Chapter 5
Biodegradation of the Explosives TNT, RDX and HMX

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5.1 Environmental Significance

In the early twentieth century, more than 60 highly explosive compounds were developed and synthesized for military and civilian use. Of these, the most widely used explosives in the world are probably hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 2,4,6-trinitrotoluene (TNT) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Fig. 5.1). These compounds are characterized by relatively high thermal stability, high density and high detonation velocity, all of which promote their extensive use (Yinon 1990; Cooper and Kurowski 1997). Some of their physical properties are summarized in Table 5.1.

Following the extensive production and use of explosive compounds in the twentieth century, their contamination of soil and groundwater has become a global problem, as reflected from reports from the US (Pennington and Brannon 2002), Canada (Darrach et al. 1998), Argentina (Fuchs et al. 2001), the UK (Seth-Smith et al. 2008), Germany (Steuckart et al. 1994; Lewin et al. 1996), Sweden (Wingfors et al. 2006), Spain (Van Dillewijn et al. 2007), Israel (Bernstein et al. 2008), and Australia (Martel et al. 2008). However, an account of explosives contamination worldwide is not available.

Soil and water contamination by explosives is related to their manufacture, the production and loading of munitions items, inappropriate waste-disposal practices during production or during demilitarization activities or military training, and unexploded residuals in the battlefield. The highest extent of contamination is

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S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_5,

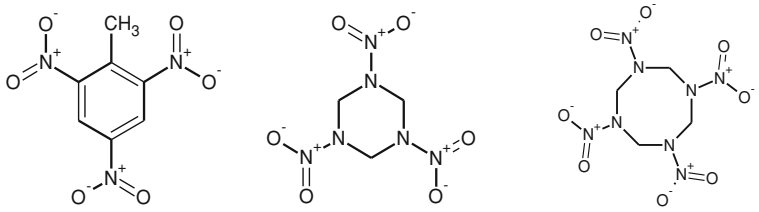

Fig. 5.1 Molecular structures of TNT, RDX and HMX

Table 5.1 Physical and chemical properties of TNT, RDX and HMX

Compound	Molecular weight (g/mol)	Melting point (°C)	Density (g/cm^3)	Vapor pressure (torr)	LogK_{ow}	Solubility (g/kg) at 20°C		
						Water	Acetone	Ethanol
TNT	227.13	80.6	1.65	5.51×10^{-6}	1.86	0.13	1,090	12.3
RDX	222.26	204	1.82	4.03×10^{-9}	0.86	0.04	83	1.5
HMX	296.20	286	1.90	3.33×10^{-14}	0.061	0.002	28	ND

ND not determined
Source Spanggord et al. (1982), Yinon and Zitrin (1993), McGrath (1995)

often associated with inappropriate wastewater handling. In particular, the discharge technique commonly used by explosives-manufacturing plants in which wastewater was discharged into unlined lagoons or streams, results in indirect contamination of soil and groundwater (Pennington and Brannon 2002). As a result, contamination often reached to high concentrations due to continuous discharge over a large surface area. Today, this method of waste disposal is prohibited throughout the western world.

In training areas where contamination by unexploded residuals is of deep concern, contamination of the upper soil layer may reach to high concentrations, but groundwater contamination may be somewhat limited since the compounds are normally spread over the soil surface in their solid phase. In this case, groundwater contamination strongly depends on precipitation, and on the compound solubility and dissolution kinetics (Morley et al. 2006).

In marine environments, contamination by explosive compounds may be caused as a result of sunken warships, military waste and navy training, as well as of dumping defective munitions. In this case, pollutant concentrations have been shown to be lower (Darrach et al. 1998).

The concentration of explosives in soil and groundwater not only depends on the polluting activities, but also on the discharge patterns, the thickness of the unsaturated zone, and natural attenuation processes which may either reduce the pollutant's point concentration (e.g., dilution) or reduce its entire mass in the environment (e.g., biodegradation). A literature review of the last few decades on soil and water pollution by explosives shows that their concentration in the

Table 5.2 Soil risk assessment screening and drinking water recommendations for explosive compounds

	Residential soil (mg/kg)	Industrial soil (mg/kg)	Drinking water (µg/l)
TNT	19	79	2
RDX	5.5	24	2
HMX	8,300	49,000	400

Source US EPA (2006, 2010)

groundwater may reach several milligrams per liter (Best et al. 1999; Charles et al. 2000; Bernstein et al. 2010), and in soils up to several or even tens of grams per kilogram (Boopathy 2000; Charles et al. 2000; Groom et al. 2001; Clark and Boopathy 2007). These concentrations exceed the recommended allowable amounts for soil and drinking water (Table 5.2).

Explosive compounds are undesirable in the environment due to their toxicity. TNT, RDX and HMX have been defined as toxic to humans and animals. Exposure to high concentrations of TNT causes anemia and abnormal liver function and is thought to promote spleen enlargement and to have other harmful effects on the blood, liver and immune system. It can also cause skin irritation after prolonged skin contact, and cataract development after long-term exposure (ATSDR 1996a). Long-term exposure to RDX can adversely affect the nervous system, and is thought to promote liver and kidney damage (ATSDR 1996b). Information on the adverse health effects of HMX is limited, but it is thought to be of lower toxicity than the other two. Nevertheless, studies in rats, mice, and rabbits indicate that HMX may be harmful to the liver and central nervous system if it is swallowed or gets on the skin (ATSDR 1997). TNT and RDX are classified by the US EPA as degree C carcinogenic, i.e., potential carcinogens in humans (US EPA 2006).

5.2 Biodegradation of Explosives

In contrast to natural attenuation processes such as dilution or sorption, which reduce the pollutant concentration, but do not reduce its overall mass in the environment, biodegradation is a natural attenuation process that promotes complete removal of the pollutant from the environment. Research on the biodegradation mechanisms of the explosive compounds that are the focus of this review has been carried out for the last four decades, starting with early works that studied degradation pathways for TNT (Won et al. 1974; McCormick et al. 1976) and RDX (McCormick et al. 1981) in sludge. These were followed by an increasing number of studies that identified additional catabolic pathways, isolated and identified increasing amounts of explosives-degrading bacteria, and studied the biochemical aspects of the processes at enzyme and genomic levels.

Research on biodegradation clearly shows the potential for reducing the concentrations of explosives in the environment via microbial activity. This can be

achieved by different degradation pathways that are strongly dictated by the redox potential of the surrounding environment and nutrient availability. The degradation pathways for the three most common explosives, RDX, HMX and TNT, are reviewed herein. The readers are also referred to some excellent review articles published in the last decade on the degradation pathways of the explosive compounds. Esteve-Núñez et al. (2001) and Stenuit et al. (2005) have focused on the biodegradation of TNT, Hawari et al. (2000a) on the biodegradation of both RDX and TNT, and Crocker et al. (2006) on the biodegradation of both RDX and HMX.

5.2.1 TNT

TNT can be biodegraded via various pathways, which mainly involve transformation of the nitro functional group, while the aromatic ring remains intact (Hawari et al. 2000a). The stability of the aromatic ring results from the strong electron-withdrawing properties of the nitro substituents which promote high electron deficiency and electrophilic characteristics on the π-electron system (Rieger and Knackmuss 1995). In addition, steric effects resulting from the symmetric position of the four functional groups protect the aromatic ring bonds from enzymatic attack (Stenuit et al. 2005).

