DMSO.
[b] SEM = Standard error of the mean.
[c] n = Number of observations for each strain and S9 combination.

Table 3.3: Mean number of revertants induced by the positive controls.

Strain	S9	Chemical	Concentration (µg/plate)	Mean revertants per plate ± SEM[a]	n[b]
TA98	N	2-nitrofluorene	3.5	78.1 ± 2.2	36
	Y	2-aminoanthracene	0.5	552.4 ± 21.2	
TA100	N	Methylmethane sulfonate	0.5[c]	606.3 ± 28.8	
	Y	2-aminoanthracene	0.5	605.0 ± 19.6	
YG1041	N	2-nitrofluorene	1.0	892.5 ± 60.9	
	Y	2-aminoanthracene	0.1	2069.5 ± 121.7	
YG1042	N	2-nitrofluorene	0.1	869.8 ± 50.8	
	Y	2-aminoanthracene	0.05	2027.3 ± 79.8	

[a] SEM = Standard error of the mean.
[b] n = Number of observations for each strain and S9 combination.
[c] Concentration in µl/plate.

3.2 Mutagenic Activity of Explosive Compounds

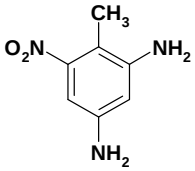
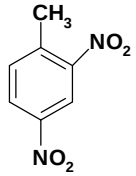
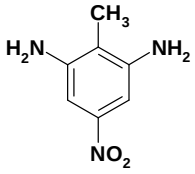
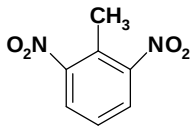
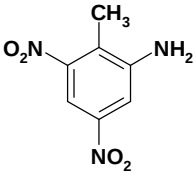
Twelve explosive compounds were evaluated for mutagenic activity using the *Salmonella* mutagenicity assay. TNT, HMX, RDX and tetryl were selected for analysis due to their high usage patterns for current and past military and civilian applications. The remainder were selected because of their noteworthy occurrence as breakdown products in explosives-contaminated soils, as well as their presence as manufacturing impurities and/or their occasional use in specific munition formulations.

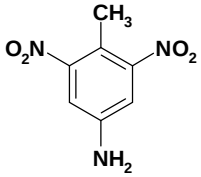
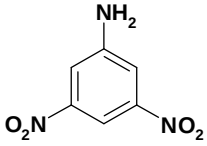
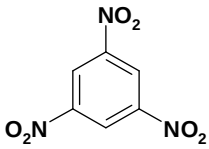
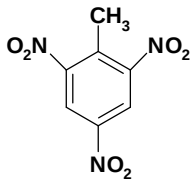
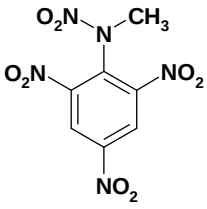
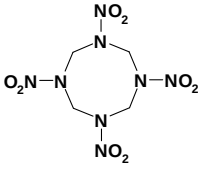
Table 3.4 summarizes the mutagenic activities for the twelve compounds examined in this thesis, for each strain and S9 combination (Appendix A provides expanded results for individual assays). The results show that ten of the twelve compounds elicited significantly positive responses. Only HMX and RDX were negative on all four strains. Three compounds, tetryl, 3,5-DNA and 1,3,5-TNB, elicited the highest mutagenic responses on the four strains. For TA98, tetryl and 3,5-DNA elicited the highest mutagenic activity with and without S9, respectively. For YG1041, tetryl and 1,3,5-TNB elicited the highest mutagenic activity with and without S9, respectively. For TA100 and YG1042, tetryl elicited the highest mutagenic activity regardless of the S9 condition.

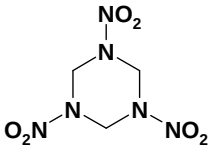
The four dinitrotoluene compounds largely exhibited the lowest mutagenic activity on the four strains, with and without S9. However, three other breakdown products, 2,6-DANT, 2,4-DANT and 3,5-DNA, also elicited low or non-mutagenic responses. For TA98, 2,4-DNT, 2,6-DNT, 4a-DNT, 2,4-DANT and 3,5-DNA all elicited non-mutagenic responses with S9, and 2,4-DNT and 2,6-DNT elicited non-mutagenic responses without S9. For TA100, 2,4-DNT, 2,6-DNT, 2a-DNT, 2,4-DANT and 3,5-

DNA all elicited non-mutagenic responses with S9, and 2,4-DNT, 2,6-DNT and 2,4-DANT elicited non-mutagenic responses without S9. For YG1041, 2,4-DNT and 2,6-DNT elicited non-mutagenic responses with S9, and 2,6-DNT elicited the lowest mutagenic activity without S9. For YG1042, 3,5-DNA elicited a non-mutagenic response with S9, and 2,6-DNT elicited the lowest mutagenic activity without S9.

Table 3.4: Mean mutagenic potencies of explosive compounds using the *Salmonella* mutagenicity assay.

Compound	Strain	S9	Mean Mutagenic Potency[a,b]	SEM[c]	n[d]
2,4-DANT 	TA98	N	0.80	0.05	3
		Y	NM[e]	-	
	TA100	N	NM	-	
		Y	NM	-	
	YG1041	N	15.1	0.72	
		Y	1.52	0.10	
	YG1042	N	64.0	2.67	
		Y	47.6	2.50	
2,4-DNT 	TA98	N	NM	-	3
		Y	NM	-	
	TA100	N	NM	-	
		Y	NM	-	
	YG1041	N	0.35	0.04	
		Y	NM	-	
	YG1042	N	21.8	0.57	2
		Y	7.39	0.39	
2,6-DANT 	TA98	N	3.85	0.10	3
		Y	1.02	0.04	
	TA100	N	2.22	0.08	
		Y	1.61	0.09	
	YG1041	N	17.6	1.06	
		Y	6.78	0.33	
	YG1042	N	116.1	3.90	
		Y	29.2	1.61	
2,6-DNT 	TA98	N	NM	-	3
		Y	NM	-	
	TA100	N	NM	-	
		Y	NM	-	
	YG1041	N	0.34	0.04	
		Y	NM	-	
	YG1042	N	9.14	0.40	2
		Y	2.12	0.53	
2a-DNT 	TA98	N	1.14	0.04	3
		Y	0.21	0.03	
	TA100	N	0.62	0.05	
		Y	NM	-	
	YG1041	N	17.3	0.52	
		Y	1.62	0.05	
	YG1042	N	56.4	4.23	
		Y	20.3	1.47	
4a-DNT	TA98	N	0.42	0.04	3

	TA100	Y	NM	-	
		N	0.29	0.06	
		Y	0.79	0.09	
	YG1041	N	5.36	0.38	
		Y	0.93	0.08	
	YG1042	N	49.1	1.07	
		Y	24.3	1.18	2
3,5-DNA	TA98	N	**37.1**[f]	3.78	
		Y	NM	-	
	TA100	N	17.6	2.09	
		Y	NM	-	
	YG1041	N	193.6	22.9	3
		Y	3.20	0.40	
	YG1042	N	122.3	10.1	
		Y	NM	-	
1,3,5-TNB	TA98	N	12.8	0.35	
		Y	1.24	0.05	
	TA100	N	15.5	0.50	
		Y	3.68	0.09	
	YG1041	N	**272.2**	7.35	3
		Y	7.12	0.20	
	YG1042	N	98.1	3.18	
		Y	42.7	1.34	
TNT	TA98	N	1.26	0.05	
		Y	NM	-	
	TA100	N	2.87	0.12	
		Y	2.85	0.13	3
	YG1041	N	13.9	0.58	
		Y	1.89	0.08	
	YG1042	N	98.9	2.40	
		Y	34.5	1.13	2
Tetryl	TA98	N	3.12	0.15	
		Y	**1.33**	0.05	
	TA100	N	**38.6**	1.34	
		Y	**7.83**	0.29	3
	YG1041	N	19.1	0.86	
		Y	**9.65**	0.21	
	YG1042	N	**125.0**	7.20	
		Y	**51.8**	3.27	
HMX	TA98	N	NM	-	3
		Y	NM	-	
	TA100	N	NM	-	
		Y	NM	-	
	YG1041	N	NM	-	
		Y	NM	-	

	YG1042	N	NM	-	
		Y	NM	-	
RDX 	TA98	N	NM	-	3
		Y	NM	-	
	TA100	N	NM	-	
		Y	NM	-	
	YG1041	N	NM	-	
		Y	NM	-	
	YG1042	N	NM	-	
		Y	NM	-	

[a] Mutagenic potency was determined by calculating the slope of the linear portion of the concentration-response function for each compound, strain and S9 combination. The revertant counts for each plate at each concentration were used for calculating mean mutagenic potency.
[b] Mutagenic potency is stated as revertants/µg compound.
[c] SEM = Standard error of the mean.
[d] n = Number of separate experiments conducted for each compound, strain and S9 combination.
[e] NM = Not mutagenic.
[f] Bolded numbers indicate the highest value for a particular strain and S9 combination.

Comparisons of the mean mutagenic potencies across S9 conditions for all compounds in each of the four strains are presented in Figures 3.1 to 3.4. The results clearly show that the strongest responses were always observed without S9, regardless of the strain used or compound tested. More specifically, the highest mutagenic responses observed for TA98, TA100, YG1041 and YG1042 are approximately 28-, 5-, 28- and 2.5-fold higher, respectively, without S9 than with S9. For example, the TA98 results showed mutagenic potency values up to 1.33 revertants/μg compound with S9, whereas potencies without S9 reached 37.1 revertants/μg compound. Similarly, TA100 results showed mutagenic potencies up to 7.83 revertants/μg compound with S9, whereas potencies without S9 reached 38.6 revertants/μg compound. YG1041 results showed mutagenic potencies up to 9.65 revertants/μg compound with S9, in comparison with values up to 272.2 revertants/μg compound without S9. YG1042 results showed mutagenic potencies up to 51.8 revertants/μg compound with S9, in comparison with values up to 125.0 revertants/μg compound without S9.

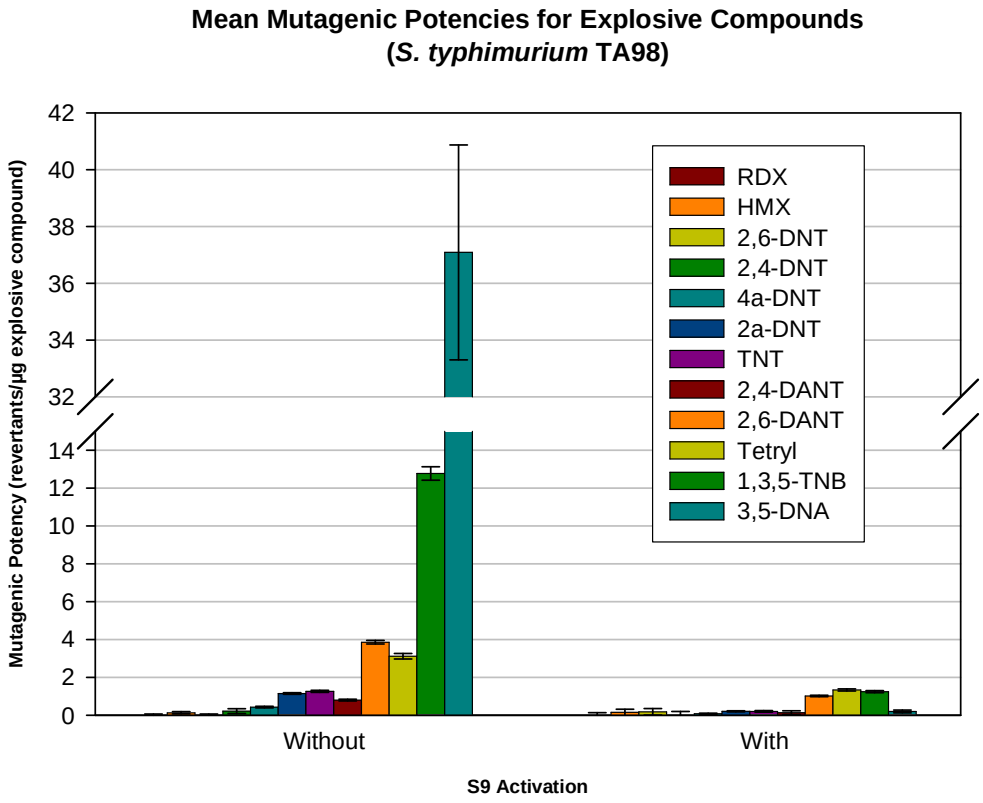


Figure 3.1: Mean mutagenic potencies of the explosive compounds on strain TA98.

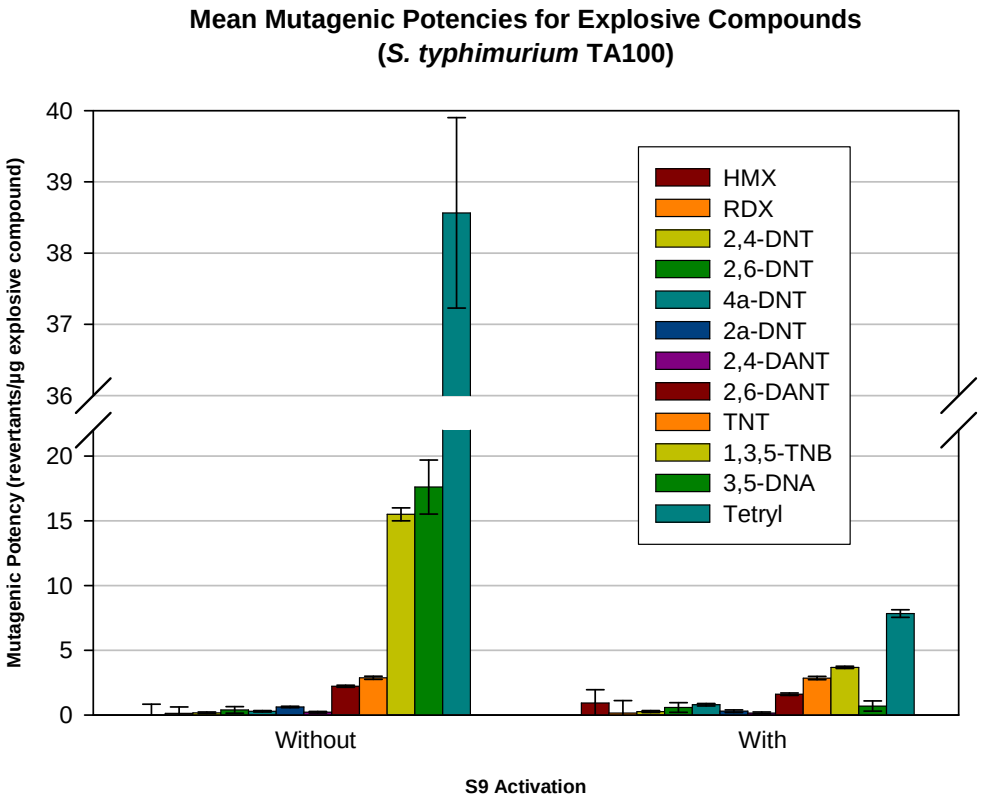


Figure 3.2: Mean mutagenic potencies of the explosive compounds on strain TA100.

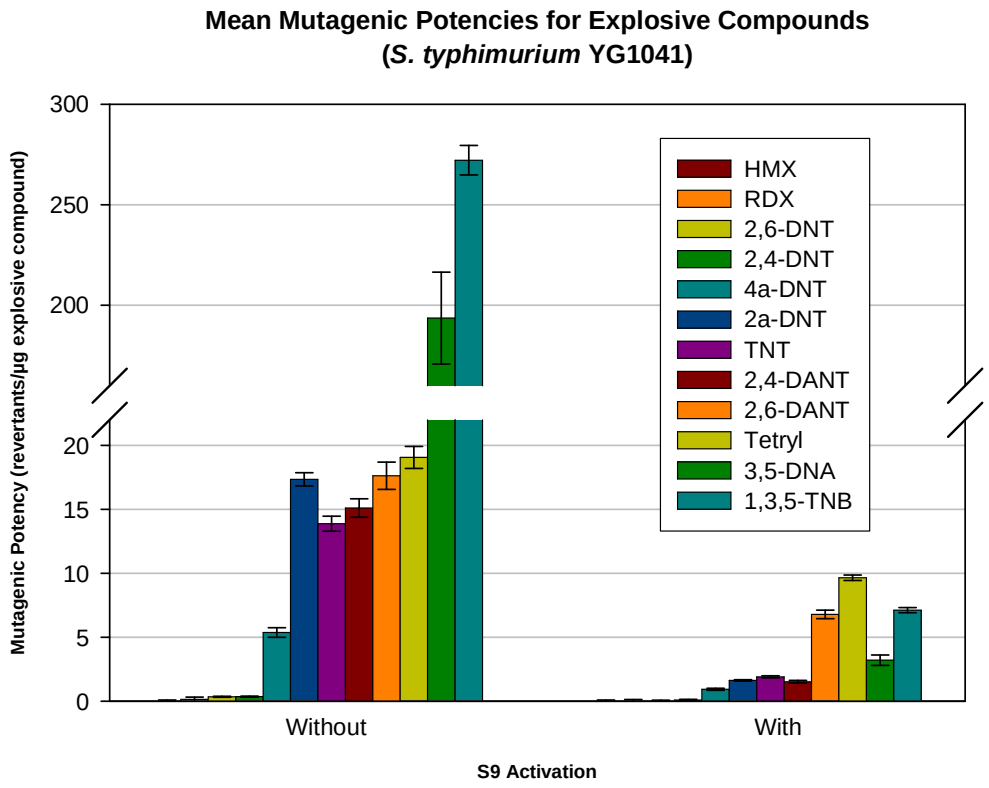


Figure 3.3: Mean mutagenic potencies of the explosive compounds on strain YG1041.

