8
Biotransformations of Explosives

CHRIS E. FRENCH*[1], SUSAN J. ROSSER[2] AND NEIL C. BRUCE[2]

[1]*Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JR, U.K. and* [2]*Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, U.K.*

Introduction

During the past century, explosives have been produced in large quantities for both military and industrial purposes. Many explosives have been found to be highly resistant to biodegradation in the environment, and considerable areas of land have become contaminated with residues resulting from their manufacture, storage, use, and disposal. Such contamination is thought to affect at least 75,000 sites in the U.S. (Rosenblatt *et al.*, 1991). An estimated 0.82 million cubic metres of soil at former ordinance sites and military proving grounds in the U.S. are contaminated with TNT and its metabolites (USATHAMA, 1989). Increased awareness of environmental problems has resulted in a considerable volume of research into the fate of explosives in the environment and into means for the cost-effective removal of explosive residues from soil and water. The cleanup costs are enormous; estimated costs for incineration are \$400 to \$600/yd^3 of soil (Griest *et al.*, 1998). Bioremediation, and most particularly *in situ* bioremediation, could potentially offer a relatively low cost method for decontamination. This review summarizes recent developments in research concerning transformations of important secondary high explosives in living systems.

*To whom correspondence may be addressed (C.French@ed.ac.uk)

Abbreviations: ADNT, aminodinitrotoluenes (unspecified mixture of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene); 2A46DNT, 2-amino-4,6-dinitrotoluene; 4A26DNT, 4-amino-2,6-dinitrotoluene; 24DNP, 2,4-dinitrophenol; DNT, dinitrotoluenes (unspecified mixture of 2,4-dinitrotoluene and 2,6-dinitrotoluene); 24DNT, 2,4-dinitrotoluene; 26DNT, 2,6-dinitrotoluene; DANT, diaminonitrotoluenes (unspecified mixture of 2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene); 24DA6NT, 2,4-diamino-6-nitrotoluene; 26DA4NT, 2,6-diamino-4-nitrotoluene; EGDN, ethylene glycol dinitrate; GDN, glycerol dinitrate (unspecified mixture of glycerol-1,2-dinitrate and glycerol-1,3-dinitrate); 12GDN, glycerol-1,2-dinitrate; 13GDN, glycerol-1,3-dinitrate; GMN, glycerol mononitrate (unspecified mixture of glycerol-1-mononitrate and glycerol-2-mononitrate); 1GMN, glycerol-1-mononitrate; 2GMN, glycerol-2-mononitrate; GTN, glycerol trinitrate; HADNT, hydroxylaminodinitrotoluenes (unspecified mixture of 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-2,6-dinitrotoluene); 2HA46DNT, 2-hydroxylamino-4,6-dinitrotoluene; 4HA26DNT, 4-hydroxylamino-2,6-dinitrotoluene; HMX, High Melting Explosive (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine); PETN, pentaerythritol tetranitrate; PGDN, propylene glycol dinitrate; RDX, Royal Demolition Explosive (hexahydro-1,3,5-trinitro-1,3,5-triazine); TAT, 2,4,6-triaminotoluene; TNB, 1,3,5-trinitrobenzene; TNP, 2,4,6-trinitrophenol (picric acid); TNT, 2,4,6-trinitrotoluene.

Biotechnology and Genetic Engineering Reviews – Vol. 18, July 2001
0264–8725/01/18/171–217 \$20.00 + \$0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, U.K.

THE NATURE AND CHEMISTRY OF EXPLOSIVES

Energetic compounds, including propellants and explosives, are capable of rearrangement with rapid conversion of a small volume of cold solid or liquid to a large volume of hot gas. This may occur by a process of combustion (deflagration), in which such rearrangement occurs relatively slowly, at a surface or interface, or detonation, in which shock-induced rearrangement is propagated at supersonic speed throughout the solid mass of explosive. In propellants, deflagration rather than detonation occurs during normal use; in high explosives, normal use involves detonation (Rosenblatt *et al.*, 1991; Lindner, 1993; Coursen, 1997).

Explosives which are sufficiently sensitive to be detonated by a local hotspot or shock are called primary explosives. Less sensitive materials, which require an

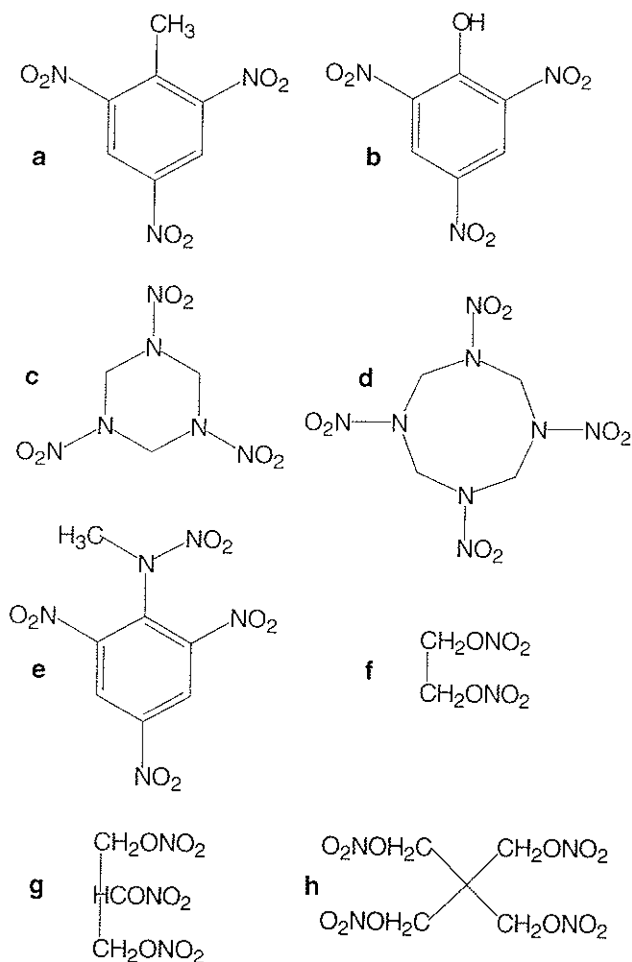

Figure 8.1. Structures of important explosives. A: 2,4,6-trinitrotoluene (TNT); B: 2,4,6-trinitrophenol (picric acid); C: Royal Demolition Explosive (RDX); D: High Melting Explosive (HMX); E: 2,4,6-trinitrophenylmethylnitramine (Tetryl); F: ethylene glycol dinitrate (EGDN); G: glycerol trinitrate (GTN, nitroglycerin); H: pentaerythritol tetranitrate (PETN).

explosive shock wave for detonation, are called secondary explosives. For reasons of controllability, a small amount of primary explosive, detonated by a small shock or an electrical spark, is normally used to detonate a much larger volume of secondary explosive. Important primary explosives include lead azide and lead styphnate. Large-scale contamination of land and water is principally associated with the most widely used secondary explosives. The principal components of most secondary high explosives are organic nitro compounds. The nitro group effectively provides an internal source of oxygen for oxidation of the carbon present, with nitrogen being converted to nitrogen gas. The most important high explosives belong to three chemical families: nitroaromatic compounds, nitramines, and nitrate esters. The structures of some important explosives are shown in *Figure 8.1*.

Nitroaromatic explosives typically possess a single aromatic ring with three nitro groups. The most important is 2,4,6-trinitrotoluene (TNT), historically the most important military high explosive. TNT is probably the most important explosive found in contaminated land, and is remarkably recalcitrant to biodegradation in the environment. Picric acid (2,4,6-trinitrophenol) and its ammonium salt are also explosives; ammonium picrate is relatively insensitive to shock and has been used, for example, as a burster explosive in armour-piercing naval projectiles.

Nitramine explosives contain one or more N-nitro groups. The most important examples are RDX (Royal Demolition Explosive, Research Department Explosive, hexogen, cyclonite, hexahydro-1,3,5-trinitro-1,3,5-triazine, cyclotrimethylenetrinitramine), HMX (High Melting Explosive, octogen, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, cyclotetramethylenetetranitramine), and tetryl (2,4,6-trinitrophenylmethylnitramine), which contains both nitramine and aromatic nitro groups. RDX is reportedly the most important military high explosive currently in use, being about 40% more powerful than TNT.

Nitrate ester explosives are esters of nitric acid, thus effectively containing one or more O-nitro groups. The most important examples include EGDN (ethylene glycol dinitrate), PGDN (propylene glycol dinitrate), GTN (glycerol trinitrate, 'nitroglycerin'), PETN (pentaerythritol tetranitrate), and nitrocellulose (cellulose nitrate). GTN and nitrocellulose are components of industrial dynamites, PETN is used in plastic explosives and blasting cord, and PGDN and EGDN are liquid explosives used as propellants for certain military applications. Nitrocellulose is also used in varnishes and lacquers. GTN and PETN are used medicinally as vasodilators for the control of coronary angina, as well as a variety of other conditions. The vasodilatant action of nitrate esters appears to be due to release of nitric oxide after enzymic attack.

Many important high explosives are blends of several of these, together with other components such as ammonium nitrate, fuel oil, or plasticizers. For example, TNT may be blended with RDX, PETN, or ammonium nitrate, and plastic explosives may contain a mixture of RDX and PETN together with plasticizers.

OCCURRENCE OF SIMILAR COMPOUNDS IN NATURE

Nitro groups are rather rare in naturally occurring compounds. Aromatic compounds with a single nitro group, such as chloramphenicol, are secondary metabolites of some bacteria; aliphatic nitro compounds such as 3-nitropropionic acid occur in plants (Venulet and van Etten, 1970). Nitrate esters appear to be even rarer; one report (Hall

et al., 1992) describes an insect sex pheromone as a long-chain nitrate ester, and nitrate esters may also be produced in small amounts through atmospheric processes (Roberts, 1990). Nitramines do not appear to occur in nature. Thus, compounds with the multiple nitro groups typical of high explosives are xenobiotic in the strict sense, and occur in the environment only as a result of relatively recent human activity. It is therefore a matter of considerable interest to study the interaction of microorganisms with such compounds. Any enzymes specifically adapted to degrade explosives must be assumed to have evolved this activity within the past century or so.

BIODEGRADATION OF EXPLOSIVES

Recent general reviews of the environmental fate, biodegradation and bioremediation of explosives and related compounds include Rosenblatt *et al.* (1991), Kaplan (1990, 1992, 1996), Gorontzy *et al.* (1994) and Keehan and Sisk (1996). Reviews covering particular subsets of this group of compounds are cited under the appropriate subheadings.

Biological transformations of nitroaromatic compounds

SUMMARY OF RELEVANT CHEMISTRY

Trinitroaromatic compounds are extremely susceptible to reduction of the nitro groups. This proceeds via the nitroso group (rarely isolated) and the hydroxylamino group to the amine. Aromatic hydroxylamines and amines are also rather reactive and form a variety of covalent adducts. For example, during the reduction process, a hydroxylaminodinitroaromatic compound may attack a nitrosodinitroaromatic compound to form a tetranitro-azoxy dimer. It appears that these reduced intermediates may also attack a variety of biological molecules to form covalent attachments (discussed below). Some typical products of nitro-group reduction of TNT are shown in *Figure 8.2*.

In trinitroaromatic compounds, and dinitrophenols, the aromatic ring is rather electron-deficient due to the electron-withdrawing nature of the nitro groups, and is highly susceptible to attack by nucleophiles to form Meisenheimer complexes (sigma complexes) (Crampton, 1969; Buncel, 1982). These are generally negatively charged and often brightly coloured due to the presence of an extensive delocalized electron system. Where the attacking species is a hydride ion, the result is a hydride-Meisenheimer complex (Kaplan and Siedle, 1971; Buncel, 1982). A second hydride ion may add to give a dihydride-Meisenheimer complex. In the case of TNT, initial hydride attack is typically at position 3 (*Figure 8.3*). Recent evidence (discussed below) suggests that hydride-Meisenheimer complexes may play an important role in the productive degradation of TNT and picric acid in some bacteria.

Due again to the electron-withdrawing nature of the nitro groups, the aromatic ring of trinitroaromatics is rather resistant to electrophilic attack. This has significant consequences for their biodegradation, since, under aerobic conditions, aromatic compounds are normally metabolized by oxygen attack. For example, dinitrotoluenes are degraded via initial oxygen attack (Spain, 1995a,b), but this type of reaction has not been demonstrated for trinitroaromatics.

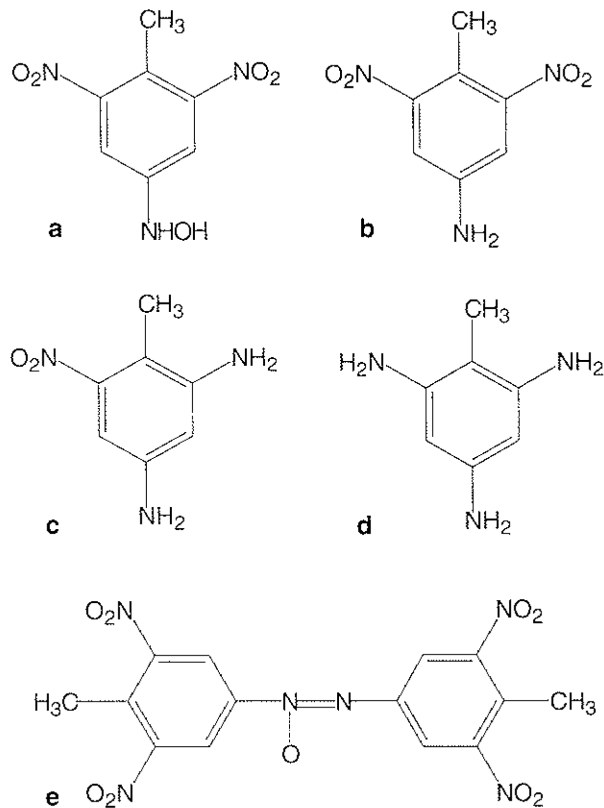

Figure 8.2. Representative products of nitro group reduction of TNT. A: 4-hydroxylamino-2,6-dinitrotoluene; B: 4-amino-2,6-dinitrotoluene; C: 2,4-diamino-6-nitrotoluene; D: 2,4,6-triaminotoluene; E: 2,2',6,6'-tetranitro-4,4'-azoxytoluene.

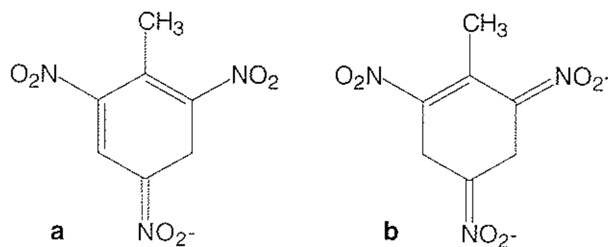

Figure 8.3. Hydride-Meisenheimer complexes of TNT. A: 3-hydride-Meisenheimer complex of TNT; B: 3,5-dihydride-Meisenheimer complex of TNT.

AROMATIC NITROREDUCTASES

The most characteristic reaction of nitroaromatic compounds, including TNT, in biological systems is reduction of the nitro groups. This is of considerable biological significance since it may convert nitroaromatic compounds to the hydroxylamino

derivatives, which can react with DNA, causing mutations. This type of activation is responsible for the toxicity and mutagenicity of many nitroaromatic compounds (Bryant and McElroy, 1991). Reduction of nitro groups is a fortuitous side-reaction catalyzed by a wide variety of enzymes possessing redox-active flavins or metal ions. Such enzymes are referred to as nitroreductases. In the great majority of cases, reduction of aromatic nitro groups is not their physiological function, although nitroreductases have been shown to occur as part of the microbial degradation pathways for mononitroaromatic compounds such as nitrobenzoate (Groenewegen *et al.*, 1992; Ybannavar and Zylstra, 1995; Chauhan and Jain, 2000), nitrobenzene (Peres *et al.*, 1998; Park and Kim, 2000), nitrophenol (Blasco and Castillo, 1993; Schenzle *et al.*, 1997), and chloronitrophenol (Schenzle *et al.*, 1999), in which degradation proceeds after reduction of the aromatic nitro group to the hydroxylamine or amine.

Reduction of aromatic nitro groups can proceed via one- or two-electron reduction. One-electron reduction results initially in the radical nitro anion species which is readily re-oxidized by molecular oxygen, with formation of superoxide. Enzymes catalyzing such one-electron reduction are referred to as oxygen-sensitive nitroreductases, or Type II nitroreductases, since no overall reduction of the nitro group occurs in the presence of oxygen. Two-electron reduction of an aromatic nitro group gives the nitroso group, which can be reduced by a further two-electron reduction to the hydroxylamino group. The nitroso compound is rarely isolated, but is presumed to occur as an intermediate in reduction of the nitro group to the hydroxylamino group. Further reduction gives the amine. Both hydroxylamino and amino derivatives of nitroaromatic compounds are readily isolated in a variety of systems. Enzymes catalyzing such two-electron reduction are referred to as oxygen-insensitive nitroreductases, or type I nitroreductases. In general, the more aromatic nitro groups present, the better the substrate; for example, TNT is a considerably better substrate than DNT for the major oxygen-insensitive nitroreductase (NfnB) of *Enterobacter cloacae* (Bryant and DeLuca, 1991). This may be due partly to the presence of more target nitro groups and partly to the greater electron deficiency caused by multiple nitro groups.

A wide variety of enzymes possessing a flavin or iron centre show oxygen-sensitive (one-electron) nitroreductase activity. In animal and plant cells, such activity has been reported in cytochrome c reductase, ferredoxin-NADP oxido-reductase, cytochrome-P450 reductase, cytochrome P450, xanthine oxidase, glutathione reductase, dihydrolipoamide dehydrogenase, and thioredoxin reductase (Bryant and McElroy, 1991). It seems that one-electron reduction of aromatic nitro groups is a facile reaction and requires little more than an available electron at a suitable potential; indeed, for representative oxygen-sensitive nitroreductases, it has been shown that the V_{max}/K_m for a set of nitroaromatic substrates shows a linear relationship to the one-electron reduction potential (Orna and Mason, 1989).

In contrast, oxygen-insensitive (two-electron) nitroreductase activity is less common, and is typically associated with a characteristic subset of flavoproteins. The best-studied mammalian enzyme to demonstrate such activity is NAD(P)H-quinone oxidoreductase (DT-diaphorase) (Bryant and McElroy, 1991). This widely distributed enzyme is a soluble, cytoplasmic flavoprotein, with two identical subunits of M_r approximately 30,000, each with one FAD prosthetic group (Tedeschi *et al.*, 1995).

By catalyzing two-electron reduction of quinones without formation of the semiquinone, this enzyme is believed to play a role in preventing the formation of reactive oxygen species which might otherwise arise through interactions of semiquinones with molecular oxygen.

In bacteria, a number of oxygen-insensitive nitroreductases of uncertain physiological function have been reported. The best studied of these are the 'classical' oxygen-insensitive nitroreductases of enteric bacteria. The existence of these enzymes became obvious at an early stage due to the use of *Escherichia coli* and *Salmonella typhimurium* in mutagenicity testing. Nitroaromatic compounds were found to be 'activated' to mutagenic species (hydroxylamino derivatives) in these organisms by endogenous reductases (Bryant and McElroy, 1991). The oxygen-insensitive nitroreductases of enteric bacteria have attracted considerable attention due to their potential use in therapeutic applications such as ADEPT (Antibody-Directed Enzyme Prodrug Therapy) and GDEPT (Gene-Directed Enzyme Prodrug Therapy), in which a nitroreductase is targeted to a specific site within the body and there activates a systemically administered nitroaromatic prodrug, such as CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide), to produce the active, toxic form of the drug (see, for example, Anlezark *et al.*, 1992).

Enteric bacteria possess multiple enzymes with nitroreductase activity, both oxygen-sensitive and oxygen-insensitive. In *Escherichia coli* K12, three oxygen-insensitive nitroreductases, designated nitroreductase A (NfsA), nitroreductase B1 (NfsB), and nitroreductase B2 (NfsC), have been reported (Bryant *et al.*, 1981). The major nitroreductase under standard growth conditions, NfsA, requires NADPH as electron donor, whereas the minor nitroreductases NfsB and NfsC use either NADH or NADPH. NfsA (Zenno *et al.*, 1996a) and NfsB (Zenno *et al.*, 1996b) have been purified and characterized, and the corresponding genes, *nfsA* (*mdaA*) and *nfsB* (*nfnB*), cloned and sequenced. The nature of NfsC is still unclear. Both NfsA and NfsB are small flavoproteins, with M_r of 27,000 and 24,000 respectively; both bind FMN non-covalently. There is little sequence similarity between NfsA and NfsB, but, interestingly, NfsA shows high sequence identity to Frp, a flavin reductase of *Vibrio harveyi*, a luminescent bacterium in which flavin reductase provides reduced free FMN as a cofactor for bacterial luciferase (Zenno *et al.*, 1996a). Indeed, random mutagenesis of NfsA resulted in a mutant with a single amino acid change resulting in high flavin reductase activity (Zenno *et al.*, 1998). Similarly, NfsB shows high identity to the major flavin reductase of *Vibrio fischeri*, another luminescent bacterium, and a single amino acid change is sufficient to confer high flavin reductase activity (Zenno *et al.*, 1996c).

The major oxygen-insensitive nitroreductases of *E. coli* B (NfnB; Anlezark *et al.*, 1992; Michael *et al.*, 1994), *Salmonella typhimurium* (Watanabe *et al.*, 1990, 1998) and *Enterobacter cloacae* (Bryant and DeLuca, 1991; Bryant *et al.*, 1991) have also been purified and characterized and the corresponding genes cloned and sequenced. All show high sequence identity and strong similarity to NfsB of *E. coli* K12. Like *E. coli*, both *S. typhimurium* and *E. cloacae* also possess minor oxygen-insensitive nitroreductases which have not yet been characterized. The crystal structures of two *E. coli* nitroreductases have recently been determined (Skelly *et al.*, 1994; Kobori *et al.*, 1999).

The physiological roles of these nitroreductases have not been clearly established.