In general, the degradation of TNT is mainly initiated by either reduction of the nitro group or C–NO_2 bond cleavage and denitration. Both can occur aerobically as well as anaerobically, yet each has unique catabolic steps. The reduction of the nitro group is often suggested to occur co-metabolically, implying that it is a non-beneficial process for the cell in terms of energy or nutrient yield. Thus, in this case, the microorganism will not derive carbon, nitrogen or energy (Stenuit et al. 2005). Nevertheless, it has been shown that TNT reduction may indeed be beneficial to the microbial cell, where it acts as a terminal electron acceptor in respiratory chains, as presented for *Pseudomonas* sp. strain JLR11 under anoxic conditions (Esteve-Núñez et al. 2000). In contrast to the presumed co-metabolic reduction pathway of the nitro group, the pathway, in which denitration of the nitro group occurs, has been shown to be clearly beneficial to the microorganism, as the nitrogen originating from the nitro group is available for further incorporation by the cell (Stenuit et al. 2005).

Normally, it is not the methyl group that plays a role in the key initial catabolic step, but the nitro groups, due to their strong electrophilic character. Nevertheless, additional possible pathways, albeit rarely documented, involve the removal or transformation of the methyl functional group at an initial transformation stage, as elaborated here.

5.2.1.1 Reduction of the Nitro Group

Anaerobic Reduction

The most common degradation pathway for TNT proceeds along the sequential reduction of the nitro groups. Following sequential steps of two-electron transfers, the corresponding mononitroso, monohydroxylamino and monoamino derivatives

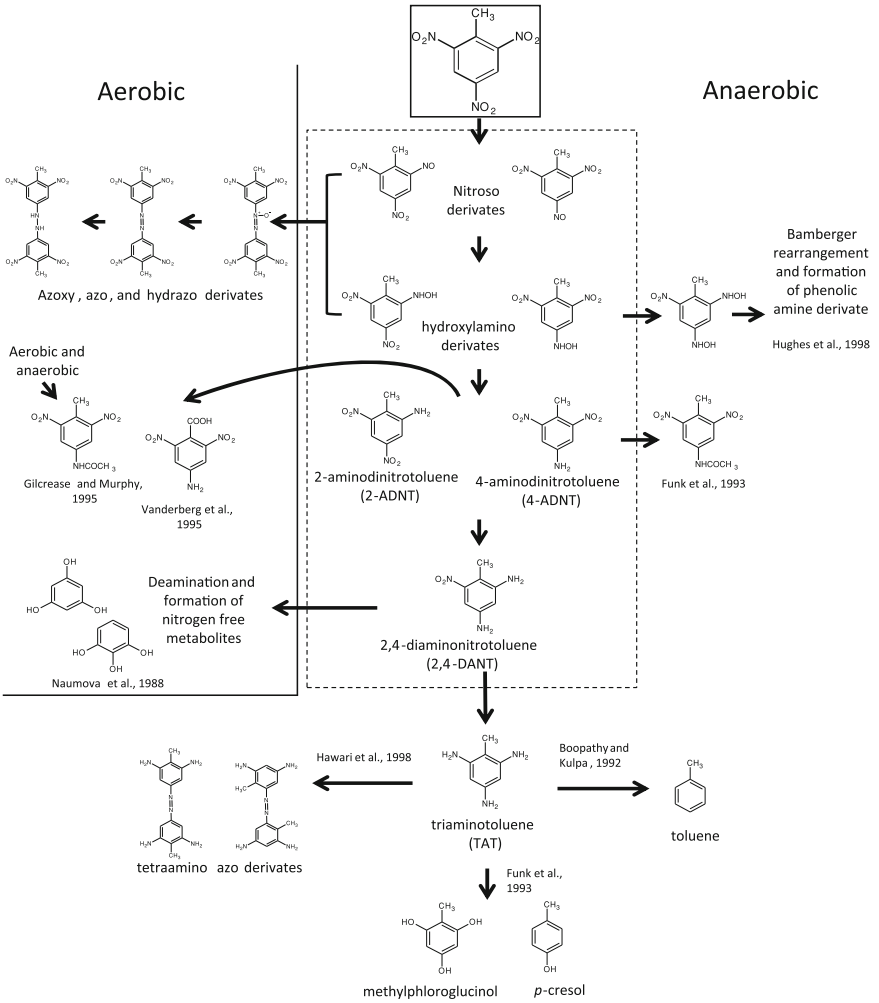

Fig. 5.2 Reduction pathway of TNT, and further transformation of the reduced derivatives under aerobic and anaerobic conditions

of TNT are sequentially formed (Fig. 5.2). Reduction of the monoamino derivatives can then proceed further to produce a diamino derivative.

The initial reduction toward the formation of diamino derivatives occurs under both aerobic and anaerobic conditions. It is performed by a large variety of microbial strains (Table 5.3) and it can be catalyzed by various enzymes, such as nitroreductase, aldehyde oxidase, dihydrolipic amide dehydrogenase, cytochrome b5 reductase, diaphorases, hydrogenases, xanthine oxidase, and carbon monoxide dehydrogenase (Esteve-Núñez et al. 2001). The reduction of the nitro group has also been shown to occur alternatively via two sequential steps of single-electron