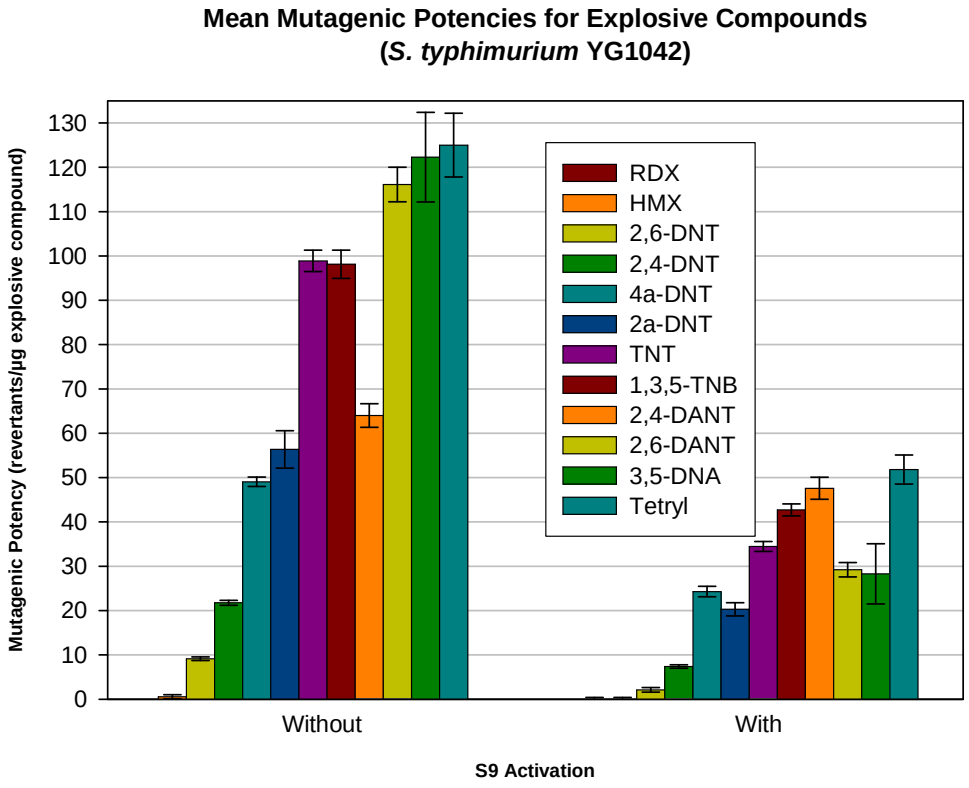


Figure 3.4: Mean mutagenic potencies of the explosive compounds on strain YG1042.

Statistical analyses (i.e., Student's t-test with appropriate multiple test correction) were conducted to determine if the mutagenic potencies for each compound and strain combination were significantly different across S9 conditions. The results are summarized in Table 3.5. The results show statistically significant differences for the mutagenic potencies between the S9 conditions, for essentially all compound and strain combinations. The addition of S9 was associated with a lower mutagenic response. The only exception was TNT on TA100, which yielded a lower response with S9, but the difference was not statistically significant.

Table 3.5: Statistical analysis of the differences in mutagenic potencies of explosive compounds across S9 conditions.

Compound	Strain	Calculated t-value	Critical t-value[a]	Significantly different at p <0.05[a]
2,4-DANT	TA98	5.97	2.78	Y
	TA100	N/A[b]	-	-
	YG1041	18.8	2.78	Y
	YG1042	4.49	2.81	Y
2,4-DNT	TA98	N/A	-	-
	TA100	N/A	-	-
	YG1041	4.07	2.78	Y
	YG1042	20.8	2.80	Y
2,6-DANT	TA98	27.0	2.78	Y
	TA100	5.04	2.78	Y
	YG1041	9.73	2.78	Y
	YG1042	20.6	2.80	Y
2,6-DNT	TA98	N/A	-	-
	TA100	N/A	-	-
	YG1041	5.99	2.78	Y
	YG1042	10.6	2.80	Y
2a-DNT	TA98	19.2	2.78	Y
	TA100	2.84	2.78	Y
	YG1041	29.9	2.78	Y
	YG1042	8.06	2.79	Y
4a-DNT	TA98	6.83	2.78	Y
	TA100	4.76	2.78	Y
	YG1041	11.5	2.78	Y
	YG1042	15.6	2.83	Y
3,5-DNA	TA98	9.75	2.80	Y
	TA100	7.96	2.81	Y
	YG1041	8.32	2.81	Y
	YG1042	7.72	2.80	Y
1,3,5-TNB	TA98	32.3	2.78	Y
	TA100	23.1	2.78	Y
	YG1041	36.0	2.79	Y
	YG1042	16.1	2.79	Y
TNT	TA98	15.9	2.78	Y
	TA100	0.13	2.78	N
	YG1041	20.3	2.78	Y
	YG1042	24.3	2.81	Y
Tetryl	TA98	11.5	2.78	Y
	TA100	22.5	2.78	Y
	YG1041	10.6	2.78	Y
	YG1042	9.26	2.79	Y
HMX	TA98	N/A	-	-

	TA100	N/A	-	-
	YG1041	N/A	-	-
	YG1042	N/A	-	-
RDX	TA98	N/A	-	-
	TA100	N/A	-	-
	YG1041	N/A	-	-
	YG1042	N/A	-	-

[a] Critical *p*-value = 0.0063 (two-tailed Student's t-test with appropriate Bonferroni correction).
[b] N/A = Not applicable (e.g., neither S9 condition elicited a positive response).

In addition to the compounds eliciting greater responses without S9, all ten of the compounds yielding positive responses elicited the highest response on the metabolically-enhanced strains, YG1041 and YG1042, in comparison to the parent strains TA98 and TA100. Comparisons of the mean mutagenic potencies for the parent and the enhanced strains, for each compound and S9 combination, are presented in Figures 3.5 to 3.8. The results clearly show large increases in mutagenic activity on the metabolically-enhanced strains relative to the parent strains. The highest responses observed on the metabolically-enhanced YG1041, with and without S9, are approximately 7- and 21-fold higher, respectively, than those obtained using the corresponding parent strain TA98. The highest mutagenic responses observed on the metabolically-enhanced YG1042, with and without S9, are approximately 6.5- and 3-fold higher, respectively, than those obtained using the corresponding parent strain TA100.

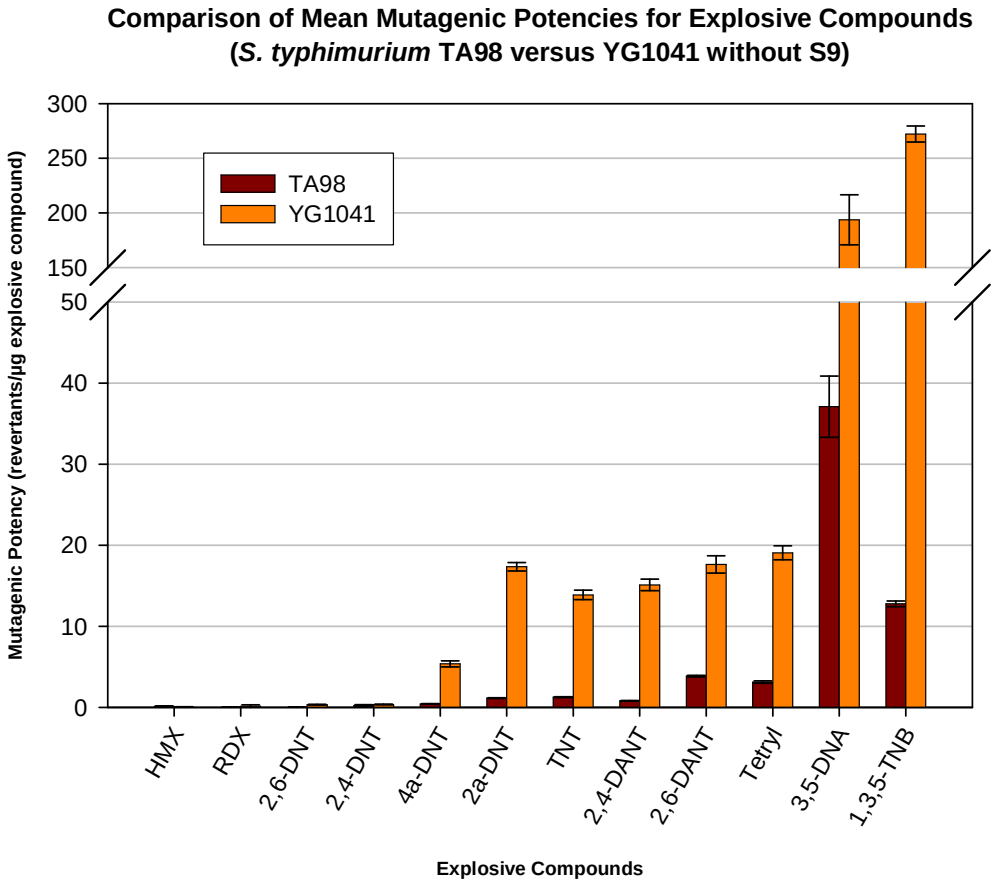


Figure 3.5: Comparisons of the mean mutagenic potencies of explosive compounds between strains TA98 and YG1041, without S9.

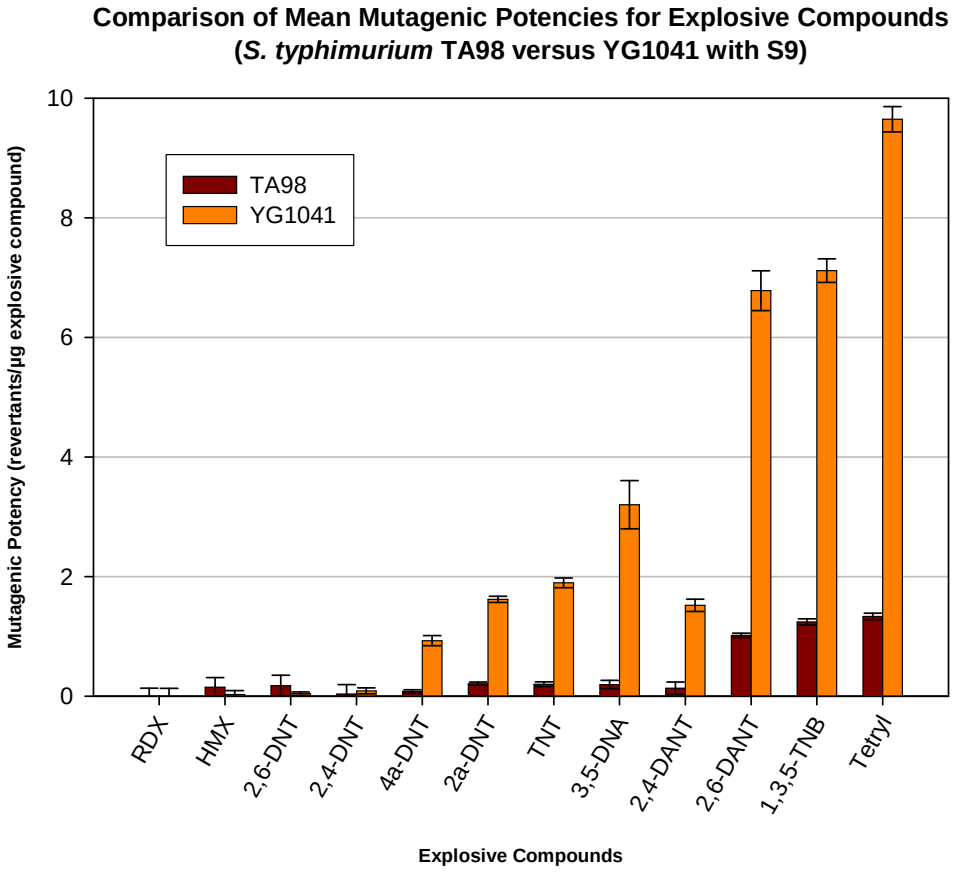


Figure 3.6: Comparisons of the mean mutagenic potencies of explosive compounds between strains TA98 and YG1041, with S9.

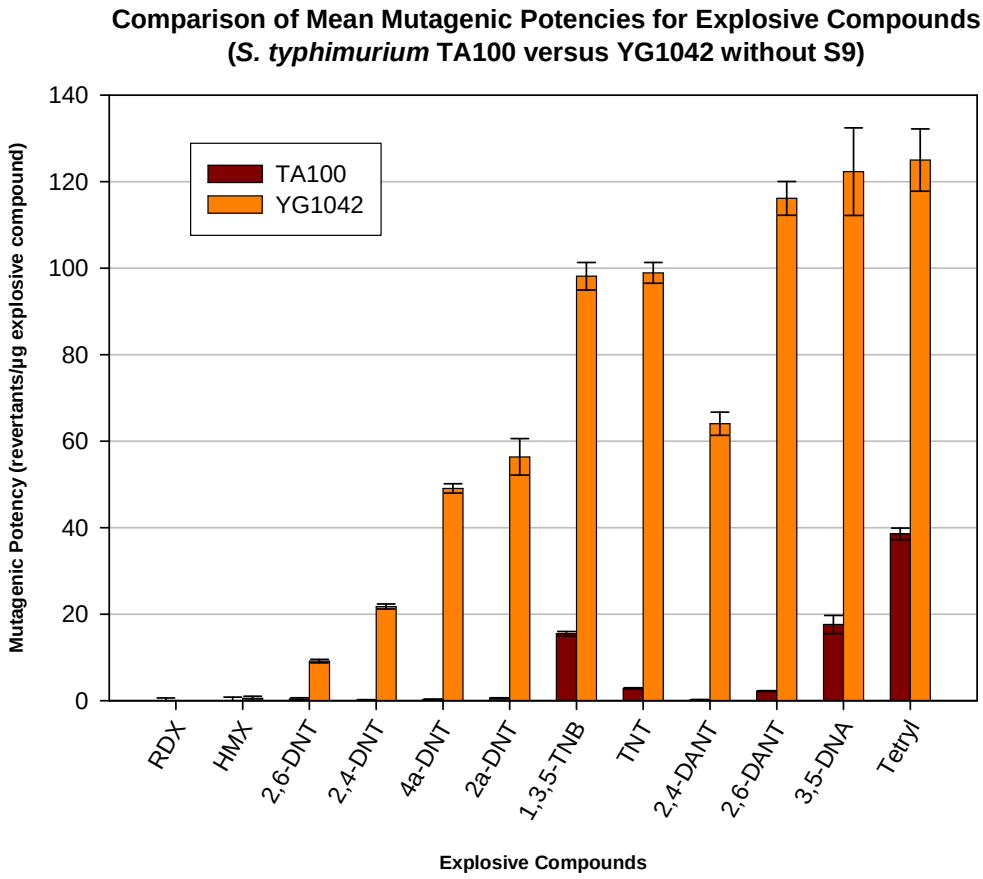


Figure 3.7: Comparisons of the mean mutagenic potencies of explosive compounds between strains TA100 and YG1042, without S9.

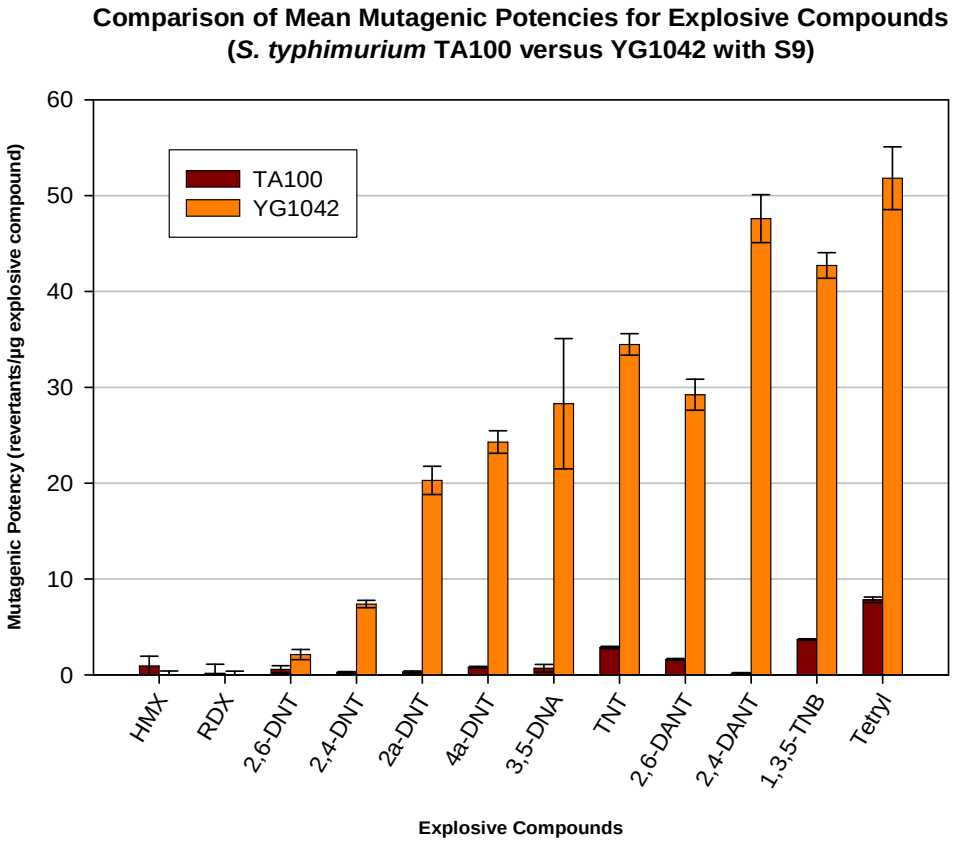


Figure 3.8: Comparisons of the mean mutagenic potencies of explosive compounds between strains TA100 and YG1042, with S9.