However, in addition to nitroaromatic compounds, they also show high activity against quinones. In *E. cloacae*, the major nitroreductase (NfnB) is induced tenfold by the presence of TNT (Bryant and DeLuca, 1991), and it has recently been demonstrated that NfsA in *E. coli* K12 is induced as part of the *soxRS* regulon, which is involved in the response to oxidative stress (Liochev *et al.*, 1999). It has therefore been proposed that their function is to prevent the formation of superoxide which would otherwise occur through one-electron reduction of susceptible compounds by ubiquitous enzymes with oxygen-sensitive nitroreductase activity (described above).

Several nitroreductases involved in bacterial degradative pathways have also been purified and characterized, although no sequences for these enzymes have yet been reported. Properties reported for these enzymes are generally similar to those described for the oxygen-insensitive nitroreductases of enteric bacteria, with minor but interesting differences. For example, the NAD(P)H-dependent nitrophenol nitroreductase of the purple phototrophic bacterium *Rhodobacter capsulatus* (Blasco and Castillo, 1993) was reported to have a subunit M_r of 27,000 and to bind FMN as prosthetic group, although, in contrast to the nitroreductases described above, it was active as a dimer rather than a monomer, and showed greater substrate specificity, reducing mono-, di- and trinitrophenols, but not dinitrotoluenes or dinitrobenzenes. The nitrobenzene reductase of the nitrobenzene-utilizing bacterium *Pseudomonas pseudoalcaligenes* JS45 (Somerville *et al.*, 1995) was reported to be an NADPH-dependent monomeric FMN-binding flavoprotein of M_r 33,000. The N-terminal sequence of this enzyme does not resemble those of NfsA, NfsB or their known relatives. Interestingly, this enzyme reduces nitrobenzene to hydroxylaminobenzene (presumably via nitrosobenzene) but does not further reduce hydroxylaminobenzene to aniline (aminobenzene). Similarly, the nitrobenzoate reductases of nitrobenzoate-utilizing *Pseudomonas picketii* YH105 (Yabannavar and Zylstra, 1995) and *Comamonas acidovorans* NBA-10 (Groenewegen *et al.*, 1992) reduce *p*-nitrobenzoate to *p*-hydroxylaminonitrobenzoate. This may be a general characteristic of the simple flavoprotein nitroreductases; purified NfnB of *Enterobacter cloacae* reduces TNT principally to hydroxylaminodinitrotoluene under aerobic conditions (R.E. Williams and N.C. Bruce, unpublished information).

Bacterial nitroreductases have also attracted attention due to their role in resistance to antibacterial agents: in general, strains lacking a major nitroreductase activity are resistant to nitroaromatic compounds. Early in the study of nitroreductases, it was noted that mutants of *S. typhimurium* lacking the major nitroreductase showed decreased sensitivity to nitroaromatic compounds (Watanabe *et al.*, 1998). In *E. coli*, resistance to nitrofurazone and related compounds involves an initial inactivating mutation in *nfsA*, with enhanced resistance if *nfsB* is also inactivated (Whiteway *et al.*, 1998). More recently, and with perhaps greater medical significance, it has been found that resistance to the antimicrobial drug metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) in the stomach microorganism *Helicobacter pylori*, associated with peptic ulcers and gastric cancer, is associated with disruption of the gene *rdxA*, encoding an NADPH-dependent oxygen-insensitive nitroreductase homologous to NfsB of *E. coli* and the major nitroreductases (NfnB) of *E. cloacae* and *S. typhimurium* (Goodwin *et al.*, 1998). In the cyanobacterium *Synechocystis*, a model system for study of photosynthesis-inhibiting herbicides, resistance to dinoseb (2-*sec*-butyl-4,6-dinitrophenol) and other nitroaromatic herbicides, as well as

metronidazole, is associated with disruption of the gene *drgA*, which encodes another NfsB-related protein (Elenskaya *et al.*, 1998).

Another reason for interest in bacterial nitroreductases is that nitroreduction of environmental nitroaromatics by intestinal bacteria may activate these compounds, and may therefore be at least partially responsible for their toxicity and mutagenicity to mammals (Rafii *et al.*, 1991). Both intracellular (Kinouchi and Ohnishi, 1983) and extracellular (Rafii *et al.*, 1991) nitroreductases have been demonstrated in intestinal anaerobic bacteria.

FATE OF TNT IN THE ENVIRONMENT

Typical biotransformation products of TNT are aminodinitrotoluenes (ADNT), diaminonitrotoluenes (DANT) and tetranitroazoxytoluenes, as produced by nitroreductase reactions. Triaminotoluene (TAT) may also be formed under anaerobic conditions. It seems likely that the majority of environmental biotransformation of TNT is due to fortuitous nitroreductase activity of microbial and plant enzymes. Reduction of TNT may also be indirect in some cases; it has been reported that nitroaromatic compounds can be effectively reduced by products of anaerobic microbial respiration such as H_2S (Gorontzy *et al.*, 1993) and Fe^{2+} (Heijman *et al.*, 1995). TNT is also significantly photolabile; major photoproducts include 2,4,6-trinitrobenzaldehyde, ADNT, and azoxy dimers (Rosenblatt *et al.*, 1991).

A large number of reports, particularly over the past five years, have described investigations of TNT transformations in soil columns, soil slurry reactors, and compost systems. Generally, the best transformation is found with anaerobic treatment in the presence of a fermentable carbon source such as sucrose, followed by aerobic treatment to encourage oxidation of reduced products (see, for example, Funk *et al.*, 1993; Roberts *et al.*, 1996).

Following biological reduction, reduced TNT metabolites become irreversibly bound to soil components (Daun *et al.*, 1998; Lenke *et al.*, 1998). Studies using [^{14}C]TNT (Pennington *et al.*, 1995; Hundal *et al.*, 1997; Drzyzga *et al.*, 1998a, 1999; Achtnich *et al.*, 1999a) have demonstrated that reduced TNT derivatives become incorporated into humic and fulvic acids, and studies using [^{15}N]TNT together with ^{15}N-NMR spectroscopy (Achtnich *et al.*, 1999b; Knicker *et al.*, 1999; Bruns-Nagel *et al.*, 2000) have given some insight into the nature of the condensation products. Remaining nitro groups in TNT derivatives which have become incorporated into macromolecules are also subject to microbial reduction, although at a slower rate than that seen for nitro groups on free nitro compounds (Achtnich *et al.*, 1999c).

It has generally been held that TNT is not mineralized to a significant degree during such processes. However, several recent studies have reported significant mineralization of [^{14}C]TNT in anaerobic–aerobic treatment processes (Widrig *et al.*, 1997; Boopathy *et al.*, 1998a; van der Loop *et al.*, 1998; Boopathy and Manning, 1999) and in soil microcosms (Bradley *et al.*, 1994; Bradley and Chapelle, 1995). It should be noted that other similar studies have found no evidence for significant mineralization of TNT (for example, Harvey *et al.*, 1997a; Shen *et al.*, 1997). Gunnison *et al.* (1997) reported that an aerobic or microaerophilic microbial mixed culture could be acclimated to TNT degradation through continuous culture, eventually developing the ability to

use TNT as sole carbon and nitrogen source, with up to 60% mineralization of TNT observed. However, pure cultures able to use TNT as sole carbon and nitrogen source could not be isolated.

MICROBIAL TRANSFORMATION OF TNT

A number of reports have described the ability of various microorganisms in pure or mixed cultures to transform TNT and other aromatic nitro compounds. Recent reviews of this subject include those of Higson (1992), Marvin-Sikkema and de Bont (1994), Crawford (1995), Lewis *et al.* (1995), Spain (1995a,b), Preuss and Rieger (1995), Rieger and Knackmuss (1995), Funk *et al.* (1996) and Nishino *et al.* (2000). Mono- and dinitroaromatics are typically degraded via oxygenase-mediated cleavage like other aromatic compounds. However, due to the electron-withdrawing nature of the nitro groups, trinitroaromatics appear to be much less susceptible to oxygenase attack, and generally much more resistant to biodegradation; where biotransformation of TNT has been shown to occur, the first steps are almost invariably reductive, leading to HADNT, ADNT and DANT. This review will concentrate on reports of the transformation of trinitroaromatics; for details of breakdown pathways for other nitroaromatic compounds, the interested reader is referred to the reviews cited above.

ANAEROBIC TRANSFORMATION OF TNT BY BACTERIA

Reduction of TNT under anaerobic conditions is generally more extensive than that seen under aerobic conditions. Degradation of explosives under anaerobic conditions has been reviewed by Crawford (1995), Preuss and Rieger (1995) and Boopathy *et al.* (1998b).

Methanogens have been reported to reduce TNT. Boopathy and Kulpa (1994) reported that a methanogen, resembling *Methanococcus*, was able to reduce TNT to 24DA6NT when provided with formate or carbon dioxide and hydrogen for methanogenesis. Further studies (Boopathy, 1994) demonstrated that various other nitro compounds were also reduced to amines; interestingly, several other species of methanogen tested failed to transform nitroaromatics.

Reduction of explosives has also been reported in fermentative bacteria. Regan and Crawford (1994) reported that a strain of *Clostridium bifermentans* was able to reduce both TNT and RDX. Further study (Lewis *et al.*, 1996) revealed that the main products of TNT reduction were TAT, phenolic products apparently derived from TAT hydrolysis, and an adduct of TAT formed by condensation with methyl glyoxal (pyruvic aldehyde). Since TAT readily forms covalent adducts with humus substances, this was considered to be a potentially useful route for bioremediation of TNT-contaminated soil, and methods for the production of *C. bifermentans* spores, for use as inocula, were developed (Sembries and Crawford, 1997). Ederer *et al.* (1997) compared the reductive abilities of several strains of *Clostridium* from a munition-fed enrichment culture to those of strains of *Clostridium*, *Lactobacillus* and enteric bacteria which had never been exposed to explosives. All *Clostridium* strains tested, whether from the explosives-fed enrichment or from culture collections, reduced TNT to DANT and then to TAT and another, unidentified, product; *Lactobacillus* and enteric strains reduced TNT to DANT but did not reduce these further.

Khan *et al.* (1997) reported reduction of TNT and other nitroaromatic compounds to water-soluble, unidentified endproducts by *Clostridium acetobutylicum* cells. Hughes *et al.* (1998a,b) conducted a study of polar metabolites produced from TNT by this organism, and demonstrated that 2-amino-4-hydroxylamino-5-hydroxy-6-nitrotoluene was formed by cells and by cell extracts in the presence of H_2 via rearrangement of 2,4-dihydroxylamino-6-nitrotoluene. Dinitrotoluenes were also reduced mainly to dihydroxylaminotoluenes (Hughes *et al.*, 1999) although rearrangements were not observed. The purified carbon monoxide dehydrogenase of *Clostridium thermoaceticum* was shown to reduce TNT to a similar spectrum of products in the presence of carbon monoxide (Huang *et al.*, 2000); Preuss *et al.* (1993) also reported reduction of DANT to diaminohydroxylaminotoluene by this enzyme, as well as by purified hydrogenase from *Clostridium pasteurianum*.

In contrast to methanogenic and fermentative systems, sulphate-reducing bacteria have been reported to degrade TNT extensively and to use it as a sole source of nitrogen. Boopathy and Kulpa (1992) reported that a strain of *Desulfovibrio* was able to use TNT as a nitrogen source (following a rather long lag phase) or as a terminal electron acceptor for respiration. The main reduction product observed was DANT, which, under nitrogen-limited conditions, was converted to toluene; this was presumed to proceed via TAT, although TAT was not itself detected. Conversion to toluene occurred only in the absence of ammonium. A variety of other nitroaromatic compounds could also be used as terminal electron acceptors or sources of nitrogen (Boopathy and Kulpa, 1993). Further characterization of this strain (Boopathy *et al.*, 1993) suggested that it might be useful, in conjunction with a toluene-degrading organism, for bioremediation of TNT-contaminated soil and water under anaerobic conditions.

Similarly, Preuss *et al.* (1993) reported isolation of a sulphate-reducing bacterium able to utilize TNT as sole nitrogen source. TNT was reduced to TAT with reduction of DANT via diaminohydroxylaminotoluene to TAT being the rate-limiting step. TAT was further degraded to unidentified products, presumably with release of nitrogen. Inhibition studies suggested that dissimilatory sulphite reductase might be resposible for reduction of DANT and/or diaminohydroxylaminotoluene. The authors also demonstrated that degradation of TAT with elimination of 1 mol ammonium occurred spontaneously in the presence of oxygen and certain metal ions.

Costa *et al.* (1996) reported the isolation of a sulphate- and nitrate-reducing organism, tentatively identified as a strain of *Desulfovibrio desulfuricans*. In a cometabolic process with pyruvate as carbon source, under sulphate-reducing conditions, this organism reduced TNT to ADNT and 24DA6NT, but under nitrate-reducing conditions, TNT was apparently converted to butyrate. In neither case did TNT seem to be used as a carbon source. Boopathy and Manning (1996) later described degradation of TNT by a consortium of *Desulfovibrio* spp. from a creek bed. In the presence of a suitable supplementary carbon source, such as pyruvate, TNT was reduced to ADNT and then 24DA6NT. This was apparently converted to nitrobenzoate, and then sequentially to cyclohexanone, 2-methylpentanoate, butyrate and acetate. All of these compounds, with the exception of acetate, could also serve as sole carbon sources. Experiments with [^{14}C]TNT showed 50% of the radioactivity appearing in acetate, 28% in biomass, and the remainder as intermediates, with no $^{14}CO_2$ detected. This consortium was also able to use 1,3,5-trinitrobenzene, RDX, and HMX as nitrogen sources (Boopathy *et al.*, 1998c,d).

AEROBIC TRANSFORMATION OF TNT BY BACTERIA

Many aerobic bacteria have been shown to possess enzymes with nitroreductase activity. When incubated with TNT, such strains typically reduce it to ADNT or DANT, but typically no further. For example, Schackmann and Muller (1991) reported that several *Pseudomonas* strains could reduce TNT to ADNT and DANT. An NAD(P)H-dependent enzyme in crude extracts was found to catalyze these reductive processes; this was presumably an oxygen-insensitive nitroreductase (discussed above under 'aromatic nitroreductases').

Similarly, Pasti-Grigsby *et al.* (1996) reported that actinomycetes derived from sites contaminated with TNT, or from uncontaminated sites, reduced TNT but did not mineralize it to a significant degree. Little difference was seen between isolates from contaminated environments and those from uncontaminated environments, consistent with the view that these transformations are essentially fortuitous and involve widely distributed enzymes.

Acetylation of amines derived from TNT has also been reported. Gilcrease and Murphy (1995) reported that a strain of *Pseudomonas fluorescens* was able to reduce TNT to ADNT and then to 24DA6NT and 26DA4NT. The former was acetylated to produce 4-N-acetylamino-2,6-dinitrotoluene. There was no evidence of further degradation of either 26DA4NT or 4-N-acetylamino-2,6-dinitrotoluene. Noguera and Freedman (1996) reported a similar reduction and N-acetylation of 2,4-dinitrotoluene by a strain of *Pseudomonas aeruginosa*.

Oxidation of the methyl group of TNT has also been described. Vanderberg *et al.* (1995) reported that a strain of *Mycobacterium vaccae*, when grown with propane as sole carbon source, generated a variety of oxidized products including 4-amino-2,6-dinitrobenzoic acid. Oxidation of the methyl group in this case may have been initiated by the rather promiscuous propane monooxygenase. Incubation with [^{14}C]-TNT and propane resulted in the incorporation of approximately half of the radioactivity in cellular lipids, suggesting that ring cleavage might have occurred.

A number of other studies have also reported that radioactivity from [^{14}C]TNT can become incorporated into the biomass of bacterial cultures, although in most cases, it is not clear whether this is due to productive degradation of TNT to central intermediates or simply to binding of reactive, reduced TNT derivatives such as HADNT to cell materials. For example, Drzyzga *et al.* (1998b) compared the capacities of aerobic bacteria, represented by *Serratia plymuthica*, and anaerobic bacteria, represented by *Desulfovibrio* sp., to reduce TNT. The former reduced TNT to HADNT and ADNT, the latter to DANT and TAT. In both cases, radioactivity from [^{14}C]TNT was observed to become associated with cell biomass (approximately 32% for *Serratia* and 42% for *Desulfovibrio*, with the remainder being present as soluble products).

Some aerobic bacteria have shown indications of genuine productive degradation of these nitroreductase-like products. For example, Boopathy *et al.* (1994a) reported that a consortium of several *Pseudomonas* sp. was able to reduce TNT to ADNT. Prolonged incubation in the presence of another carbon source, such as succinate, resulted in the incorporation of a small amount of [^{14}C]TNT into biomass, and release of some radioactivity as $^{14}CO_2$. Four *Pseudomonas* spp. were isolated from this consortium (Boopathy *et al.*, 1994b). All four transformed TNT, with the principal products detected being aminodinitrotoluenes, as in the mixed culture. However,

nitrite was also produced, in levels up to 1 mol per mol TNT, and varying amounts of ^{14}C from TNT, up to 13%, were incorporated into biomass after prolonged incubation.

Alvarez *et al.* (1995) similarly reported aerobic degradation of DANT derived by reduction of TNT. In this case, *Pseudomonas aeruginosa* MAO1, isolated for its ability to use athranilate (2-aminobenzoate) as a sole carbon source, was shown to reduce TNT to ADNT and then to degrade these in an oxygen-dependent process, presumably attack by an oxygenase, to generate unidentified polar products. This required the presence of an alternative carbon source such as succinate. It was reported that up to 45% of radioactivity from [^{14}C]TNT appeared in polar products. DANT were not produced; when the organism was incubated with these compounds, 24DA6NT was N-acetylated, and not further metabolized, and 26DA4NT remained unaltered, as reported by Gilcrease and Murphy (1995) (described above). Fiorella and Spain (1997) reported a novel transformation pathway for TNT in *Pseudomonas pseudoalcaligenes* JS52. This strain uses nitrobenzene as a sole carbon source, and possesses a nitroreductase which reduces aromatic nitro groups to the hydroxylamino form without further reducing them to amine groups (discussed above under 'aromatic nitroreductases'). Nitrobenzene-grown cells (and cell extracts, in the presence of NADPH) transformed TNT to 4HA26DNT, 4A26DNT, 2,4-dihydroxylamino-6-nitrotoluene, and 4-amino-2-hydroxylamino-6-nitrotoluene. Under anaerobic conditions, these last two products accumulated, but under aerobic conditions, they were apparently further transformed with the production of a polar yellow product and a small amount of nitrite.

A number of reports have described a productive metabolic pathway in pseudomonads in which TNT is denitrated to DNT, with nitrogen liberated as nitrite. Duque *et al.* (1993) reported the isolation of *Pseudomonas* strain C1S1, able to grow with TNT or dinitrotoluenes as sole nitrogen sources, with nitrite accumulating in the medium, and derivation of *Pseudomonas* strain A, which grew more rapidly and did not accumulate nitrite. TNT degradation resulted in sequential formation of dinitrotoluenes, 2-nitrotoluene and toluene. Introduction of a TOL-plasmid derivative allowed the use of TNT as sole carbon and nitrogen source. The hydride-Meisenheimer complex of TNT was identified in culture supernatants, and cell extracts were reported to transform the chemically synthesized hydride-Meisenheimer complex to 2,4-dinitrotoluene and another, unidentified, product, possibly 1,3,5-trinitroheptane or 4-methyl-1,3,5-trinitrohexane (Haïdour and Ramos, 1996). *Pseudomonas* sp. strain A also produced typical nitroreductase products from TNT, including tetranitroazoxy dimers. These accumulated in the culture medium and appeared to be dead-end products, presumably arising through fortuitous reduction of TNT by various redox enzymes.

The proposed pathway for TNT degradation by *Pseudomonas* sp. A was later disputed. Vorbeck *et al.* (1998) reported that this organism, and another Gram negative organism similarly able to grow with TNT as sole nitrogen source, did not produce dinitrotoluenes from TNT or from its hydride-Meisenheimer complex; rather, use of TNT as a nitrogen source appeared to proceed via HADNT and an unidentified polar product, possibly a phenolic rearrangement product; phenolic rearrangement products of dihydroxylaminonitrotoluenes have been reported in anaerobic bacteria (Hughes *et al.*, 1998b; discussed above under 'anaerobic transformation of TNT by bacteria').

However, degradation of TNT via DNT was also reported by Martin *et al.* (1997), who described the isolation of a strain identified as *Pseudomonas savastanoi* from contaminated soil at the former Nebraska Ordnance Plant. This isolate was able to denitrate TNT with production of 2,4-dinitrotoluene and nitrite. Typical nitroreductase products, ADNT, were also produced, particularly in the presence of glucose; denitration of TNT was favoured by absence of ammonium and presence of nitrite. There was no significant production of $^{14}CO_2$ from labelled TNT.

Several other organisms have been reported to reduce TNT to its hydride-Meisenheimer complex. Vorbeck *et al.* (1994) noted that a strain of *Mycobacterium* sp., designated HL-4-NT-1, which had been isolated on the basis of its ability to grow with 4-nitrotoluene as a sole source of nitrogen, would transform TNT under aerobic conditions, with production of a dark red, water soluble metabolite, identified as the hydride-Meisenheimer complex. Liberation of nitrite was also observed, but DNT did not appear to be a product. It was subsequently demonstrated (Vorbeck *et al.*, 1998) that this strain, as well as the picric acid-degrading strain *R. erythropolis* HLPM-1, could reduce TNT to the hydride-Meisenheimer complex and then further to the dihydride-Meisenheimer complex. Similarly, French *et al.* (1998) demonstrated that *Enterobacter cloacae* PB2, isolated for its ability to grow with the nitrate esters GTN and PETN as sole nitrogen sources (discussed further below under 'bacterial degradation of nitrate esters'), could also use TNT as a sole source of nitrogen for growth. Furthermore, it was demonstrated that purified PETN reductase, the enzyme responsible for denitration of PETN and GTN, was capable of catalyzing NADH-dependent reduction of TNT to the hydride and dihydride-Meisenheimer complexes. The latter appeared to undergo further degradation, either spontaneous or catalyzed at a low rate by PETN reductase, to yield 1 mol nitrite per mol TNT, plus unidentified, water-soluble, non-aromatic products. PETN reductase also produced typical nitroreductase products from reduction of TNT, but at a far lower rate than that seen with the well-characterized oxygen-insensitive nitroreductases of *E. cloacae* and other enteric bacteria (discussed above under 'aromatic nitroreductases'). In contrast to the results reported by Haïdour and Ramos (1996), none of these reports described breakdown of the hydride-Meisenheimer complex of TNT to produce DNT.