Table 5.3 Microbial isolates of TNT degraders

Isolate	Conditions	Suggested pathway	Reference
Acinetobacter johnsonii	Aerobic	Pathway was not determined	Fuller and Manning (1997)
A. junii A8	Aerobic	TNT transforms to 2,6-DN-4-nitrosotoluene, 4-AM-2,6-dinitrotoluene, 4-AM-2,6-dinitrobenzoic acid	Soojhawon et al. (2005)
Agrobacterium sp. 2PC	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Alcaligenes eutrophus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Anabaena sp.		Detection of azoxy-tetranitrotoluene isomers and hydroxylaminodinitrotoluene	Pavlostathis and Jackson (1999)
Arthrobacter globiformis	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Arthrobacter sp. RP17	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Bacillus cereus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Bacillus sp.	Aerobic	(1) Release of nitrite, with final formation of toluene; (2) sequential reduction to the final formation of TAT	Kalafut et al. (1998)
B. subtilis	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Cellulomonas sp. ES6	Anaerobic	Amino and hydroxylamino derivatives	Borch et al. (2005)
Clostridium acetobutylicum	Anaerobic	Degradation via Bamberger rearrangement of 2,4-diHA-6-nitrotoluene to either 2-AM-4-HA-5-hydroxyl-6-nitrotoluene (4-AM-6-HA-3-methyl-2 nitrophenol) or 2-HA-4-AM-5-hydroxyl-6-nitrotoluene (6-AM-4-HA-3-methyl-2-nitrophenol).	Hughes et al. (1998)
C. acetobutylicum	Anaerobic	(1) Partial reduction by Bamberger rearrangement to HA intermediate; (2) TNT-TAT	Khan et al. (1997), Hughes et al. (1998)
C. bifermentans	Anaerobic	Detection of TAT, phenolic products of TAT hydrolysis, and TAT and pyruvic aldehyde condensation products	Lewis et al. (1996)
C. bifermentans	Anaerobic	Reductive TNT transformations to form TAT and phenolic products of TAT hydrolysis	Regan and Crawford (1994), Lewis et al. (1996)
C. bifermentans ATCC 638	Anaerobic	Reductive pathway	Ederer et al. (1997)
C. bifermentans KMR-1	Anaerobic	Reductive pathway	Ederer et al. (1997)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
C. pasteurianum DSM 525	Anaerobic	Reductive TNT transformations to form TAT and further transformation of TAT to undefined products	Preuss et al. (1993)
C. sordellii	Anaerobic	Reductive pathway	Ederer et al. (1997)
C. sporogenes	Anaerobic	Reductive pathway	Ederer et al. (1997)
C. thermoaceticum	Anaerobic		Huang et al. (2000)
Corynebacterium glutamicum	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Corynebacterium sp. Nap2	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Cytophaga pectinovora	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Desulfovibrio gigas	Anaerobic		Boopathy and Manning (1996)
D. indolicum	Anaerobic		Boopathy et al. (1997)
Desulfovibrio sp.	Anaerobic	Detection of TNT, DANT and TAT isomers	Drzyzga et al. (1999)
Desulfovibrio sp. strain B	Anaerobic	In the absence of external N sources, reductive deamination of amino derivatives to the final formation of toluene	Boopathy et al. (1993)
D. vulgaris	Anaerobic		Boopathy and Kulpa (1994)
Enterobacter cloacae PB2	Aerobic	Reduction to hydride-Meisenheimer complex with release of nitrite	French et al. (1998)
Escherichia coli	Aerobic	Pathway was not determined	Fuller and Manning (1997)
E. coli	Anaerobic	Reductive pathway	Ederer et al. (1997)
Flavobacterium odoratume	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Klebsiella sp. 1PC	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Klebsiella sp. C1	Anaerobic	Reduced to hydroxylamino dinitrotoluenes, aminodinitrotoluenes and to nitrite via denitration	Kim et al. (2002)
Lactobacillus acidophilus	Anaerobic	Reductive pathway	Ederer et al. (1997)
L. casei	Anaerobic	Reductive pathway	Ederer et al. (1997)
L. lactis	Anaerobic	Reductive pathway	Ederer et al. (1997)
Methanococcus deltae			Boopathy (1994)
Methanococcus sp. strain B	Anaerobic		Boopathy (1994)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
M. thermolithotrophicus			Boopathy (1994)
Methylobacterium sp. BJ001	Anaerobic	Reduced to ADNT	Van Aken et al. (2004)
Micrococcus luteus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Mycobacterium sp. strain HL 4-NT-1	Aerobic	Formation of a hydride-Meisenheimer complex	Vorbeck et al. (1994)
M. vaccae strain JOB-5	Aerobic	(1) Detection of products as 4-AM-2,6-dinitrobenzoic acid and 2,4-diAM-6-nitrobenzyl methyl ether; (2) detection of azoxy compounds; (3) evidence of ring cleavage	Vanderberg et al. (1995)
Myxococcus xanthus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Nocardiodes CB22-2	Aerobic	Dinitration leads to formation of Meisenheimer complex	Behrend and Heesche-Wagner (1999)
Pseudomonas	Aerobic	Production of hydride-Meisenheimer complex and transformation to DNT, NT and toluene	Duque et al. (1993)
P. aeruginosa	Aerobic	(1) Release of nitrite, with final formation of toluene; (2) sequential reduction to the final formation of TAT	Kalafut et al. (1998)
P. aeruginosa	Aerobic	Detection of HA and AM derivatives	Oh et al. (2001)
P. aeruginosa	Aerobic	Pathway was not determined	Fuller and Manning (1997)
P. aeruginosa strain MA01	Aerobic	Oxidation of ADNT isomers to N(4)-acetyl-2,4-diAM-6-nitrotoluene and tetranitroazoxytoluene isomers	Alvarez et al. (1995)
P. cepacia	Aerobic	Pathway was not determined	Fuller and Manning (1997)
P. fluorescens	Aerobic	Reduction by addition of hydride to form dihydride Meisenheimer complex to catalyze reduction of nitro group	Pak et al. (2000)
P. fluorescens	Aerobic	Pathway was not determined	Fuller and Manning (1997)
P. pseudoalcaligenes JS52	Aerobic and anaerobic	Detection of mono- and di-HADNTs, monoAM monoHA NT, and monoAM DNT	Fiorella and Spain (1997)
P. putida	Aerobic	Pathway was not determined	Fuller and Manning (1997)
P. putida HK-6	Aerobic	Reductive pathway	Cho et al. (2008)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
P. putida KP-T20 1	Aerobic	Amino derivatives of TNT, and denitrated products (DNT)	Park et al. (2003)
Pseudomonas sp. clone A	Aerobic	Formation of hydride-Meisenheimer complex, followed by denitration to form 2,4-DNT and an unidentified compound. AM and HA products were also detected, and condensed to azoxytoluenes	Haïdour and Ramos (1996)
Pseudomonas sp. DFC49	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Pseudomonas sp. JLR11	Anaerobic	Mineralization, via the formation of products such as 2,4,6-trinitrobenzaldehyde, 2-nitro-4-hydroxybenzoic acid, and 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid	Esteve-Núñez and Ramos (1998)
Pseudomonas sp. JS150	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Pseudomonas sp. Tol1A	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Rahnella aquitilis BFB	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Raoultella terrigena HB	Aerobic	AM derivatives and azoxy dimers	Claus et al. (2007)
Rhodococcus erythropolis	Aerobic	Dinitration leads to formation of Meisenheimer complex by F420 reductase and hydride transferase	Rieger et al. (1999)
R. erythropolis	Aerobic	Formation of hydride and dihydride TNT-Meisenheimer complexes	Vorbeck et al. (1998)
R. erythropolis	Aerobic	Pathway was not determined	Fuller and Manning (1997)
R. globerulus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
R. rhodocrous	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Rhodococcus sp. TF2	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Salmonella typhimurium	Anaerobic	Reductive pathway	Ederer et al. (1997)
Serratia marcescens	Aerobic	Not defined. ADNT isomers were detected	Montpas et al. (1997)
SP1b (coryneform)	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Sphingomonas capsulata	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Staphylococcus sp.	Aerobic	(1) Release of nitrite, with final formation of toluene; (2) sequential reduction to final formation of TAT	Kalafut et al. (1998)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
Streptomyces albus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
S. chromofuscus A11	Aerobic		Pasti-Grigsby et al. (1996)
S. griseus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Sulphate-reducing bacterium	Anaerobic	TNT reduction to TAT and further transformation to unknown products	Preuss et al. (1993)
Veillonella alkalescens			McCormick et al. (1976)
Yarrowia lipolytica AN-L15	Aerobic	Detection of eight different hydride complexes and nitrite release from the ring	Ziganshin et al. (2007)

DN dinitro, *AM* amino, *HA* hydroxylamino, *DANT* diaminonitrotoluene, *ADNT* aminodinitrotoluene, *TAT* triaminotoluene

transfers, forming first a nitroanion radical and then the corresponding nitroso derivative. This is catalyzed by the oxygen-sensitive nitroreductase enzymes, which are found in bacteria such as *Clostridium* (Angermaier and Simon 1983) and *Escherichia coli* (Peterson et al. 1979).

Because of the high electron deficiency on the nitro groups of TNT, its microbial degradation is often initiated by reductive rather than oxidative reactions, even under aerobic conditions (Vorbeck et al. 1998). Nevertheless, further transformation of the mono and diamino derivatives toward the formation of the most reduced product—triaminotoluene (TAT)—proceeds only under strictly anaerobic conditions in which redox potential values are essentially below −200 mV (Hawari et al. 2000a). Thus, under oxic conditions, diamino derivatives tend to accumulate, while the presence of TAT is indicative of strictly reduced conditions.

A few studies have aimed to identify further transformation steps of TAT under anaerobic conditions. Hawari et al. (1998) identified its further transformation to tetraaminoazo derivatives in an anaerobic sludge, which, in turn, disappeared from the solution and was suspected of polymerization (Hawari et al. 1998). Boopathy and Kulpa (1992) detected toluene as a product in TNT degradation by *Desulfovibrio* sp. strain B. They proposed that the toluene was formed by reductive elimination of the amino groups from TAT, but this pathway was never verified. Funk et al. (1993) identified the formation of para-hydroxytoluene (*p*-cresol) and methylphloroglucinol (2,4,6-trihydroxytoluene) in anaerobic mixed cultures. They suggested that these products were formed from TAT.

The reductive pathway of TNT towards the formation of amino derivatives may be desirable in contaminated environments, since the toxicity and mutagenic characteristics of the amino derivatives are found to be morally lower than those of TNT (Drzyzga et al. 1995; Lachance et al. 1999; Neuwoehner et al. 2007). In addition, the amino derivatives, and most significantly TAT, present higher sorption characteristics than TNT itself, with TAT even presenting irreversible sorption to soil particles (Daun et al. 1998; Achtnich et al. 1999). This strong affinity to soil material decreases the pollutant concentration in water. Nevertheless, it remains in the environment.