Statistical analysis was conducted to determine if the mutagenic potencies for each compound and S9 combination were significantly different between the parent and enhanced strains. The results are summarized in Table 3.6. The results show statistically significant differences for the mutagenic potencies between the metabolically-enhanced and the parent strains, for essentially all compound and S9 combinations. The parent strain was associated with a lower mutagenic response. The only exceptions were 2,4-DNT (without S9) and 2,6-DNT (with S9), which yielded lower responses on TA98 and TA100, respectively, but the differences were not statistically significant.

Table 3.6: Statistical analysis of the differences in mutagenic potencies of explosive compounds between parent and metabolically-enhanced strains.

Compound	Comparison	S9	Calculated t-value	Critical t-value[a]	Statistically different at p<0.05[a]
2,4-DANT	TA98 / YG1041	N	19.9	2.78	Y
	TA98 / YG1041	Y	9.55	2.78	Y
	TA100 / YG1042	N	23.9	2.79	Y
	TA100 / YG1042	Y	19.0	2.79	Y
2,4-DNT	TA98 / YG1041	N	1.04	2.79	N
	TA98 / YG1041	Y	N/A[b]	-	-
	TA100 / YG1042	N	37.8	2.79	Y
	TA100 / YG1042	Y	17.9	2.79	Y
2,6-DANT	TA98 / YG1041	N	12.9	2.78	Y
	TA98 / YG1041	Y	17.2	2.78	Y
	TA100 / YG1042	N	29.2	2.79	Y
	TA100 / YG1042	Y	17.1	2.79	Y
2,6-DNT	TA98 / YG1041	N	5.98	2.78	Y
	TA98 / YG1041	Y	N/A	-	-
	TA100 / YG1042	N	18.6	2.81	Y
	TA100 / YG1042	Y	2.37	2.80	N
2a-DNT	TA98 / YG1041	N	30.9	2.79	Y
	TA98 / YG1041	Y	24.0	2.78	Y
	TA100 / YG1042	N	13.2	2.78	Y
	TA100 / YG1042	Y	13.6	2.78	Y
4a-DNT	TA98 / YG1041	N	13.1	2.78	Y
	TA98 / YG1041	Y	9.51	2.78	Y
	TA100 / YG1042	N	45.7	2.79	Y
	TA100 / YG1042	Y	19.9	2.79	Y
3,5-DNA	TA98 / YG1041	N	6.75	2.81	Y
	TA98 / YG1041	Y	7.34	2.80	Y
	TA100 / YG1042	N	10.1	2.80	Y
	TA100 / YG1042	Y	N/A	-	-
1,3,5-TNB	TA98 / YG1041	N	35.3	2.78	Y
	TA98 / YG1041	Y	28.7	2.78	Y
	TA100 / YG1042	N	25.7	2.79	Y
	TA100 / YG1042	Y	29.1	2.78	Y
TNT	TA98 / YG1041	N	21.5	2.78	Y
	TA98 / YG1041	Y	18.9	2.78	Y
	TA100 / YG1042	N	39.9	2.79	Y
	TA100 / YG1042	Y	27.9	2.79	Y
Tetryl	TA98 / YG1041	N	18.3	2.78	Y
	TA98 / YG1041	Y	37.9	2.78	Y
	TA100 / YG1042	N	11.8	2.79	Y
	TA100 / YG1042	Y	13.4	2.78	Y
HMX	TA98 / YG1041	N	N/A	-	-

	TA98 / YG1041	Y	N/A	-	-
	TA100 / YG1042	N	N/A	-	-
	TA100 / YG1042	Y	N/A	-	-
RDX	TA98 / YG1041	N	N/A	-	-
	TA98 / YG1041	Y	N/A	-	-
	TA100 / YG1042	N	N/A	-	-
	TA100 / YG1042	Y	N/A	-	-

[a] Critical *p*-value = 0.0063 (two-tailed Student's t-test with appropriate Bonferroni correction).
[b] N/A = Not applicable (e.g., neither S9 condition yielded a positive response).

3.3 Soil Analysis

3.3.1 Quantification Limits for Explosive Compounds in Soil

The HPLC limits of quantification (LOQs) for each of the fourteen compounds found in EPA 8330 Mixes A and B were determined using an analytical calibration standard, and observed to be approximately 0.1 µg/ml. These values were based on a 10:1 signal:noise ratio. The corresponding soil LOQs for each of the compounds were calculated using the instrument LOQs, and assumed ideal extraction conditions. The soil LOQs for the compounds are presented in Table 3.7. Any compound that was present in the three soil samples examined in this thesis at a level above the soil LOQ was quantified.

Table 3.7: Soil limits of quantification for fourteen explosive compounds, as determined using the Acclaim® Explosives E1 column.

Compound	Limit of Quantification (ppm)*
HMX	0.2721
RDX	0.2086
1,3,5-TNB	0.2095
1,3-DNB	0.1992
Nitrobenzene	0.2020
Tetryl	0.1687
TNT	0.1996
4a-DNT	0.2123
2a-DNT	0.1475
2,6-DNT	0.2584
2,4-DNT	0.1919
2-nitrotoluene	0.2014
4-nitrotoluene	0.1926
3-nitrotoluene	0.1840

* ppm = parts per million

3.3.2 Characterization of Soil Samples

Concentrations of the different compounds present in the three soil samples were determined using HPLC, and were found to vary by up to four orders of magnitude within a sampling site. In PET 1, for example, explosive concentrations ranged from 0.289 ppm for 1,3,5-TNB to 1135.5 ppm for HMX. Concentrations of the different compounds also varied by up to three orders of magnitude across sampling sites. HMX, for example, ranged from 1.06 ppm in PET 3 to 1145.4 ppm in PET 2. In total, eight compounds were detected in the soils examined, and the results are presented in Table 3.8.

Table 3.8: Concentrations of explosive compounds in the three soil samples examined in this thesis.*

Compound	PET 1	PET 2	PET 3
HMX	1135.5 (0.7633)	1145.4 (0.5787)	1.06 (0.0006)
RDX	-	0.651 (0.0003)	-
TNT	6.02 (0.0040)	5.88 (0.0030)	-
1,3,5-TNB	0.289 (0.0002)	0.702 (0.0004)	0.374 (0.0002)
4a-DNT	1.86 (0.0012)	1.74 (0.0009)	-
2a-DNT	0.325 (0.0002)	0.336 (0.0002)	-
Nitrobenzene	-	-	0.505 (0.0003)
2,4-DNT	-	-	5.79 (0.0035)

* Values in ppm. Values in parentheses in μg compound/mg dry soil equivalents.

3.4 Mutagenic Activity of Soil Extracts

The soil sample extracts were evaluated for mutagenic activity using the *Salmonella* mutagenicity assay, and the results are summarized in Table 3.9. The results show that all three soil extracts elicited positive responses. PET 2 elicited the highest responses on all four strains, while PET 3 exhibited the lowest responses, both with and without S9. The strongest responses were always observed without S9 and on the metabolically-enhanced frameshift strains, regardless of the soil sample tested.

Table 3.9: Mean mutagenic potencies of soil extracts using the *Salmonella* mutagenicity assay.

Soil Sample	Strain	S9	Mutagenic Potency[a,b]	Standard Error	r^2	*p*-value	Mutation Ratio[c]	Result
PET 1	TA98	N	1.82	0.08	0.94	<0.0001	18.0	Pos
		Y	0.33	0.04	0.70	<0.0001	2.30	Pos
	TA100	N	0.58	0.12	0.47	<0.0001	1.49	MP[d]
		Y	NM[e]	-	-	-	-	Neg
	YG1041	N	31.9	0.42	1.00	<0.0001	104.0	Pos
		Y	6.33	0.17	0.98	<0.0001	20.5	Pos
	YG1042	N	8.56	0.39	0.96	<0.0001	2.80	Pos
		Y	3.12	0.20	0.90	<0.0001	2.01	MP
PET 2	TA98	N	5.41	0.17	0.98	<0.0001	19.7	Pos
		Y	0.91	0.07	0.85	<0.0001	6.06	Pos
	TA100	N	0.75	0.18	0.44	0.0004	1.43	MP
		Y	NM	-	-	-	-	Neg
	YG1041	N	67.4	2.50	0.97	<0.0001	89.8	Pos
		Y	13.1	0.36	0.98	<0.0001	30.8	Pos
	YG1042	N	12.3	0.61	0.96	<0.0001	3.79	Pos
		Y	5.27	0.25	0.94	<0.0001	2.77	Pos
PET 3	TA98	N	0.13	0.04	0.30	0.0018	1.99	MP
		Y	0.26	0.04	0.58	<0.0001	2.38	MP
	TA100	N	0.47	0.20	0.20	0.0270	1.19	MP
		Y	NM	-	-	-	-	Neg
	YG1041	N	1.99	0.10	0.94	<0.0001	8.18	Pos
		Y	1.29	0.06	0.94	<0.0001	3.72	Pos
	YG1042	N	1.33	0.10	0.87	<0.0001	2.35	MP
		Y	0.57	0.14	0.38	0.0003	1.20	MP

[a] Mutagenic potency was determined by calculating the slope of the linear portion of the concentration-response function for each soil extract, strain and S9 combination.
[b] Mutagenic potency is stated as revertants/mg dry soil equivalents.
[c] Mutation ratio is defined as the mean number of revertants at the highest concentration used in the calculation of mutagenic potency, divided by the mean number of spontaneous revertants.
[d] MP = Marginally positive mutagenic response (significant *p*-value <0.05, but fewer than 2 consecutive concentrations eliciting response 2-fold greater than spontaneous).
[e] NM = Not mutagenic.

The mean mutagenic potencies of the soil sample extracts on each of the four strains are presented in Figures 3.9 to 3.12. The results clearly show that the greatest responses for TA98, YG1041 and YG1042 are approximately 6-, 5- and 2-fold higher, respectively, without S9 than with S9. The soil extracts were not mutagenic on TA100 with S9, therefore a similar calculation could not be made. More specifically, TA98 results showed mutagenic potencies up to 0.91 revertants/mg dry soil equivalents with S9, whereas potencies without S9 reached 5.41 revertants/mg dry soil equivalents. TA100 results showed no mutagenic activity for any soil sample with S9, and up to 0.75 revertants/mg dry soil equivalents without S9. YG1041 results showed mutagenic potencies up to 13.1 revertants/mg dry soil equivalents with S9, whereas potencies without S9 reached 67.4 revertants/mg dry soil equivalents. YG1042 results showed mutagenic potencies up to 5.27 revertants/mg dry soil equivalents with S9, whereas potencies without S9 reached 12.3 revertants/mg dry soil equivalents.

The highest responses observed on the metabolically-enhanced YG1041, with and without S9, are approximately 14- and 12.5-fold higher, respectively, than on the corresponding parent strain TA98. Similarly, the greatest response observed on YG1042, without S9, is approximately 16.5-fold higher than on the corresponding parent strain TA100. TA100 displayed no mutagenic activity with S9, therefore a similar calculation could not be made; however, YG1042 with S9 did show a mutagenic response.

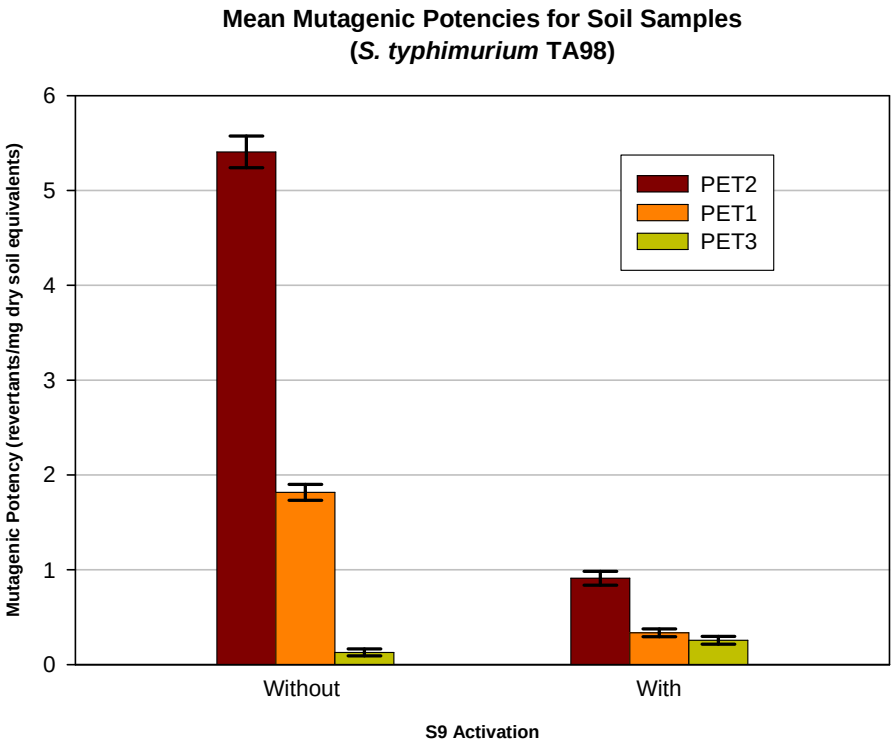


Figure 3.9: Mean mutagenic potencies of the soil extracts on strain TA98.

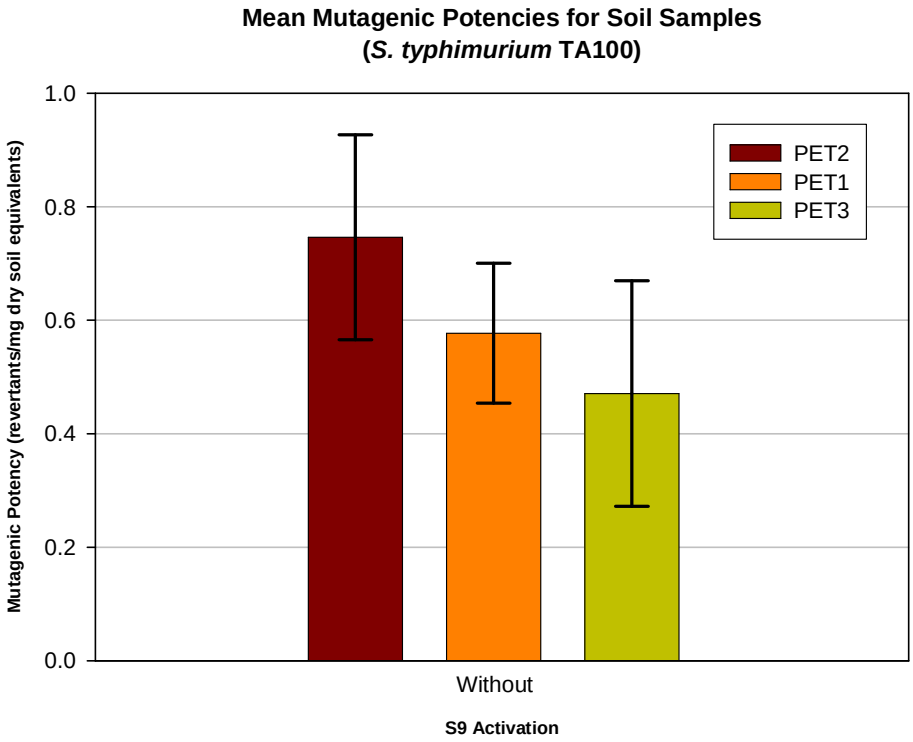


Figure 3.10: Mean mutagenic potencies of the soil extracts on strain TA100.

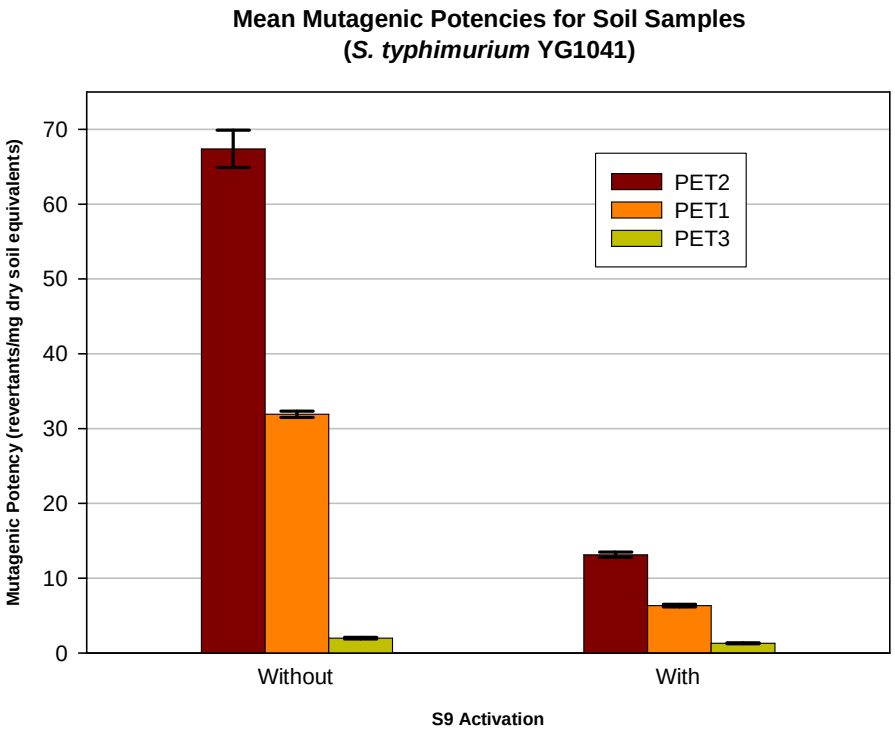


Figure 3.11: Mean mutagenic potencies of the soil extracts on strain YG1041.