There are indications that other productive pathways for TNT degradation may exist in aerobic bacteria, although these are so far less well characterized. For example, Esteve-Nuñez and Ramos (1998) described a novel TNT degradation pathway in *Pseudomonas* sp. JLR11, which is able to use TNT as sole nitrogen source for aerobic growth. Products detected included 2,4,6-trinitrobenzaldehyde, 2-nitro-4-hydroxybenzoate, 4-hydroxybenzaldehyde and 4-hydroxybenzoate. Approximately 85% of the nitrogen supplied as TNT was incorporated into cell biomass, and only 1% of carbon from [^{14}C]TNT was recovered as $^{14}CO_2$. Further study (Esteve-Nuñez *et al.*, 2000) revealed that, under anoxic conditions, this strain could also use TNT effectively as terminal electron acceptor for respiration, reducing it to aminonitrotoluenes; this reduction was coupled to ATP synthesis.

Other reports also describe productive degradation of TNT, but with insufficient detail to propose a degradative pathway. For example, Montpas *et al.* (1997) reported that a strain of *Serratia marcescens*, isolated from contaminated soil, possessed the unusual ability to use TNT as sole source of carbon and energy, provided that a surfactant, Tween 80, was present. Small amounts of ADNT were observed, but were

apparently dead-end side products. Kalafut *et al.* (1998) reported that a strain of *Bacillus* sp. was able to denitrate TNT.

DEGRADATION OF 2,4,6-TRINITROPHENOL AND 1,3,5-TRINITROBENZENE BY BACTERIA

The microbial degradation of picric acid has not undergone such extensive study as that of TNT. However, a number of reports have now described productive degradation of picric acid by bacteria of the high-GC Gram-positive group. In all cases, evidence suggests that picric acid is initially reduced to its hydride-Meisenheimer complex, which eliminates nitrite to produce 2,4-dinitrophenol (24DNP). This is then further reduced to its hydride-Meisenheimer complex, which is further transformed via an unknown route, possibly requiring oxygen, with liberation of the remaining nitro groups as nitrite.

This pathway was first described in *Rhodococcus erythropolis* HL24-1 and HL24-2, isolated on the basis of their ability to use 24DNP as sole nitrogen source (Lenke *et al.*, 1992). Under aerobic conditions, 24DNP was metabolized with liberation of nitrite and formation of a small amount of 4,6-dinitrohexanoate; under anaerobic conditions, essentially complete conversion to 4,6-dinitrohexanoate occurred. Substituted dinitrophenols were also attacked (Lenke and Knackmuss, 1996). These strains could not use picric acid as sole nitrogen source, but dinitrophenol-grown cells transformed picric acid with release of nitrite (Lenke and Knackmuss, 1992). A spontaneous mutant strain, HLPM-1, was able to grow with picric acid as sole nitrogen source, apparently due to altered regulation of the relevant genes. With both HL24-2 and HLPM-1, picric acid was initially reduced to the distinctive orange-red hydride-Meisenheimer complex (Rieger *et al.*, 1999), which was then transformed to 24DNP with release of nitrite; elimination of nitrite apparently occurred from the protonated rather than the anionic form of the hydride-Meisenheimer complex. The formation of small amounts of 2,4,6-trinitrocyclohexanone, converted under acid conditions to 1,3,5-trinitropentane, indicated that the hydride-Meisenheimer complex of picric acid could be further reduced to the dihydride adduct in an apparently unproductive side-reaction. Under aerobic conditions, nitrite (60 to 80%) and trinitrocyclohexanone (20 to 40%) accounted for essentially all of the nitrogen of picrate; however, under anaerobic conditions, transient accumulation of 2,4-dinitrophenol was observed, followed by conversion to 4,6-dinitrohexanoate.

In a second example, Rajan *et al.* (1996) reported isolation of four strains, all identified as close relatives of *Nocardioides simplex* on the basis of 16S RNA sequence, which could use picric acid as a sole source of carbon and energy. Nitrogen was released stoichiometrically as nitrite, with 2,4-dinitrophenol appearing as an intermediate. Up to 65% of carbon from [^{14}C]TNP was recovered as $^{14}CO_2$. Subsequent investigation of one of these strains (Ebert *et al.*, 1999) resulted in the substantial purification of a two-component enzyme system catalyzing hydride transfer from NADPH to picric acid or 2,4-dinitrophenol. As in *R. erythropolis*, picrate was converted initially to 2,4-dinitrophenol, which was converted to products including 4,6-dinitrohexanoate. Activity was dependent on cofactor F420, an unusual redox cofactor found in Archaea and some high-GC Gram-positive bacteria. One of the two enzyme components was identified as an NADPH-F420 reductase, the N-terminal sequence of which showed similarity to that of archaeal F420 reductases.

The second component apparently transferred hydride from F420 to the aromatic substrate; its N-terminal sequence was not related to those of known proteins.

In a third example, Behrend and Heesche-Wagner (1999) reported isolation of a strain able to use picric acid as sole carbon and nitrogen source, also related by 16S RNA sequence to *Nocardioides simplex*. Resting cells and cell extracts transformed picric acid with stoichiometric release of nitrite. The hydride-Meisenheimer complex of picric acid was detected by its distinctive spectral characteristics; other products were identified as 2,4-dinitrophenol and its hydride-Meisenheimer complex (with addition of hydride at position 3). Further degradation of this complex in cell extracts required NADPH and did not appear to yield nitrophenols. Fractionation of extracts resulted in a fraction capable of reducing picric acid to its hydride-Meisenheimer complex, but in contrast to the results of Ebert *et al.* (1999) this fraction did not appear to catalyze degradation of this complex or reduction of dinitrophenol. The authors suggested that productive degradation of 2,4-dinitrophenol might proceed via oxygenase attack on the hydride-Meisenheimer complex. Dinitrotoluenes are typically degraded by oxygenase attack, whereas this is not known to occur for dinitrophenols. Reduction of dinitrophenol to the hydride-Meisenheimer complex might add sufficient electron density to render the compound susceptible to oxygenase attack. This is consistent with the observation that dinitrophenol-degrading *R. erythropolis* strains, under anaerobic conditions, convert 2,4-dinitrophenol quantitatively to 4,6-dinitrohexanoate, without the nitrite liberation observed when oxygen is present (Lenke *et al.*, 1992; Lenke and Knackmuss, 1996).

To date, all isolates capable of degrading picric acid and 2,4-DNP have been identified as high G+C rich Gram-positive organisms. Due to a deficiency in genetic tools for these genera there has been little progress in identifying the genes responsible for the picric acid degradative pathways. Recently, however, Russ *et al.* (2000) used the technique of mRNA differential display to identify genes involved in picric acid degradation by *R. erythropolis* strain HL PM-1 (Lenke and Knackmuss, 1992) which contained an inducible picric acid degradation pathway (Russ, unpublished results). A 12.5 kb sequence of *R. erythropolis* HL PM-1 genome was identified which encoded 10 genes transcribed in the same direction suggesting an operon structure. The identification of this picric acid degradation cluster and the sequences of its genes allowed Russ *et al.* (2000) to propose function by comparison with other genes in the databases. Three of the genes were similar in sequence to oxidoreduction enzymes which use F420 as a cofactor. One of the genes is homologous to an F420:NADPH oxidoreductase of methanogenic bacteria and its N-terminal sequence was almost identical to the enzyme purified by Ebert *et al.* (1999). Two of the genes are closely related to members of the F420 dependent reductase/dehydrogenase family and the cell extracts of *E. coli* expressing one of these ORFs were demonstrated to reduce picric acid using electrons from reduced F420. The pathway is inducible and two of the genes had homologies to transcription factors which are likely to have a role in this regulation. Three genes have homologies to enzymes typical of the beta oxidation pathway of fatty acids suggesting that they might function in the oxidation of 4,6-dinitrohexanoate. Russ *et al.* (2000) postulated that removal of the nitro group from the picric acid-Meisenheimer complex could be carried out by a gene product with a predicted homology to a variety of dehydratases and racemases involved in group transfer. An alternative role for the product of this

open reading frame was the enzymatic hydrolysis of 2,4-dinitrocyclohexanone if the nonenzymatic reaction was too slow under physiological conditions. The remaining open reading frame corresponded to an aldehyde dehydrogenase which might be involved in the further oxidation of an aldehyde produced by the oxidation of 4,6-dinitrohexanoate by a nitroalkane oxidase as described for the oxidation of 1-nitropropane to 1-propanone (Kido *et al.*, 1978). The identification of metabolic intermediates is inherently difficult and the cloning of the picric acid degradation gene cluster and subsequent expression studies is likely to lead to the more rapid elucidation of the picric degradation pathway.

Relatively little has been reported regarding the biodegradation of 1,3,5-trinitrobenzene (TNB); however, its degradation, like that of picric acid, appears to be more facile than that of TNT. For example, Boopathy *et al.* (1994c) reported that a TNT-degrading aerobic consortium was able to degrade TNB via dinitroaniline, dinitrobenzene, and nitroaniline to nitrobenzene, which was not further degraded. Nitrogen was released as ammonium and used as a nitrogen source for growth. An isolated *Pseudomonas vesicularis* strain was shown to degrade trinitrobenzene by the same pathway (Davis *et al.*, 1997). Degradation of TNB by a TNT-degrading consortium under sulphate-reducing conditions has also been reported (Boopathy *et al.*, 1998c,d).

DEGRADATION OF TNT BY FUNGI

Many studies have demonstrated transformation of TNT by fungi. Parrish (1977) screened 190 fungal strains from 98 genera, and found that 183 of these, grown in shake-flask culture in liquid medium supplemented with 100 mg/l TNT, were able to transform TNT to a significant degree. The major products detected were 4A26DNT, 4HA26DNT and 2,2'-6,6'-tetranitro-4,4'-azoxytoluene, suggesting that transformation was effected through nitroreductase activities as seen in bacteria. Use of [^{14}C]TNT gave no evidence for ring cleavage products. Interestingly, the ability to transform 24DNT was much less common, occurring to a significant degree in only 5 of the strains tested. Scheibner *et al.* (1997) screened 91 strains of 32 genera. All rapidly reduced TNT to ADNT, but significant mineralization of [^{14}C]TNT occurred only with basidiomycetes involved in wood and litter decay; the best of these gave 42% mineralization of 0.1 mM (23 mg/l) TNT over 64 days. Bayman and Radkar (1997) tested 8 strains belonging to various taxonomic groups. Again, all reduced TNT to HADNT, ADNT and tetranitroazoxytoluenes; several also produced water-soluble products, but none mineralized TNT to a significant degree. Samson *et al.* (1998) also reported transformation of TNT to ADNT by selected fungi; in some cases, ADNT were apparently further metabolized. Meharg *et al.* (1997) reported that ectomycorrhizal basidiomycetes and their extracellular enzymes were able to transform TNT. Thus, evidence suggests that a wide variety of fungi are able to catalyze nitroreductase-like transformation of TNT, but that mineralization of TNT is essentially restricted to wood-decaying basidiomycetes.

Many studies have concentrated on white rot fungi such as *Phanerochaete chrysosporium* and *Phlebia radiata*; recent reviews include Stahl and Aust (1995a) and Michels and Gottschalk (1995). These fungi are able to degrade complex substrates such as lignin via non-specific oxygen attack using extracellular peroxidases such as lignin peroxidase and manganese peroxidase, and also fortuitously degrade a wide

variety of recalcitrant pollutants. These fungi are also able to mineralize TNT. For example, Fernando *et al.* (1990) and Fernando and Aust (1991) reported that *P. chrysosporium* rapidly transformed high levels of [^{14}C]TNT in liquid cultures and in soil. In liquid culture, with a starting concentration of 100 mg/l TNT, 14% of label was recovered as $^{14}CO_2$, 52% as water-soluble products, 5% associated with the mycelium, and 22% was recovered as TNT after 30 days. In soil with 10,000 mg TNT/kg soil, after 90 days, 18% of label was recovered as $^{14}CO_2$, 15% as TNT, and 12% was bound to the matrix and could not be extracted. Partial mineralization of TNT by growing mycelia of *P. chrysosporium* was also reported by Spiker *et al.* (1992); interestingly, pre-grown mycelia transformed TNT but did not mineralize it. Mineralization of TNT may be enhanced by the presence of surfactants such as Tween 80 (Hodgson *et al.*, 2000). Treatment of 'red water' or 'pink water', waste streams from munitions manufacture, has been reported using *P. chrysosporium* in various types of suspended-growth and fixed-film bioreactor (Tsai, 1991; Bumpus *et al.*, 1992; Sublette *et al.*, 1992).

More detailed studies have shown that *P. chrysosporium* initially reduces TNT to typical aerobic nitroreductase products such as HADNT and ADNT, and that these are further degraded with partial release of CO_2 (Bumpus and Tatarko, 1994). Mineralization of such reduced products, which may also be produced by photocatalytic reduction of TNT, is considerably more effective than mineralization of TNT itself (Hess *et al.*, 1998). Conversion of TNT to ADNT with subsequent further degradation has also been reported in other white-rot fungi (Donnelly *et al.*, 1997).

Initial reduction of TNT in *P. chrysosporium* may be due to a membrane-associated nitroreductase; both NADPH-dependent (Rieble *et al.*, 1994) and membrane-potential dependent (Stahl and Aust, 1993a, 1995b) activities have been reported.

Subsequent degradation of reduced products appears to involve extracellular peroxidases. Stahl and Aust (1993b) reported that conversion of TNT to ADNT preceded the development of peroxidase activities; disappearance of ADNT coincided with the development of manganese peroxidase activity in nitrogen-limited cultures. Mineralization of TNT was also associated with the presence of lignin peroxidases. In nitrogen-sufficient cultures, peroxidase activity did not develop, and ADNT were partially reduced to DANT. Similarly, Van Aken *et al.* (1997) reported that *Phl. radiata* transformed TNT to 4HA26DNT and 4A26DNT in a process which did not involve extracellular peroxidases, but that peroxidases appeared to be involved in further degradation of these products, since their disappearance was much more rapid under ligninolytic than non-ligninolytic conditions. Hawari *et al.* (1999) reported that conversion of TNT to HADNT, ADNT, and phenolamines (resulting from rearrangement of HADNT) by *Phanerochaete chrysosporium* occurred prior to the development of lignin peroxidase activity, and that mineralization, to a maximum level of 10%, coincided with the development of lignin peroxidase activity. Other products observed included N-acetylated and N-formylated derivatives, tetranitroazoxy dimers, and reduced derivatives thereof, all of which subsequently disappeared.

HADNT has been shown to be both a substrate and an inhibitor of lignin peroxidase in *P. chrysosporium* (Bumpus and Tatarko, 1994; Michels and Gottshalk, 1994), being oxidized to nitrosodinitrotoluene with consequent formation of tetranitroazoxytoluenes. Similarly, Van Aken *et al.* (1999a) reported that cell-free manganese peroxidase of *Phl. radiata* oxidized 4HA26DNT to its nitroso form, with subsequent production of tetranitroazoxytoluenes, TNT, and 4A26DNT; a small amount of

mineralization (4%) was observed during the reaction. Inclusion of glutathione increased the degree of mineralization to 27% and resulted in the formation of unidentified polar products. It was proposed that this might be due to the formation of highly reactive thiol radicals. Further study (Van Aken *et al.*, 1999b) showed that this enzyme, in the presence of 10 mM glutathione, attacked TNT, ADNT, and DANT, in order of increasing activity. Over 5 days, 22% mineralization of TNT and 76% mineralization of 2A46DNT were observed. Similarly, Scheibner and Hofrichter (1998) reported that cell-free extracellular enzymes from *Nematoloma frowardii* and *Stropharia rugosoannulata* degraded 2A46DNT, 4A26DNT and 26DA4NT to unidentified products with partial mineralization. Again, the reaction was enhanced in the presence of thiol compounds (glutathione and L-cysteine).

By contrast, Eilers *et al.* (1999) found that degradation of TNT by another white-rot fungus, *Bjerkandera adusta*, did not appear to be related to manganese peroxidase levels, but rather, was associated with a microsomal cytochrome P450, apparently induced by the presence of TNT. Addition of cytochrome-P450 inhibitors decreased the extent of TNT mineralization from 21% to 1%, and also decreased the extent of conversion of TNT to polar products and its incorporation into biomass.

Finally, it should be noted that extracellular fungal enzymes may also be involved in the humification of TNT-reduction products. For example, Dawel *et al.* (1997) reported that a fungal laccase catalyzed coupling of 24DA6NT to guaiacol (2-methoxyphenol), a model humus constituent.

TRANSFORMATION OF TNT BY PLANTS

Study of interactions of explosives with plants has been motivated by two interacting concerns. Firstly, agricultural plants may take up toxic explosive components from contaminated soil and accumulate these, or transform them to toxic or mutagenic products, rendering the plants unfit for consumption. Concern over the presence of explosive residues in agricultural plants has led to the development of sensitive techniques for detection of explosives in plant tissues (Harvey *et al.*, 1990, 1997b; Larson, 1997; Larson *et al.*, 1999a). Secondly, plants may be able to transform explosives to harmless products, and may therefore be useful in bioremediation of contaminated land and water. This type of process, known as phytoremediation, has been studied for a variety of pollutants (Schnoor *et al.*, 1995).

TNT-contaminated soil is significantly toxic to plants. This has been investigated extensively in various grasses (Peterson *et al.*, 1996, 1998; Krishnan *et al.*, 2000). Inoculation of soil with bacteria known to reduce TNT to ADNT and DANT was reported to increase toxicity to grasses in some cases, presumably due to the known toxicity of HADNT; in other cases, the plant–bacterial combination showed potential as a bioremediation system (Siciliano and Greer, 2000). Gong *et al.* (1999) investigated the sensitivity of various agricultural plants to TNT; interestingly, sub-inhibitory TNT levels apparently stimulated growth. Oats (*Avena sativa*) was most TNT-resistant, tolerating 1.6 g TNT/kg soil.

Many types of terrestrial plants have been shown to take up TNT and transform it to typical aerobic nitroreductase products such as ADNT. This was first demonstrated by Palazzo and Leggett (1986) using yellow nutsedge (*Cyperus esculentus*) grown in hydroponic systems with up to 20 mg/l TNT. TNT was toxic to plants at 5 mg/l; the

major extractable product was 4A26DNT, which accumulated particularly in roots. Subsequent studies have shown a number of other products, as well as binding of TNT metabolites to plant macromolecules. For example, Scheidemann *et al.* (1998) investigated uptake and transformation of TNT by agricultural plants. The principal extractable products were ADNT isomers. Only one species, bean (*Phaseolus vulgaris*) tolerated TNT at 500 mg/kg. It was also noted that four of six cultivars of *Triticum aestivum* (wheat) caused significant reductions in rhizosphere TNT levels. Further study of *T. aestivum* (Sens *et al.*, 1999) with [^{14}C]TNT showed that 57% of radioactivity ended up in the cell wall (27% apparently covalently bound to the lignin fraction) and 43% was detected in the cytoplasm; cytoplasmic metabolites detected included 2ADNT, 4ADNT, one other non-polar product and ten unidentified polar products. Similar studies in *P. vulgaris* (Sens *et al.*, 1998) showed approximately equal amounts of radioactivity in cell wall and cytoplasm.

Poplar has attracted attention for bioremediation purposes due to its deep roots. Hybrid poplar (*Populus deltoides* × *nigra*) has been reported to take up TNT in soil and hydroponic systems (Thompson *et al.*, 1998a,b). About 75% of radioactivity from [^{14}C]TNT remained in roots; about 10% was detected in leaves. The majority of plant-associated label was not extractable; ADNT and several unidentified polar products were detected. Concentrations of TNT exceeding 5 mg/l were toxic, causing diminished transpiration, bleaching, and loss of leaves.

Other plant-associated transformations of TNT have also been proposed. Schneider *et al.* (1996) tested plants growing at a contaminated ammunition plant site, and reported that TNT, ADNT and DNT accumulated in roots, and to a lesser extent in leaves and stems, with little variation between species. They suggested that transformation of TNT to DNT might be occurring in plant tissues. This has been reported in some bacteria (discussed above), but other studies have not demonstrated such a process in plants; since DNT may also be present in TNT-contaminated soil, it would be necessary to exclude the possibility that plants had simply taken up and accumulated DNT, which is generally less susceptible to fortuitous biotransformation than TNT (see, for example, Parrish, 1977).

Larson *et al.* (1999b) described uptake and transformation of TNT from water in several agricultural plant species, as well as the aquatic plant *Myriophyllum aquaticum* (parrot-feather). TNT was taken up and transformed with production of unidentifed polar, high M_r products. TNT transformation has also been reported in *Myriophyllum spicatum* (Pavlostathis *et al.*, 1998). Another study involving *M. aquaticum* may cast light on the nature of some of these unidentified polar products. Bhadra *et al.* (1999b) tested *M. aquaticum* for the ability to produce oxidized TNT derivatives. Products detected included 2-amino-4,6-dinitrobenzoic acid, 2,4-dinitro-6-hydroxybenzyl alcohol, 2-N-acetoxyamino-4,6-dinitrobenzaldehyde, 2,4-dinitro-6-hydroxytoluene, and two binuclear products clearly distinct from tetranitroazoxytoluenes. These products accounted for a substantial fraction of total TNT transformation products. It should be noted that trinitrobenzaldehyde, 2,4,6-trinitrobenzyl alcohol and 2,4,6-trinitrobenzoic acid are significant products of abiotic photodegradation of TNT (Rosenblatt *et al.*, 1991; Gorontzy *et al.*, 1994).