Some studies have shown that the anaerobic reductive pathway does not necessarily proceed to TAT, but to the alternative transformation of the partly reduced monohydroxylamine derivatives to dihydroxylamine derivatives which may be further transformed to phenolic amine products (Hughes et al. 1998). This was shown to occur anaerobically via Bamberger rearrangement of 2,4-dihydroxylamino-6-nitrotoluene by anaerobic *Clostridium acetobutylicum* cell extracts, as well as in whole-cell systems. The products of this mechanism were identified as either 2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene (4-amino-6-hydroxylamino-3-methyl-2-nitrophenol) or 2-hydroxylamino-4-amino-5-hydroxyl-6-nitrotoluene (6-amino-4-hydroxylamino-3-methyl-2-nitrophenol). Similar products were identified during incubation of TNT with the enzyme CO dehydrogenase purified from *Clostridium thermoaceticum* (Huang et al. 2000).

Aerobic Reduction

Reduced TNT derivatives are also commonly detected under aerobic conditions (Table 5.3). Nevertheless, under these conditions, the mono and diamino derivatives often accumulate in the medium without further metabolism (Esteve-Núñez et al. 2001) and the formation of TAT is halted. In the presence of oxygen, however, it has been shown that both the nitroso and the monohydroxylamino metabolites can follow an alternative abiotic transformation pathway to form tetranitroazoxytoluene dimmers, also referred as azoxytetranitrotoluene as shown in Fig. 5.2 (McCormick et al. 1976; Haïdour and Ramos 1996). These azoxy products were shown to cause a higher rate of mutations than TNT (George et al. 2001) and were thought to suppress the degradation of RDX and HMX which may coexist with TNT in the environment (Sagi-Ben Moshe et al. 2009). The accumulation of azoxy derivatives in the environment is hence clearly undesirable, but laboratory experiments have shown that these derivatives themselves may further degrade in microcosm experiments with the fungal strain *Phanerochaete chrysosporium* (Hawari et al. 1999), or in soil slurries with a mixed culture (Sagi-Ben Moshe et al. 2009), as well as in other experimental systems. In these two examples, complete disappearance of tetranitroazoxytoluene was achieved within a few weeks, indicating the non-recalcitrant nature of the compound. Its removal was followed by its further transformation to azo derivatives (tetranitroazotoluene), which, in turn, formed hydrazo derivatives (tetranitrohydrazotoluene), that also disappeared from the solution. Sorption of the azoxy metabolite to soil material in slurries was shown to occur in trace amounts (Sagi-Ben Moshe et al. 2009).

Under aerobic conditions, the amino derivatives may alternatively follow deaminization (Naumova et al. 1988), and be transformed to benzoic acid (Vanderberg et al. 1995) or *N*-acetylamino derivatives (Gilcrease and Murphy 1995). Thus, although the amino derivatives are often accumulated under aerobic conditions, they should not be treated as dead-end products.

5.2.1.2 Denitration

Aerobic Denitration

A variety of strains have been found capable of aerobic growth on TNT as sole nitrogen source. This unique phenomenon is coupled to their ability to denitrate the molecule with the subsequent release of nitrite to the medium (Table 5.3). A number of studies show that under aerobic conditions, the denitration pathway proceeds via the nucleophilic addition of a hydride ion to the aromatic ring in the presence of NAD(P)H and the formation of a hydride-Meisenheimer complex, which can be further transformed to a dihydride complex with the subsequent release of nitrite as depicted in Fig. 5.3 (Lenke and Knackmuss 1992). The formation of the Meisenheimer complex as a key metabolite that promotes denitration

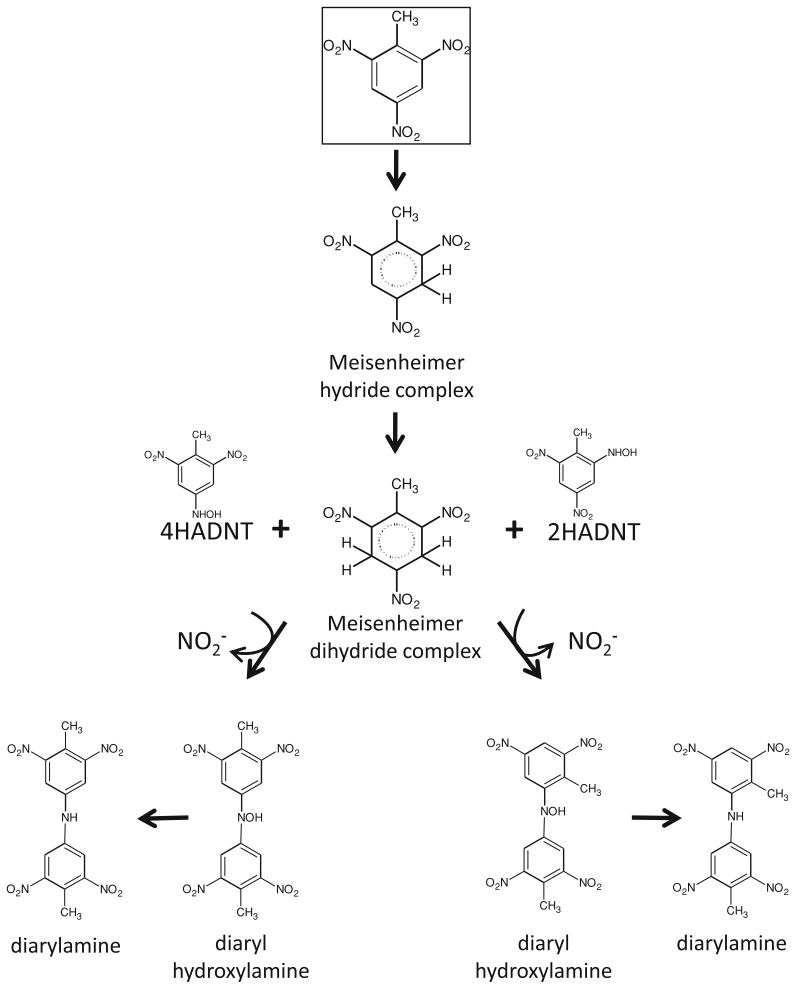

Fig. 5.3 The aerobic release of nitrite following the dimerization of dihydride complex with HADNTs

was first shown to occur with the bacterial strain *Mycobacterium* sp. strain HL 4-NT-1 (Vorbeck et al. 1994), and later proven for other strains also (Table 5.3). Several different enzymes of the type II hydride transferases have been identified capable of performing the nucleophilic addition of hydride ions (Stenuit et al. 2006): pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2 (French et al. 1998), xenobiotic reductase B (XenB) from *Pseudomonas fluorescens* I-C (Pak et al. 2000), and N-ethylmaleimide (NEM) reductase from *E. coli* (Williams et al. 2004).

It was suggested that once the non-aromatic Meisenheimer complex is formed, aromaticity is restored upon nitrite release (Rieger and Knackmuss 1995).

This hypothesis was adopted to explain the detection of 2,4-dinitrotoluene as a metabolic product of TNT's hydride-Meisenheimer complexes during incubation with the white-rot fungus *Irpex lacteus* (Kim and Song 2000). Nevertheless, in the last few years, more and more studies have shown that the release of nitrite from the TNT molecule likely occurs following dimerization of the dihydride complex with coexisting hydroxylaminodinitrotoluenes (HADNTs) with the formation of the secondary diarylamines, accompanied by the release of stoichiometric amounts of nitrite as shown in Fig. 5.3 (Pak et al. 2000; Stenuit et al. 2006; Van Dillewijn et al. 2008; Wittich et al. 2008). The condensation of the two molecules and the release of nitrite were shown to be a potentially chemically catalyzed reaction rather than an enzymatic one, and the nitrite that is released during the condensation was shown to originate from the Meisenheimer-dihydride complex rather than from the hydroxylamine (Wittich et al. 2009).