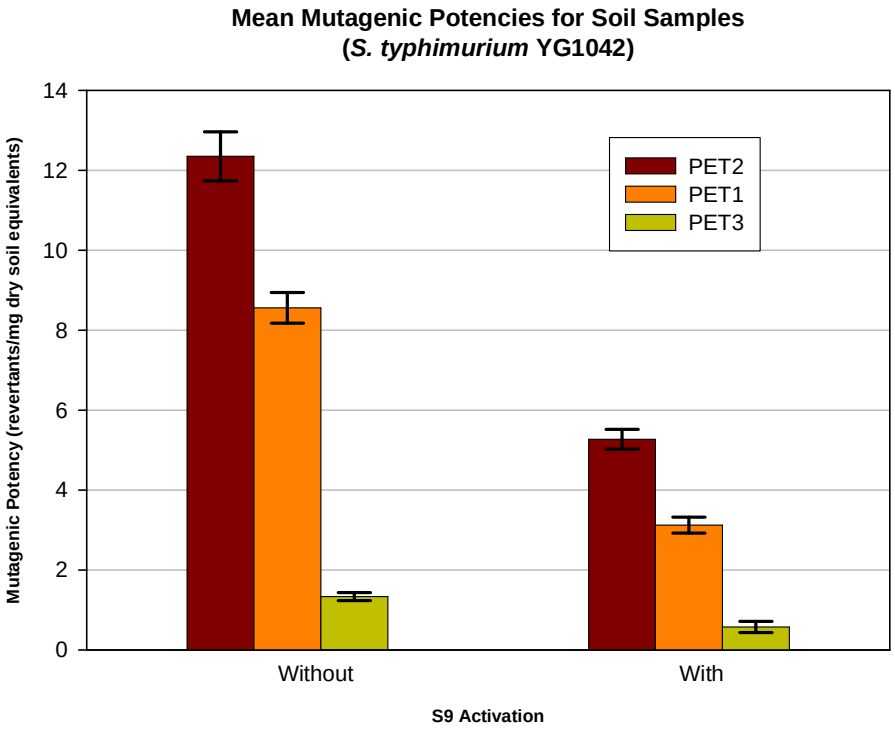


Figure 3.12: Mean mutagenic potencies of the soil extracts on strain YG1042.

3.4.1 Predicted *Salmonella* Mutagenic Activity of Soil Extracts

Predicted mutagenic potencies of the three soil sample extracts were determined using the observed concentrations and mutagenic potencies of the individual explosive compounds (refer to Tables 3.8 and 3.4, respectively). These values were subsequently compared to the observed activity. Predicted, or rather expected values, are simply the sum of the expected mutagenic contributions from each of the measured compounds, for each strain and S9 combination (refer to Section 2.5.6 in Materials and Methods). The predicted mutagenic potencies, based on an assumption of additivity, are presented in Table 3.10. In the majority of cases (i.e., 21 of 24), the predicted mutagenic potencies of the three soil samples, on all four strains with and without S9, highly underestimated the actual mutagenic activity by up to four orders of magnitude. The remaining three predicted values were greater than the observed values; however, in these three cases the *Salmonella* mutagenicity assay was unable to detect any observable mutagenic activity.

Table 3.10: Observed and predicted mutagenic potencies of soil samples.

Soil Sample	Strain	S9	Observed Mutagenic Potency[a]	Standard Error	Predicted Mutagenic Potency[a]
PET 1	TA98	N	1.82	0.08	0.0084
		Y	0.33	0.04	0.0003
	TA100	N	0.58	0.12	0.0151
		Y	NM[b]	-	0.0132
	YG1041	N	31.9	0.42	0.1195
		Y	6.33	0.17	0.0106
	YG1042	N	8.56	0.39	0.4926
		Y	3.12	0.20	0.1825
PET 2	TA98	N	5.41	0.17	0.0088
		Y	0.91	0.07	0.0005
	TA100	N	0.75	0.18	0.0144
		Y	NM	-	0.0105
	YG1041	N	67.4	2.50	0.1454
		Y	13.1	0.36	0.0092
	YG1042	N	12.3	0.61	0.3812
		Y	5.27	0.25	0.1423
PET 3	TA98	N	0.13	0.04	0.0029
		Y	0.26	0.04	0.0003
	TA100	N	0.47	0.20	0.0035
		Y	NM	-	0.0008
	YG1041	N	1.99	0.10	0.0633
		Y	1.29	0.06	0.0016
	YG1042	N	1.33	0.10	0.0992
		Y	0.57	0.14	0.0358

[a] Mutagenic potency is stated as revertants/mg dry soil equivalents.
[b] NM = Not mutagenic.

3.5 FE1 Muta^{TM}Mouse *in vitro* Transgene Mutation Assay

The Muta^{TM}Mouse assay was employed to assess the mutagenic activity of the explosive compounds and soil extracts, both with and without exogenous S9 metabolic activation.

3.5.1 Positive and Negative Controls

Positive and negative controls were tested to ensure that the appropriate spontaneous or chemically-induced mutant frequencies were obtained. Due to the fact that the explosive standards and the soil extracts examined in this thesis were prepared in ACN, this solvent was used as the negative control. PhIP and B[a]P were used as positive controls, with and without the presence of S9, respectively. The spontaneous and chemically-induced mutant frequencies are presented in Table 3.11.

Table 3.11: Spontaneous and chemically-induced mean mutant frequencies.

Chemical	S9	Minimum Mutant Frequency ($x10^{-5}$)[a]	Maximum Mutant Frequency ($x10^{-5}$)[b]	Mean Mutant Frequency[c] ± SEM[d] ($x10^{-5}$)	n[e]
Acetonitrile (1%)	N	16.6	186.8	45.8 ± 5.60	29
	Y	25.3	75.7	44.6 ± 2.49	29
Acetonitrile (2%)	N	11.7	47.9	29.5 ± 4.53	8
	Y	31.1	67.8	42.4 ± 5.85	6
Acetonitrile (4%)	N	43.4	99.6	69.7 ± 6.04	10
	Y	37.2	69.7	53.3 ± 3.04	9
B[*a*]P	N	562.1	1237.9	750.8 ± 74.9	8
PhIP	Y	139.7	175.7	154.6 ± 6.45	6

[a] Minimum mutant frequency is defined as the lowest of all mutant frequency observations for a particular chemical and S9 combination. Mutant frequency is the ratio of *lacZ* mutants to total plaque forming units.
[b] Maximum mutant frequency is defined as the highest of all mutant frequency observations for a particular chemical and S9 combination. Mutant frequency is the ratio of *lacZ* mutants to total plaque forming units.
[c] Mean mutant frequency is defined as the average of all mutant frequency observations for a particular chemical and S9 combination. Mutant frequency is the ratio of *lacZ* mutants to total plaque forming units.
[d] SEM = Standard error of the mean.
[e] n = Number of observations (i.e., biological replicates per chemical and S9 combination).

3.5.2 Mutagenic Activity of Explosive Compounds

The twelve compounds examined for mutagenic activity in the *Salmonella* mutagenicity assay were also tested in the Muta^{TM}Mouse assay, and the results are summarized in Table 3.12. Table 3.13 provides expanded results for those compounds eliciting significant positive responses at one or more concentrations. The results show that four of the twelve compounds, namely 1,3,5-TNB, TNT, HMX and RDX, elicited significant positive responses. RDX and TNT exhibited mutagenic activity without S9, and HMX elicited a response both with and without S9. Due to the lack of a concentration-related trend and the large standard error associated with these positive responses, however, it is unlikely that they are treatment-related. 1,3,5-TNB exhibited mutagenic activity with S9, and although there appears to be some evidence of a concentration-related increase in response, the trend is not linear. It is possible that at higher concentrations, 1,3,5-TNB could induce a more distinguishable trend.

Table 3.12: Mutagenicity of explosive compounds using the Muta^{TM}Mouse assay.

Compound	S9	*p*-value[a]	Chi-square	Concentrations Inducing Significant Positive Responses (µg compound/ml)
2a-DNT	N	NS[b]	-	-
	Y	NS	-	-
4a-DNT	N	NS	-	-
	Y	NS	-	-
3,5-DNA	N	NS	-	-
	Y	NS	-	-
2,4-DNT	N	NS	-	-
	Y	NS	-	-
2,6-DNT	N	NS	-	-
	Y	NS	-	-
2,6-DANT	N	NS	-	-
	Y	NS	-	-
2,4-DANT	N	NS	-	-
	Y	NS	-	-
Tetryl	N	NS	-	-
	Y	NS	-	-
1,3,5-TNB	N	NS	-	-
	Y	**0.0887**	**4.84**	**10**
TNT	**N**	**0.0686**	**10.24**	**-**
	Y	NS	-	-
HMX	**N**	**<0.0001**	**39.46**	**150**
	Y	**0.0069**	**17.73**	**-**
RDX	**N**	**0.0356**	**15.03**	**-**
	Y	NS	-	-

[a] Critical *p*-value = 0.10 (i.e., 0.05 for a one-tailed chi-square test).
[b] NS = Not significant.

Table 3.13: Expanded results of the mutagenic analysis for explosive compounds eliciting significant positive responses.

Compound	Concentration (µg compound/ml)	S9	Mean Mutant Frequency[a] (x10^{-5})	SEM[b]	*p*-value	Chi-square	n[c]
1,3,5-TNB	0	**Y**	41.7	-	-	-	1
	1		51.0	4.66	NS[d,e]	-	2
	10		**53.9**	**2.12**	**0.0277**	**4.84**	**2**
TNT[f]	0	N	31.0	5.32	-	-	6
	0.1		37.5	1.30	NS[g]	-	2
	1		34.2	2.51	NS	-	2
	10		53.5	9.55	NS	-	6
	20		72.6	1.57	NS	-	2
	40		50.7	-	NS	-	1
HMX[f]	0	**N**	41.8	3.81	-	-	6
	0.1		29.1	2.42	NS[g]	-	2
	1		29.7	1.43	NS	-	2
	10		29.2	1.78	NS	-	2
	150		**61.5**	**1.25**	**0.0027**	**9.00**	**2**
	300		47.0	2.89	NS	-	2
	0	Y	40.6	5.30	-	-	5
	0.1		28.5	0.89	NS[h]	-	2
	1		31.7	2.20	NS	-	2
	10		38.1	4.09	NS	-	4
	20		42.4	0.09	NS	-	2
	150		59.9	14.4	NS	-	2
	300		60.3	9.36	NS	-	2
RDX[f]	0	N	35.8	5.17	-	-	8
	0.1		28.8	1.34	NS[i]	-	2
	1		28.8	4.02	NS	-	2
	10		40.0	5.07	NS	-	6
	20		65.1	14.0	NS	-	2
	40		52.2	2.42	NS	-	2
	250		45.6	1.27	NS	-	2
	500		50.4	2.60	NS	-	2

[a] Mean mutant frequency is defined as the average of all mutant frequency observations for a particular compound, concentration and S9 combination. Mutant frequency is the ratio of *lacZ* mutants to total plaque forming units.
[b] SEM = Standard error of the mean.
[c] n = Number of observations (i.e., biological replicates per compound, concentration and S9 combination).
[d] NS = Not significant.
[e] Critical *p*-value = 0.05 (one-tailed chi-square test with appropriate Bonferroni correction).
[f] No individual comparisons were found to be significant for TNT, HMX (with S9) and RDX. Results are reported to show trend in mutant frequencies.
[g] Critical *p*-value = 0.02 (one-tailed chi-square test with appropriate Bonferroni correction).
[h] Critical *p*-value = 0.0167 (one-tailed chi-square test with appropriate Bonferroni correction).
[i] Critical *p*-value = 0.0143 (one-tailed chi-square test with appropriate Bonferroni correction).

3.5.3 Mutagenic Activity of Soil Extracts

The three soil extracts tested for mutagenic activity in the *Salmonella* mutagenicity assay were also tested in the Muta^{TM}Mouse assay, and the results are summarized in Table 3.14. Table 3.15 provides expanded results for those samples eliciting significant positive responses at one or more concentrations. The results show that all three soil extracts elicited significant positive responses. PET 2 elicited a significant positive response with S9 at the highest concentration tested, but the lower concentrations yielded mutant frequencies similar to the negative control. PET 1 and PET 3 elicited significant positive responses for two and three consecutive concentrations, respectively, without S9. There appears to be some evidence of a concentration-related trend for mutagenic activity and thus it seems likely that, at higher concentrations, PET 1 and PET 3 extracts would induce higher mutant frequency values.

Table 3.14: Mutagenicity of soil extracts using the Muta^{TM}Mouse assay.

Soil Sample	S9	*p*-value[a]	Chi-square	Concentrations Inducing Significant Positive Responses (mg dry soil equivalents/ml)
PET 1	**N**	**0.0086**	**11.68**	**2.114, 21.14**
	Y	NS[b]	-	-
PET 2	N	NS	-	-
	Y	**0.0215**	**9.68**	**16.73**
PET 3	**N**	**<0.0001**	**23.05**	**0.194, 1.94, 19.40**
	Y	NS	-	-

[a] Critical *p*-value = 0.10 (i.e., 0.05 for a one-tailed chi-square test).
[b] NS = Not significant.

Table 3.15: Expanded results of the mutagenic analysis for soil extracts eliciting significant positive responses.

Soil Sample	Concentration (mg dry soil equivalents/ml)	S9	Mean Mutant Frequency[a] (x10^{-5})	SEM[b]	*p*-value[c]	Chi-square	n[d]
PET 1	0	**N**	17.8	6.11	-	-	**2**
	0.2114		32.7	2.40	NS[e]	-	
	2.114		**38.2**	**5.46**	**0.0037**	**8.41**	
	21.14		**39.2**	**1.73**	**0.0023**	**9.26**	
	0	Y	31.5	0.48	-	-	2
	0.2114		44.5	6.51	NS	-	
	2.114		32.5	4.84	NS	-	
	21.14		41.4	9.78	NS	-	
PET 2	0	N	36.3	-	-	-	1
	0.1673		21.0	2.32	NS	-	
	1.673		24.3	0.51	NS	-	2
	16.73		29.0	6.83	NS	-	
	0	**Y**	31.5	0.48	-		**2**
	0.1673		31.9	1.63	NS		
	1.673		30.8	6.02	NS		
	16.73		**43.0**	**0.55**	**0.0133**	**6.13**	
PET 3	0	**N**	19.8	1.22	-	-	**2**
	0.194		**26.4**	**3.26**	**0.0112**	**6.44**	
	1.94		**27.2**	**2.18**	**0.0069**	**7.29**	
	19.4		**31.9**	**0.24**	**<0.0001**	**21.95**	
	0	Y	36.8	3.71	-	-	2
	0.194		32.4	0.01	NS	-	
	1.94		32.9	1.09	NS	-	
	19.4		34.0	0.41	NS	-	

[a] Mean mutant frequency is defined as the average of all mutant frequency observations for a particular soil sample, concentration and S9 combination. Mutant frequency is the ratio of *lacZ* mutant frequency to total plaque forming units.
[b] SEM = Standard error of the mean.
[c] Critical *p*-value = 0.0333 (one-tailed chi-square test with appropriate Bonferroni correction).
[d] n = Number of observations (i.e., biological replicates per soil sample, concentration and S9 combination).
[e] NS = Not significant.

4.0 DISCUSSION

4.1 Mutagenic Activity of Explosive Compounds

4.1.1 Ames/*Salmonella* Reverse Mutation Assay Results

Mutagenic activity of the twelve explosive compounds examined in this thesis was evaluated using four *S. typhimurium* strains, both with and without exogenous S9 metabolic activation. Testing of each compound on all four strains, with and without S9, not only allows a greater chance for detection of mutagenic activity, but also provides insight into the mutagenic mechanism(s) of action. The results obtained indicate that ten of the twelve compounds investigated elicited significant positive responses on one or more strains. Only HMX and RDX were negative on all four strains.

Heterocyclic Amines

HAAs are formed, among other activities, during the process of cooking meat, and are known to be highly mutagenic and carcinogenic [69, 70]. These compounds, however, are different from non-aromatic heterocyclic amines that do not appear to exhibit mutagenic activity [15]. The results obtained for the two heterocyclic amines examined in this thesis, RDX and HMX, are in agreement with this generalization, and neither compound induced mutations in the frameshift or base-pair substitution strains. This is consistent with other studies examining the mutagenic potential of these two nitramine explosive compounds. For example, Tan et al. (1992) [33], Lachance et al. (1999) [71], Whong et al. (1980) [72] and Whong and Edwards (1984) [73] examined the mutagenicity of RDX and HMX, with and without S9, on strains TA98 and TA100 and noted that these two compounds did not induce a positive response.

Nitroaromatics and Aromatic Amines

Frameshift versus base-pair substitution

Although nitroaromatics and aromatic amines can cause either frameshift or base-pair substitution mutations [37, 39, 41, 74], they are often found to have more potent frameshift activity. For example, Watanabe et al. (1989, 1990) [41, 39] and Ames et al. (1972) [75] examined the mutagenicity of a variety of nitroaromatic and aromatic amine compounds and found that they consistently yielded higher responses on the frameshift strains (e.g., TA98, YG1021 and YG1024) compared with base-pair substitution strains. Consistent with this trend, the two highest mutagenic potency values observed in this study (i.e., 1,3,5-TNB and 3,5-DNA) were elicited on the metabolically-enhanced frameshift strain, YG1041. The majority of compounds examined, however, were observed to be more potent on the metabolically-enhanced base-pair substitution strain, YG1042. For example, in the absence of S9, this study observed a mutagenic potency for TNT of 98.9 revertants/μg compound on YG1042 versus 13.9 revertants/μg compound on YG1041. The observation of higher base-pair substitution activity is consistent with other studies that assessed the mutagenic potential of explosive compounds on both frameshift and base-pair substitution strains [32, 33, 72, 73, 76, 77]. For example, in the absence of S9, a study conducted by George et al. (2001) [77] observed a mutagenic potency for TNT of 1.2 revertants/μg compound on TA98 versus 3.4 revertants/μg compound on TA100 using the plate incorporation version of the Ames assay. Using the microsuspension version, this trend was even more noticeable, and mutagenic potencies of 1.22 and 10.35 revertants/μg compound were observed for TA98 and TA100, respectively.