Many other studies have also investigated transformation of TNT by aquatic plants; this is potentially relevant to the treatment of contaminated lagoons which had been long used to hold munitions wastewater. For example, Qaisi *et al.* (1996) studied the

kinetics of TNT biotransformation by *M. aquaticum* and other aquatic plants, and reported that TNT desorption and transport appeared to be rate-limiting. A number of studies have also investigated the role of aquatic plants in biotransformation of TNT and other explosives in wetland systems (Best *et al.*, 1997, 1999a,b; Sikora *et al.*, 1997; Rivera *et al.*, 1998). TNT at 1 mg/l was biodegraded in the presence of any of a number of submerged and emergent aquatic plant species, with transient appearance of ADNT and a number of unidentified polar products.

Several studies have reported transformation of TNT in axenic (particularly, microorganism-free) plant systems. In perhaps the simplest possible case, the cyanobacterium *Anabaena*, a model system for plant-associated biotransformation, has been reported to transform TNT at concentrations below 10 mg/l, higher concentrations being toxic (Pavlostathis and Jackson, 1999). At pH 7.5 to 8.5, the main products detected were tetranitroazoxytoluenes, accounting for about 25% of TNT transformed. At pH 5.6 to 5.9, essentially complete conversion to HADNT was observed, followed by slow transformation to unidentified products.

Hughes *et al.* (1997) compared the fate of TNT in three systems: *Myriophyllum spicatum* with associated microorganisms; axenic *M. aquaticum*; and root cultures of *Catharanthus roseus* (Madagascar periwinkle). In all cases, TNT was completely transformed over 7 days, with some production of ADNT (maximum of 16% of transformed TNT). No other metabolites were identified. No mineralization of [^{14}C]TNT was observed; about 50% of label was eventually associated with plant biomass. Plant-associated radioactivity was found in roots, stems and leaves, and became progressively more resistant to extraction (Vanderford *et al.*, 1997). Further study (Bhadra *et al.*, 1999a) showed that, in *C. roseus* root cultures, ADNT became conjugated via the amine group to small molecules within plant cells; such conjugates may account for some of the unidentified polar products commonly observed in plant biotransformation of TNT, and may also be intermediates in the production of non-extractable products. Transformation of TNT has also been reported in cell suspension cultures of *Datura innoxia* (Lucero *et al.*, 1999).

It has been speculated that transgenic plants expressing microbial degradative enzymes may offer an optimal system for *in situ* bioremediation. French *et al.* (1999) reported construction of transgenic tobacco (*Nicotiana tabacum*) expressing PETN reductase from *Enterobacter cloacae*; as discussed above (under 'aerobic degradation of TNT by bacteria'), this enzyme reduces TNT to the dihydride-Meisenheimer adduct which is apparently further transformed to unidentified water-soluble non-aromatic products with elimination of nitrite. These transgenic plants were more tolerant of TNT than wild-type *N. tabacum*; germination and growth of wild-type seeds were strongly inhibited in agar containing 0.05 mM TNT, whereas transgenic seeds germinated and grew normally in this medium. This implies that the products of PETN-reductase mediated degradation of TNT, as yet unidentified, are less toxic than TNT or its common reduction products.

Biotransformations of nitramines

RELEVANT CHEMISTRY OF NITRAMINES

Nitramines are highly susceptible to alkaline hydrolysis; in the case of RDX, the

initial step is elimination of nitrite to yield an unstable product which breaks down to release products such as ammonia and formaldehyde (Croce and Okamoto, 1979). Nitramines are also susceptible to reductive denitration. For example, in HMX and tetryl, nitro groups can be replaced by hydrogen using mild reducing agents such as 1-benzyl-1,4-dihydronicotinamide, an analogue of NAD(P)H; the reaction requires radical initiation which may be provided by photolysis or an agent such as dithionite (Chapman *et al.*, 1996). Cleavage occurred via the radical anion. In the case of tetryl, the product was N-methylpicramide (N-methyl-2,4,6-trinitroaniline); in the case of HMX, the apparent product was not octahydro-1,3,5,7-tetrazocine; apparently this unstable compound dissociated to formaldehyde and ammonia which formed the more stable adduct hexamethylenetetramine. These results have obvious implications for possible biochemical breakdown pathways of nitramine explosives.

FATE OF RDX AND HMX IN THE ENVIRONMENT

RDX is probably the explosive of greatest environmental concern after TNT. RDX is released into the environment at up to 12 mg/l in manufacturing process wastewaters (Jackson *et al.*, 1978), whilst the levels of HMX contamination resulting from open burning and open detonation vary considerably from only 10 mg/kg to 1640 mg/kg at one antitank firing range (Jenkins *et al.*, 1998; Thiboutot *et al.*, 1998). RDX and HMX are toxic, having adverse effects on the central nervous system, and are classified as class C carcinogens (Yinon, 1990; Rocheleau *et al.* 1999; Talmage *et al.*, 1999). Under aerobic conditions, RDX is highly persistent in soil and groundwater; it is much less susceptible than TNT to biotransformation, is less strongly adsorbed to soil, and undergoes much less immobilization (Singh *et al.*, 1998). Under anaerobic conditions, RDX is susceptible to biological reduction, but, in contrast to the case with TNT (described above), persistent reduced metabolites are not observed. For example, Shen *et al.* (1997) investigated transformation of [^{14}C]RDX in aerobic or anaerobic soil slurry bioreactors. RDX was recalcitrant under aerobic conditions, but under anaerobic conditions, 15% mineralization to $^{14}CO_2$ was observed due to the action of indigenous soil microorganisms; supplementation with anaerobic sewage sludge resulted in up to 60% mineralization.

RDX does not appear to be effectively reduced under nitrate-reducing conditions; for example, Freedman and Sutherland (1998) reported that, in an anoxic biological treatment system designed to remediate RDX- and nitrate-containing wastewater, RDX was not transformed as long as nitrate was present. Similarly, Ronen *et al.* (1998) reported biodegradation of munitions wastewater containing RDX and nitrate, using a sequential anaerobic (denitrifying) and aerobic system. Removal of RDX in the aerobic stage was dependent on complete removal of nitrate in the anoxic stage. Interestingly, it appeared that RDX was completely degraded to undetectable products under aerobic conditions, in contrast to its usual recalcitrance; RDX was apparently used as a nitrogen source, with cyclohexanone (a common component of RDX process wastewaters) supplied as a supplementary carbon source. Conceivably, RDX was attacked by enzymes of cyclohexanone degradation.

Both RDX and HMX can be effectively degraded under sulphate-reducing conditions (Boopathy *et al.*, 1998c,d). Composting has also been reported to remove RDX and HMX effectively (Williams *et al.*, 1992; Keehan and Sisk, 1996).

BIODEGRADATION OF RDX UNDER ANAEROBIC CONDITIONS

McCormick *et al.* (1981) reported that, under anaerobic conditions in nutrient broth, mixed cultures from sewage sludge rapidly degraded RDX, with transient accumulation of the mono-, di-, and trinitroso derivatives. These were apparently converted to products including formaldehyde, methanol, hydrazine, 1,1-dimethylhydrazine and 1,2-dimethylhydrazine. Essentially no radioactivity from [^{14}C]RDX was recovered as $^{14}CO_2$. The authors proposed that reduction of a nitroso group to the dihydroxylamino group resulted in an unstable product subject to hydrolytic cleavage and rearrangement. Young *et al.* (1997b) reported a similar degradation process using a soil slurry reactor supplemented with corn steep liquor. Indigenous soil microorganisms effectively catalyzed RDX breakdown and introduction of known RDX-degrading isolates had little effect.

These results demonstrate that RDX, the most significant nitramine pollutant, can be effectively broken down. However, it should be noted that nitrosamines and hydrazines are toxic, mutagenic, carcinogenic and generally undesirable compounds, and for bioremediation purposes, it would be desirable to ensure that they were subsequently transformed to harmless products, for example by including a subsequent aerobic stage to promote their oxidation (Roberts *et al.*, 1996).

More recently, Hawari *et al.* (2000) reported the degradation of RDX in liquid cultures with municipal sludge. At least two routes of degradation were suggested. The first route involved the production of the nitroso derivatives hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine. The second route produced two presumed ring cleavage products methylenedinitramine and bis(hydroxymethyl)nitramine. None of the metabolites described persisted in the system, disappearing to give rise to nitrous oxide, formaldehyde, methanol and formic acid which in turn was broken down to produce methane and carbon dioxide. The two degradation pathways are shown in *Figure 8.4*.

Several studies have reported the anaerobic degradation of RDX by pure bacterial cultures. The results are generally similar to those reported by McCormick *et al.* (1981). For example, Kitts *et al.* (1994) isolated organisms from soil contaminated with RDX and HMX. Some isolates, in pure culture, failed to transform RDX; three which effectively degraded RDX were all identified as members of the Enterobacteriaceae, specifically strains of *Providencia rettgeri*, *Morganella morganii* and *Citrobacter freundii*. All failed to transform RDX under aerobic conditions but degraded it under anaerobic conditions, with transient accumulation of nitroso derivatives; in contrast to previous reports, some radioactivity (5 to 9%) from [^{14}C]RDX was recovered as $^{14}CO_2$, possibly due to less strict anaerobiosis resulting in oxidation of some of the breakdown products. HMX was also transformed to nitroso derivatives, although at a lower rate, presumably due at least partially to its lower solubility. Young *et al.* (1997a) investigated RDX transformation by bacteria isolated from an RDX-degrading consortium derived from horse manure. The isolates investigated were strains of *Pseudomonas putida*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Xanthomonas maltophilia* (= *Pseudomonas maltophilia* = *Stenotrophomonas maltophilia*) and *Escherichia coli*. In anaerobic phases (following aerobic growth), all transformed RDX, with *S. marcescens* most effective. Kitts *et al.* (2000) have demonstrated that RDX

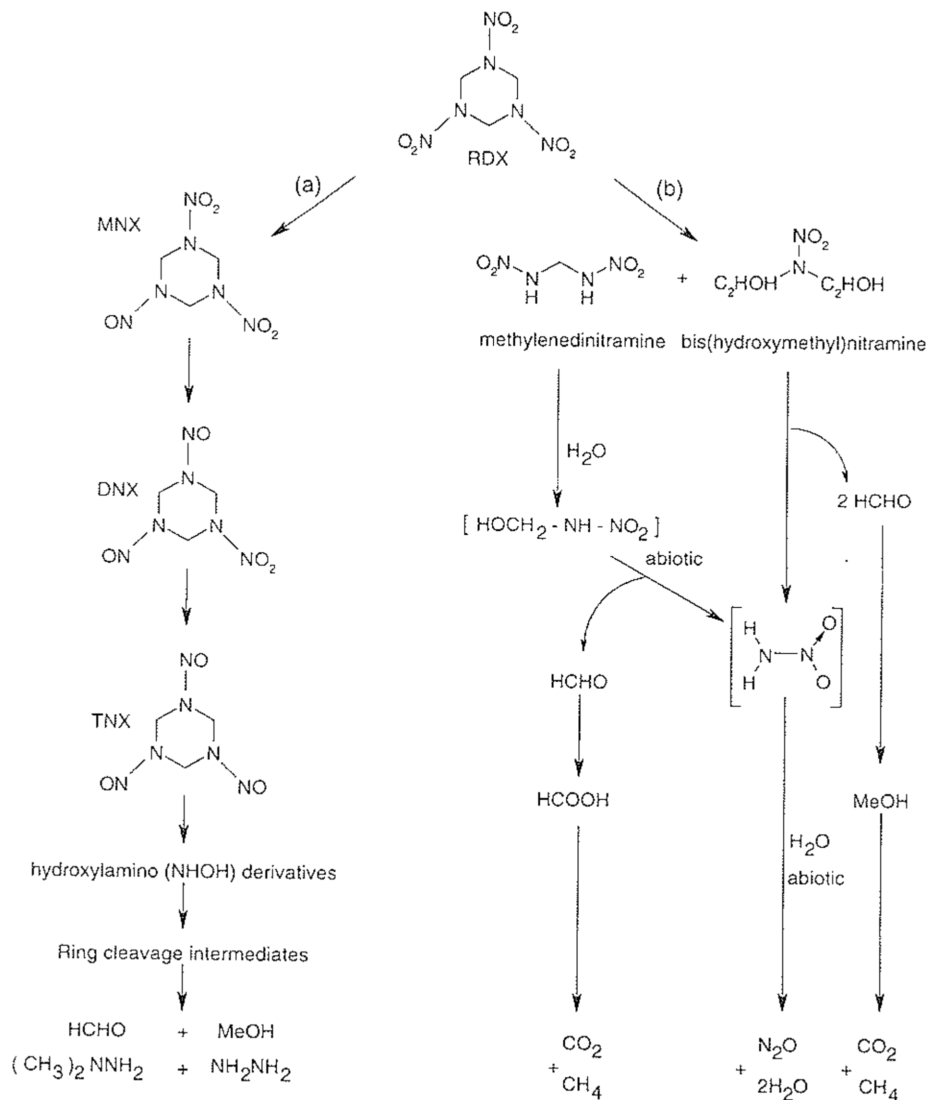

Figure 8.4. Pathways for the biodegradation of RDX with municipal anaerobic sludge (modified from Hawari *et al.*, 2000). Pathway a, degradation via reduction to the nitroso derivative (McCormik *et al.*, 1981); Pathway b, degradation via direct ring cleavage (Hawari *et al.*, 2000).

reduction in soil bacteria of the family Enterobacteriaceae is due to the same oxygen-insensitive nitroreductases involved in TNT reduction (discussed above under 'aromatic nitroreductases').

RDX and HMX can also be degraded by sulphate-reducing bacteria. Boopathy *et al.* (1998c,d) reported that a consortium of several *Desulfovibrio* spp. was able to degrade RDX or HMX as sole nitrogen sources. Nitrogen released from these compounds was apparently converted to ammonia prior to assimilation.

DEGRADATION OF RDX BY BACTERIA UNDER AEROBIC CONDITIONS

Most studies, including those described above, have found that RDX is persistent under aerobic conditions. However, as noted above, Ronen *et al.* (1998) reported mineralization of RDX in an aerobic wastewater treatment process with cyclohexanone supplied as carbon source. Two recent reports have demonstrated aerobic breakdown of RDX by pure cultures of bacteria able to utilize it as sole nitrogen source. Binks *et al.* (1995) reported aerobic degradation of RDX by a strain of *Stenotrophomonas maltophilia* (formerly *Pseudomonas maltophilia* and *Xanthomonas maltophilia*) designated PB1. This organism, isolated from RDX-contaminated soil, apparently used three of the six available nitrogen atoms of RDX for growth, as judged by the growth yield. RDX degradation was inhibited by the presence of ammonium nitrate. A putative metabolite of RDX was detected, and was tentatively identified as methylene-N-(hydroxymethyl)-hydroxylamine-N'-(hydroxymethyl)-nitramine; a second metabolite was tentatively identified as methylene-N-nitroamino-N'-acetoxy-ammonium chloride, a breakdown product of 1-acetyl-hexahydro-3,5-dinitro-1,3,5-triazine, a known minor component of RDX preparations. In a recent study of anaerobic RDX degradation using municipal sludge, Hawari *et al.* (2000) identified methylenedinitramine as one of the intermediates in RDX degradation. Hawari *et al.* (2000) noted that the mass spectrum obtained by Binks *et al.* (1995) contained a strong mass ion with m/z at 136 Da which is characteristic of the molecular mass ion of methylenedinitramine. They went on to suggest that since the reaction of methylenedinitramine with HCl (the acid used by Binks *et al.*, 1995) would give a salt with the same molecular mass ion as methylene-N-nitroamino-N'-acetoxyammonium chloride, the methylenedinitramine chloride salt was more likely to have been the product Binks *et al.* observed.

Coleman *et al.* (1998) also reported aerobic degradation of RDX. *Rhodococcus erythropolis* DN22, isolated from RDX-contaminated soil, grew aerobically with RDX as sole nitrogen source, with transient accumulation of nitrite to a maximum level of 1.8 mol nitrite per mol RDX initially present. Growth yield studies suggested that, as in *S. maltophilia* PB1, 3 of the 6 available nitrogen atoms were ultimately assimilated. The presence of ammonium, but not nitrate or nitrite, inhibited RDX degradation; RDX was not transformed in the absence of oxygen. Addition of *R. erythropolis* DN22 to contaminated soil provoked significant disappearance of RDX, with essentially no aerobic RDX degradation occurring in controls. Recent work on RDX degradation by *R. erythropolis* DN22 implicated the involvement of cytochrome P-450 (Coleman and Duxbury, 1999). This enzyme was also implicated in RDX degradation by another *Rhodococcus* sp. YH11 in a recent study by Tekoah and Abeliovich (1999). Neither study identified any metabolites other than nitrite.

We are also currently working with a strain of *Rhodococcus* sp., which degrades RDX via a membrane-bound enzyme with production of nitrite, ammonia and formaldehyde (A. Basran and N.C. Bruce, unpublished information). The nature of this enzyme has not yet been elucidated, but the similarity of the reaction products to those observed under alkaline hydrolysis has led to speculation that the initial attack may be via elimination of nitrite to form an unstable product as seen in alkaline hydrolysis (Croce and Okamoto, 1979).

TRANSFORMATION OF RDX AND HMX BY FUNGI

Relatively little has been reported about transformations of RDX in fungi. Fernando and Aust (1991) reported that the white-rot fungus *Phanerochaete chrysosporium* was able to mineralize RDX; in liquid cultures, 67% of 0.028 mg/l [^{14}C]RDX was converted to $^{14}CO_2$ over 30 days, with only 4% of the initial RDX being recovered; in soil, 76% conversion to $^{14}CO_2$ was seen. No metabolites were detected. RDX removal by *P. chrysosporium* was also reported by Sublette *et al.* (1992), in a rotating biological contactor system treating 'pink water' contaminated with approximately 150 mg/l TNT and 25 mg/l RDX. Bayman *et al.* (1995) compared the RDX-degrading ability of *P. chrysosporium* with that of several other fungi: another lignin-degrading basidiomycete, *Cyanthus pallidus*; a zygomycete, *Cunninghamella echinulata* var *elegans*; and an ascomycete, *Cladosporium resinae*, using a nutrient poor non-ligninolytic medium with 100 mg/l RDX. RDX was found to be much less toxic than TNT to these fungi. Over the first 3 days, extractable RDX diminished by 22% with *P. chrysosporium*, with the other fungi showing decreases ranging from 12% (*Cu. echinulata*) to 31% (*Cl. resinae*). Little change was seen over the subsequent 14 days. For all four fungi, radioactivity from [^{14}C]RDX was recovered essentially only in organic fractions, with little or no radioactivity in aqueous or gaseous fractions or associated with cell wall material. In particular, and in contrast to the results of Fernando and Aust (1991), in which much lower RDX levels were used, there was no evidence of mineralization, although in the case of *P. chrysosporium* only about 75% of the label was eventually accounted for.

TRANSFORMATION OF RDX BY PLANTS

RDX transformation in plants is less well-studied than that of TNT. Harvey *et al.* (1991) reported that hydroponically grown bush bean plants (*Phaseolus vulgaris*) took up [^{14}C]RDX from the medium; accumulation in aerial tissues, and production of unidentified polar metabolites, was detected, but production of $^{14}CO_2$ or volatile organics did not occur. Thompson *et al.* (1999) reported similar experiments with hydroponically grown hybrid poplar trees (*Populus deltoides* × *nigra*); 60% of uptaken RDX accumulated in leaves, and, in contrast to the results of Harvey *et al.* (1991), no significant transformation occurred over 7 days. Larson *et al.* (1999b) reported uptake and transformation of RDX in several agricultural and aquatic plants, with production of polar, high M_r products.

RDX degradation has also been examined in constructed wetlands (Best *et al.*, 1999a,b). Both submerged and emergent plants were shown to take up [^{14}C]RDX, which accumulated in sites of active growth. Biotransformation of RDX to unidentified products was also observed. Mineralization was very low (less than 5%) and production of volatile organics negligible. From these results it is clear that plants can take up and transform RDX, but the nature of the transformation products remains unclear.

BIOTRANSFORMATIONS OF TETRYL

Tetryl (2,4,6-trinitrophenylmethylnitramine) possesses both aromatic nitro groups and an N-nitro group, and therefore may undergo reactions typical of both classes of

compound. For example, Harvey *et al.* (1992) reported that tetryl is rapidly transformed in soil, with the major product being N-methylpicramide (N-methyl-2,4,6-trinitroaniline), implying rapid N-denitration. A minor product was aminodinitrophenylmethylnitramine, resulting from reduction of one of the aromatic nitro groups.

Microbial degradation of tetryl has been relatively little-studied. Boopathy (2000) reported that a sulphate-reducing consortium, using pyruvate as substrate, converted tetryl to aniline, which underwent further degradation; this implies reductive denitration of both aromatic and N-nitro groups. Degradation of tetryl in a soil-slurry bioreactor, with molasses as carbon source, has also been reported (Boopathy and Manning, 1998). Tetryl was completely degraded over 3 months, with transient accumulation of trinitro-N-methylaniline (N-methylpicramide), trinitroaniline, diamino-dinitrobenzene, nitroaniline and aniline, suggesting initial N-denitration, followed by N-demethylation and reductive denitration.

From the results described above, it seems that tetryl is extremely susceptible to reductive N-denitration, perhaps due to the electron-withdrawing effect of the trinitrophenyl substituent. A number of studies have reported reductive N-denitration of tetryl by enzymes known to act as oxygen sensitive (one electron) nitroreductases. Shah and Spain (1996) reported that ferredoxin-NADP oxidoreductase, glutathione reductase, xanthine oxidase, and cytochrome c reductase all catalyzed NADPH-dependent elimination of nitrite from tetryl to form N-methylpicramide. This reaction was inhibited in the presence of oxygen and was shown to proceed via the nitroanion radical. Interestingly, in the presence of high concentrations of NADPH, non-enzymic reaction between tetryl and NADPH was seen, although this was negligible at lower NADPH concentration; this recalls the results of Chapman *et al.* (1996; discussed above under 'relevant chemistry of nitramines'). Similar results have been described using mammalian DT-diaphorase (NAD(P)H-quinone oxidoreductase) (Anusevicius *et al.*, 1998) and *Arabidopsis thaliana* (thale cress) thioredoxin reductase (Miskiniene *et al.*, 1998). Unsurprisingly, N-methylpicramide has also been reported as a biotransformation product of tetryl in plant tissues (Harvey *et al.*, 1993).