In addition to the increasing evidence of the importance of Meisenheimer complexes in TNT denitration, it is evident that denitration can potentially occur via other routes. An example is given by Stenuit et al. (2009), in which extracellular catalysts extracted from the supernatant of *Pseudomonas aeruginosa* ESA-5, were incubated with TNT and NAD(P)H, and significant release of nitrite was observed. The release of nitrite was coupled with the formation of two polar metabolites, which had lost two nitro groups from the parent compound. It was suggested that superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide are involved in the denitration process. This pathway, however, has not been demonstrated in living cells.

Other studies showing denitration with the release of nitrite and formation of dinitrotoluene, mononitrotoluene and even toluene were documented by Duque et al. (1993) and Kalafut et al. (1998). However, the detailed mechanisms remain still to be investigated.

Anaerobic Denitration

Under anaerobic conditions, similar to aerobic denitration, denitration of the nitro group of the aromatic ring has also been shown to occur in the absence of alternative nitrogen sources, although fewer strains have actually been shown capable of utilizing TNT as sole nitrogen source under such conditions.

One example is provided by Esteve-Núñez and Ramos (1998), who studied the metabolism of 2,4,6-trinitrotoluene by *Pseudomonas* sp. JLR11, which utilizes TNT as sole N source under anaerobic conditions. During incubation, release of nitrite was detected. This strain incorporated around 85% of the N-TNT into N-organic nitrogen. The mechanism of nitrite release by this strain remains still to be explored.

Another example is provided by Eyers et al. (2008), who identified denitration in an oxygen-depleted enrichment culture, where TNT served as sole nitrogen source. During incubation, significant release of nitrite was observed and *P. aeruginosa* ESA-5 was subsequently isolated as the denitrating strain. Reduced derivatives of

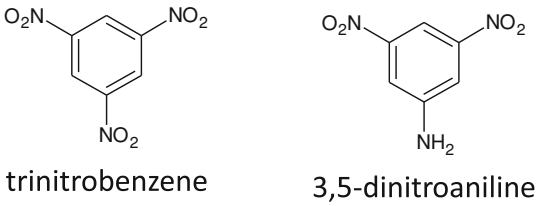

Fig. 5.4 Methyl-group transformation products detected by Esteve-Núñez and Ramos (1998)

TNT and several unidentified metabolites were detected as well. Nevertheless, the exact mechanism of the anaerobic denitration was not traced out. It should be noted that the facultative anaerobe *P. aeruginosa* had already been reported to promote TNT denitration under aerobic conditions (Kalafut et al. 1998; Oh et al. 2003), but whether the pathway in both cases was similar is still unknown. Denitration was also shown to be catalyzed by extracellular catalysts of this strain as discussed earlier (Stenuit et al. 2009), which would be governing catalysts of the denitration.

5.2.1.3 Pathways Involving the Methyl Group

The methyl group is not normally involved in the early degradation steps of TNT. Nevertheless, a few exceptional studies have shown its removal in an initial catabolic reaction. Involvement of the methyl group as an initial step in the degradation of TNT under anaerobic conditions was shown by Esteve-Núñez and Ramos (1998), who studied the metabolism of TNT by *Pseudomonas* sp. JLR11, which utilizes TNT as a sole N source under anaerobic conditions. Of the products detected, 1,3,5-trinitrobenzene and 3,5-dinitroaniline were identified (Fig. 5.4), thus indicating the potential removal of the methyl group from TNT under similar conditions.

A different pathway showing the involvement of the methyl group in TNT degradation by *Mycobacterium vaccae* was reported by Vanderberg et al. (1995), who detected the transformation of the methyl group to carboxylic acid, and the consequent formation of 4-amino-2,6-dinitrobenzoic acid (Fig. 5.2). Nevertheless, the transformation of the methyl group in this pathway was unlikely the initial transformation step, but reduction to an amino derivative was likely.

5.2.2 RDX

RDX is composed of a triazinic ring to which three nitro functional groups are perpendicularly attached. In RDX, similar to TNT, the nitro groups are the main targets of the first degradation steps by sequential reduction or denitration as key steps (Fig. 5.5, Table 5.4).

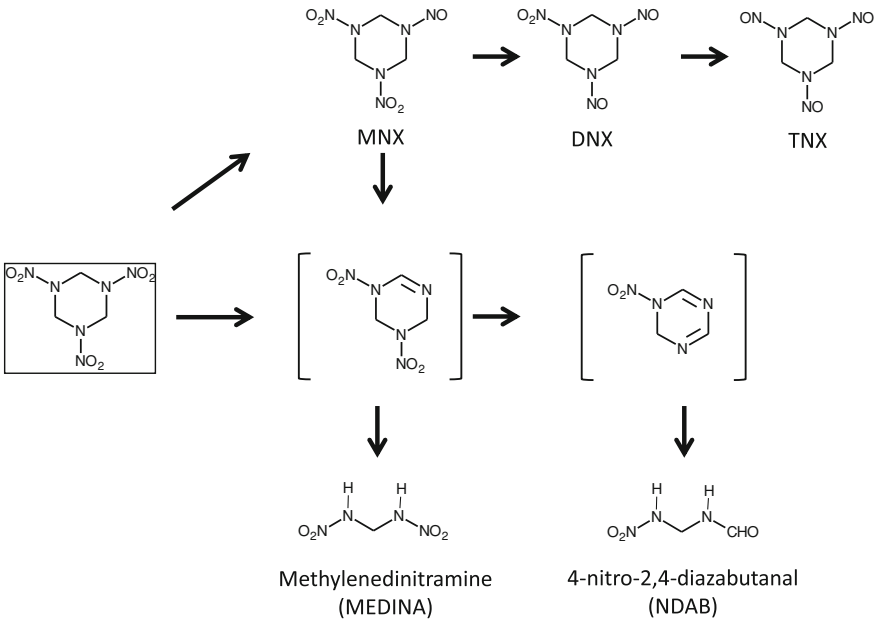

Fig. 5.5 The common degradation pathways of RDX. Compounds in *brackets* are postulated intermediates

5.2.2.1 Sequential Reduction Pathway

Anaerobic Reduction

In contrast to TNT, sequential reduction of RDX occurs mostly under anaerobic conditions, as initially presented by McCormick et al. (1981). This pathway proceeds through reduction of the nitro groups to nitroso derivatives by subsequent two-electron transfer steps, followed by accumulation of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) derivatives. Again in contrast to TNT, the nitroso derivatives do not tend to undergo further reduction to stable detectable product. Although this degradation pathway leads to the desired decrease in RDX concentrations in the contaminated environment, the accumulation of the nitroso derivatives themselves is undesirable due to their toxicity (Zhang et al. 2006). Nevertheless, the nitroso derivatives can further degrade via ring cleavage, after being transferred to the corresponding hydroxylamine metabolites, and produce more simple compounds that can undergo complete mineralization (McCormick et al. 1981).