Higher base-pair substitution activity was observed for the majority of compounds examined in this thesis, as compared to frameshift activity. This trend could be explained by the fact that these nitro-substituted compounds are methylated (i.e., a single methyl group attached to the aromatic ring). Several studies have demonstrated that methyl-substituted nitroaromatic compounds elicit markedly higher mutagenic activity in TA100 as compared to TA98 [78-80]. This idea is further supported by the fact that the two explosive compounds examined in this thesis that elicited the strongest activity on the frameshift strains (i.e., 1,3,5-TNB and 3,5-DNA) are not methylated. A similar trend of strong base-pair substitution activity for contaminated soil samples containing di- and trinitrotoluene explosive residues was also noted in a review by White and Claxton (2004) [2].

Effect of exogenous S9 metabolic activation

With respect to the compounds examined in this thesis, there are two important bacterial biochemical processes. Direct-acting mutagenicity is induced by nitroreduction and subsequent acetylation of a substance via nitroreductase and *O*-acetyltransferase, respectively. These enzymes are endogenous to *Salmonella*. *Salmonella* are limited, however, in terms of their ability to metabolize substances that ordinarily require mammalian enzymes such as the CYP450 mixed function oxidases. Thus, indirect-acting mutagenicity occurs in the presence of an exogenous S9 metabolic activation system. Oxidation of the amino group on a substance can be catalyzed by CYP450 enzymes, and this is followed by subsequent acetylation of the resulting arylhydroxylamine via *O*-acetyltransferase.

The mutagenicity of nitroaromatic compounds depends on reduction of the nitro moiety by nitroreductase, and acetylation of the resulting arylhydroxylamine intermediate by *O*-acetyltransferase, to generate an electrophilic DNA-reactive nitrenium or carbenium [37-39, 41]. Thus nitroaromatics are considered to be direct-acting mutagens as they only require endogenous enzymes to be converted to their active form. With the exception of RDX and HMX, all compounds examined in this thesis have nitroaromatic properties. Not surprisingly, these nitroaromatics elicited the highest mutagenic responses without the addition of S9.

In contrast, aromatic amines generally require the addition of S9 to catalyze the oxidation of the amino group by CYP450 enzymes [38, 42]. Acetylation of the resulting arylhydroxylamine intermediate by *O*-acetyltransferase generates the electrophilic nitrenium or carbenium [37, 39]. Thus aromatic amines are considered to be indirect-acting mutagens as they require exogenous enzymes to be converted to their active form. Several of the compounds examined in this thesis, namely tetryl, 3,5-DNA, 2a-DNT, 4a-DNT, 2,4-DANT and 2,6-DANT, possess the structural properties of an aromatic amine. These compounds did elicit a mutagenic response with the addition of S9; however, the S9-mediated response was less potent relative to the direct-acting response, and there are several explanations for this phenomenon. First, the presence of relatively high concentrations of protein may create a physical interference that could restrict contact between the bacterial cell and the compound, entry into the cell, and eventual formation of the electrophilic DNA-reactive intermediate. Such an effect was observed in a study conducted by Courtois et al. (1992) [81] who noted a decrease in the genotoxicity of pro-mutagens as a function of increasing protein content in the S9 metabolic activation

mixture. Second, the CYP450 enzymes (specifically CYP1A1) can oxidize the aromatic ring of a substance. This has been shown to increase detoxification and reduce mutagenic activity [82, 83]. Third, it is possible that the CYP450 enzymes are not able to oxidize the amino groups on these compounds. Certainly the tertiary amine in tetryl cannot be readily converted to a hydroxylamine. Moreover, given the efficiency at which nitroreductase in *Salmonella* is able to reduce nitro moieties [43, 45] it is not surprising that the mutagenic responses for the six aromatic amines (which also possess properties of a nitroaromatic) were consistently higher without the addition of S9.

Some of the compounds possessing solely nitroaromatic properties, namely 1,3,5-TNB and TNT, also exhibited potent mutagenic responses with the addition of S9. For TNT, this mutagenic response may, in part, be due to the presence of the methyl group. When tested with the addition of S9, methyl groups attached to nitroaromatics are known to be oxidised to benzyl alcohols and further metabolized to sulfate compounds in *Salmonella*. These sulfates are strong electrophilic DNA-reactive intermediates [80]. The S9-mediated response, however, again does not appear to be as potent as the direct-acting response. This is likely due to the aforementioned high protein content of the S9 metabolic activation mixture physically interfering with exposure, ring oxidation leading to detoxification, or an inability to oxidize the amino groups.

Parent versus metabolically-enhanced strains

Previous studies have shown that nitroreduction and acetyl group transfer are the rate-limiting factors controlling the metabolism of nitroaromatics and aromatic amines in strains TA98 and TA100 [37]. These endogenous enzymes are critical to the metabolic

transformation of nitroaromatics and aromatic amines into their active form, and therefore limit the detection of mutagenic activity. Strains YG1041 and YG1042 harbour the plasmid pYG233, which contains the *cnr* and *OAT* genes that encode the classical nitroreductase and *O*-acetyltransferase enzymes, respectively. Thus, over-expression of these genes in YG1041 and YG1042 confers extraordinary sensitivity to chemicals that are substrates for these enzymes [37].

Dependence of nitroaromatic mutagenicity on nitro-reduction and subsequent acetylation is exemplified by the significantly increased mutagenic responses observed in the nitroreductase/*O*-acetyltransferase-enhanced strains YG1041 and YG1042, relative to the parent strains. The most significant difference in mutagenic potency for a nitroaromatic compound examined in this thesis, without the addition of S9, was observed for 1,3,5-TNB, which elicited mutagenic potencies of 12.8 and 15.5 revertants/μg compound on TA98 and TA100, respectively, and potencies of 272.2 and 98.1 revertants/μg compound on YG1041 and YG1042, respectively.

Dependence of aromatic amine mutagenicity on oxidation and subsequent acetylation is exemplified by the significantly increased mutagenic responses observed in the *O*-acetyltransferase-enhanced strains YG1041 and YG1042, as compared to the parent strains. The most significant difference in mutagenic potency for an aromatic amine compound examined in this thesis, with the addition of S9, was observed for tetryl, which elicited mutagenic potencies of 1.33 and 7.83 revertants/μg compound on TA98 and TA100, respectively, and potencies of 9.65 and 51.8 revertants/μg compound on YG1041 and YG1042, respectively.

Nitroaromatics, Aromatic Amines and Heterocyclic Amines

Observed trends in mutagenic activity

An interesting trend observed in this study indicates that the four dinitrotoluene compounds (i.e., 2,4-DNT, 2,6-DNT, 2a-DNT and 4a-DNT), as well as the two diamino-nitrotoluenes (i.e., 2,4-DANT and 2,6-DANT) yield the lowest mutagenic potency values on all four *Salmonella* strains, despite the fact that they are classified as nitroaromatics and/or aromatic amines that are similar in structure to the potent *Salmonella* mutagens 1,3,5-TNB and tetryl. This is in agreement with other studies that have examined the response of these compounds using the Ames assay [19, 31-33, 73], and there are several possible explanations for the trend. These include competing detoxification reactions leading to the formation of excretable metabolites, steric factors affecting binding of these compounds to the activating enzymes, electronic, steric or hydrophilic/hydrophobic factors affecting transport of these compounds and/or active metabolites to the genetic material, and shape and positioning of the active metabolite for reaction with the DNA [42]. 2a-DNT, 4a-DNT, 2,4-DANT and 2,6-DANT are not used as primary explosives themselves, but rather are created as a result of the degradation of primary explosive compounds such as TNT [11]. 2,4-DNT and 2,6-DNT are occasionally employed in specific munition formulations and are well-known manufacturing impurities, but have also been observed as degradation products of TNT [10-12]. Because TNT is known to rapidly degrade to form these metabolites (2a-DNT and 4a-DNT being energetically favoured [12]), the degradation process could be expected to decrease the mutagenic activity of contaminated soil and water samples via conversion of the mutagenic parent compound to the less mutagenic or benign metabolites. This may accordingly decrease

the human health hazard associated with exposure to the dinitrotoluene and diamino-nitrotoluene compounds in contaminated environmental media such as soil.

Another interesting observation is the fact that the two heterocyclic amines examined in this thesis, RDX and HMX, did not elicit a significant mutagenic response on any of the four strains. Thus, these compounds do not appear to be DNA-reactive and likely constitute a negligible human health hazard relative to other explosives that yielded a significant positive response (e.g., TNT, tetryl, etc.). This is indeed fortunate because RDX and HMX are rapidly becoming the most highly used explosive substances for both military and civilian applications [9, 10].

4.1.2 FE1 Muta^{TM}Mouse *in vitro* Transgene Mutation Assay Results

Although the *Salmonella* mutagenicity assay is useful as a screening tool for identifying mutagenic substances that may pose a hazard to mammalian receptors (e.g., humans), extrapolation of results from the bacterial assay to humans is difficult and tenuous. Despite the fact that addition of an exogenous S9 metabolic activation system allows *in vitro* simulation of mammalian metabolism, there remain other important physiological differences between mammalian and bacterial cells that govern the manifestation of a mutagenic effect (as will be discussed below). Thus, the *in vitro* Muta^{TM}Mouse assay, which utilizes the FE1 cell line, was employed to confirm the ability of the explosive compounds to induce mutations in a metabolically-competent mammalian cell.

Only four of the twelve compounds elicited significant positive responses in the Muta^{TM}Mouse assay. Two of these compounds, HMX and RDX, failed to elicit positive responses in the *Salmonella* mutagenicity assay. HMX exhibited mutagenic activity both

with and without the addition of S9, and RDX elicited a positive response only in the absence of S9; however, neither of these compounds exhibited clear concentration-related trends. When tested with the mammalian cell line, TNT elicited a positive response without the addition of S9. Although this does mirror the potent direct-acting mutagenic activity observed for the *Salmonella* mutagenicity assay, this compound also failed to exhibit a clear concentration-related trend. Lastly, when tested with the mammalian cell line, 1,3,5-TNB exhibited mutagenic activity with the addition of S9. Mutagenic activity was observed for 1,3,5-TNB on all four *Salmonella* strains in the presence of S9; however, the highest responses were observed without the addition of S9. Although there does appear to be some evidence of a concentration-related increase in response, only a single concentration yielded a significant increase above the control. It is possible that at higher concentrations, 1,3,5-TNB would induce a more distinguishable trend.

4.1.3 Comparative Analysis of the *Salmonella* Mutagenicity and Muta^{TM}Mouse Assay Results

The apparent inconsistency between the *Salmonella* mutagenicity and Muta^{TM}Mouse assay results illustrates the drawback, with regards to human hazard, of using only a bacterial assay to identify mutagenic hazard. Several structural and metabolic features of human or other mammalian cell lines cannot be approximated using bacterial mutagenicity assays. For example, bacterial nitroreductases are remarkably effective at reducing nitro moieties in comparison with mammalian cells. This is due to the fact that bacteria contain Type I nitroreductase enzymes that are oxygen-insensitive. The Type I group catalyzes a two-electron reduction of the nitro moiety to produce an arylhydroxylamine intermediate. This intermediate can subsequently be acetylated, via *O*-acetyltransferase, to generate an electrophilic DNA-reactive intermediate. Mammalian

cells also contain these Type I nitroreductase enzymes, however they further utilize Type II nitroreductases that are oxygen-sensitive. The Type II group catalyses a one-electron reduction of the nitro moiety to produce a nitroaromatic anion free radical. This radical can rapidly react with oxygen to regenerate the parent nitroaromatic compound [43, 44]. As a result, mammalian cells likely have great difficulty metabolizing the nitro moiety of the explosive compounds examined in this thesis, preventing the creation of an arylhydroxylamine intermediate that is essential for mutagenic activity.

Furthermore, mammalian metabolism is highly complex when compared with bacterial metabolism. For example, nitroreduction in mammalian cells has been associated with several enzymes including xanthine oxidase, DT-diaphorase, aldehyde oxidase, microsomal NADPH:P450 reductase and cytosolic NAD(P)H:quinine oxidoreductase [45]. It is a multi-step process that can involve numerous enzymes and intermediates.

4.2 Characterization of Soil Samples

The explosive compounds found to be present in the three soil samples examined in this thesis (refer to Section 3.3.2 in Results) are expected based on the function and use of the sites from which they were collected. HMX and TNT were present in samples PET 1 and PET 2, and RDX was present in PET 2. The TNT concentration was three orders of magnitude less than that of HMX, and RDX was present at one order of magnitude less than that of TNT. This ratio of explosive compounds is characteristic of anti-tank firing positions and target areas such as PET 1 and PET 2. A concentration of 2,4-DNT similar

to that of TNT was detected in sample PET 3. This is typical of what would be expected for a small arms munitions firing point such as PET 3 [57].

Non-cancer or threshold-based soil quality guidelines for human health risk at military training areas were established by the National Research Council Canada (2005) [84]. These guidelines state limits of 9, 20, 52 and 915 ppm for TNT, 2,4-DNT, RDX and HMX, respectively. Levels of TNT present in PET 1 and PET 2, RDX present in PET 2, and HMX and 2,4-DNT present in PET 3, do not exceed the values stated in these guidelines. However, HMX was present at levels of 1135.5 and 1145.5 ppm in PET 1 and PET 2, respectively. These levels do exceed the values stated in the guidelines and thus these soils may constitute a risk to human health.

With regards to the comparison of other explosives-contaminated sites, significant concentrations of the same explosive compounds identified in the soil samples obtained from CFB Petawawa were also observed at the Camp Edwards military base. The mean concentrations of RDX, HMX, TNT and 2,4-DNT determined to be present in Camp Edwards contaminated soil samples were approximately 9273, 1710, 1586 and 98 μg/kg, respectively [17]. These concentrations were generally observed to be considerably lower than levels detected for the three soil samples examined in this thesis. For example, levels of HMX present in PET 1 and PET 2, as well as levels of 2,4-DNT present in PET 3, were three and two orders of magnitude higher, respectively, than the concentrations observed in the Camp Edwards soil samples.

Contrary to the observations above, a munitions site that was included in the review by White and Claxton (2004) [2] cited concentrations of TNT and DNT isomers at 156 and 6.6 ppm, respectively, and an additional site noted concentrations of TNT at 12

200 ppm. Thus, the levels of explosive compounds detected at those sites are far higher than the levels detected in the three soil samples examined in this thesis.

4.3 Mutagenic Activity of Soil Extracts

4.3.1 Ames/*Salmonella* Reverse Mutation Assay Results

The three soil extracts exhibited positive mutagenic responses in all four *Salmonella* strains, both with and without exogenous S9 metabolic activation. The only exception was TA100 with S9. This suggests the presence of a variety of compounds in these contaminated soil samples, including both direct- and indirect-acting frameshift and base-pair substitution mutagens. For all soil extracts, however, the strongest responses were observed on the frameshift strains, TA98 and YG1041, as compared with the base-pair substitution strains, TA100 and YG1042. The samples also elicited the strongest activity in the absence of S9. Furthermore, higher mutagenic activity was observed on the metabolically-enhanced strains, YG1041 and YG1042, as compared to the parent strains, TA98 and TA100.

The highest overall response for all soil extracts was observed on the metabolically-enhanced frameshift strain YG1041, without S9. This suggests a predominance of direct-acting mutagens, capable of eliciting frameshift mutations in the presence of nitroreductase and *O*-acetyltransferase. More specifically, this response is strongly indicative of nitroaromatics.

Although the highest mutagenic responses were observed without exogenous metabolic activation, the three soil samples also elicited significant activity in the presence of S9. The highest S9-activated mutagenic response was again observed on the

metabolically-enhanced frameshift strain YG1041. This activity may indicate the presence of aromatic amines. Several other factors may also play a role, however, in the observed S9-mediated mutagenic activity. More specifically, in the reduction of mutagenicity relative to the response observed without S9. These include: interference of proteins with bacterial exposure, aromatic ring oxidation leading to detoxification, and/or the possibility that CYP450 enzymes are unable to oxidize the amino group on these compounds.

Published versus Observed Mutagenic Activity of Soil Extracts

There is little published research regarding the exact composition and corresponding mutagenic activity of soils contaminated with explosive compounds. A few examples, however, were noted in the review by White and Claxton (2004) [2]. This review examined three different classes of soil sites: rural, urban/suburban and industrial. The authors found that on *Salmonella* strain TA98 without S9, nineteen industrial sites were considered to be positive outliers of the distribution of mutagenic activity; yielding extremely potent extracts (e.g., above 30 revertants/mg dry soil equivalents). Eighteen of the nineteen outliers were identified as soils contaminated with munitions and explosive wastes. On the same strain with S9, approximately twenty-two industrial sites were considered to be positive outliers, with mutagenic potency values ranging from 56.9 to 376 revertants/mg dry soil equivalents. Several of these outliers were also samples obtained from sites heavily contaminated with explosives. Similarly, on TA100 without S9, two industrial sites were considered to be positive outliers, with potency values ranging from 6 to 259 revertants/mg dry soil equivalents. Both of these outliers were soils

contaminated with explosive residues or munitions wastes. On the same strain with S9, six industrial sites were considered to be positive outliers, with potency values ranging from 87 to 925 revertants/mg dry soil equivalents. One of these outliers was an explosive contaminated soil.