Biotransformations of nitrate esters

RELEVANT CHEMISTRY OF NITRATE ESTERS

Nitrate esters are readily hydrolysed under acidic or alkaline conditions. However, typical products are the aldehyde and nitrite, rather than (or in addition to) the alcohol and nitrate (Boschan *et al.*, 1955; Urbanski, 1965). Hydrolysis in the presence of mild reducing agents yields the alcohol and nitrite. Nitrate esters can also be reduced at an electrode, yielding the alcohol and nitrite. These reactions are in contrast to those of sulphate and phosphate esters, which are easily hydrolysed to the alcohol and sulphate or phosphate (White and Snape, 1993). This presumably reflects the strong electron-withdrawing nature of the nitrate ester group. Unlike sulphate esters, nitrate esters are uncharged and show relatively low solubility in aqueous solution (for data, see Rosenblatt *et al.*, 1991).

FATE OF NITRATE ESTERS IN THE ENVIRONMENT

Nitrate esters have found a variety of applications and have been produced over the

past 100 years for use as high explosives and medicinally as vasodilators. They are used therapeutically at low doses but at higher concentrations nitrate esters and their metabolites are toxic and have significant environmental impact. Mammalian toxicity data suggest that GTN has acute toxicity levels of between 30 and 1300 mg/kg (Wendt *et al.*, 1978) and an LD_{50} of 1 mg/l for fish is quoted by Urbanski (1984). The major environmental problems arise as a result of wastewater streams from GTN manufacturing plants and the disposal of munitions containing nitrocellulose and GTN once they have exceeded their shelf life. GTN and its related incompletely nitrated products are fairly soluble in water and conventional chemical treatments of waste streams include strong acid, strong alkali, or denitration with sodium sulphide. The products obtained by such treatments may include the partially denitrated GTN, glycidyl nitrate (2-nitrooxymethyl-oxirane), and glycidol (oxiranyl methanol) along with nitrite and nitrate, which can require further denitrifying treatment prior to final discharge.

DEGRADATION OF NITRATE ESTERS IN MAMMALIAN TISSUES

Nitrate esters such as GTN, PETN and isosorbide dinitrate, administered as vasodilators for control of various medical conditions, are denitrated to produce the parent alcohols. Nitrogen may be released as nitrite or nitric oxide, only the latter possessing significant pharmacological activity (Bennett *et al.*, 1994). A large body of literature exists concerning the nature of the biochemical processes involved in nitrate ester metabolism in mammalian tissues. Enzymes implicated in these reactions, sometimes designated nitrate ester reductases or organic nitrate reductases, include xanthine oxidoreductase (O'Byrne *et al.*, 2000), cytochrome P450 reductase (McGuire *et al.*, 1998), cytochromes P540 (Yuan *et al.*, 1997), glutathione-S-transferases (Kenkare and Benet, 1996; Nigam *et al.*, 1996; Singhal *et al.*, 1996), and other thiol-dependent enzymes (Ogawa *et al.*, 1995a,b). The question of which enzymes are most significant in terms of the pharmacological effect of nitrate esters has not yet been resolved. Interestingly, several of these enzymes have also been reported to catalyze one-electron reduction of aromatic nitro compounds in mammalian tissues (discussed above under 'aromatic nitroreductases'). By analogy, it might be suggested that one-electron reduction of nitrate esters results in liberation of nitric oxide, and two-electron reduction yields nitrite ion, as seen in some bacterial flavoprotein nitrate ester reductases (discussed below), which, interestingly, may also display two-electron (oxygen-insensitive) aromatic nitroreductase activity (French *et al.*, 1998). However, this may be an over-simplification of a complex process.

DEGRADATION OF NITRATE ESTERS BY MICROORGANISMS

A variety of microorganisms have been shown to degrade nitrate esters, typically by sequential denitration with production of the parent alcohol, and release of nitrogen which can be detected as nitrate, nitrite or nitric oxide. Degradation of nitrate esters by microorganisms has been reviewed by White and Snape (1993).

A number of studies have shown that mixed microbial cultures such as activated sludge sequentially denitrate GTN to produce glycerol-1,2-dinitrate (12GDN) and glycerol-1,3-dinitrate (13GDN), followed by glycerol-1-mononitrate (1GMN) and

glycerol-2-mononitrate (2GMN) and eventually glycerol (Wendt *et al.*, 1978; Bhaumik *et al.*, 1997, 1998; Christodoulatos *et al.*, 1997; Accashian *et al.*, 1998). Generally, sequential denitration steps proceed at decreasing rates, with GMN often accumulating, and a co-substrate is usually required, since GTN is not generally effective as a sole carbon source, probably due to the low rate of removal of the last nitrate group. Accashian *et al.* (1998), however, reported use of GTN as sole carbon and nitrogen source by a mixed microbial culture. The stereospecificity of denitration varies, but generally all isomers of GDN and GMN are observed at detectable levels.

BIODEGRADATION OF NITRATE ESTERS BY BACTERIA

A variety of bacteria have been shown to degrade GTN and PETN and, in some cases, to use them as sole nitrogen sources for growth.

Meng *et al.* (1995) isolated a number of bacteria which could either use GTN as a sole nitrogen source or grow in the presence of high GTN concentrations. The best of these, identified as *Bacillus thuringiensis* or *Bacillus cereus* (closely related species, difficult to distinguish) and *Enterobacter agglomerans*, were further investigated. In both cases, resting cells denitrated GTN with nitrite production; to judge by the nitrite levels detected, removal of the second nitrate group was considerably slower than that of the first. Expression of the enzymes involved was constitutive. In the *Bacillus* sp., activity in cell extracts was soluble; in *E. agglomerans*, activity was associated with the membrane pellet. In both cases, activity was not diminished through dialysis, suggesting that small soluble cofactors such as NADPH were not required. Using [^{14}C]GTN, the metabolites were identified as 12GDN, 13GDN, 1GMN, 2GMN and glycerol; essentially all nitrogen released was accounted for as nitrite and/or nitrate (not distinguished in cell free experiments). The authors suggested that nitrogen was initially released as nitrate and then reduced to nitrite. The same *Bacillus* strain was also found to denitrate propylene glycol dinitrate (PGDN) to propylene glycol mononitrate (PGMN), which was eventually denitrated to propylene glycol after prolonged incubation (Sun *et al.*, 1996). Again, the denitration activity was constitutive and did not require dialysable cofactors.

Other studies have found that bacterial denitration of GTN and PETN in strains of *Enterobacter*, *Agrobacterium* and *Pseudomonas* is reductive, requiring NADPH or NADH and releasing nitrogen as nitrite, as shown in *Figure 8.5*. For example, Binks *et al.* (1996) described a strain of *Enterobacter cloacae*, designated PB2, capable of growth with GTN or PETN as sole nitrogen source. The enzyme responsible for denitration of GTN and PETN was found to be a soluble NADPH-dependent monomeric FMN binding flavoprotein of M_r 40,000, designated PETN reductase. The gene encoding this enzyme, designated *onr* (for organic nitrate reductase) was cloned and sequenced (French *et al.*, 1996); the sequence showed that PETN reductase was related to the Old Yellow Enzyme family of flavoenzymes. Like its closest relative, morphinone reductase from *Pseudomonas putida* M10 (French and Bruce, 1995), PETN reductase was also able to reduce α,β-unsaturated ketones such as 2-cyclohexen-1-one. Interestingly, later study showed that PB2, and purified PETN reductase, could also reduce TNT to its hydride-Meisenheimer complex and further to the dihydride adduct (described above under 'aerobic degradation of TNT by bacteria'); this was the first report of a purified enzyme able to catalyze this

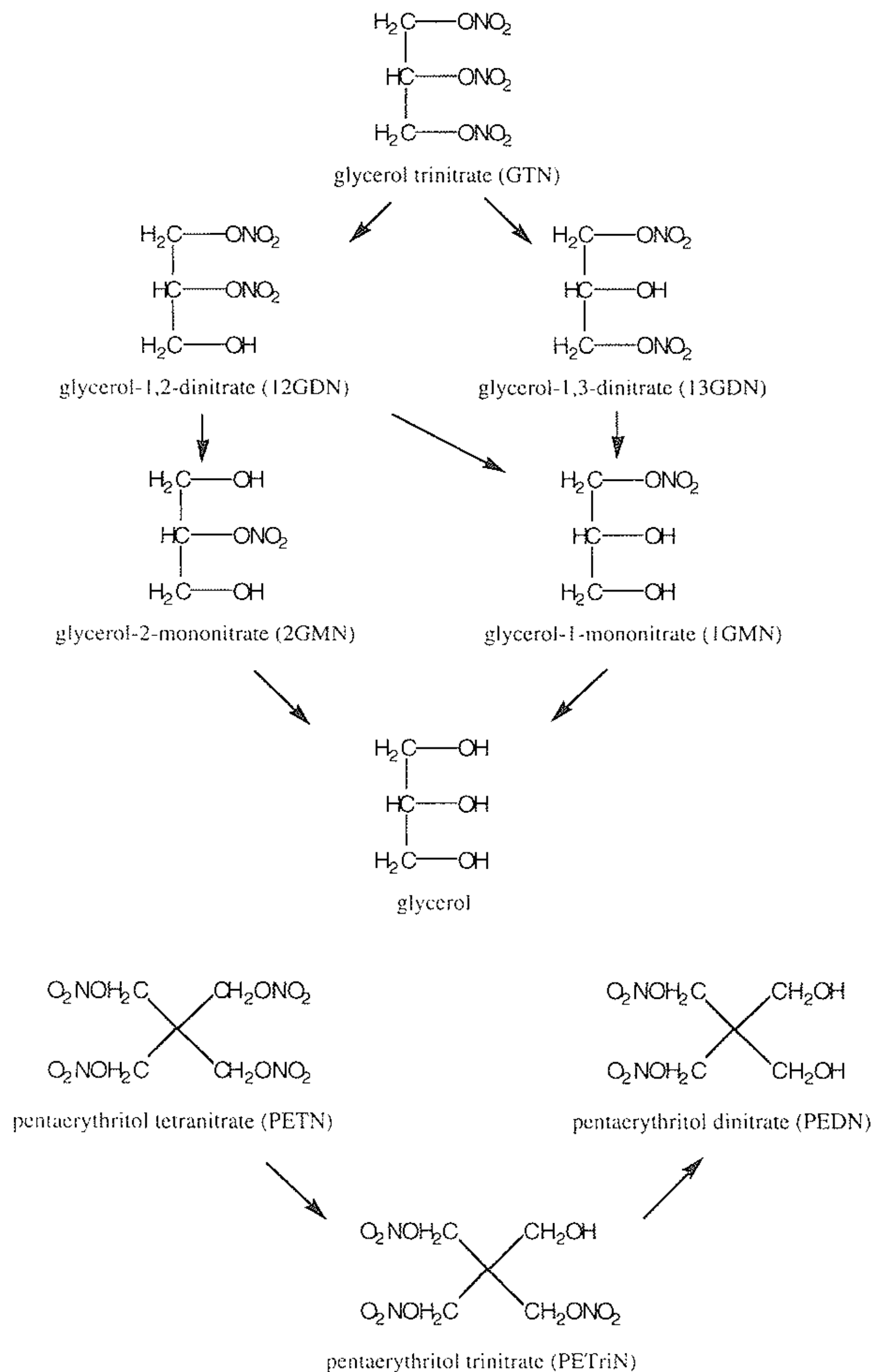

Figure 8.5. Bacterial transformations of GTN and PETN. Each step involves oxidation of NAD(P)H to $NAD(P)^{+}$ and release of nitrite.

reaction. Since this pathway shows considerable promise for bioremediation of TNT, this enzyme has undergone considerable further study, and a crystal structure has been determined (Moody *et al.*, 1998).

Similarly, White *et al.* (1996a) reported that a strain of *Agrobacterium radiobacter*, isolated from explosives-contaminated soil, could grow with GTN as sole nitrogen source. The enzyme responsible for GTN denitration, designated GTN reductase, was purified and the structural gene cloned and sequenced (Snape *et al.*, 1997). In sequence and properties, GTN reductase was very similar to PETN reductase of *E. cloacae* PB2, except that it was specific for NADH rather than NADPH as cofactor; also, GTN reductase was induced by the presence of GTN, whereas PETN reductase in *E. cloacae* PB2 was produced constitutively. A similar degradation pathway for GTN was reported for *Pseudomonas* sp. strain R1-GTN1 (White *et al.*, 1996b,c).

Enzymes similar to PETN reductase and GTN reductase have also been reported in two other *Pseudomonas* strains able to grow with GTN as sole nitrogen source (Blehert *et al.*, 1997) (interestingly, 9 of 13 laboratory strains of *Pseudomonas* were also able to degrade GTN). Cloning and sequencing of the structural genes encoding these enzymes, designated *xenA* and *xenB*, revealed that both enzymes were closely related to PETN reductase, GTN reductase, morphinone reductase and other bacterial, fungal and plant flavoproteins of this family (Blehert *et al.*, 1999). It now appears that enzymes of this group are widespread in bacteria; for example, an *Escherichia coli* homologue, NemA, purified as an N-ethylmaleimide reductase, has been reported (Miura *et al.*, 1997). These enzymes may serve a role in detoxification of pro-oxidant substances (Blehert *et al.*, 1999), as previously suggested for the bacterial oxygen-insensitive nitroreductases (described above under 'aromatic nitroreductases'), which are also small FMN-binding flavoproteins of broad substrate specificity.

The denitration of GTN can result in the production of two isomers of the dinitrate and mononitrate. The variation in isomeric composition of GTN breakdown products has often been suggested as evidence of multiple degradation pathways (Bennett *et al.*, 1994) or the result of multiple enzymes with differing regiospecificities (White and Snape, 1993). However, it is apparent that the regiospecificity of bacterial nitrate ester reductases varies from species to species despite significant similarity in enzyme structure. When *A. radiobacter* was grown on GTN as sole nitrogen source, the major product was 1,3-GDN with a small amount of 1,2-GDN, which were eventually broken down to 1-GMN and a small quantity of 2-GMN. Detnitration of the C2 nitrate group by resting cells was around ten times faster than denitration of the terminal nitrate groups (White *et al.*, 1996a).

When the purified nitrate ester reductase from the same organism was used, the ratio of GDN products was the same (Snape *et al.*, 1997). A similar bias towards denitration at the 2-position of GTN and 1,2-GDN was observed in the products obtained with the purified enzyme from *P. fluorescens* (Blehert *et al.*, 1999). However, denitration of GTN by the *P. putida* enzyme gave the 2:1 ratio of 1,2- to 1,3-GDN expected if there were no regioselective bias. It is therefore clear that subtle differences in the enzyme substrate recognition influence regioselectivity.

Degradation of EGDN by bacterial cutures has also been reported. Ramos *et al.* (1996) isolated a strain of *Arthrobacter ilicis* capable of denitration of EGDN to ethylene glycol mononitrate (EGMN) and ethylene glycol, which could be mineral-

ized; mineralization was enhanced in co-culture with a strain of *Agrobacterium radiobacter* capable of oxidizing ethylene glycol.

DEGRADATION OF NITRATE ESTERS BY FUNGI AND PLANTS

Given that nitrate esters are readily denitrated by mammalian tissues, it is not surprising that they are also degraded in a similar way by fungal and plant tissues. DuCrocq *et al.* (1989) reported that the filamentous fungus *Geotrichum candidum* was able to denitrate GTN with production of GDN and GMN, as seen in bacteria. DuCrocq *et al.* (1990) investigated the ability of the white-rot fungus *Phanerochaete chrysosporium* to degrade GTN. As in other organisms, GTN was transformed to GDN and GMN. A greater degreee of site-specificity of attack was noted than in most other organisms, with glycerol-2-moninitrate the predominant product. More detailed investigation (Servent *et al.*, 1991, 1992) showed that GTN was attacked by at least two different classes of enzyme: an oxygen-insensitive glutathione-dependent enzyme which liberated nitrite, and an oxygen-sensitive NADPH-dependent cytochrome P450-like activity which liberated nitric oxide. No evidence was seen for a hydrolytic mechanism producing nitrate. Zhang *et al.* (1997) reported that *Penicillium corylophilum* was able to denitrate GTN with complete disappearance of GDN and GMN in the presence of suitable carbon and nitrogen sources.

Relatively little has been reported about the transformation of nitrate esters by plant tissues, probably because of their relatively high biodegradability. Goel *et al.* (1997) reported that cell cultures of sugar beet (*Beta vulgaris* L.) were able to denitrate GTN with production of GDN and GMN. Cell extracts also catalyzed this reaction; dialysis did not cause loss of activity, suggesting that dissociable cofactors were not required. French *et al.* (1999) demonstrated that tobacco seedlings in axenic liquid culture were able to denitrate GTN with production of GDN and a small amount of GMN. Transgenic tobacco plants expressing PETN reductase of *Enterobacter cloacae* PB2 were also prepared. These transgenic plants tolerated concentrations of GTN and TNT which were strongly inhibitory to wild-type seedlings, and showed considerably enhanced denitration of GTN to GDN and GMN.

Conclusions

To conclude, work published in recent years has established beyond doubt that xenobiotic poly-nitro compounds, formerly thought immune to biodegradation, can be degraded by microorganisms, and degradative pathways are now being elucidated. This offers the prospect for effective methods of bioremediation of contaminated land and water. One promising method is sequential anaerobic–aerobic treatment to promote microbial transformations of explosives to less harmful products; this should be highly effective for TNT and RDX, the explosives of greatest current environmental concern. Another approach, potentially more cost-effective for *in situ* bioremediation, is the transfer of genes encoding microbial aerobic degradation pathways into transgenic plants (French *et al.*, 1999). A great amount of work has been published during the past five years; the next five years will no doubt see significant progress in clarifying the chemistry and biochemistry of biotransformations of explosives, as well as application of this knowledge to the problem of bioremediation.

References

ACCASHIAN, J.V., VINOPAL, R.T., KIM, B.J. AND SMETS, B.F. (1998). Aerobic growth on nitroglycerin as the sole carbon, nitrogen, and energy source by a mixed bacterial culture. *Applied and Environmental Microbiology* **64**, 3300–3304.

ACHTNICH, C., SIEGLEN, U., KNACKMUSS, H.-J. AND LENKE, H. (1999a). Irreversible binding of biologically reduced 2,4,6-trinitrotoluene to soil. *Environmental Toxicology and Chemistry* **18**, 2416–2423.

ACHTNICH, C., FERNANDES, E., BOLLAG, J.M., KNACKMUSS, H.-J. AND LENKE, H. (1999b). Covalent binding of reduced metabolites of [$^{15}N_3$]TNT to soil organic matter during a bioremediation process analysed by ^{15}N-NMR spectroscopy. *Environmental Science and Technology* **33**, 4448–4456.

ACHTNICH, C., PFORTNER, P., WELLER, M.G., NIESSNER, R., LENKE, H. AND KNACKMUSS, H.-J. (1999c). Reductive transformation of bound trinitrophenyl residues and free TNT during a bioremediation process analysed by immunoassay. *Environmental Science and Technology* **33**, 3421–3426.

ALVAREZ, M.A., KITTS, C.L., BOTSFORD, J.L. AND UNKEFER, P.J. (1995). *Pseudomonas aeruginosa* strain MAO1 aerobically metabolizes the aminodinitrotoluenes produced by 2,4,6-trinitrotoluene nitro-group reduction. *Canadian Journal of Microbiology* **41**, 984–991.

ANLEZARK, G.M., MELTON, R.G., SHERWOOD, R.F., COLES, B., FRIEDLOS, F. AND KNOX, R.J. (1992). The bioactiviation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) I: purification and characterization of a nitroreductase enzyme from *Escherichia coli* – a potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). *Biochemical Pharmacology* **44**, 2289–2295.

ANUSEVICIUS, Z., SARLAUSKAS, J., NIVINSKAS, H., SEGURA-AGUILAR, J. AND CENAS, N. (1998). DT-diaphorase catalyses N-denitration and redox cycling of tetryl. *FEBS Letters* **436**, 144–148.

BAYMAN, P. AND RADKAR, G.V. (1997). Transformation and tolerance of TNT (2,4,6-trinitrotoluene) by fungi. *International Biodeterioration & Biodegradation* **39**, 45–53.

BAYMAN, P., RUTCHEY, S.D. AND BENNET, J.W. (1995). Fungal interactions with the explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). *Journal of Industrial Microbiology* **15**, 418–423.

BEHREND, C. AND HEESCHE-WAGNER, K. (1999). Formation of hydride-Meisenheimer complexes of picric acid (2,4,6-trinitrophenol) and 2,4-dinitrophenol during mineralization of picric acid by *Nocardioides* sp. strain CB 22-2. *Applied and Environmental Microbiology* **65**, 1372–1377.

BENNETT, B.M., MCDONALD, B.J., NIGAM, R. AND SIMON, W.C. (1994). Biotransformation of organic nitrates and vascular smooth muscle cell function. *Trends in Pharmacological Science* **15**, 245–249.

BEST, E.P.H., ZAPPI, M.E., FREDRICKSON, H.L., SPRECHER, S.L., LARSON, S.L. AND OCHMAN, M. (1997). Screening of aquatic and wetland plant species for phytoremediation of explosives-contaminated groundwater from the Iowa Army Ammunition Plant. *New York Academy of Sciences. Annals* **829**, 179–194.