Table 5.4 Microbial isolates of RDX degraders

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
Bacillus strains HPB2, HPB3	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
Acetobacterium malicum strain HAAP-1	Anaerobic	Denitration of RDX and MNX followed by ring cleavage. Detection of MEDINA	Adrian and Arnett (2004)
A. paludosum	Anaerobic	Ring cleavage of RDX. nitroso derivatives were not detected. Other intermediates were not identified	Sherburne et al. (2005)
Citrobacter freundii	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
C. freundii NS2	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
Clostridium acetobutylicum	Anaerobic	Reduction and formation of amino products. Detection of mononitroso-, monohydroxylamino-, mononitrosomonohydroxylamino-, monoamino-, diamino-, and triamino-compounds	Zhang and Hughes (2003)
C. bifermentans HAW-1	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003a, b)
Clostridium sp. EDB2	Anaerobic	Denitration pathway. Products were not reported	Bhushan et al. (2004)
Clostridium sp. HAW-1, HAW-G3, HAW-G4, HAW-E3, HAW-HC1, and HAW-ES2	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003b)
Clostridium sp. HAW-E3	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003b)
Clostridium sp. HAW-EB17	Anaerobic	Sequential reduction, denitration and ring cleavage of RDX and MNX. Detection of MNX, DNX, TNX. With resting cells, detection of NDAB and MEDINA	Zhao et al. (2004a)
Clostridium sp. HAW-G4	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003b)
Clostridium sp. HAW-HC1	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003b)

(continued)

Table 5.4 (continued)

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
Desulfovibrio sp. HAW-EB18	Anaerobic	Sequential reduction, denitration and ring cleavage of RDX and MNX. Detection of MNX, DNX, TNX. With resting cells, detection of NDAB and MEDINA	Zhao et al. (2004a)
Desulfovibrio sp.	Anaerobic	Pathway not defined. Sequential reduction or direct ring cleavage were excluded as major pathways following low nitroso derivative concentrations and no detection of MEDINA	Arnett and Adrian (2009)
Enterobacter cloacae	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (2000), Pudge et al. (2003)
Fusobacteria isolate HAW-EB21	Anaerobic	Sequential reduction, denitration and ring cleavage of RDX and MNX. Detection of MNX, DNX, TNX. With resting cells, detection of NDAB and MEDINA	Zhao et al. (2004a, b)
Geobacter metallireducens strain *GS-15*	Anaerobic	Reduction to MNX, RDX and MNX denitration and ring cleavage. Detection of MEDINA	Kwon and Finneran (2008)
Gordonia sp. KTR9	Aerobic	Pathway not defined, products not identified	Thompson et al. (2005)
Gorodnia strain YY1	Aerobic	RDX denitration and ring cleavage. Detection of NDAB	Ronen et al. (2008)
Klebsiella pneumoniae SCZ1	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2002)
Methylobacterium extorquens	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
M. organophilum	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
M. rhodesianum	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
Methylobacterium sp. BJ001	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
Morganella morganii B2	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
M. morganii	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)

(continued)

Table 5.4 (continued)

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
Providencia rettgeri B1	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
P. rettgeri	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
Pseudomonas putida HK-6	Aerobic	Pathway not defined, products not identified	Cho et al. (2008)
Pseudomonas strain HPB1	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
Rhodococcus rhodochrous sp. 11Y	Aerobic	Two denitration steps followed by ring cleavage. Detection of NDAB	Seth-Smith et al. (2002)
Rhodococcus sp. DN22	Aerobic	Two denitration steps followed by ring cleavage. Detection of NDAB	Coleman et al. (1998), Fournier et al. (2002), Bhushan et al. (2003b)
Rhodococcus sp. YH1	Aerobic	Two denitration steps followed by ring cleavage. Detection of NDAB	Nejidat et al. (2008)
Serratia marcescens	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Young et al. (1997)
Shewanella halifaxensis HAW-EB4	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Zhao et al. (2004a, 2006)
S. sediminis HAW-EB3	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Zhao et al. (2004a, 2005)
Shewanella sp. HAW-EB1, HAW-EB2, HAW-EB5	Anaerobic	Sequential reduction, denitration and ring cleavage of RDX and MNX. Detection of MNX, DNX, TNX, NDAB, MEDINA	Zhao et al. (2004a)
Stenotrophomonas maltophilia PB1	Aerobic	Pathway not determined, detection of methylene-N-(hydroxymethyl)-hydroxylamine-N'-(hydroxymethyl)nitroamine	Binks et al. (1995)
Williamsia sp. KTR4	Aerobic	Pathway not defined, products not identified	Thompson et al. (2005)

Aerobic Reduction

Under aerobic conditions, the formation of nitroso derivatives is normally not observed. This may be expected from thermodynamic considerations, where the calculated E^0 shows a decrease from trinitroaromatics to nitramine, suggesting thermodynamic control of the reduction of RDX under aerobic conditions (Uchimiya et al. 2010).

Only a few exceptions indicate formation of mononitroso derivatives under aerobic conditions: the first, reported for incubation of the white-rot fungus *P. chrysosporium* with RDX (Sheremata and Hawari 2000) which showed formation of MNX. This was followed by ring cleavage and the subsequent formation of methylene dinitramine (MEDINA). The second, presented by Van Aken et al. (2004), reported the detection of MNX during RDX biodegradation under aerobic conditions by a phytosymbiotic *Methylobacterium* sp. that was associated with poplar tissue. Latter on these authors observed a polar metabolite which was produced by the ring cleavage of MNX, and had the molecular formula of MEDINA. The detection of di- or tri-nitro derivatives under aerobic conditions has never been documented.

5.2.2.2 Denitration

Aerobic Denitration

Denitration appears to be the most important aerobic degradation pathway for RDX, and was initially demonstrated for *Rhodococcus* strain DN22 (Fournier et al. 2002; Bhushan et al. 2003b). Later, it was also shown to be the governing pathway for *Rhodococcus* strains 11Y (Seth-Smith et al. 2002) and YH1 (Nejidat et al. 2008). It was also assumed to occur in *Williamsia* sp. strain KTR4, and *Gordonia* sp. strain KTR9 (Thompson et al. 2005), with the observation of a degradation product which was thought to be 4-nitro-2,4-diazabutanal (NDAB).

In recent years, considerable effort has been made in delineating the biochemical pathway of RDX biodegradation carried out by the various *Rhodococcus* species. In contrast to denitration of TNT, which is not accompanied by ring cleavage due to the relatively high stability of the aromatic ring, denitration of RDX has been shown to be accompanied by spontaneous ring cleavage, and the denitrated RDX intermediate prior to ring cleavage is thus never observed. Under aerobic conditions, the denitration pathway has been suggested to be promoted by two subsequent single-electron transfer steps which promote the release of two nitro groups, after which ring cleavage proceeds, with the subsequent formation of NDAB as the ring-cleavage product. Recently, MEDINA was detected as well as a ring-cleavage product under aerobic conditions, in experiments conducted with *Rhodococcus* strain DN22 (Halasz et al. 2010), as well as under microaerophilic conditions by various other *Rhodococcus* strains (Fuller et al. 2010).

The denitration of RDX by *Rhodococcus* species was reported to be catalyzed by a unique form of the enzyme cytochrome P450 (Coleman et al. 2002) which was later found to be encoded by the gene *XplA* and to promote denitration with NADPH as an electron donor (Indest et al. 2007; Jackson et al. 2007; Seth-Smith et al. 2008; Roh et al. 2009; Rylott et al. 2011). The activity of cytochrome P450 in the denitration of RDX was demonstrated in vivo for the *Rhodococcus* strains DN22 (Coleman et al. 2002; Fournier et al. 2002; Bhushan et al. 2003b), 11Y (Seth-Smith et al. 2008) and YH1 (Tekoah et al. 1999; Nejidat et al. 2008).

The cytochrome P450 enzymes catalyze a vast array of chemical reactions. These enzymes are notable for both the diversity of the reactions they catalyze and the range of chemically dissimilar substrates upon which they act. Cytochrome P450s support the oxidative, peroxidative and reductive metabolism of such endogenous and xenobiotic substrates as environmental pollutants, agrochemicals, plant allelochemicals, steroids, prostaglandins and fatty acids (Danielson 2002). Encoded by *XplA/XplB*, the arrangement of the enzyme's subunits is unique: it comprises a flavodoxin domain fused to an N-terminal cytochrome P450 domain (Hlavica 2009; Indest et al. 2010; Rylott et al. 2011). *XplB* serves as a partner NADH-utilizing flavodoxin reductase (Jackson et al. 2007). Since it has been detected in RDX-degrading *Rhodoccocus* strains worldwide and since it was absent in *Rhodoccocus* species that were not exposed to RDX, it is assumed to have evolved in response to RDX exposure (Seth-Smith et al. 2008).