These potency values are far higher than the potencies elicited for the three soil extracts examined in this thesis, which ranged from 0.13 to 5.41 revertants/mg dry soil equivalents on TA98 without S9, 0.26 to 0.91 revertants/mg dry soil equivalents on TA98 with S9, and 0.47 to 0.75 revertants/mg dry soil equivalents on TA100 without S9. For example, when comparing the highest mutagenic response observed for the three soil extracts examined in this thesis (PET 2 on TA98 without S9) to the highest response observed for the thirty-one contaminated munitions sites analysed in the aforementioned review (for the same strain and S9 combination), the potency value elicited by PET 2 was determined to be greater than 50-fold lower than the contaminated munitions site reviewed in White and Claxton. In fact, PET 2 on TA98 without S9 only exhibited higher mutagenic activity than three of the thirty-one sites reviewed [84-86]. The exact nature of contamination at the sites described in White and Claxton, however, was often unknown, and many of the most mutagenic samples were obtained from heavily contaminated sites that may have far higher concentrations of explosives than the three samples currently being assessed. Furthermore, it is likely that, given the heavy contamination at these sites, there may be several classes of compounds other than explosives that are contributing to the high mutagenic potencies observed.

Although the mutagenic activity of the soil extracts examined in this thesis was significantly lower when compared to the activity of the majority of munitions sites

analysed in the review, the potency value elicited by PET 2 (on TA98 without S9) was determined to be 7-fold higher than the geometric mean of the potency values elicited by all industrial sites reviewed in White and Claxton. Thus, the mutagenic activity of the samples examined in this thesis is significantly higher when compared to the activity of the average industrial site; however, they appear to be below what may be regarded as typical for military sites heavily contaminated with explosives or explosive residues.

4.3.2 Observed versus Predicted *Salmonella* Mutagenic Activity

The predicted mutagenic potencies for each of the three soil samples examined in this thesis were determined, using an assumption of additivity, by summing the relative contributions of each explosive compound to the total mutagenic activity (refer to Section 2.5.6 in Materials and Methods). This was done for each *Salmonella* strain and S9 combination. The predicted mutagenic potency values were subsequently compared to the observed mutagenic activity to determine if the observed activity could be explained by the quantities of known mutagens in the soil samples. In the majority of cases, the predicted mutagenic potencies of the three soil samples, on all four strains, with and without S9, were far lower (i.e., up to four orders of magnitude) than the observed mutagenic activity. There are several possible explanations for this trend. First, because only the quantifiable explosive compounds were used in calculating the predicted mutagenic activity, it is not at all surprising that the predicted values are far lower than the observed, and it seems reasonable that the difference is related to the presence of hitherto unknown mutagenic compounds (e.g., explosive metabolites) in the complex organic extracts. Indeed, the HPLC chromatograms revealed peaks that could not be readily identified. The increased activity of the soil extracts on the metabolically-

enhanced strains YG1041 and YG1042, relative to the parent strains, suggests the presence of nitroaromatics and aromatic amines. It therefore seems reasonable that the compounds quantified by HPLC only constitute a fraction of the total mutagenic nitroaromatic and aromatic amine content in the complex soil extract. In addition, it should be noted that the profile of observed mutagenic activity is different from what would be expected if the quantified compounds were solely responsible for the effect. When tested as pure chemicals, the majority of explosive compounds identified in the soil samples elicited the highest mutagenic activity on the metabolically-enhanced, base-pair substitution strain YG1042, without S9. Although the three soil extracts examined also elicited the highest mutagenic activity without the addition of S9, the highest responses were observed on the metabolically-enhanced frameshift strain YG1041.

Alternatively, it is possible that the mutagenic activity of the complex soil extract cannot be predicted using an assumption of additivity. Because the observed activity far exceeds the predicted, based on the concentrations and mutagenicity of the identified components, it is possible that synergistic interactions between the putative mutagens contribute to the high level of observed activity. For example, Berthe-Corti et al. (1998) [84] suggested the possibility of a synergistic effect to explain the mutagenic activity of a nitroaromatic- and RDX-contaminated soil that could not be accounted for by the identified substances present in the complex sample. The extreme difference between the observed and the predicted activity (i.e., predicted is <1% of observed), however, suggests that it is unlikely that synergism can account for the differential. It is possible that a synergistic effect between the explosive compounds is occurring to some degree, but it is more likely that other compounds present in the sample are interacting, either

with each other or with the explosive compounds, and are responsible for altering the mutagenic activity. An example of this phenomenon was noted in White (2002) [87] and Kawalek and Andrews (1981) [88]. Both studies reported that various aromatic hydrocarbons (e.g., benzene and a variety of PAHs) significantly inhibited the mutagenic response of 2-aminoanthracene when tested using the SOS chromotest and Ames assays, respectively.

Despite the possibility of synergistic interactions, it should be noted that the use of an assumption of additivity is supported by regulatory agencies that routinely employ an additivity assumption to calculate the total toxicological risk posed by a chemical mixture [89-91]. For example, the U.S. EPA recommends that in the absence of a sufficiently similar mixture, the toxicity of each individual component should be evaluated, and the overall toxicity of the mixture should be assumed to be equivalent to the sum of the individual component toxicity values [90, 91]. The nature of the interactions between the compounds monitored in this study could be assessed by examining the mutagenic activity of reconstituted simple mixtures.

4.3.3 FE1 Muta^{TM}Mouse *in vitro* Transgene Mutation Assay Results

Although the results show that all three soil extracts elicited significant positive responses, as well as some evidence of a concentration-related trend for mutagenic activity (i.e., PET 1 and PET 3 without S9), the responses were weak. In addition, when comparing the patterns of mutagenic activity for the soil samples to those of several of the individual explosive compounds observed to be present, there are unexplained inconsistencies. For example, 1,3,5-TNB was detected in the PET 1 and PET 3 soil samples, and 1,3,5-TNB elicited a significant mutagenic response in the Muta^{TM}Mouse

assay only in the presence of S9. However, both PET 1 and PET 3 extracts elicited positive responses only in the absence of S9. RDX and TNT were detected in the PET 2 soil sample, and these compounds elicited a positive response in the Muta^{TM}Mouse assay only in the absence of S9. The PET 2 extract, however, only elicited a positive response in the presence of S9. Additionally, in cases where the profile of the mutagenic activity of the soil extracts is similar to that observed for the individual explosive compounds detected, the concentrations of the compounds were not sufficient to induce the observed mutagenic responses. In PET 1, for example, HMX and TNT were detected, and, when tested using the Muta^{TM}Mouse assay, were both found to exhibit mutagenic activity without the addition of S9; a similar trend to the soil extract. The concentrations of these two compounds in the soil extract, however, were far lower than that required to elicit a significant positive mutagenic response in the Muta^{TM}Mouse assay. Furthermore, there is inconsistency in the relative responses obtained for PET 1 and PET 2 extracts. PET 1 and PET 2 are quite similar in terms of the explosive compounds detected, and the concentrations observed (refer to Table 3.8 in Results). However, PET 1 was found to elicit a positive mutagenic response only in the absence of S9, and PET 2 was positive only in the presence of S9.

These inconsistencies suggest that compounds other than the detected explosives are, at least in part, responsible for the mutagenic activity of the soil extracts. This is consistent with earlier statements regarding the pattern of *Salmonella* mutagenicity assay results that also suggest the presence of hitherto unidentified nitroaromatics in the contaminated military soils. Nitroaromatics are known to be mutagenic in various transgenic rodent assays, including both the *in vitro* and *in vivo* versions of the

Muta^{TM}Mouse assay [68, 92]. Thus, it is likely that unidentified nitroaromatic contamination present in the soil samples is at least partially responsible for the mutagenic activity observed using this assay. It is also possible that the mutagenic activity observed for soil extracts PET 1 and PET 3 are, in part, due to the presence of PAHs. These two samples were found to exhibit mutagenic activity only in the absence of S9, and White et al. (2003) [46] noted that PAHs elicit a positive response in the Muta^{TM}Mouse FE1 cell assay.

4.3.4 Comparative Analysis of the *Salmonella* Mutagenicity and Muta^{TM}Mouse Assay Results

The results obtained for the soil extracts using the Muta^{TM}Mouse assay were consistent with the *Salmonella* mutagenicity assay in terms of indicating the likely presence of hitherto unidentified mutagenic compounds, including other nitroaromatics. There are, however, several instances that emphasize inconsistency between the two assays. For example, using the *Salmonella* mutagenicity assay, all three soil extracts elicited the strongest mutagenic activity without the addition of S9, and the highest response was observed for PET 2. Although the Muta^{TM}Mouse results for PET 1 and PET 3 showed a similar pattern, exhibiting mutagenic activity in the absence of S9, PET 2 only elicited a positive response in the presence of S9.

Although these observations are interesting, it should be noted that any comparisons between bacterial and mammalian mutagenicity results are tenuous given the profound mechanistic differences between the metabolism of compounds, such as nitroaromatics, in bacterial and mammalian cells. This is likely due to the aforementioned difference between bacterial and mammalian nitroreductases, and the complex multi-step metabolic process in mammalian relative to bacterial cells. Thus, extrapolation of the

results presented for the determination of human hazard should be executed with caution because the processes in mammalian cells that metabolize many of the compounds investigated are poorly understood.

4.4 Preliminary Cancer Risk Assessment

A preliminary quantitative risk assessment was conducted to estimate the excess lifetime cancer risk from oral exposure to specific explosive compounds (i.e., TNT, RDX and 2,4-DNT) present in the soil samples examined in this thesis, employing a daily soil ingestion rate of 265 mg/day and an exposure frequency and duration of 100 days/year for 38 years, to represent a maximal exposure scenario for a training instructor [52, 53]. The resulting excess lifetime cancer risk from exposure to current concentrations of the three explosive compounds is less than 1×10^{-5} (i.e., less than one in 100 000). At these concentrations, risk above 1×10^{-5} would require ingestion of more than 10 g/day of contaminated soil. Furthermore, because only specialized personnel would have contact with soils in the target areas, risk to the general military population is likely to be substantially lower. Nevertheless, it is important to note that such an analysis only includes the risk attributable to compounds that have been identified and quantified. Analysis of the *Salmonella* mutagenicity assay results suggests that the monitored compounds may only account for a small fraction of the total hazard. However, because of the extreme sensitivity of *Salmonella* to the effects of nitroaromatics and aromatic amines, any conclusions regarding carcinogenic hazard posed by contamination at military sites, which would be attributable to a mutagenic mode of action, would need to be validated by further testing in mammalian systems, both *in vitro* and *in vivo*. The

mammalian results presented in this thesis, indeed would suggest negligible hazard and risk because, under the conditions of the assay, a clear pattern of mutagenic activity was not detected. However, *in vitro*, as well as *in vivo*, mutagenic hazard cannot be ruled out at this time, particularly in light of the fact that other researchers, including Dillon et al. (1994) [93], have clearly demonstrated that intestinal bacteria can metabolize and activate similar nitroaromatics.

5.0 CONCLUSION

This thesis evaluated the mutagenic activity of twelve explosive compounds using the *Salmonella* mutagenicity and Muta^{TM}Mouse assays. The analysis of mutagenic activity using four *S. typhimurium* strains indicates that ten of the twelve explosive compounds, all possessing properties of a nitroaromatic and/or an aromatic amine, elicited significant positive responses. Only HMX and RDX, the two heterocyclic compounds, were negative on all four *Salmonella* strains. Tetryl, 1,3,5-TNB and 3,5-DNA, as well as TNT, consistently induced the strongest activity, while the six dinitrotoluene and diamino-nitrotoluene compounds exhibited the weakest. The two highest mutagenic potency values were observed on the frameshift strain YG1041; however, the majority of compounds examined were observed to be more potent on the base-pair substitution strain YG1042. The ten mutagenic compounds were consistently observed to elicit higher responses without the addition of exogenous S9 metabolic activation. Furthermore, the highest activity was observed on the metabolically-enhanced strains YG1041 and YG1042, when compared to the corresponding parent strains TA98 and TA100. The assessment of mutagenic activity using pulmonary FE1 epithelial cells from the transgenic Muta^{TM}Mouse indicates that only 1,3,5-TNB, TNT, RDX and HMX elicited significant positive responses; however, the responses fail to show a clear mutagenic trend. The differential response between the bacterial and mammalian assays is likely due to differences in the metabolic capacity of bacterial versus mammalian cells. More specifically, bacterial cells contain Type I nitroreductase enzymes, whereas mammalian cells contain Type I and Type II nitroreductases; having a significant impact on the metabolism of compounds such as nitroaromatics. Furthermore, mammalian

metabolism of compounds such as nitroaromatics and aromatic amines (i.e., the compounds depicted in Figures 1.1 and 1.2) is complex and relatively poorly understood.

This thesis further evaluated the mutagenic activity of three soil extracts using the same two assays. The analysis of mutagenic activity using the *Salmonella* mutagenicity assay revealed that all extracts elicited significant positive responses. PET 2 (i.e., from an anti-tank target area) induced the strongest activity on all four strains, while PET 3 (i.e., from a small arms munitions firing point) exhibited the weakest, with and without S9. The highest potency values were observed on the frameshift strain YG1041, and were consistently observed to elicit higher responses without the addition of S9. Furthermore, the highest activity was observed on the metabolically-enhanced strains, as compared to the parent strains. When comparing the predicted *Salmonella* mutagenic activity to the observed, the results suggest that there are unidentified compounds present in these soil samples that are, at least in part, responsible for the mutagenic activity. The assessment of mutagenic activity using the Muta^{TM}Mouse assay revealed that all three soil extracts elicited significant positive responses (PET 1 [i.e., from an anti-tank firing position] and PET 3 without S9, and PET 2 with S9) at one or more concentrations. There appears to be some evidence of a concentration-related trend for mutagenic activity, and thus it seems likely that at higher concentrations these extracts would induce higher *lacZ* mutant frequency values. However, when comparing the patterns of mutagenic activity for the soil samples to those of the individual explosive compounds observed to be present, there are unexplained inconsistencies. This is consistent with results obtained using the *Salmonella* mutagenicity assay, and suggests that compounds other than the detected explosives are present and are, at least in part, responsible for the mutagenic activity.

However, it should be noted that any comparisons between bacterial and mammalian mutagenicity results are tenuous given the profound mechanistic differences between the metabolism of various compounds such as nitroaromatics and aromatic amines in bacterial and mammalian cells.

The mammalian cell results suggest that the risk to human health from exposure to explosive compounds is limited due to the inability of mammalian cells to metabolize and activate potential mutagens. However, the complexity of mammalian metabolism prohibits any definitive conclusions about actual mutagenic and carcinogenic hazard. Any quantitative assessments of hazard and/or risk would clearly require additional information regarding mammalian metabolism of these compounds. In this regard, it would be advisable to follow this work with several projects, including *in vitro* exposure of FE1 cells following pre-treatment with caecal bacteria (similar to Dillon et al. 1994 [93]), and chronic *in vivo* studies in the Muta^{TM}Mouse to quantify target tissue exposure and effect.

6.0 REFERENCES

[1] Treasury Board of Canada Secretariat. 2010. The Federal Contaminated Sites and Solid Waste Landfills Inventory. Online. Internet. [cited 2010 Dec 22]. Available from: http://www.tbs-sct.gc.ca/fcsi-rscf/home-accueil-eng.aspx

[2] White, P.A. and L. Claxton. 2004. Mutagens in contaminated soil: a review. *Mutat. Res.* 567(2-3): 227-345.

[3] Ad Hoc International Working Group on Contaminated Land. 2002. "Report of the 5th Meeting, 16-18.9.2001, Geneva, Switzerland" National Research Council Canada, Montreal, QC.

[4] Bhushan, B., A. Halasz and J. Hawari. 2006. Effects of iron(III), humic acids and anthraquinone-2,6-disulfonate on biodegradation of cyclic nitramines by *Clostridium* sp. EDB2. *J. Appl. Microbiol.* 100(3): 555-563.

[5] Bearden, D.M. 2008. "CRS Report for Congress – Military Base Closures: Cleanup of Contaminated Properties for Civilian Reuse," Order Code RS22065, Congressional Research Service, Washington, DC.

[6] Prokop, G., M. Schamann and I. Edelgaard. 2000. "Management of contaminated sites in Western Europe," Topic report No 13/1999, European Environment Agency, Copenhagen, DK.

[7] Ad Hoc International Working Group on Contaminated Land. 2000. "Management of Contaminated Sites and Land in Central and Eastern Europe," Danish Cooperation for Environment in Eastern Europe, Ministry of Environment and Energy, Copenhagen, DK.

[8] Global Security. 2006. Explosives – Nitroaromatics. Online. Internet. [cited 2010 Dec 29]. Available from: http://www.globalsecurity.org/military/systems/munitions/explosives-nitroaromatics.htm

[9] Global Security. 2006. Explosives – Nitramines. Online. Internet. [cited 2010 Dec 29]. Available from: http://www.globalsecurity.org/military/systems/munitions/explosives-nitramines.htm

[10] Thiboutot, S., G. Ampleman and A.D. Hewitt. 2002. "Guide for characterization of sites contaminated with energetic materials," ERDC/CRREL TR-02-1, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

[11] Clausen, J.L., N. Korte, M. Dodson, J. Robb and S. Rieven. 2006. "Conceptual Model for the Transport of Energetic Residues from Surface Soil to Groundwater by Range Activities," ERDC/CRREL TR-06-18, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

[12] Brannon, J.M. and J. Pennington. 2002. “Environmental Fate and Transport Process Descriptors for Explosives,” ERDC/EL TR-02-10, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

[13] ATSDR. 1995. “Toxicological profile for tetryl,” U.S. Agency for Toxic Substances and Disease Registry, Department of Health and Human Services, Atlanta, GA.

[14] Esteve-Núñez, A., A. Caballero and J.L. Ramos. 2001. Biological Degradation of 2,4,6-Trinitrotoluene. *Microbiol. Mol. Biol. Rev.* 65(3): 335-352.