BEST, E.P.H., SPRECHER, S.L., LARSON, S.L., FREDRICKSON, H.L. AND BADER, D.F. (1999a). Environmental behaviour of explosives in groundwater from the Milan army ammunition plant in aquatic and wetland plant treatments. Removal, mass balances, and fate in groundwater of TNT and RDX. *Chemosphere* **38**, 3383–3396.

BEST, E.P.H., SPRECHER, S.L., LARSON, S.L., FREDRICKSON, H.L. AND BADER, D.F. (1999b). Environmental behaviour of explosives in groundwater from the Milan army ammunition plant in aquatic and wetland plant treatments. Uptake and fate of TNT and RDX in plants. *Chemosphere* **39**, 2057–2072.

BHADRA, R., WAYMENT, D.G., HUGHES, J.B. AND SHANKS, J.V. (1999a). Confirmation of conjugation processes during TNT metabolism by axenic plant roots. *Environmental Science and Technology* **33**, 446–452.

BHADRA, R., SPANGGORD, R.J., WAYMENT, D.G., HUGHES, J.B. AND SHANKS, J.V. (1999b). Characterization of oxidation products of TNT metabolism in aquatic phytoremediation

systems of *Myriophyllum aquaticum*. *Environmental Science and Technology* **33**, 3354–3361.

BHAUMIK, S., CHRISTODOULATOS, C., KORFIATIS, G.P. AND BRODMAN, B.W. (1997). Aerobic and anaerobic biodegradation of nitroglycerin in batch and packed bed bioreactors. *Water Science and Technology* **36**, part 3–4, 139–146.

BHAUMIK, S., CHRISTODOULATOS, C., BRODMAN, B.W. AND PAL, N. (1998). Biodegradation of glycerol trinitrate by activated sludge: cosubstrate requirements, inhibition and kinetics. *Journal of Environmental Science and Health Part A – Toxic/Hazardous Substances and Environmental Engineering* **33**, 547–571.

BINKS, P.R., NICKLIN, S. AND BRUCE, N.C. (1995). Degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Stenotrophomonas maltophilia* PB1. *Applied and Environmental Microbiology* **61**, 1318–1322.

BINKS, P.R., FRENCH, C.E., NICKLIN, S. AND BRUCE, N.C. (1996). Degradation of pentaerythritol tetranitrate by *Enterobacter cloacae* PB2. *Applied and Environmental Microbiology* **62**, 1214–1219.

BLASCO, R. AND CASTILLO, F. (1993). Characterization of a nitrophenol reductase from the phototrophic bacterium *Rhodobacter capsulatus*. *Applied and Environmental Microbiology* **59**, 1774–1778.

BLEHERT, D.S., KNOKE, K.L., FOX, B.G. AND CHAMBLISS, G.H. (1997). Regioselectivity of nitroglycerin denitration by flavoprotein nitroester reductases purified from two *Pseudomonas* species. *Journal of Bacteriology* **179**, 6912–6920.

BLEHERT, D.S., FOX, B.G. AND CHAMBLISS, G.H. (1999). Cloning and sequence analysis of two *Pseudomonas* flavoprotein xenobiotic reductases. *Journal of Bacteriology* **181**, 6254–6263.

BOOPATHY, R. (1994). Transformation of nitroaromatic compounds by a methanogenic bacterium, *Methanococcus* sp. (strain B). *Archives of Microbiology* **162**, 167–172.

BOOPATHY, R. (2000). Formation of aniline as a transient metabolite during the metabolism of tetryl by a sulfate-reducing consortium. *Current Microbiology* **40**, 190–193.

BOOPATHY, R. AND KULPA, C.F. (1992). Trinitrotoluene (TNT) as a sole nitrogen source for a sulfate-reducing bacterium *Desulfovibrio* sp. (B strain) isolated from an anerobic digester. *Current Microbiology* **25**, 235–241.

BOOPATHY, R. AND KULPA, C.F. (1993). Nitroaromatic compounds serve as nitrogen sources for *Desulfovibrio* sp. (B-strain). *Canadian Journal of Microbiology* **39**, 430–433.

BOOPATHY, R. AND KULPA, C.F. (1994). Biotransformation of 2,4,6-trinitrotoluene (TNT) by a *Methanococcus* sp. (strain-B) isolated from a lake sediment. *Canadian Journal of Microbiology* **40**, 273–278.

BOOPATHY, R. AND MANNING, J.F. (1996). Characterization of partial anaerobic pathway for 2,4,6-trinitrotoluene degradation by a sulfate-reducing bacterial consortium. *Canadian Journal of Microbiology* **42**, 1203–1208.

BOOPATHY, R. AND MANNING, J. (1998). Biodegradation of tetryl (2,4,6-trinitrophenylmethylnitramine) in a soil-slurry reactor. *Water Environment Research* **70**, 1049–1055.

BOOPATHY, R. AND MANNING, J. (1999). Surfactant-enhanced bioremediation of soil contaminated with 2,4,6-trinitrotoluene in soil slurry reactors. *Water Environment Research* **71**, 119–124.

BOOPATHY, R., KULPA, C.F. AND WILSON, M. (1993). Metabolism of 2,4,6-trinitrotoluene by *Desulfovibrio* sp. (B-strain). *Applied Microbiology and Biotechnology* **39**, 270–275.

BOOPATHY, R., MANNING, J., MONTEMAGNO, C. AND KULPA, C. (1994a). Metabolism of 2,4,6-trinitrotoluene by a *Pseudomonas* consortium under aerobic conditions. *Current Microbiology* **28**, 131–137.

BOOPATHY, R., WILSON, M., MONTEMAGNO, C., MANNING, J.F. AND KULPA, C.F. (1994b). Biological transformation of 2,4,6-trinitrotoluene (TNT). by soil bacteria isolated from TNT-contaminated soil. *Bioresource Technology* **47**, 19–24.

BOOPATHY, R., MANNING, J., MONTEMAGNO, C. AND RIMKUS, K. (1994c). Metabolism of trinitrobenzene by a *Pseudomonas* consortium. *Canadian Journal of Microbiology* **40**, 787–790.

BOOPATHY, R., MANNING, J. AND KULPA, C.F. (1998a). A laboratory study of the bioremediation

of 2,4,6-trinitrotoluene-contaminated soil using an aerobic/anoxic soil slurry reactor. *Water Environment Research* **70**, 80–86.

BOOPATHY, R., KULPA, C.F. AND MANNING, J. (1998b). Anaerobic degradation of explosives and related compounds by sulfate-reducing and methanogenic bacteria: a review. *Bioresource Technology* **63**, 81–89.

BOOPATHY, R., GURGAS, M., ULLIAN, J. AND MANNING, J.F. (1998c). Metabolism of explosive compounds by sulfate-reducing bacteria. *Current Microbiology* **37**, 127–131.

BOOPATHY, R., MANNING, J. AND KULPA, C.F. (1998d). Biotransformation of explosives by anaerobic consortia in liquid culture and in soil slurry. *International Biodeterioration & Biodegradation* **41**, 67–74.

BOSCHAN, R., MERROW, R.T. AND VAN DOLAN, R.W. (1955). The chemistry of nitrate esters. *Chemical Reviews* **55**, 485–510.

BRADLEY, P.M. AND CHAPELLE, F.H. (1995). Factors affecting 2,4,6-trinitrotoluene mineralization in contaminated soil. *Environmental Science and Technology* **29**, 802–806.

BRADLEY, P.M., CHAPPELLE, F.H., LANDMEYER, J.E. AND SCHUMACHER, J.G. (1994). Microbial transformation of nitroaromatics in surface soils and aquifer materials. *Applied and Environmental Microbiology* **60**, 2170–2175.

BRUNS-NAGEL, D., KNICKER, H., DRZYZGA, O., BUTEHORN, U., STEINBACH, K., GEMSA, D. AND VON LOW, E. (2000). Characterization of ^{15}N-TNT residues after an anaerobic/aerobic treatment of soil/molasses mixtures by solid state ^{15}N-NMR spectroscopy. 2. Systematic investigation of whole soil and different humic fractions. *Environmental Science and Technology* **34**, 1549–1556.

BRYANT, C. AND DELUCA, M. (1991). Purification and characterization of an oxygen-insensitive NAD(P)H nitroreductase from *Enterobacter cloacae*. *Journal of Biological Chemistry* **266**, 4119–4125.

BRYANT, C. AND MCELROY, W.D. (1991). Nitroreductases. In: *Chemistry and biochemistry of flavoproteins*, volume II. Ed. F. Müller, pp 291–304. Boca Raton, USA: CRC Press.

BRYANT, D.W., MCCALLA, D.R., LEEKSMA, M. AND LANEUVILLE, P. (1981). Type I nitroreductases of *Escherichia coli*. *Canadian Journal of Microbiology* **27**, 81–86.

BRYANT, C., HUBBARD, L. AND MCELROY, W.D. (1991). Cloning, nucleotide sequence, and expression of the nitroreductase gene from *Enterobacter cloacae*. *Journal of Biological Chemistry* **266**, 4126–4130.

BUMPUS, J.A. AND TATARKO, M. (1994). Biodegradation of 2,4,6-trinitrotoluene by *Phanerochaete chrysosporium* – identification of initial degradation products and the discovery of a TNT metabolite that inhibits lignin peroxidases. *Current Microbiology* **28**, 185–190.

BUMPUS, J.A., VENKATADRI, R., IRVINE, R.L., WENSITS, K. AND TATARKO, M. (1992). Biodegradation of 2,4,6-trinitrotoluene (TNT) by a wood-rotting fungus in a fixed-film bioreactor. *Abstracts of Papers of the American Chemical Society* **203**, 58–ENVR.

BUNCEL, E. (1982). Role of Meisenheimer or sigma complexes in nitroarene-base interactions. In: *The chemistry of functional groups, supplement F: The chemistry of amino, nitroso and nitro compounds and their derivatives*. Ed. S. Patai, pp 1225–1260. New York: John Wiley and Sons.

CHAPMAN, R.D., O'BRIEN, R.A. AND KONDRACKI, P.A. (1996). N-denitration of nitramines by dihydronicotinamides. *Tetrahedron* **52**, 9655–9664.

CHAUHAN, A. AND JAIN, R.K. (2000). Degradation of *o*-nitrobenzoate via anthranilic acid (o-aminobenzoate) by *Arthrobacter protophormiae*: a plasmid encoded new pathway. *Biochemical and Biophysical Research Communications* **267**, 236–244.

CHRISTODOULATOS, C., BHAUMIK, S. AND BRODMAN, B.W. (1997). Anaerobic biodegradation of nitroglycerin. *Water Research* **31**, 1462–1470.

COLEMAN, N.V. AND DUXBURY, T. (1999). Biodegradation of RDX by *Rhodococcus* sp., Strain DN22. Second International Symposium Biodegradation of Nitroaromatic Compounds and Explosives. Leesburg, VA, September 8–9, Abstract p 13.

COLEMAN, N.V., NELSON, D.R. AND DUXBURY, T. (1998). Aerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source by a *Rhodococcus* sp., strain DN22. *Soil Biology and Biochemistry* **30**, 1159–1167.

COSTA, V., BOOPATHY, R. AND MANNING, J. (1996). Isolation and characterization of a sulfate-reducing bacterium that removed TNT (2,4,6-trinitrotoluene). under sulfate- and nitrate-reducing conditions. *Bioresource Technology* **56**, 273–278.

COURSEN, D.L. (1997). Explosives. In: *McGraw-Hill encyclopaedia of science and technology*, 8th ed., vol 6, pp 632–639. New York: McGraw-Hill.

CRAMPTON, M.R. (1969). Meisenheimer complexes. *Advances in Physical Organic Chemistry* **7**, 211–257.

CRAWFORD, R.L. (1995). Biodegradation of nitrated munitions compounds and herbicides by obligately anaerobic bacteria. In: *Biodegradation of nitroaromatic compounds*. Ed. J.C. Spain, pp 87–98. New York: Plenum Press.

CROCE, M. AND OKAMOTO, Y. (1979). Cationic micellar analysis of the aqueous alkaline hydrolyses of 1,3,5-triaza-1,3,5-trinitrocyclohexane and 1,3,5,7-tetraaza-1,3,5,7-tetranitrocyclooctane. *Journal of Organic Chemistry* **44**, 2100–2103.

DAUN, G., LENKE, H., REUSS, M. AND KNACKMUSS, H.-J. (1998). Biological treatment of TNT-contaminated soil. 1. Anaerobic cometabolic reduction and interaction of TNT and metabolites with soil components. *Environmental Science and Technology* **32**, 1956–1963.

DAVIS, E.P., BOOPATHY, R. AND MANNING, J. (1997). Use of trinitrobenzene as a nitrogen source by *Pseudomonas vesicularis* isolated from soil. *Current Microbiology* **34**, 192–197.

DAWEL, G., KASTNER, M., MICHELS, J., POPPITZ, W., GUNTHER, W. AND FRITSCHE, W. (1997). Structure of a laccase-mediated product of coupling of 2,4-diamino-6-nitrotoluene to guaiacol, a model for coupling of 2,4,6-trinitrotoluene metabolites to a humic organic soil matrix. *Applied and Environmental Microbiology* **63**, 2560–2565.

DONNELLY, K.C., CHEN, J.C., HUEBNER, H.J., BROWN, K.W., AUTENREITH, R.L. AND BONNER, J.S. (1997). Utility of four strains of white-rot fungi for the detoxification of 2,4,6-trinitrotoluene in liquid culture. *Environmental Toxicology and Chemistry* **16**, 1105–1110.

DRZYZGA, O., BRUNS-NAGEL, D., GORONTZY, T., BLOTEVOGEL, K.H., GEMSA, D. AND VON LOW, E. (1998a). Incorporation of ^{14}C-labelled 2,4,6-trinitrotoluene metabolites into different soil fractions after anaerobic and anaerobic–aerobic treatment of soil/molasses mixtures. *Environmental Science and Technology* **32**, 3529–3535.

DRZYZGA, O., BRUNS-NAGEL, D., GORONTZY, T., BLOTEVOGEL, K.H., GEMSA, D. AND VON LOW, E. (1998b). Mass balance studies with ^{14}C-labelled 2,4,6-trinitrotoluene (TNT) mediated by an anaerobic *Desulfovibrio* species and an aerobic *Serratia* species. *Current Microbiology* **37**, 380–386.

DRZYZGA, O., BRUNS-NAGEL, D., GORONTZY, T., BLOTEVOGEL, K.H. AND VON LOW, E. (1999). Anaerobic incorporation of the radiolabelled explosive TNT and metabolites into the organic soil matrix of contaminated soil after different treatment processes. *Chemosphere* **38**, 2081–2095.

DUCROCQ, C., SERVY, C. AND LENFANT, M. (1989). Bioconversion of glyceryl trinitrate into mononitrates by *Geotrichum candidum*. *FEMS Microbiology Letters* **65**, 219–222.

DUCROCQ, C., SERVY, C. AND LENFANT, M. (1990). Formation of glyceryl-2-mononitrate by regioselective bioconversion of glyceryl trinitrate: efficiency of the filamentous fungus *Phanerochaete chrysosporium*. *Biotechnology and Applied Biochemistry* **12**, 325–330.

DUQUE, E., HAIDOUR, A., GODOY, F. AND RAMOS, J.L. (1993). Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. *Journal of Bacteriology* **175**, 2278–2283.

EBERT, S., RIEGER, P.-G. AND KNACKMUSS, H.-J. (1999). Function of coenzyme F420 in aerobic catabolism of 2,4,6-trinitrophenol and 2,4-dinitrophenol by *Nocardioides simplex* FJ2-1A. *Journal of Bacteriology* **181**, 2669–2674.

EDERER, M.M., LEWIS, T.A. AND CRAWFORD, R.L. (1997). 2,4,6-Trinitrotoluene (TNT) transformation by clostridia isolated from a munition-fed bioreactor: comparison with non-adapted bacteria. *Journal of Industrial Microbiology and Biotechnology* **18**, 82–88.

EILERS, A., RUNGELING, E., STUNDL, U.M. AND GOTTSCHALK, G. (1999). Metabolism of 2,4,6-trinitrotoluene by the white-rot fungus *Bjerkandera adusta* DSM 3375 depends on cytochrome P450. *Applied Microbiology and Biotechnology* **53**, 75–80.

ELENSKAYA, I.V., CHESNAVICHENE, E.A., VERNOTTE, C. AND ASTIER, C. (1998). Resistance to nitrophenolic herbicides and metronidazole in the cyanobacterium *Synechocystis* sp.

PCC 6803 as a result of the inactivation of a nitroreductase-like protein encoded by *drgA* gene. *FEBS Letters* **428**, 188–192.

ESTEVE-NUÑEZ, A. AND RAMOS, J.L. (1998). Metabolism of 2,4,6-trinitrotoluene by *Pseudomonas* sp. JLR11. *Environmental Science and Technology* **32**, 3802–3808.

ESTEVE-NUÑEZ, A., LUCCHESI, G., PHILIPP, B., SCHINK, B. AND RAMOS, J.L. (2000). Respiration of 2,4,6-trinitrotoluene by *Pseudomonas* sp. strain JLR11. *Journal of Bacteriology* **182**, 1352–1355.

FERNANDO, T. AND AUST, S.D. (1991). Biodegradation of munition waste, TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by *Phanerochaete chrysosporium*. *ACS Symposium Series* **468**, 214–232.

FERNANDO, T., BUMPUS, J.A. AND AUST, S.D. (1990). Biodegradation of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **56**, 1666–1671.

FIORELLA, P.D. AND SPAIN, J.C. (1997). Transformation of 2,4,6-trinitrotoluene by *Pseudomonas pseudoalcaligenes* JS52. *Applied and Environmental Microbiology* **63**, 2007–2015.

FREEDMAN, D.L. AND SUTHERLAND, K.W. (1998). Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine under nitrate-reducing conditions. *Water Science and Technology* **38**, part 7, 33–40.

FRENCH, C.E. AND BRUCE, N.C. (1995). Morphinone reductase of *Pseudomonas putida* M10 is related to Old Yellow Enzyme. *Biochemical Journal* **312**, 671–678.

FRENCH, C.E., NICKLIN, S. AND BRUCE, N.C. (1996). Sequence and properties of pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2. *Journal of Bacteriology* **178**, 6623–6627.

FRENCH, C.E., NICKLIN, S. AND BRUCE, N.C. (1998). Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Applied and Environmental Microbiology* **64**, 2864–2868.

FRENCH, C.E., ROSSER, S.J., DAVIES, G.J., NICKLIN, S. AND BRUCE, N.C. (1999). Biodegradation of explosives by transgenic plants expressing pentaerythritol tetranitrate reductase. *Nature Biotechnology* **17**, 491–494.

FUNK, S.B., ROBERTS, D.J., CRAWFORD, D.L. AND CRAWFORD, R.L. (1993). Initial phase optimization for bioremediation of munition compound-contaminated soils. *Applied and Environmental Microbiology* **59**, 2171–2177.

FUNK, S.B., CRAWFORD, D.L. AND CRAWFORD, R.L. (1996). Bioremediation of nitroaromatic compounds. In: *Bioremediation: principles and applications*. Eds. R.L. Crawford and D.L. Crawford, pp 195–207. Cambridge, UK: Cambridge University Press.

GILCREASE, P.C. AND MURPHY, V.G. (1995). Bioconversion of 2,4-diamino-6-nitrotoluene to a novel metabolite under anoxic and aerobic conditions. *Applied and Environmental Microbiology* **61**, 4209–4214.

GOEL, A., KUMAR, G., PAYNE, G.F. AND DUBE, S.K. (1997). Plant cell degradation of a xenobiotic nitrate ester, nitroglycerin. *Nature Biotechnology* **15**, 174–177.

GONG, P., WILKE, B.M. AND FLEISCHMANN, S. (1999). Soil-based phytotoxicity of 2,4,6-trinitrotoluene (TNT) to terrestrial higher plants. *Archives of Environmental Contamination and Toxicology* **36**, 152–157.

GOODWIN, A., KERSULYTE, D., SISSON, G., VAN ZANTEN, S.J.O.V., BERG, D.E. AND HOFFMAN, P.S. (1998). Metronidazole resistance in *Helicobacter pylori* is due to null mutations in a gene (*rdxA*) that encodes an oxygen-insensitive NADPH nitroreductase. *Molecular Microbiology* **28**, 383–393.

GORONTZY, T., KUVER, J. AND BLOTEVOGEL, K.H. (1993). Microbial transformation of nitroaromatic compounds under anaerobic conditions. *Journal of General Microbiology* **139**, 1331–1336.

GORONTZY, T., DRZYZGA, O., KAHL, M.W., BRUNS-NAGEL, D., BREITUNG, J., VON LOEW, E. AND BLOTEVOGEL, K.H. (1994). Microbial degradation of explosives and related compounds. *Critical Reviews in Microbiology* **20**, 265–284.

GRIEST, W.H., STEWART, A.J., VASS, A.A. AND HO, C.-H. (1998). Chemical and toxicological characterization of slurry reactor biotreatment of explosives-contaminated soils. Final

SFIM-AEC-ET-CR-96186. U.S. Army Environmental Center (USAEC), Aberdeen Proving Ground, MD.

GROENEWEGEN, P.E.J., BREEUWER, P., VAN HELVOORT, J.M.L.M., LANGENHOFF, A.A.M., DE VRIES, F.P. AND DE BONT, J.A.M. (1992). Novel degradative pathway pf 4-nitrobenzoate in *Comamonas acidovorans* NBA-10. *Journal of General Microbiology* **138**, 1599–1605.

GUNNISON, D., FREDRICKSON, H.L., KAPLAN, D.L., ALLEN, A.L., MELLO, C.M., WALKER, J.E., MYRICK, G., EVANS, W.E. AND OCHMAN, M. (1997). Application of continuous culture technology for the development of explosives-degrading microorganisms. *Annals of the New York Academy of Sciences* **829**, 230–241.

HAÏDOUR, A. AND RAMOS, J.L. (1996). Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene by *Pseudomonas* sp. *Environmental Science and Technology* **30**, 2365–2370.