In most studies, RDX-degrading *Rhodococcus* strains have been isolated from explosive-contaminated soils: strain DN22 was isolated from contaminated soils in Australia (Coleman et al. 1998), strain 11Y and strains HS1-HS19 from contaminated soils in England (Seth-Smith et al. 2002, 2008), and strains YH1 and YY1 from contaminated soils in Israel (Tekoah et al. 1999; Brenner et al. 2000; Ronen et al. 2008). Recently, *Rhodococcus* strains have also been isolated from explosive-contaminated groundwater (Bernstein et al. 2011).

Anaerobic Denitration

Anaerobic denitration has been reported to be carried out by two distinct strains: *Klebsiella pneumoniae* strain SZC-1 (Zhao et al. 2002) and *Clostridium bifermentans* strain HAW-1 (Zhao et al. 2003a), both isolated from the anaerobic sludge. The RDX denitration under anaerobic conditions was accompanied by ring cleavage and the formation of MEDINA rather than the NDAB which is normally observed following denitration under aerobic conditions. This implies that during the anaerobic denitration, as postulated by Zhao et al. (2003a), only one single-electron transfer step occurs with the formation of a free-radical anion ($RDX^{\bullet -}$) and the subsequent release of a single nitro group and ring cleavage.

Anaerobic denitration has also been detected for strains isolated from cold marine sediments: *Shewanella sediminis* strain HAW-EB3 (Zhao et al. 2005) and *Shewanella halifaxensis* HAW-EB4 (Zhao et al. 2006), with the detection of both NDAB and MEDINA as ring-cleavage products. The degradation mechanism

involved a specific c-type cytochrome in the anaerobic RDX metabolism, which degraded RDX by mono-denitration (Zhao et al. 2010).

Denitration was studied at the enzyme level by Jackson et al. (2007) under anoxic conditions focusing on the activity of *XplA* which plays an important role in the aerobic degradation of RDX. With *XplA* expression under anoxic conditions, denitration was also shown to be an initial catabolic step. Nevertheless, under anaerobic conditions, following only a single denitration and a single hydration step, ring cleavage of RDX was already observed with the detection of MEDINA as a ring-cleavage product. Under aerobic conditions, on the other hand, RDX was proposed to follow two denitration and two hydration steps before ring cleavage occurs, leading to the formation of NDAB (Jackson et al. 2007). Nevertheless, in contrast to the in vitro study by Jackson et al. (2007), it was noted in in vivo studies with various *Rhodococcus* strains that denitration is actually halted under anaerobic conditions (Fuller et al. 2010).

The transformation of RDX by *Rhodococcus* strains in general and activity of the *XplA* system in particular have garnered much attention. However, RDX denitration by a different system–the xenobiotic reductase enzymes, has been studied recently (Fuller et al. 2010). Xenobiotic reductase enzymes capable of degrading TNT (Blehert et al. 1999; Pak et al. 2000) have been also shown to degrade RDX in addition to other energetic compounds. The enzymes XenA (in *Pseudomonas putida* II-B) and XenB (in *Pseudomonas fluorescens* I-C) were observed capable of transforming RDX, especially at low oxygen concentrations (and to a small extent under aerobic conditions as well), when external sources of carbon (as succinate) and nitrogen (as ammonium) are added. The primary degradation product for RDX by these studied enzymes was identified as MEDINA, but an additional minor pathway produced NDAB during transformation by whole cells of *P. putida* II-B and by purified XenA. In accordance to detected degradation products, it is likely that ring cleavage was the result of the previous denitration. The exact mechanism of the reaction, however, has not yet been studied.

5.2.2.3 Other Pathways

Ring-cleavage of RDX has also been shown to occur under anoxic conditions, via a route that is not initiated by denitration (Hawari et al. 2000b). This pathway was documented in biodegradation experiments carried out with municipal anaerobic sludge under measured Eh values of −250 to −300 mV. The anoxic ring-cleavage route was postulated to involve enzymatic hydrolysis of an inner C–N bond as the initial step. This was followed by ring cleavage, in which the triazinic ring was divided into two detectable products: MEDINA and bis(hydroxymethyl)nitramine. The ring-cleavage products were further degraded with eventual formation of simpler products. Halasz et al. (2002) suggested that during incubation of RDX with the above sludge, water is involved in the formation of MEDINA. Following experiments with deuterated water, they observed deuterated MEDINA products, but could not determine whether inclusion of water occurred through the initial

enzymatic attack on RDX with enzymatic cleavage of the inner C–N bond or was simply caused by subsequent hydrolysis of the ring-cleavage product.

Another very different RDX-transformation pathway was suggested by Zhang and Hughes (2003) who performed experiments with crude cell extract of *C. acetobutylicum* and demonstrated the transformation of RDX with H_2 as an electron donor. The degradation was accompanied by the formation of hydroxylamino compounds, analogous to the transformation of TNT. Nevertheless, this pathway was not found with whole cells, and thus not yet confirmed to be of environmental relevance.

5.2.3 HMX

HMX is less amenable to biodegradation in the environment than RDX due to its lower water solubility and its relative chemical stability (Hawari et al. 2000a). It is structurally similar to RDX, and thus follows analogous degradation pathways: both sequential reduction of the nitro group to nitroso derivatives and anaerobic denitration followed by ring cleavage have been documented as HMX-degradation pathways (Fig. 5.6). Nevertheless, the study of these degradation pathways has not been as extensive as for RDX, and limited microbial strains were found capable of degrading this compound (Table 5.5). Low documentation may be due to the more recalcitrant nature of the compound, or also because it is of less environmental significance and interest (HMX is less frequently found in the environment, and its contamination is of little concern: drinking water recommendations for HMX are more than two orders of magnitude higher than those for RDX) (Table 5.2). The degradation pathways which have been presented for HMX are summarized as below:

5.2.3.1 Sequential Reduction of the Nitro Group

Similar to the sequential reduction of RDX to its corresponding nitroso derivatives following subsequent two-electron transfer steps (McCormick et al. 1981), nitro groups of the HMX molecule can be reduced to nitroso derivatives. Nevertheless, while for RDX, all three nitroso derivatives are observed in high abundance, in HMX, only less reduced nitroso derivatives are normally detected. For example, the detection of only a mononitroso derivative was documented by Fournier et al. (2004) with the fungal strain *P. chrysosporium*. In this case, the mononitroso derivative did not continue along the sequential pathway, but underwent ring cleavage accompanied by NDAB formation. Similarly, ring cleavage of the mononitroso derivative was also observed by Zhao et al. (2007) with *C. bifermentans* strain HAW-1 isolated from an anaerobic sludge.