[15] Lai, D.Y., Y. Woo, M.F. Argus and J.C. Arcos. 1996. Cancer Risk Reduction Through Mechanism-Based Molecular Design of Chemicals. *In*: DeVito, S.C. and R.L. Garrett (eds.) *Designing Safer Chemicals*. American Chemical Society, Washington, Vol. 640, pp. 62-73.

[16] Brochu, S. 2008. “Environmental Assessment of 100 Years of Military Training at Canadian Force Base Petawawa,” DRDC Valcartier TR-2008-118, Defence R&D Canada – Valcartier, Valcartier, QC.

[17] Pennington, J.C., T.F. Jenkins, G. Ampleman, S. Thiboutot, A.D. Hewitt, S. Brochu, J. Robb, E. Diaz, J. Lewis, H. Colby, R. Martel, K. Poe, K. Groff, K.L. Bjella, C.A. Ramsey, C.A. Hayes, S. Yost, A. Marois, A. Gagnon, B. Silverblatt, T. Crutcher, K. Harriz, K. Heisen, S.R. Bigl, T.E. Berry, Jr., J. Muzzin, D.J. Lambert, M.J. Bishop, B. Rice, M. Wojtas, M.E. Walsh, M.R. Walsh and S. Taylor. 2006. “Distribution and Fate of Energetics on DoD Test and Training Ranges: Interim Report 6,” ERDC TR-06-12, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

[18] U.S. EPA. 2004. Overview of Environmental Issues Associated with Residues of Energetic Materials. United States Environmental Protection Agency, Office of Superfund Remediation and Technology Innovation. Online. Internet. [cited 2011 Jan 31]. Available from: http://www.clu-in.org/characterization/technologies/exp.cfm

[19] ATSDR. 1995. “Toxicological profile for 2,4,6-trinitrotoluene,” U.S. Agency for Toxic Substances and Disease Registry, Department of Health and Human Services, Atlanta, GA.

[20] ATSDR. 2010. “Toxicological profile for RDX,” U.S. Agency for Toxic Substances and Disease Registry, Department of Health and Human Services, Atlanta, GA.

[21] ATSDR. 1997. “Toxicological profile for HMX,” U.S. Agency for Toxic Substances and Disease Registry, Department of Health and Human Services, Atlanta, GA.

[22] ATSDR. 1995. “Toxicological profile for 1,3-dinitrobenzene and 1,3,5-trinitrobenzene,” U.S. Agency for Toxic Substances and Disease Registry, Department of Health and Human Services, Atlanta, GA.

[23] ATSDR. 1998. “Toxicological profile for 2,4- and 2,6-dinitrotoluene,” U.S. Agency for Toxic Substances and Disease Registry, Department of Health and Human Services, Atlanta, GA.

[24] U.S. EPA. 2008. “Drinking Water Health Advisory for 2,4-Dinitrotoluene and 2,6-Dinitrotoluene,” United States Environmental Protection Agency, Office of Science and Technology; Office of Water, Washington, DC.

[25] Hanahan, D. and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell* 100(1): 57-70.

[26] Environment Canada. 2007. Existing Substances Evaluation – Domestic Substances List Categorization and Screening Program. Online. Internet. [cited 2011 May 7]. Available from: http://www.ec.gc.ca/substances/ese/eng/dsl/dslprog.cfm

[27] IARC. 1996. “Printing Processes and Printing Inks, Carbon Black and Some Nitro Compounds. 2,4,6-trinitrotoluene,” IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. International Agency for Research on Cancer, World Health Organization, Lyon, France. Vol. 65, pp. 449-475.

[28] IARC. 1996. “Printing Processes and Printing Inks, Carbon Black and Some Nitro Compounds. 2,4-dinitrotoluene, 2,6-dinitrotoluene and 3,5-dinitrotoluene,” IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. International Agency for Research on Cancer, World Health Organization, Lyon, France. Vol. 65, pp. 309-368.

[29] European Commission. 2008. “European Union Risk Assessment Report. 2,4-dinitrotoluene,” European Commission, Joint Research Centre, Institute for Health and Consumer Protection, Luxembourg.

[30] ESIS. European Chemical Substances Information System [database on the Internet]. C1995-2009. European Chemicals Bureau (ECB). [cited 2011 Feb 14]. Available from: http://ecb.jrc.it/esis/

[31] Won, W.D., L.H. DiSalvo and J. Ng. 1976. Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Appl. Environ. Microbiol.* 31(4): 576-580.

[32] Spanggord, R.J., K.E. Mortelmans, A.F. Griffin and V.F. Simmon. 1982. Mutagenicity in *Salmonella typhimurium* and Structure-Activity Relationships of Wastewater Components Emanating From the Manufacture of Trinitrotoluene. *Environ. Mutagen.* 4(2): 163-179.

[33] Tan, E.L., C.H. Ho, W.H. Griest and R.L. Tyndall. 1992. Mutagenicity of trinitrotoluene and its metabolites formed during composting. *J. Toxicol. Environ. Health* 36(3): 165-175.

[34] Mortelmans, K. and E. Zeiger. 2000. The Ames *Salmonella*/microsome mutagenicity assay. *Mutat. Res.* 455(1-2): 29-60.

[35] Maron, D.M. and B.N. Ames. 1983. Revised methods for the Salmonella mutagenicity test. *Mutat. Res.* 113(3-4): 173-215.

[36] Carroll, C.C., D. Warnakulasuriyarachchi, M.R. Nokhbeh and I.B. Lambert. 2002. *Salmonella typhimurium* mutagenicity tester strains that overexpress oxygen-insensitive nitroreductases *nfsA* and *nfsB*. *Mutat. Res.* 501(1-2): 79-98.

[37] Hagiwara, Y., M. Watanabe, Y. Oda, T. Sofuni and T. Nohmi. 1993. Specificity and sensitivity of *Salmonella typhimurium* YG1041 and YG1042 strains possessing elevated levels of both nitroreductase and acetyltransferase activity. *Mutat. Res.* 291(3): 171-180.

[38] Debnath, A.K., G. Debnath, A.J. Shusterman and C. Hansch. 1992. A QSAR Investigation of the Role of Hydrophobicity in Regulating Mutagenicity in the Ames Test: 1. Mutagenicity of Aromatic and Heteroaromatic Amines in *Salmonella typhimurium* TA98 and TA100. *Environ. Mol. Mutagen.* 19(1): 37-52.

[39] Watanabe, M., M. Ishidate and T. Nohmi. 1990. Sensitive method for the detection of mutagenic nitroarenes and aromatic amines: new derivatives of *Salmonella typhimurium* tester strains possessing elevated *O*-acetyltransferase levels. *Mutat. Res.* 234(5): 337-348.

[40] Lemieux, C. 2006. Evaluating the Mutagenic Activities of Complex PAH Mixtures in Soil. Master of Science thesis, Carleton University, Ottawa, ON.

[41] Watanabe, M., M. Ishidate and T. Nohmi. 1989. A sensitive method for the detection of mutagenic nitroarenes: construction of nitroreductase-overproducing derivatives of *Salmonella typhimurium* strains TA98 and TA100. *Mutat. Res.* 216(4): 211-220.

[42] Hatch, F.T., M.G. Knize and J.S. Felton. 1991. Quanititative Structure-Activity Relationships of Heterocyclic Amine Mutagens Formed During the Cooking of Food. *Environ. Mol. Mutagen.* 17(1): 4-19.

[43] Chen, G., I.B. Lambert, G.R. Douglas and P.A. White. 2005. Assessment of 3-nitrobenzanthrone reductase activity in mammalian tissues by normal-phase HPLC with fluorescence detection. *J. Chrom. B* 824(1-2): 229-237.

[44] Peterson, F.J., R.P. Mason, J. Hovsepian and J.L. Holtzman. 1979. Oxygen-sensitive and -insensitive Nitroreduction by *Escherichia coli* and Rat Hepatic Microsomes. *J. Biol. Chem.* 254(10): 4009-4014.

[45] Chen, G., J. Gingerich, L. Soper, G.R. Douglas and P.A. White. 2008. Tissue-Specific Metabolic Activation and Mutagenicity of 3-Nitrobenzanthrone in Muta^{TM}Mouse. *Environ. Mol. Mutagen.* 49(8): 602-613.

[46] White, P.A., G.R. Douglas, J. Gingerich, C. Parfett, P. Shwed, V. Seligy, L. Soper, L. Berndt, J. Bayley, S. Wagner, K. Pound and D. Blakey. 2003. Development and characterization of a stable epithelial cell line from Muta^{TM}Mouse lung. *Environ. Mol. Mutagen.* 42(3): 166-184.

[47] Vijg, J. and G.R. Douglas. 1996. Bacteriophage lambda and plasmid *lacZ* transgenic mice for studying mutations *in vivo*. *In*: Pfeifer, G. (ed.) *Technologies for Detection of DNA Damage and Mutations*. Plenum Press, New York, pp. 391-410.

[48] Gossen, J.A., W.J. de Leeuw, C.H. Tan, E.C. Zwarthoff, F. Berends, P.H. Lohman, D.L. Knook and J. Vijg. 1989. Efficient rescue of integrated shuttle vectors from transgenic mice: A model for studying mutations *in vivo*. *Proc. Natl. Acad. Sci. Unit. States Am.* 86(20): 7971-7975.

[49] Berndt-Weis, M.L., L.M. Kauri, A. Williams, P. White, G. Douglas and C. Yauk. 2009. Global transcriptional characterization of a mouse pulmonary epithelial cell line for use in genetic toxicology. *Toxicol. In Vitro* 23(5): 816-833.

[50] Stratagene. 2006. Transpack packaging extract for lambda transgenic shuttle vector recovery. Stratagene, LaJolla, CA.

[51] Gossen, J.A., A.C. Molijn, G.R. Douglas and J. Vijg. 1992. Application of galactose-sensitive *E. coli* strains as selective hosts for LacZ$^-$ plasmids. *Nucleic Acids Res.* 20(12): 3254.

[52] Robidoux, PY., B. Lachance, L. Didillon, F-O. Dion and G.I. Sunahara. 2005. "Development of Ecological Criteria and Human Health Criteria for Energetic Materials to Ensure Training Sustainability of Canadian Forces," NRC # 45936, National Research Council Canada, Montreal, QC.

[53] Hauschild, V. and J. Johnson. 2003. "Chemical Exposure Guidelines for Deployed Military Personnel," Reference Document 230, Version 1.3, U.S. Army Center for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, MD.

[54] Subcommittee on the Toxicological Risks to Deployed Military Personnel, Committee on Toxicology, Board of Environmental Studies and Toxicology, Division on Earth and Life Studies, National Research Council of the National Academies. 2004. "Review of the Army's Technical Guides on Assessing and Managing Chemical Hazards to Deployed Personnel," The National Academies Press, Washington, DC.

[55] U.S. EPA. 2006. "Method 8330B – Nitroaromatics, Nitramines, and Nitrate Esters by High Performance Liquid Chromatography (HPLC), Revision 2," United States Environmental Protection Agency, Washington, DC.

[56] Jenkins, T.F., S. Thiboutot, G. Ampleman, A.D. Hewitt, M.E. Walsh, T.A. Ranney, C.A. Ramsey, C.L. Grant, C.M. Collins, S. Brochu, S.R. Bigl and J.C. Pennington. 2005.

"Identity and Distribution of Residues of Energetic Compounds at Military Live-Fire Training Ranges," ERDC TR-05-10, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

[57] Sylvie Brochu, Defence Scientist, DRDC Valcartier, personal communication (2009).

[58] Thiboutot, S., G. Ampleman, S. Brochu, R. Martel, G. Sunahara, J. Hawari, S. Nicklin, A. Provatas, J.C. Pennington, T.F. Jenkins and A. Hewitt. 2003. "Protocol for Energetic Materials-Contaminated Sites Characterization – KTA 4-28 Final Report, Volume II," The Technical Cooperation Program, Subcommittee on Non-atomic Military Research and Development, WPN Group – Conventional Weapon Technology, Technical Panel 4 – Energetic Materials and Propulsion Technology.

[59] Crockett, A.B, H.D. Craig, T.F. Jenkins and W.E. Sisk. 1996. "Guidance for Characterizing Explosives Contaminated Soils: Sampling and Selecting On-site Analytical Methods," United States Environmental Protection Agency, Office of Research and Development, Washington, DC.

[60] Walsh, M.E., C.A. Ramsey and T.F. Jenkins. 2002. The effect of particle size reduction by grinding on subsampling variance for explosives residues in soil. *Chemosphere* 49(10): 1267-1273.

[61] U.S. EPA. 1994. "Method 8330 – Nitroaromatics and Nitramines by High Performance Liquid Chromatography (HPLC), Revision 0," United States Environmental Protection Agency, Washington, DC.

[62] Dionex. 1997. "Extraction of Explosives from Soils by Accelerated Solvent Extraction (ASE) – Application Note 328," Dionex Corporation, Sunnyvale, CA.

[63] Onuska, F.I., A.H. El-Shaarawi, K. Terry and E.M. Vieira. 2001. Optimization of Accelerated Solvent Extraction for the Analysis of Munitions Residues in Sediment Samples. *J. Microcolumn Sep.* 13(2): 54-61.

[64] Dionex. 2006. "Acclaim Explosives Product Manuel," Dionex Corporation, Sunnyvale, CA.

[65] White, P.A. and D.M. DeMarini. Water-borne mutagens. In: Möller, L., (ed.). Environmental Medicine. Stockholm: Karolinska Institute, 2000, pp. 102-123.

[66] Maron, D., J. Katzenellenbogen and B.N. Ames. 1981. Compatibility of organic solvents with the *Salmonella*/microsome test. *Mutat. Res.* 88(4): 343-350.

[67] Kim, B.S. and B.H. Margolin. 1999. Statistical methods for the Ames *Salmonella* assay: a review. *Mutat. Res.* 436(1): 113-122.

[68] Lambert, I.B., T.M. Singer, S.E. Boucher and G.R. Douglas. 2005. Detailed review of transgenic rodent mutation assays. *Mutat. Res.* 590(1-3): 1-280.

[69] Turesky, R.J., N.P. Lang, M.A. Butler, C.H. Teitel and F.F. Kadlubar. 1991. Metabolic activation of carcinogenic heterocyclic aromatic amines by human liver and colon. *Carcinogenesis* 12(10): 1839-1845.

[70] Gross, G.A. and A. Grüter. 1992. Quantitation of mutagenic/carcinogenic heterocyclic aromatic amines in food particles. *J. Chrom.* 592(1-2): 271-278.

[71] Lachance, B., P.Y. Robidoux, J. Hawari, G. Ampleman, S. Thiboutot and G.I. Sunahara. 1999. Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro. *Mutat. Res.* 444(1): 25-39.

[72] Whong, W., N.D. Speciner and G.S. Edwards. 1980. Mutagenic activity of tetryl, a nitroaromatic explosive, in three microbial test systems. *Toxicol. Lett.* 5(1): 11-17.

[73] Whong, W. and G.S. Edwards. 1984. Genotoxic activity of nitroaromatic explosives and related compounds in *Salmonella typhimurium*. *Mutat. Res.* 136(3): 209-215.

[74] McCann, J., E. Choi, E. Yamasaki and B.N. Ames. 1975. Detection of carcinogens as mutagens in the *Salmonella*/microsome test: Assay of 300 chemicals. *Proc. Natl. Acad. Sci.* 72(12): 5135-5139.

[75] Ames, B.N., E.G. Gurney, J.A. Miller and H. Bartsch. 1972. Carcinogens as Frameshift Mutagens: Metabolites and Derivatives of 2-Acetylaminofluorene and Other Aromatic Amine Carcinogens. *Proc. Nat. Acad. Sci.* 69(11): 3128-3132.

[76] Karamova, N.S., O.N. Il'inskaia and O.B. Ivanchenko. 1994. Mutagenic activity of 2,4,6-trinitrotoluene: the role of metabolizing enzymes. *Genetika* 30(7): 898-902.

[77] George, S.E., G. Huggins-Clark and L.R. Brooks. 2001. Use of a *Salmonella* microsuspension bioassay to detect the mutagenicity of munitions compounds at low concentrations. *Mutat. Res.* 490(1): 45-56.

[78] Ludolph, B., M. Klein, L. Erdinger and G. Boche. 2001. The effects of 4-'alkyl substituents on the mutagenicity activity of 4-amino- and 4-nitrostilbenes in *Salmonella typhimurium*. *Mutat. Res.* 491(1-2): 195-209.

[79] Hooberman, B.H., M.D. Brezzell, S.K. Das, Z. You and J.E. Sinsheimer. 1994. Substituent effects on the genotoxicity of 4-nitrostilbene derivatives. *Mutat. Res.* 341(1): 57-69.

[80] Klein, M., L. Erdinger and G. Boche. 2000. From mutagenic to non-mutagenic nitroarenes: effect of bulky alkyl substituents on the mutagenic activity of nitroaromatics

in *Salmonella typhimurium* Part II. Substituents far away from the nitro group. *Mutat. Res.* 467(1): 69-82.

[81] Courtois, Y.A., M.L. Pesle and B. Festy. 1992. Activation of pro-mutagens in complex mixtures by rat liver S9 systems. *Mutat. Res.* 276(1-2): 133-137.

[82] Zeiger, E., R.S. Chhabra and B.H. Margolin. 1979. Effects of the hepatic S9 fraction from Aroclor-1254-treated rats on the mutagenicity of benzo[*a*]pyrene and 2-aminoanthracene in the Salmonella/microsome assay. *Mutat. Res.* 64(6): 379-389.

[83] Jemnitz, K., Z. Veres, G. Torok, E. Toth and L. Vereczkey. 2004. Comparative study in the Ames test of benzo[*a*]pyrene and 2-aminoanthracene metabolic activation using rat hepatic S9 and hepatocytes following *in vivo* or *in vitro* induction. *Mutagenesis* 19(3): 245-250.