HALL, D.R., BEEVOR, P.S., CAMPION, D.G., CHAMBERLAIN, D.J., CORK, A., WHITE, R.D., ALMESTAR, A. AND HENNEBERRY, T.J. (1992). Nitrate esters: novel sex pheromone components of the cotton leafperforator, *Bucculatrix thurberiella* Busk. (Lepidoptera: Lyonetiidae). *Tetrahedron Letters* **33**, 4811–4814.

HARVEY, S.D., FELLOWS, R.J., CATALDO, D.A. AND BEAN, R.M. (1990). Analysis of 2,4,6-trinitrotoluene and its transformation products in soils and plant tissues by high performance liquid chromatography. *Journal of Chromatography* **518**, 361–374.

HARVEY, S.D., FELLOWS, R.J., CATALDO, D.A. AND BEAN, R.M. (1991). Fate of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in soil and bioaccumulation in bush bean hydroponic plants. *Environmental Toxicology and Chemistry* **10**, 845–855.

HARVEY, S.D., FELLOWS, R.J., CAMPBELL, J.A. AND CATALDO, D.A. (1992). Determination of the explosive 2,4,6-trinitrophenylmethylnitramine (tetryl) and its transformation products in soil. *Journal of Chromatography* **605**, 227–240.

HARVEY, S.D., FELLOWS, R.J., CATALDO, D.A. AND BEAN, R.M. (1993). Analysis of the explosive 2,4,6-trinitrophenylmethylnitramine in bush bean plants. *Journal of Chromatography* **630**, 167–177.

HARVEY, S.D., FREDRICKSON, H.L., EVANS, W.E., ZAPPI, M.E. AND HILL, D.O. (1997a). Evaluation of bioslurry ecosystems for removal of TNT from contaminated soil using a variety of process amendments. *Annals of the New York Academy of Sciences* **829**, 142–159.

HARVEY, S.D., GALLOWAY, H. AND KRUPSHA, A. (1997b). Trace analysis of military high explosives (2,4,6-trinitrotoluene and hexahydro-1,3,5-trinitro-1,3,5-triazine) in agricultural crops. *Journal of Chromatography A* **775**, 117–124.

HAWARI, J., HALASZ, A., BEAUDET, S., PACQUET, L., AMPLEMAN, G. AND THIBOUTOT, S. (1999). Biotransformation of 2,4,6-trinitrotoluene with *Phanerochaete chrysosporium* in agitated cultures at pH 4.5. *Applied and Environmental Microbiology* **65**, 2977–2986.

HAWARI, J., HALASZ, A., SHEREMATA, T., BEAUDET, S., GROOM, C., PAQUET, L., RHOFIR, C., AMPLEMAN, G. AND THIBOUTOT, S. (2000). Characterization of metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with municipal anaerobic sludge. *Applied and Environmental Microbiology* **66**, 2652–2657.

HEIJMAN, C.G., GRIEDER, E., HOLLIGER, C. AND SCHWARZENBACH, R.P. (1995). Reduction of nitroaromatic compounds coupled to microbial iron reduction in laboratory aquifer columns. *Environmental Science and Technology* **29**, 775–783.

HESS, T.F., LEWIS, T.A., CRAWFORD, R.L., KATAMNENI, S., WELLS, J.H. AND WATTS, J.R. (1998). Combined photocatalytic and fungal treatment for the destruction of 2,4,6-trinitrotoluene. *Water Research* **32**, 1481–1491.

HIGSON, F.K. (1992). Microbial degradation of nitroaromatic compounds. *Advances in Applied Microbiology* **37**, 1–19.

HODGSON, J., RHO, D., GUIOT, S.R., AMPLEMAN, G., THIBOUTOT, S. AND HAWARI, J. (2000). Tween-80 enhanced TNT mineralization by *Phanerochaete chrysosporium*. *Canadian Journal of Microbiology* **46**, 110–118.

HUANG, S.Q., LINDAHL, P.A., WANG, C.Y., BENNETT, G.N., RUDOLPH, F.B. AND HUGHES, J.B. (2000). 2,4,6-trinitrotoluene reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. *Applied and Environmental Microbiology* **66**, 1474–1478.

HUGHES, J.B., SHANKS, J., VANDERFORD, M.J. LAURITZEN, J. AND BHADRA, R. (1997). Transformation of TNT by aquatic plants and plant tissue cultures. *Environmental Science and Technology* **31**, 266–271.

HUGHES, J.B., WANG, C.Y., BHADRA, R., RICHARDSON, A., BENNER, G.N. AND RUDOLPH, F.B. (1998a). Reduction of 2,4,6-trinitrotoluene by *Clostridium acetobutylicum* through hydroxylaminonitrotoluene intermediates. *Environmental Toxicology and Chemistry* **17**, 343–348.

HUGHES, J.B., WANG, C., YESLAND, K., RICHARDSON, A., BHADRA, R., BENNET, G. AND RUDOLPH, F. (1998b). Bamberger rearrangement during TNT metabolism by *Clostridium acetobutylicum*. *Environmental Science and Technology* **32**, 494–500.

HUGHES, J.B., WANG, C.Y. AND ZHANG, C.L. (1999). Anaerobic biotransformation of 2,4-dinitrotoluene and 2,6-dinitrotoluene by *Clostridium acetobutylicum*: a pathway through dihydroxylamino intermediates. *Environmental Science and Technology* **33**, 1065–1070.

HUNDAL, L.S., SHEA, P.J., COMFORT, S.D., POWERS, W.L. AND SINGH, J. (1997). Long term TNT sorption and bound residue formation in soil. *Journal of Environmental Quality* **26**, 896–904.

JACKSON, M., GREEN, J.M., HASH, R.L., LINDSTEN, D.C. AND TATYREK, A.F. (1978). Nitramine (RDX and HMX) wastewater treatment at Holston Army Ammunition Plant. Report ARLCD-77013. U.S. Army Armament Research and Development Command, Dover, NJ.

JENKINS, T.F., WALSH, M.E., THORNE, P.G., MIARES, P.H., RANNEY, T.A., GRANT, C.L. AND ESPARZA, J.R. (1998). Site characterization for explosives contamination at a military firing range impact area. Special Report 98–9. U.S. Army Corps of Engineers, CRREL, Hanover, NH.

KALAFUT, T., WALES, M.E., RASTOGI, V.K., NAUMOVA, R.P., ZARIPOVA, S.K. AND WILD, J.R. (1998). Biotransformation patterns of 2,4,6-trinitrotoluene by aerobic bacteria. *Current Microbiology* **36**, 45–54.

KAPLAN, D.L. (1990). Biotransformation pathways of hazardous energetic organo-nitro compounds. In: *Advances in biotechnology series volume 4: Biotechnology and biodegradation*. Eds. D. Kamely, A. Chakrabarty and G.S. Omenn, pp 155–181. Woodlands, Texas: Portfolio Publishing Co.

KAPLAN, D.L. (1992). Biological degradation of explosives and chemical agents. *Current Opinion in Biotechnology* **3**, 253–260.

KAPLAN, D.L. (1996). Biotechnology and bioremediation for organic energetic compounds. In: *Organic energetic compounds*. Ed. P. Marinkas, pp 373–415. New York: Nova Science Publishers, Inc.

KAPLAN, L.A. AND SIEDLE, A.R. (1971). Studies in boron hydrides IV. Stable hydride Meisenheimer adducts. *Journal of Organic Chemistry* **36**, 937–939.

KEEHAN, K.R. AND SISK, W.E. (1996). Development of composting for the remediation of explosives-contaminated soils. In: *Biotechnology in industrial waste treatment and bioremediation*. Eds. R.F. Hickey and G.Smith, pp 69–78. Boca Raton, FL: CRC Press, Inc.

KENKARE, S.R. AND BENET, L.Z. (1996). Tolerance to nitroglycerin in rabbit aorta – investigating the involvement of the mu isozyme of glutathione-S-transferases. *Biochemical Pharmacology* **51**, 1357–1363.

KHAN, T.A., BHADRA, R. AND HUGHES, J. (1997). Anaerobic transformation of 2,4,6-trinitrotoluene and related nitroaromatic compounds by *Clostridium acetobutylicum*. *Journal of Industrial Microbiology and Biotechnology* **18**, 198–203.

KIDO, T., HASHIZUME, K. AND SODA, K. (1978). Purification and properties of nitroalkane oxidase from *Fusarium oxysporum*. *Journal of Bacteriology* **133**, 53–58.

KINOUCHI, T. AND OHNISHI, Y. (1983). Purification and characterization of 1-nitropyrene nitroreductases from *Bacteroides fragilis*. *Applied and Environmental Microbiology* **46**, 596–604.

KITTS, C.L., CUNINGHAM, D.P. AND UNKEFER, P.J. (1994). Isolation of three hexahydro-1,3,5-trinitro-1,3,5-triazine degrading species of the family Enterobacteriaceae from nitramine explosive-contaminated soil. *Applied and Environmental Microbiology* **60**, 4608–4611.

KITTS, C.L., GRREN, C.E., OTLEY, R.A., ALVAREZ, M.A. AND UNKEFER, P.J. (2000). Type I

nitroreductases in soil enterobacteria reduce TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). *Canadian Journal of Microbiology* **46**, 278–282.

KNICKER, H., BRUNSNAGEL, D., DRZYZGA, O., VON LOW, E. AND STEINBACH, K. (1999). Characterization of ^{15}N-TNT residues after an anaerobic/aerobic treatment of soil/molasses mixtures by solid state ^{15}N-NMR spectroscopy. 1. Determination and optimization of relevant NMR spectroscopic parameters. *Environmental Science and Technology* **33**, 343–349.

KOBORI, T., LEE, W.C., AGAKI, T., SASAKI, H., ZENNO, S., SAIGO, K. AND TANOKURA, M. (1999). Crystallization and preliminary crystallographic analysis of major nitroreductase from *Escherichia coli*. *Acta Crystallographica Section D: Biological Crystallography* **55**, 1901–1902.

KRISHNAN, G., HORST, G.L., DARNELL, S. AND POWERS, W.L. (2000). Growth and development of smooth bromegrass and tall fescue in TNT-contaminated soil. *Environmental Pollution* **107**, 109–116.

LARSON, S.L. (1997). Fate of explosive contaminants in plants. *Annals of the New York Academy of Sciences* **829**, 195–201.

LARSON, S.L., WEISS, C.A., ESCALON, B.L. AND PARKER, D. (1999a). Increased extraction efficiency of acetonitrile/water mixtures for explosives determination in plant tissues. *Chemosphere* **38**, 2153–2162.

LARSON, S.L., JONES, R.P., ESCALON, L. AND PARKER, D. (1999b). Classification of explosive transformations in plant tissue. *Environmental Toxicology and Chemistry* **18**, 1270–1276.

LENKE, H. AND KNACKMUSS, H.-J. (1992). Initial hydrogenation during catabolism of picric acid by *Rhodococcus erythropolis* HL 24-2. *Applied and Environmental Microbiology* **58**, 2933–2937.

LENKE, H. AND KNACKMUSS, H.-J. (1996). Initial hydrogenation and extensive reduction of substituted 2,4-dinitrophenols. *Applied and Environmental Microbiology* **62**, 784–790.

LENKE, H., PIEPER, D.H., BRUHN, C. AND KNACKMUSS, H.-J. (1992). Degradation of 2,4-dinitrophenol by two *Rhodococcus erythropolis* strains, HL 24-1 and HL 24-2. *Applied and Environmental Microbiology* **58**, 2928–2932.

LENKE, H., WARRELMANN, J., DAUN, G., HUND, K., SIEGLEN, U., WALTER, U. AND KNACKMUSS, H.-J. (1998). Biological treatment of TNT contaminated soil. 2. Biologically induced immobilization of the contaminants and full scale application. *Environmental Science and Technology* **32**, 1964–1971.

LEWIS, T.A., EDERER, M.M., CRAWFORD, R.L. AND CRAWFORD, D.L. (1995). Microbial transformation of 2,4,6-trinitrotoluene. *Journal of Industrial Microbiology and Biotechnology* **18**, 89–96.

LEWIS, T.A., GOSZCZYNSKI, S., CRAWFORD, R.L., KORUS, R.A. AND ADMASSU, W. (1996). Products of anaerobic 2,4,6-trinitroluene (TNT). transformation by *Clostridium bifermentans*. *Applied and Environmental Microbiology* **62**, 4669–4674.

LINDNER, V. (1993). Explosives and propellants. In: *Kirk-Othmer encyclopaedia of chemical technology*, 4th edn, vol 10. Ed. M. Howe-Grant, pp 1–125. New York, USA: John Wiley and Sons, Inc.

LIOCHEV, S.I., HAUSLADEN, A. AND FRIDOVICH, I. (1999). Nitroreductase A is regulated as a member of the *soxRS* regulon of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3537–3539.

LUCERO, M.E., MUELLER, W., HUBSTENBERGER, J., PHILLIPS, G.C. AND CONNEL, M.A.O. (1999). Tolerance to nitrogenous explosives and metabolism of TNT by cell supensions of *Datura innoxia*. *In Vitro Cell and Developmental Biology – Plant* **35**, 480–486.

MARTIN, J.L., COMFORT, S.D., SHEA, P.J., KOKJOHN, T.A. AND DRIJBER, R.A. (1997). Denitration of 2,4,6-trinitrotoluene by *Pseudomonas savastanoi*. *Canadian Journal of Microbiology* **43**, 447–455.

MARVIN-SIKKEMA, F.D. AND DE BONT, J.A.M. (1994). Degradation of nitroaromatic compounds by microorganisms. *Applied Microbiology and Biotechnology* **42**, 499–507.

MCCORMICK, N.G., CORNELL, J.H. AND KAPLAN, K.A. (1981). Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. *Applied and Environmental Microbiology* **42**, 817–823.

McGuire, J.J., Anderson, D.J., McDonald, B.J., Narayanasami, R. and Bennett, B.M. (1998). Inhibition of NADPH-cytochrome P450 reductase and glyceryl trinitrate biotransformation by diphenyleneiodium sulfate. *Biochemical Pharmacology* **56**, 881–893.

Meharg, A.A., Dennis, G.R. and Cairney, J.W.G. (1997). Biotransformation of 2,4,6-trinitrotoluene (TNT) by ectomycorrhizal basidiomycetes. *Chemosphere* **35**, 513–521.

Meng, M., Sun, W.-Q., Geelhaar, L.A., Kumar, G., Patel, A.R., Payne, G.F., Speedie, M.K. and Stacy, J.R. (1995). Denitration of glycerol trinitrate by resting cells and cell extracts of *Bacillus thuringiensis/cereus* and *Enterobacter agglomerans*. *Applied and Environmental Microbiology* **61**, 2548–2553.

Michael, N.P., Brehm, J.K., Anlezark, G.M. and Minton, N.P. (1994). Physical characterization of the *Escherichia coli* B gene encoding nitroreductase and its overexpression in *Escherichia coli* K12. *FEMS Microbiology Letters* **124**, 195–202.

Michels, J. and Gottschalk, G. (1994). Inhibition of the lignin peroxidase of *Phanerochaete chrysosporium* by hydroxylaminodinitrotoluene, an early intermediate in the degradation of 2,4,6-trinitrotoluene. *Applied and Environmental Microbiology* **60**, 187–194.

Michels, J. and Gottschalk, G. (1995). Pathway of 2,4,6-trinitrotoluene (TNT) degradation by *Phanerochaete chrysosporium*. In: *Biodegradation of nitroaromatic compounds*. Ed. J.C. Spain, pp 117–134. New York: Plenum Press.

Miskiniene, V., Sarlauskas, J., Jacquot, J.-P. and Cenas, N. (1998). Nitroreductase reactions of *Arabidopsis thaliana* thioredoxin reductase. *Biochimica et Biophysica Acta* **1366**, 275–283.

Miura, K., Tomioka, Y., Suzuki, H., Yonezawa, M., Hishinuma, T. and Mizugaki, M. (1997). Molecular cloning of the *nemA* gene encoding N-ethylmaleimide reductase from *Escherichia coli*. *Biological and Pharmaceutical Bulletin* **20**, 110–112.

Montpas, S., Samson, J., Langlois, E., Lei, J.Y., Piche, Y. and Chenevert, R. (1997). Degradation of 2,4,6-trinitrotoluene by *Serratia marcescens*. *Biotechnology Letters* **19**, 291–294.

Moody, P.C.E., Shikotra, N., French, C.E., Bruce, N.C. and Scrutton, N.S. (1998). Crystallization and preliminary diffraction studies of pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2. *Acta Crystallographica D* **54**, 675–677.

Nigam, R., Anderson, D.J., Lee, S.F. and Bennett, B.M. (1996). Isoform-specific biotransformation of glyceryl trinitrate by rat aortic glutathione-S-transferases. *Journal of Pharmacology and Experimental Therapeutics* **279**, 1527–1534.

Nishino, S.F., Spain, J.C. and He, Z. (2000). Strategies for aerobic degradation of nitroaromatic compounds by bacteria: Process discovery to field application. In: *Biodegradation of nitroaromatic compounds and explosives*. Eds. J.C. Spain, J.B. Hughes and H.-J. Knackmuss, pp 7–61. Boca Raton, USA: CRC Press LLC.

Noguera, D.R. and Freedman, D.L. (1996). Reduction and acetylation of 2,4-dinitrotoluene by a *Pseudomonas aeruginosa* strain. *Applied and Environmental Microbiology* **62**, 2257–2263.

O'Byrne, S., Shirodaria, C., Millar, T., Stevens, C., Blake, D. and Benjamin, N. (2000). Inhibition of platelet aggregation with glyceryl trinitrate and xanthine oxidoreductase. *Journal of Pharmacology and Experimental Therapeutics* **292**, 326–330.

Ogawa, N., Hirose, T., Tsukamoto, M., Fukushima, K., Suwa, T. and Satoh, T. (1995a). Purification and characterization of glutathione-independent denitration enzyme of organic nitrate esters in rabbit hepatic cytosol. *Biological and Pharmaceutical Bulletin* **18**, 1352–1355.

Ogawa, N., Hirose, T., Tsukamoto, M., Fukushima, K., Suwa, T. and Satoh, T. (1995b). GSH-independent denitration of organic nitrate esters in rabbit hepatic and vascular cytosol. *Research Communications in Molecular Pathology and Pharmacology* **88**, 153–161.

Orna, M.V. and Mason, R.P. (1989). Correlation of kinetic parameters of nitroreductase enzymes with redox properties of nitroaromatic compounds. *Journal of Biological Chemistry* **264**, 12379–12384.

Palazzo, A.J. and Leggett, D.C. (1986). Effect and disposition of TNT in a terrestrial plant. *Journal of Environmental Quality* **15**, 49–52.

PARK, H.S. AND KIM, H.S. (2000). Identification and characterization of the nitrobenzene catabolic plasmids pNB1 and pNB2 in *Pseudomonas putida* HS12. *Journal of Bacteriology* **182**, 573–580.

PARRISH, F.W. (1977). Fungal transformation of 2,4-dinitrotoluene and 2,4,6-trinitrotoluene. *Applied and Environmental Microbiology* **34**, 232–233.

PASTI-GRIGSBY, M.B., LEWIS, T.A., CRAWFORD, D.L. AND CRAWFORD, R.L. (1996). Transformation of 2,4,6-trinitrotoluene (TNT) by actinomycetes isolated from TNT-contaminated and uncontaminated environments. *Applied and Environmental Microbiology* **62**, 1120–1123.

PAVLOSTATHIS, S.G. AND JACKSON, G.H. (1999). Biotransformation of 2,4,6-trinitrotoluene in *Anabaena* sp. cultures. *Environmental Toxicology and Chemistry* **18**, 412–419.

PAVLOSTATHIS, S.G., COMSTOCK, K.K., JACOBSON, M.E. AND SAUNDERS, F.M. (1998). Transformation of 2,4,6-trinitrotoluene by the aquatic plant *Myriophyllum spicatum*. *Environmental Toxicology and Chemistry* **17**, 2266–2273.

PENNINGTON, J.C., HAYES, C.A., MYERS, K.F., OCHMAN, M., GUNNISON, D., FELT, D.R. AND MCCORMICK, E.F. (1995). Fate of 2,4,6-trinitrotoluene in a simulated compost system. *Chemosphere* **30**, 429–438.

PERES, C.M., NAVEAU, H. AND AGATHOS, S.N. (1998). Biodegradation of nitrobenzene by its simultaneous reduction into aniline and mineralization of the aniline formed. *Applied Microbiology and Biotechnology* **49**, 343–349.

PETERSON, M.M., HORST, G.L, SHEA, P.J., COMFORT, S.D. AND PETERSON, R.K.D. (1996). TNT and 4-amino-2,6-dinotrotoluene influence on germination and early seedling development of tall fescue. *Environmental Pollution* **93**, 57–62.

PETERSON, M.M., HORST, G.L, SHEA, P.J. AND COMFORT, S.D. (1998). Germination and seedling development of switchgrass and smooth bromegrass exposed to 2,4,6-trinitrotoluene. *Environmental Pollution* **99**, 53–59.

PREUSS, A. AND RIEGER, P.-G. (1995). Anaerobic transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. In: *Biodegradation of nitroaromatic compounds*. Ed. J.C. Spain, pp 69–86. New York: Plenum Press.

PREUSS, A., FIMPEL, J. AND DIEKERT, G. (1993). Anaerobic transformation of 2,4,6-trinitrotoluene. *Archives of Microbiology* **159**, 345–353.

QAISI, K.M., THIBODEAUX, L.J. AND ADRIAN, D. (1996). Aquatic plant augmented TNT degradation – analysis of reaction kinetics. *Journal of Environmental Science and Health A – Environmental Science and Engineering and Toxic and Hazardous Substance Control* **31**, 1596–1603.

RAFII, F., FRANKLIN, W., HEFLICH, R.H. AND CERNIGLIA, C.E. (1991). Reduction of nitroaromatic compounds by anaerobic bacteria isolated from the human gastrointestinal tract. *Applied and Environmental Microbiology* **57**, 962–968.