[84] Berthe-Corti, L., H. Jacobi, S. Kleihauer and I. Witte. 1998. Cytotoxicity and mutagenicity of a 2,4,6-trinitrotoluene (TNT) and hexogen contaminated soil in S. typhimurium and mammalian cells. *Chemosphere* 37(2): 209-218.

[85] Griest, W.H., A.J. Stewart, R.L. Tyndall, J.E. Caton, C.H. Ho, K.S. Ironside, W.M. Caldwell and E. Tan. 1993. Chemical and toxicological testing of composted explosives-contaminated soil. *Environ. Toxicol. Chem.* 12(6): 1105-1116.

[86] Donnelly, K.C., K.W. Brown, C.S. Giam and B.R. Scott. 1993. Acute and genetic toxicity of extracts of munitions wastewater contaminated soils. *Chemosphere* 27(8): 1439-1450.

[87] White, P.A. 2002. The genotoxicity of priority polycyclic aromatic hydrocarbons in complex mixtures. *Mutat. Res.* 515(1-2): 85-98.

[88] Kawalek, J.C. and A.W. Andrews. 1981. Effect of aromatic hydrocarbons on the metabolism of 2-aminoanthracene to mutagenic products in the Ames assay. *Carcinogenesis* 2(12): 1367-1369.

[89] Cassee, F.R., J.P. Groten, P.J. van Bladeren and V.J. Feron. 1998. Toxicological Evaluation and Risk Assessment of Chemical Mixtures. *Crit. Rev. Toxicol.* 28(1): 73-101.

[90] U.S. EPA. 1986. *Guidelines for the Health Risk Assessment of Chemical Mixtures*. Risk Assessment Forum, United States Environmental Protection Agency, Washington, DC.

[91] U.S. EPA. 2000. *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures*. Risk Assessment Forum, United States Environmental Protection Agency, Washington, DC.

[92] Kohara, A., T. Suzuki, M. Honma, T. Oomori, T. Ohwada and M. Hayashi. 2002. Dinitropyrenes induce gene mutations in multiple organs of the lambda/lacZ transgenic mouse (Muta Mouse). *Mutat. Res.* 515(1-2): 73-83.

[93] Dillon, D., R. Combes and E. Zeiger. 1994. Activation by caecal reduction of the azo dye D & C Red No. 9 to a bacterial mutagen. *Mutagenesis* 9(4): 295-299.

Appendix A

Compound	Strain	S9	Mutagenic Potency[a]	Standard Error	r^2	*p*-value	Mutation Ratio[b]	Result
2,4-DANT	TA98	N	1.05	0.07	0.91	<0.0001	9.05	Pos
			0.64	0.06	0.82	<0.0001	3.46	Pos
			0.95	0.06	0.93	<0.0001	5.05	Pos
		Y	0.09	0.04	0.19	0.0318	1.90	MP[c]
			NM[d]	-	-	-	-	Neg
			NM	-	-	-	-	Neg
	TA100	N	0.24	0.08	0.28	0.0073	1.22	MP
			0.36	0.08	0.50	0.0004	1.17	MP
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
	YG1041	N	25.6	0.75	0.99	<0.0001	64.7	Pos
			13.7	0.55	0.97	<0.0001	45.2	Pos
			14.2	0.55	0.97	<0.0001	65.8	Pos
		Y	2.35	0.13	0.94	<0.0001	5.67	Pos
			0.96	0.07	0.90	<0.0001	2.75	Pos
			1.24	0.09	0.89	<0.0001	3.01	Pos
	YG1042	N	112.8	2.25	1.00	<0.0001	12.6	Pos
			70.6	2.18	0.99	<0.0001	18.4	Pos
			89.3	4.35	0.98	<0.0001	10.2	Pos
		Y	90.1	4.60	0.97	<0.0001	6.36	Pos
			51.1	1.37	0.99	<0.0001	4.73	Pos
			33.3	0.93	0.99	<0.0001	2.63	Pos
2,4-DNT	TA98	N	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
	TA100	N	0.38	0.07	0.56	<0.0001	1.29	MP
			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			0.20	0.09	0.20	0.0288	1.13	MP
			0.31	0.13	0.21	0.0228	1.54	MP
	YG1041	N	0.51	0.07	0.73	<0.0001	3.30	Pos
			0.35	0.05	0.68	<0.0001	2.04	MP
			0.28	0.04	0.68	<0.0001	2.54	Pos
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
	YG1042	N	27.7	0.61	0.99	<0.0001	13.7	Pos
			20.8	0.39	0.99	<0.0001	12.6	Pos
		Y	6.71	0.17	0.99	<0.0001	2.47	Pos
			9.19	0.26	0.99	<0.0001	3.97	Pos
2,6-DANT	TA98	N	4.41	0.11	0.99	<0.0001	20.1	Pos
			3.45	0.10	0.98	<0.0001	13.0	Pos
			3.36	0.13	0.97	<0.0001	34.1	Pos
		Y	1.07	0.07	0.92	<0.0001	4.50	Pos
			1.00	0.04	0.96	<0.0001	3.99	Pos

			0.98	0.04	0.96	<0.0001	5.35	Pos
			2.21	0.13	0.94	<0.0001	2.66	Pos
		N	2.62	0.15	0.94	<0.0001	2.39	Pos
			2.18	0.11	0.95	<0.0001	2.90	Pos
	TA100		1.33	0.11	0.89	<0.0001	2.02	MP
		Y	1.89	0.11	0.93	<0.0001	2.18	MP
			1.64	0.12	0.89	<0.0001	2.03	MP
			37.7	1.17	0.98	<0.0001	110.7	Pos
		N	16.8	0.60	0.98	<0.0001	47.5	Pos
			18.2	0.55	0.98	<0.0001	51.3	Pos
	YG1041		9.66	0.20	0.99	<0.0001	17.5	Pos
		Y	4.85	0.11	0.99	<0.0001	9.39	Pos
			5.84	0.10	0.99	<0.0001	10.3	Pos
			149.2	3.10	1.00	<0.0001	17.5	Pos
		N	120.0	2.88	0.99	<0.0001	18.3	Pos
			123.2	4.94	0.98	<0.0001	14.8	Pos
	YG1042		47.4	2.19	0.97	<0.0001	5.47	Pos
		Y	30.5	0.83	0.99	<0.0001	6.96	Pos
			26.6	1.09	0.97	<0.0001	2.95	Pos
			NM	-	-	-	-	Neg
		N	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
	TA98		NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
			0.79	0.34	0.30	0.0356	1.16	MP
		N	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
	TA100		NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
2,6-DNT			NM	-	-	-	-	Neg
			0.61	0.04	0.90	<0.0001	4.81	Pos
		N	0.20	0.05	0.43	0.0005	1.50	MP
			0.20	0.04	0.53	<0.0001	1.93	MP
	YG1041		0.14	0.04	0.30	0.0058	1.21	MP
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		N	10.6	0.34	0.98	<0.0001	9.56	Pos
	YG1042		7.63	0.23	0.98	<0.0001	7.52	Pos
		Y	10.1	3.26	0.42	0.0087	2.85	Pos
			2.41	0.14	0.93	<0.0001	2.25	Pos
2a-DNT			1.41	0.11	0.92	<0.0001	5.53	Pos
		N	1.06	0.06	0.95	<0.0001	5.15	Pos
			1.22	0.04	0.98	<0.0001	6.66	Pos
	TA98		0.29	0.05	0.68	<0.0001	2.09	Pos
		Y	0.24	0.04	0.63	<0.0001	2.07	MP
			0.23	0.06	0.41	0.0017	1.83	MP
			0.55	0.07	0.72	<0.0001	1.67	MP
		N	0.67	0.07	0.81	<0.0001	1.67	MP
			0.90	0.13	0.73	<0.0001	1.80	MP
	TA100		1.58	0.43	0.51	0.0029	1.29	MP
		Y	0.49	0.12	0.43	0.0005	1.36	MP
			NM	-	-	-	-	Neg
	YG1041	N	24.6	1.39	0.96	<0.0001	60.4	Pos
			18.6	0.61	0.98	<0.0001	40.7	Pos

			16.1	0.37	0.99	<0.0001	41.5	Pos
			1.88	0.07	0.97	<0.0001	7.71	Pos
		Y	1.53	0.08	0.95	<0.0001	3.82	Pos
			1.45	0.08	0.94	<0.0001	6.18	Pos
			29.2	0.93	0.98	<0.0001	8.42	Pos
		N	84.4	2.52	0.99	<0.0001	17.2	Pos
	YG1042		71.6	1.94	0.99	<0.0001	16.1	Pos
			10.4	0.84	0.89	<0.0001	6.38	Pos
		Y	26.5	1.44	0.96	<0.0001	6.42	Pos
			21.7	0.94	0.97	<0.0001	4.25	Pos
			0.51	0.06	0.75	<0.0001	3.98	Pos
		N	0.52	0.05	0.83	<0.0001	3.32	Pos
	TA98		0.35	0.06	0.62	<0.0001	2.85	Pos
			0.24	0.09	0.30	0.0197	1.50	MP
		Y	0.33	0.12	0.33	0.0120	1.88	MP
			NM	-	-	-	-	Neg
			0.32	0.08	0.39	0.0011	1.39	MP
		N	0.34	0.08	0.45	0.0003	1.41	MP
	TA100		0.20	0.07	0.25	0.0136	1.26	MP
			0.92	0.11	0.75	<0.0001	1.55	MP
		Y	0.95	0.13	0.70	<0.0001	1.68	MP
4a-DNT			0.51	0.08	0.67	<0.0001	1.26	MP
			7.46	0.20	0.98	<0.0001	38.0	Pos
		N	6.32	0.20	0.98	<0.0001	14.5	Pos
	YG1041		2.31	0.12	0.94	<0.0001	13.7	Pos
			1.36	0.10	0.90	<0.0001	3.37	Pos
		Y	0.82	0.09	0.78	<0.0001	2.95	Pos
			0.60	0.07	0.77	<0.0001	2.00	MP
		N	42.2	1.46	0.98	<0.0001	13.6	Pos
	YG1042		49.9	1.08	0.99	<0.0001	11.8	Pos
		Y	29.6	0.86	0.99	<0.0001	2.81	Pos
			25.4	2.18	0.89	<0.0001	8.97	Pos
			75.5	3.00	0.99	<0.0001	5.63	Pos
		N	71.5	10.3	0.87	0.0002	13.6	Pos
	TA98		33.0	2.34	0.95	<0.0001	9.09	Pos
			0.22	0.07	0.36	0.0082	1.57	MP
		Y	NM	-	-	-	-	Neg
			0.43	0.10	0.55	0.0004	1.86	MP
			22.4	3.31	0.87	0.0003	1.77	MP
		N	33.4	5.57	0.84	0.0005	2.03	MP
	TA100		9.73	2.12	0.75	0.0025	1.31	MP
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			0.85	0.22	0.48	0.0014	1.24	MP
			321.6	13.9	0.98	<0.0001	37.1	Pos
		N	322.6	14.0	0.99	<0.0001	12.1	Pos
	YG1041		122.9	11.8	0.92	<0.0001	32.3	Pos
			5.45	0.58	0.87	<0.0001	3.11	Pos
		Y	2.61	0.42	0.71	<0.0001	1.99	MP
			3.09	0.68	0.67	0.0011	1.71	MP
			225.0	43.7	0.73	0.0004	4.28	Pos
		N	272.6	20.3	0.96	<0.0001	2.75	MP
	YG1042		108.8	10.4	0.92	<0.0001	2.56	Pos
		Y	19.3	0.84	0.97	<0.0001	3.44	Pos
			41.6	2.83	0.94	<0.0001	4.30	Pos

			23.7	2.94	0.83	<0.0001	1.48	MP
1,3,5-TNB	TA98	N	15.7	0.23	1.00	<0.0001	47.8	Pos
			10.8	0.49	0.96	<0.0001	43.2	Pos
			14.8	0.58	0.97	<0.0001	66.6	Pos
		Y	1.45	0.09	0.92	<0.0001	8.36	Pos
			1.46	0.09	0.92	<0.0001	5.64	Pos
			1.41	0.08	0.94	<0.0001	6.24	Pos
	TA100	N	18.1	0.61	0.98	<0.0001	4.57	Pos
			14.2	0.56	0.97	<0.0001	3.78	Pos
			14.1	0.74	0.95	<0.0001	3.72	Pos
		Y	3.89	0.15	0.97	<0.0001	3.71	Pos
			3.40	0.18	0.94	<0.0001	3.42	Pos
			3.74	0.12	0.98	<0.0001	3.93	Pos
	YG1041	N	264.1	11.6	0.97	<0.0001	200.0	Pos
			268.8	13.1	0.96	<0.0001	102.8	Pos
			283.6	13.9	0.96	<0.0001	134.4	Pos
		Y	8.67	0.24	0.99	<0.0001	13.4	Pos
			6.47	0.12	0.99	<0.0001	10.9	Pos
			6.20	0.22	0.98	<0.0001	13.2	Pos
	YG1042	N	86.9	4.44	0.96	<0.0001	10.4	Pos
			98.7	3.54	0.98	<0.0001	8.61	Pos
			108.8	2.85	0.99	<0.0001	12.8	Pos
		Y	39.0	1.53	0.97	<0.0001	8.06	Pos
			43.4	1.23	0.98	<0.0001	4.02	Pos
			45.7	1.48	0.98	<0.0001	4.07	Pos
TNT	TA98	N	1.86	0.06	0.98	<0.0001	9.87	Pos
			1.07	0.08	0.89	<0.0001	4.30	Pos
			1.14	0.04	0.97	<0.0001	9.64	Pos
		Y	0.20	0.05	0.47	0.0002	1.66	MP
			0.18	0.04	0.44	0.0004	1.40	MP
			0.21	0.03	0.69	<0.0001	2.01	MP
	TA100	N	3.21	0.24	0.89	<0.0001	3.62	Pos
			2.75	0.17	0.93	<0.0001	2.72	MP
			2.67	0.14	0.94	<0.0001	3.56	Pos
		Y	3.14	0.18	0.93	<0.0001	2.60	Pos
			2.76	0.11	0.96	<0.0001	2.93	Pos
			2.66	0.13	0.95	<0.0001	2.86	Pos
	YG1041	N	18.4	0.42	0.99	<0.0001	74.1	Pos
			11.1	0.35	0.98	<0.0001	30.0	Pos
			12.1	0.26	0.99	<0.0001	50.4	Pos
		Y	2.24	0.07	0.98	<0.0001	4.44	Pos
			1.76	0.08	0.96	<0.0001	3.50	Pos
			1.69	0.09	0.94	<0.0001	6.46	Pos
	YG1042	N	112.9	2.03	1.00	<0.0001	15.9	Pos
			96.3	3.37	0.98	<0.0001	13.5	Pos
		Y	45.0	1.78	0.97	<0.0001	3.80	Pos
			33.5	0.94	0.98	<0.0001	5.03	Pos
Tetryl	TA98	N	3.80	0.19	0.95	<0.0001	19.2	Pos
			6.14	0.22	0.98	<0.0001	8.75	Pos
			2.97	0.14	0.96	<0.0001	9.56	Pos
		Y	1.21	0.07	0.93	<0.0001	7.02	Pos
			1.57	0.08	0.94	<0.0001	7.99	Pos
			1.22	0.07	0.93	<0.0001	5.43	Pos
	TA100	N	37.2	1.94	0.95	<0.0001	7.87	Pos

			44.5	2.15	0.96	<0.0001	12.4	Pos
			34.0	1.90	0.94	<0.0001	6.46	Pos
			8.38	0.37	0.96	<0.0001	4.89	Pos
		Y	5.98	0.27	0.96	<0.0001	4.15	Pos
			9.14	0.42	0.96	<0.0001	5.11	Pos
			18.1	1.01	0.94	<0.0001	27.2	Pos
		N	26.9	1.32	0.97	<0.0001	15.6	Pos
	YG1041		21.1	1.03	0.96	<0.0001	23.2	Pos
			10.9	0.21	0.99	<0.0001	22.0	Pos
		Y	9.27	0.16	0.99	<0.0001	20.1	Pos
			9.81	0.32	0.98	<0.0001	17.5	Pos
			84.6	4.61	0.95	<0.0001	8.61	Pos
		N	156.6	4.02	0.99	<0.0001	13.2	Pos
	YG1042		133.8	3.29	0.99	<0.0001	12.6	Pos
			36.3	1.11	0.98	<0.0001	3.38	Pos
		Y	61.5	1.36	0.99	<0.0001	6.81	Pos
			57.6	2.98	0.95	<0.0001	2.96	Pos
			NM	-	-	-	-	Neg
		N	NM	-	-	-	-	Neg
	TA98		NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		Y	0.64	0.19	0.53	0.0073	1.54	MP
			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		N	NM	-	-	-	-	Neg
	TA100		NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
HMX			NM	-	-	-	-	Neg
		N	NM	-	-	-	-	Neg
	YG1041		NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		N	NM	-	-	-	-	Neg
	YG1042		NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
RDX			NM	-	-	-	-	Neg
		N	NM	-	-	-	-	Neg
	TA98		NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		N	NM	-	-	-	-	Neg
	TA100		NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
	YG1041	N	NM	-	-	-	-	Neg

			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		N	NM	-	-	-	-	Neg
	YG1042		NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg
		Y	NM	-	-	-	-	Neg
			NM	-	-	-	-	Neg

[a] Mutagenic potency in revertants/µg compound.
[b] Mutation ratio is defined as the mean number of revertants at the highest concentration used in the calculation of mutagenic potency, divided by the mean number of spontaneous revertants.
[c] MP = Marginal positive response (significant *p*-value <0.05, but fewer than 2 consecutive concentrations eliciting response 2-fold above spontaneous).
[d] NM = Not mutagenic.