RAJAN, J., VALLI, K., PERKINS, R.E., SARIASLANI, F.S., BARNS, S.M., REYSENBACH, A.L., REHM, S., EHRINGER, M. AND PACE, N.R. (1996). Mineralization of 2,4,6-trinitrophenol (picric acid): characterization and phylogenetic identification of microbial strains. *Journal of Industrial Microbiology* **16**, 319–324.

RAMOS, J.L., HAÏDOUR, A., DUQUE, E., PINAR, G., CALVO, V. AND OLIVA, J-M. (1996). Metabolism of nitrate esters by a consortium of two bacteria. *Nature Biotechnology* **14**, 320–322.

REGAN, K.M. AND CRAWFORD, R.L. (1994). Characterization of *Clostridium bifermentans* and its biotransformation of 2,4,6-trinitrotoluene (TNT) and 1,3,5-triaza-1,3,5-trinitrocyclohexane (RDX). *Biotechnology Letters* **16**, 1081–1086.

RIEBLE, S., JOSHI, D.K. AND GOLD, M.H. (1994). Aromatic nitroreductase from the basidiomycete *Phanerochaete chrysosporium*. *Biochemical and Biophysical Research Communications* **205**, 298–304.

RIEGER, P.-G. AND KNACKMUSS, H.-J. (1995). Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil. In: *Biodegradation of nitroaromatic compounds*. Ed. J.C. Spain, pp 1–18. New York: Plenum Press.

RIEGER, P.-G., SINNWELL, V., PREUSS, A., FRANCKE, W. AND KNACKMUSS, H.-J. (1999).

Hydride-Meisenheimer complex formation and protonation as key reactions of 2,4,6-trinitrophenol biodegradation by *Rhodococcus erythropolis*. *Journal of Bacteriology* **181**, 1189–1195.

RIVERA, R., MEDINA, V.F., LARSON, S.L. AND MCCUTCHEON, S.C. (1998). Phytotreatment of TNT-contaminated groundwater. *Journal of Soil Contamination* **7**, 511–529.

ROBERTS, J.M. (1990). The atmospheric chemistry of organic nitrates. *Atmospheric Environment* **24A**, 243–287.

ROBERTS, D.J., AHMAD, F. AND PENDHARKAR, S. (1996). Optimization of an aerobic polishing stage to complete the anaerobic treatment of munitions-contaminated soils. *Environmental Science and Technology* **30**, 2021–2026.

ROCHELEAU, S., CIMPOIA, R., PAQUET, L., VAN KOPPEN, I., GUIOT, S.R., HAWARI, J., THIBOUTOT, S., AMPLEMAN, G. AND SUNAHARA, G.I. (1999). Ecotoxicological evaluation of a bioslurry process treating TNT and RDX contaminated soil. *Bioremediation Journal* **3**, 233–245.

RONEN, Z., BRENNER, A. AND ABELIOVICH, A. (1998). Biodegradation of RDX-contaminated wastes in a nitrogen-deficient environment. *Water Science and Technology* **38**, part 7, 219–224.

ROSENBLATT, D.H., BURROWS, E.P., MITCHELL, W.R. AND PARMER, D.L. (1991). Organic explosives and related compounds. In: *Handbook of environmental chemistry*, volume 3, part G. Ed. O. Hutzinger, pp 195–234. Berlin: Springer-Verlag.

RUSS, R., WALTERS, D.M., KNACKMUSS, H-J. AND ROUVIERE, P.E. (2000). Identification of genes involved in picric acid and 2,4-dinitrophenol degradation by mRNA differential display. In: *Biodegradation of nitroaromatic compounds and explosives*. Eds. J.C. Spain, J.B. Hughes and H-J. Knackmuss, pp 127–143. Boca Raton, FL: CRC Press LLC.

SAMSON, J., LANGLOIS, E., LEI, J., PICHE, Y. AND CHENEVERT, R. (1998). Removal of 2,4,6-trinitrotoluene and 2,4-dinitrotoluene by fungi (*Ceratocystis coerulescens*, *Lentinus lepideus*, and *Trichoderma harzianum*). *Biotechnology Letters* **20**, 355–358.

SCHACKMANN, A. AND MULLER, R. (1991). Reduction of nitroaromatic compounds by different *Pseudomonas* species under aerobic conditions. *Applied Microbiology and Biotechnology* **34**, 809–813.

SCHEIBNER, K. AND HOFRICHTER, M. (1998). Conversion of aminonitrotoluenes by fungal manganese peroxidase. *Journal of Basic Microbiology* **38**, 51–59.

SCHEIBNER, E., HOFRICHTER, M., HERRE, A., MICHELS, J. AND FRITSCHE, W. (1997). Screening for fungi intensively mineralizing 2,4,6-trinitrotoluene. *Applied Microbiology and Biotechnology* **47**, 452–457; correction/addition published *Applied Microbiology and Biotechnology* **48**, 431.

SCHEIDEMANN, P., KLUNK, A., SENS, C. AND WERNER, D. (1998). Species-dependent uptake and tolerance of nitroaromatic compounds by higher plants. *Journal of Plant Physiology* **152**, 242–247.

SCHENZLE, A., LENKE, H., FISCHER, P., WILLIAMS, P.A. AND KNACKMUSS, H.-J. (1997). Catabolism of 3-nitrophenol by *Ralstonia eutropha* JMP134. *Applied and Environmental Microbiology* **63**, 1421–1427.

SCHENZLE, A., LENKE, H., SPAIN, J.C. AND KNACKMUSS, H.-J. (1999). Chemoselective nitro-group reduction and reductive dechlorination initiate degradation of 2-chloro-5-nitrophenol by *Ralstonia eutropha* JMP134. *Applied and Environmental Microbiology* **65**, 2317–2323.

SCHNEIDER, K., OLTMANNS, J., RADENBERG, T., SCHNEIDER, T. AND PAULY-MUNDEGAR, D. (1996). Uptake of nitroaromatic compounds in plants – implications for risk assessment of ammunition sites. *Environmental Science and Pollution Research* **3**, 135–138.

SCHNOOR, J.L., LICHT, L.A., MCCUTCHEON, S.C., WOLFE, N.L. AND CARREIRA, L.H. (1995). Phytoremediation of organic and nutrient contaminants. *Environmental Science and Technology* **29**, 318A–322A.

SEMBRIES, S. AND CRAWFORD, R.L. (1997). Production of *Clostridium bifermentans* spores as inoculum for bioremediation of nitroaromatic contaminanants. *Applied and Environmental Microbiology* **63**, 2100–2104.

SENS, C., SCHEIDEMANN, P., KLUNK, A. AND WERNER, D. (1998). Distribution of ^{14}C-TNT and derivatives in different biochemical compartments of *Phaseolus vulgaris*. *Environmental Science and Pollution Research* **5**, 202–208.

SENS, C., SCHEIDEMANN, P. AND WERNER, D. (1999). The distribution of ^{14}C-TNT in different biochemical compartments of the monocotyledonous *Triticum aestivum*. *Environmental Pollution* **104**, 113–119.

SERVENT, D., DUCROCQ, C., HENRY, Y., GUISSANI, A. AND LENFANT, M. (1991). Nitroglycerin metabolism by *Phanerochaete chrysosporium* – evidence for nitric oxide and nitrite formation. *Biochimica et Biophysica Acta* **1074**, 320–325.

SERVENT, D., DUCROCQ, C., HENRY, Y., SERVY, C. AND LENFANT, M. (1992). Multiple enzymic pathways in the metabolism of glyceryl trinitrate in *Phanerochaete chrysosporium*. *Biotechnology and Applied Biochemistry* **15**, 257–266.

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

SHEN, C.F., GUIOT, S.R., THIBOUTOT, S., AMPLEMAN, G. AND HAWARI, J. (1997). Fate of explosives and their metabolites in bioslurry treatment processes. *Biodegradation* **8**, 339–347.

SICILIANO, S.D. AND GREER, C.W. (2000). Plant–bacterial combinations to phytoremediate soil contaminated with high concentrations of 2,4,6-trinitrotoluene. *Journal of Environmental Quality* **29**, 311–316.

SIKORA, F.J., BEHRENDS, L.L., PHILLIPS, W.D., CONROOD, H.S., BAILEY, E. AND BADER, D.F. (1997). A microcosm study on remediation of explosives-contaminated groundwater using contructed wetlands. *Annals of the New York Academy of Sciences* **829**, 202–218.

SINGH, J., COMFORT, S.D., HUNDAL, S.L. AND SHEA, P.J. (1998). Long term RDX sorption and fate in soil. *Journal of Environmental Quality* **27**, 572–577.

SINGHAL, S.S., PIPER, J.T., SRIVASTAVA, S.K., CHAUBEY, M., BANDAROWICZ-PIKULA, J., AWASTHI, S. AND AWASTHI, Y.C. (1996). Rabbit aorta glutathione-S-transferases and their role in bioactivation of trinitroglycerin. *Toxicology and Applied Pharmacology* **140**, 378–386.

SKELLY, J.V., COLLINS, P.J., KNOX, R.J., ANLEZARK, G.M. AND MELTON, R.G. (1994). Crystallization and preliminary diffraction data for an FMN-dependent nitroreductase from *Escherichia coli* B. *Journal of Molecular Biology* **238**, 852–853.

SNAPE, J.R., WALKLEY, N.A., MORBY, A.P., NICKLIN, S. AND WHITE, G.F. (1997). Purification, properties and sequence of glycerol trinitrate reductase from *Agrobacterium radiobacter*. *Journal of Bacteriology* **179**, 7796–7802.

SOMERVILLE, C.C., NISHINO, S.F. AND SPAIN, J.C. (1995). Purification and characterization of nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* JS45. *Journal of Bacteriology* **177**, 3837–3842.

SPAIN, J.C. (1995a). Bacterial degradation of nitroaromatic compounds under aerobic conditions. In: *Biodegradation of nitroaromatic compounds*. Ed. J.C. Spain, pp 19–36. New York: Plenum Press.

SPAIN, J.C. (1995b). Biodegradation of nitroaromatic compounds. *Annual Review of Microbiology* **49**, 523–555.

SPIKER, J.K., CRAWFORD, D.L. AND CRAWFORD, R.L. (1992). Influence of 2,4,6-trinitrotoluene (TNT) concentration on the degradation of TNT in explosive-contaminated soils by the white rot fungus *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **58**, 3199–3202.

STAHL, J.D. AND AUST, S.D. (1993a). Plasma-membrane dependent reduction of 2,4,6-trinitrotoluene by *Phanerochaete chrysosporium*. *Biochemical and Biophysical Research Communications* **192**, 471–476.

STAHL, J.D. AND AUST, S.D. (1993b). Metabolism and detoxification of TNT by *Phanerochaete chrysosporium*. *Biochemical and Biophysical Research Communications* **192**, 477–482.

STAHL, J.D. AND AUST, S.D. (1995a). Biodegradation of 2,4,6-trinitrotoluene by the white rot fungus *Phanerochaete chrysosporium*. In: *Biodegradation of nitroaromatic compounds*. Ed. J.C. Spain, pp 117–134. New York: Plenum Press.

STAHL, J.D. AND AUST, S.D. (1995b). Properties of a transplasma membrane redox system of *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics* **320**, 369–374.

SUBLETTE, K.L., GANAPATHY, E.V. AND SCHWARTZ, S. (1992). Degradation of munitions waste by *Phanerochaete chrysosporium*. *Applied Biochemistry and Biotechnology* **34**, 709–723.

SUN, W.-Q., MENG, M., KUMAR, G., GEELHAAR, L.A., PAYNE, G.F., SPEEDIE, M.K. AND STACY, J. (1996). Biological denitration of propylene glycol dinitrate by *Bacillus* sp. ATCC 51912. *Applied Microbiology and Biotechnology* **45**, 525–529.

TALMAGE, S.S., OPRESKO, D.M., MAXWEL, C.J., WELSH, C.J.E., CRETELLA, F.M., RENO, P.H. AND DANIEL, F.B. (1999). Nitroaromatic munition compounds: environmental effects and screening values. *Reviews in Environmental Contamination Toxicology* **161**, 1–156.

TEDESCHI, G., CHEN, S. AND MASSEY, V. (1995). DT diaphorase: redox potential, steady state, and reaction studies. *Journal of Biological Chemistry* **270**, 1198–1204.

TEKOAH, Y. AND ABELIOVICH, N.A. (1999). Participation of cytochrome P-450 in the biodegradation of RDX by a *Rhodococcus* strain. Second International Symposium Biodegradation of Nitroaromatic Compounds and Explosives. Leesburg, VA, September 8–9, Abstract p 7.

THIBOUTOT, S., AMPLEMAN, G., GAGNON, A., MAROIS, A., JENKINS, T.F., WALSH, C.J.E., THORNE, P.G. AND RANNEY, T.A. (1998). Characterization of anti-tank firing ranges at CFB Valcartier. Report DREV-R-9809. WATC Wainwright and CFAD Dundurn, Quebec.

THOMPSON, P.L., RAMER, L.A. AND SCHNOOR, J.L. (1998a). Uptake and transformation of TNT by hybrid poplar trees. *Environmental Science and Technology* **32**, 975–980.

THOMPSON, P.L., RAMER, L.A., GUFFEY, A.P. AND SCHNOOR, J.L. (1998b). Decreased transpiration in poplar trees exposed to 2,4,6-trinitrotoluene. *Environmental Toxicology and Chemistry* **17**, 902–906.

THOMPSON, P.L., RAMER, L.A. AND SCHNOOR, J.L. (1999). Hexahydro-1,3,5-trinitro-1,3,5-triazine translocation in poplar trees. *Environmental Toxicology and Chemistry* **18**, 279–284.

TSAI, T.S. (1991). Biotreatment of red water – a hazardous waste stream from explosive manufacture – with fungal systems. *Hazardous Waste and Hazardous Materials* **8**, 231–244.

URBANSKI, T. (1965). *Chemistry and technology of explosives*, volume 2. Oxford: Pergamon Press Ltd.

URBANSKI, T. (1984). *Chemistry and technology of explosives*, volume 3. Oxford: Pergamon Press.

U.S. ARMY TOXIC AND HAZARDOUS MATERIALS AGENCY (USATHAMA). (1989). Proceeding for the workshop on composting of explosives in contaminated soils. Report No. CETHA-TS-SR 89276. New Orleans, LA.

VAN AKEN, B., SKUBISZ, K., NAVEAU, H. AND AGATHOS, S.N. (1997). Biodegradation of 2,4,6-trinitrotoluene (TNT). by the white-rot basidiomycete *Phlebia radiata*. *Biotechnology Letters* **19**, 813–817.

VAN AKEN, B., GODEFROID, L.M., PERES, C.M., NAVEAU, H. AND AGATHOS, S.N. (1999a). Mineralization of ^{14}C-U-ring labelled 4-hydroxylamino-2,6-dinitrotoluene by manganese-dependent peroxidase of the white-rot basidiomycete *Phlebia radiata*. *Journal of Biotechnology* **68**, 159–169.

VAN AKEN, B., HOFRICHTER, M., SCHEIBNER, K., HATAKKA, A.I., NAVEAU, H. AND AGATHOS, S.N. (1999b). Transformation and mineralization of 2,4,6-trinitrotoluene by manganese peroxidase from the white-rot basidiomycete *Phlebia radiata*. *Biodegradation* **10**, 83–91.

VANDERBERG, L.A., PERRY, J.J. AND UNKEFER, P.J. (1995). Catabolism of 2,4,6-trinitrotoluene by *Mycobacterium vaccae*. *Applied Microbiology and Biotechnology* **43**, 937–945.

VANDERFORD, M., SHANKS, J.V. AND HUGHES, J.B. (1997). Phytotransformation of trinitrotoluene (TNT). and distribution of metabolic products in *Myriophyllum aquaticum*. *Biotechnology Letters* **19**, 277–280.

VAN DER LOOP, S.L., SUIDAN, M.T., MOTELEB, M.A. AND MALONEY, S.W. (1998). Two-stage biotransoformation of 2,4,6-trinitrotoluene under nitrogen-rich and nitrogen-limiting conditions. *Water Environment Research* **70**, 189–196.

VENULET, J. AND VAN ETTEN, R.L. (1970). Biochemistry and pharmacology of the nitro and nitroso groups. In: *The chemistry of the nitro and nitroso groups*. Ed. H. Feuer, pp 201–287. New York: Interscience Publishers, a division of John Wiley & Sons.

VORBECK, C., LENKE, H., FISCHER, P. AND KNACKMUSS, H.-J. (1994). Identification of a hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene by a *Mycobacterium* strain. *Journal of Bacteriology* **176**, 932–934.

VORBECK, C., LENKE, H., FISCHER, P., SPAIN, J.C. AND KNACKMUSS, H.-J. (1998). Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. *Applied and Environmental Microbiology* **64**, 246–252.

WATANABE, M., ISHIDATE, M. AND NOHMI, T. (1990). Nucleotide sequence of *Salmonella typhimurium* nitroreductase gene. *Nucleic Acids Research* **18**, 1059.

WATANABE, M., NISHINO, T., TAKIO, K., SOFUNI, T. AND NOHMI, T. (1998). Purification and characterization of wild-type and mutant 'classical' nitroreductases of *Salmonella typhimurium* – L33R mutation greatly diminishes binding of FMN to the nitroreductase of *S. typhimurium*. *Journal of Biological Chemistry* **273**, 23922–23928.

WENDT, T.M., CORNELL, J.H. AND KAPLAN, A.M. (1978). Microbial degradation of glycerol nitrates. *Applied and Environmental Microbiology* **36**, 693–699.

WHITE, G.F. AND SNAPE, J.R. (1993). Microbial cleavage of nitrate esters: defusing the environment. *Journal of General Microbiology* **139**, 1947–1957.

WHITE, G.F., SNAPE, J.R. AND NICKLIN, S. (1996a). Biodegradation of glycerol trinitrate and pentaerythritol tetranitrate by *Agrobacterium radiobacter*. *Applied and Environmental Microbiology* **62**, 637–642.

WHITE, G.F., SNAPE, J.R. AND NICKLIN, S. (1996b). Bacterial biodegradation of glycerol trinitrate. *International Biodeterioration & Biodegradation* **38**, 77–82.

WHITE, G.F., SNAPE, J.R. AND NICKLIN, S. (1996c). Bacterial biodegradation of nitrate ester explosives. In: *Sea dumped chemical weapons: Aspects, problems, and solutions*. Ed. A.V. Kaffka, pp 145–156. Netherlands: Kluwer Academic Publishers.

WHITEWAY, J., KOZIARZ, P., VEALL, J., SANDHU, N., KUMAR, P., HOECHER, B. AND LAMBERT, I.B. (1998). Oxygen-sensitive nitroreductases: analysis of the roles of *nfsA* and *nfsB* in development of resistance to 5-nitrofuran derivatives in *Escherichia coli*. *Journal of Bacteriology* **180**, 5529–5539.

WIDRIG, D.L., BOOPATHY, R. AND MANNING, J.F. (1997). Bioremediation of TNT-contaminated soil: a laboratory study. *Environmental Toxicology and Chemistry* **16**, 1141–1148.

WILLIAMS, R.T., ZIEGENFUSS, P.S. AND SISK, W.E. (1992). Composting of explosives and propellant contaminated soils under thermophilic and mesophilic conditions. *Journal of Industrial Microbiology* **9**, 137–144.

YABANNAVAR, A.V. AND ZYLSTRA, G.J. (1995). Cloning and characterization of the genes for *p*-nitrobenzoate degradation from *Pseudomonas picketii* YH105. *Applied and Environmental Microbiology* **61**, 4284–4290.

YINON, J. (1990). *Toxicity and metabolism of explosives*. Boca Raton, FL: CRC Press.

YOUNG, D.M., UNKEFER, P.J. AND OGDEN, K.L. (1997a). Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-trinazine (RDX) by a prospective consortium and its most effective isolate *Serratia marcescens*. *Biotechnology and Bioengineering* **53**, 515–522.

YOUNG, D.M., KITTS, C.L., UNKEFER, P.J. AND OGDEN, K.L. (1997b). Biological breakdown of RDX in slurry reactors proceeds with multiple kinetically distinguishable paths. *Biotechnology and Bioengineering* **56**, 258–267.

YUAN, R., SUMI, M. AND BENET, L.Z. (1997). Investigation of aortic CYP3A bioactivation of nitroglycerin *in vivo*. *Journal of Pharmacology and Experimental Therapeutics* **281**, 1499–1505.

ZENNO, S., KOIKE, H., KUMAR, A.N., JAYARAMAN, R., TANOKURA, M. AND SAIGO, K. (1996a). Biochemical characterization of NfsA, the *Escherichia coli* major nitroreductase exhibiting a high amino acid sequence homology to Frp, a *Vibrio harveyi* flavin oxidoreductase. *Journal of Bacteriology* **178**, 4508–4514.

ZENNO, S., KOIKE, H., TANOKURA, M. AND SAIGO, K. (1996b). Gene cloning, purification, and characterization of NfsB, a minor oxygen-insensitive nitroreductase from *Escherichia coli*, similar in biochemical properties to Frase I, the major flavin reductase in *Vibrio fischeri*. *Journal of Biochemistry* **120**, 736–744.

ZENNO, S., KOIKE, H., TANOKURA, M. AND SAIGO, K. (1996c). Conversion of NfsB, a minor *Escherichia coli* nitroreductase, to a flavin reductase similar in biochemical properties to Frase I, the major flavin reductase in *Vibrio fischeri*, by a single amino acid substitution. *Journal of Bacteriology* **178**, 4731–4733.

ZENNO, S., KOBORI, T., TANOKURA, M. AND SAIGO, K. (1998). Conversion of NfsA, the major

Escherichia coli nitroreductase, to a flavin reductase with an activity similar to that of Frp, a flavin reductase in *Vibrio harveyi*, by a single amino acid substitution. *Journal of Bacteriology* **180**, 422–425.

ZHANG, Y.Z., SUNDARAM, S.T., SHARMA, A. AND BRODMAN, B.W. (1997). Biodegradation of glyceryl trinitrate by *Penicillium corylophilum* Dierckx. *Applied and Environmental Microbiology* **63**, 1712–1714.