The formation of the first two nitroso derivatives: mono- and dinitroso HMX, was observed by Hawari et al. (2001) following incubation of HMX with

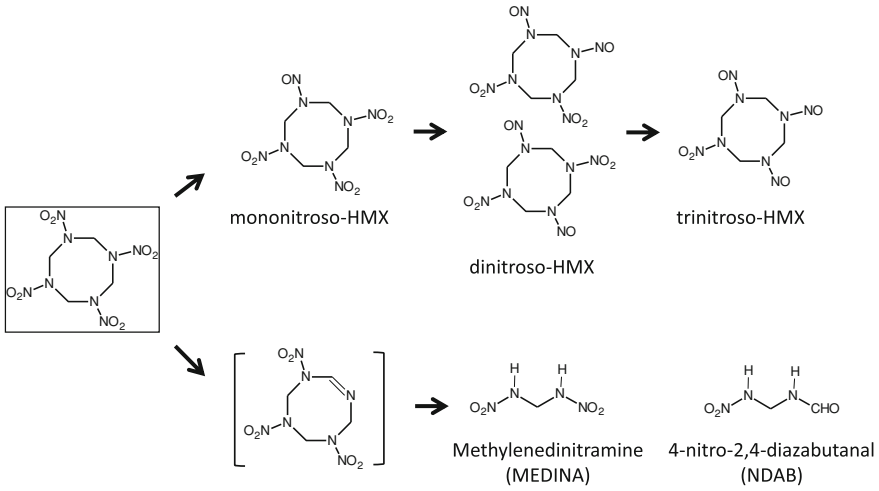

Fig. 5.6 Postulated degradation pathways of HMX. Compounds in *brackets* are postulated intermediates

anaerobic sludge. Further degradation of these nitroso derivatives was not identified in this study.

The formation of the first three nitroso derivatives: mono-, di-, and trinitroso HMX, was detected with various anaerobic strains isolated from marine sediments: strain HAW-EB21 (Zhao et al. 2004a, b), and resting-cell incubations of *Paenibacillus* strain UXO5-11 and strain UXO5-19 (which is not phylogenetically affiliated), and of *Desulfovibrion* strain midref-29 (Zhao et al. 2007).

Formation of most reduced nitroso derivative of HMX: tetranitroso-HMX has not yet been documented.

5.2.3.2 Initial Denitration Followed by Ring Cleavage

Anaerobic denitration of HMX as a result of a single-electron transfer, followed by ring cleavage and MEDINA formation, was detected by resting cells of *C. bifermentans* strains HAW-1 and HAW-EB21 (Zhao et al. 2004a, b). NDAB was not detected in this study. Bhushan et al. (2003a) studied the transformation pathway of HMX with the metallo-flavo enzyme xanthine oxidase. Based on the detected products, they proposed that HMX undergoes a single denitration step. They observed this step under anaerobic conditions, whereas under aerobic conditions, HMX competed with O_2 for binding to the enzyme's active site, and thus resulted in smaller yield. The denitrated product was found unstable and subsequent ring cleavage resulted in the formation of both NDAB and MEDINA. However, their formation was not demonstrated for a whole-cell system.

In the absence of oxygen, HMX was degraded by *P. fluorescens* I-C (Fuller et al. 2009). Microbial isolates capable of performing aerobic denitration and

Table 5.5 Microbial isolates of HMX degraders

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
Bacillus strain HPB2, strain HPB3	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
Citrobacter freundii NS2	Anaerobic	Sequential reduction. Detection of mono- and dinitroso HMX	Kitts et al. (1994)
Clostridiale strain UXO5-19	Anaerobic	(1) Denitration followed by ring cleavage; (2) reduction of one or more nitro groups. Detection of mononitroso HMX, MEDINA	Zhao et al. (2007)
Clostridium bifermentans HAW-1	Anaerobic	(1) Sequential reduction; (2) denitration	Zhao et al. (2004b)
Clostridium sp. EDB2	Anaerobic	Pathway not defined, Products not identified	Bhushan et al. (2004)
Clostridium sp. HAW-E3	Anaerobic	(1) Sequential reduction; (2) denitration	Zhao et al. (2004b)
Clostridium sp. HAW-EB17	Anaerobic	Pathway not defined	Zhao et al. (2004b)
Clostridium sp. HAW-G4	Anaerobic	(1) Sequential reduction; (2) denitration	Zhao et al. (2004b)
Clostridium sp. HAW-HC1	Anaerobic	(1) Sequential reduction; (2) denitration. Detection of mono-, di- and trinitroso HMX	Zhao et al. (2004b)
Desulfovibrio sp. HAW-EB18	Anaerobic	Pathway not defined. Detection of mononitroso HMX	Zhao et al. (2004b)
Desulfovibrio strain Midref-29	Anaerobic	(1) Denitration followed by ring cleavage; (2) reduction of one or more nitro groups. Detection of mononitroso HMX, MEDINA	Zhao et al. (2007)
Fusobacteria isolate HAW-EB21	Anaerobic	Pathway not defined. Detection of mononitroso HMX	Zhao et al. (2004b)
Klebsiella pneumoniae SCZ1	Anaerobic	(1) Sequential reduction; (2) denitration. Detection of mononitroso HMX, MEDINA	Zhao et al. (2004b)
Methylobacterium extorquens	Aerobic	Pathway not defined, products not identified	Van Aken et al. (2004)
M. organophilum	Aerobic	Pathway not defined, products not identified	Van Aken et al. (2004)
M. rhodesianum	Aerobic	Pathway not defined, products not identified	Van Aken et al. (2004)
Methylobacterium sp. BJ001	Aerobic	Reduction	Van Aken et al. (2004)
Morganella morganii B2	Anaerobic	Sequential reduction. Detection of mono- and dinitroso HMX	Kitts et al. (1994)

(continued)

Table 5.5 (continued)

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
Paenibacillus strain UXO5-11	Anaerobic	(1) Denitration followed by ring cleavage; (2) reduction of one or more nitro groups. Detection of mononitroso HMX, MEDINA	Zhao et al. (2007)
Providencia rettgeri B1	Anaerobic	Sequential reduction. Detection of mono- and dinitroso HMX	Kitts et al. (1994)
Pseudomonas strain HPB1	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
Pseudomonas strain HPB1	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)

ring cleavage, similar to the common aerobic pathway of RDX, have not been detected.

5.3 Influence of Chemical and Physical Properties on Biodegradation Rate

Looking at the reductive transformation of the nitro group from a purely energetic compounds, one would expect the decrease in reduction rate from TNT to RDX, and finally to HMX (Uchimiya et al. 2010). On the other hand, in complex biotic systems such as the sub-surface, factors other than energetic yield will dictate the rate at which explosives degrade.

Nutrient availability is one important factor that plays a significant role in the rate of explosives biodegradation. Compared to other common pollutants, explosives, particularly RDX and HMX, are characterized by a higher N/C ratio. Although they may theoretically serve as sources of both carbon and nitrogen, only nitrogen is used by some organisms, and hence, another carbon source must be added (Allard and Neilson 1997). Various strains have been shown to degrade TNT and RDX as sole nitrogen sources (Tables 5.3 and 5.4), but evidence for their degradation as sole carbon source is scarce. Somewhat exceptional are growths of a *Gordonia* strain and *Williamsia* spp. on RDX as sole carbon source (Thompson et al. 2005), or growth of a transconjugant *Pseudomonas* sp. on TNT as sole carbon source (Duque et al. 1993).

Since denitration is frequently carried out by strains that are capable of utilizing the explosive compound as a nitrogen source, a negative response is often observed in microcosm experiments when external nitrogen sources (either ammonium or nitrate) are added, as these nitrogen-containing compounds may compete with degradation of the explosives for nitrogen (Binks et al. 1995; Coleman et al. 1998; Nejidat et al. 2008; Ronen et al. 2008; Bernstein et al. 2011). On the other hand, a positive response can be observed with additional carbon sources, as additional carbon supports microbial growth without competing with the explosive compounds for metabolism of RDX (Speitel et al. 2001; Waisner et al. 2002; Ronen et al. 2008) and TNT (Boopathy and Manning 1996).

Nitrate may not only compete with the explosive compounds as an alternative nitrogen source, but under anaerobic conditions, it may also compete as an electron acceptor. This was observed for RDX by Wani and Davis (2003) who demonstrated a significant decrease in the first-order biotransformation rate of RDX in a column study with the addition of nitrate, or by Freedman and Sutherland (1998) who reported similar observations for microcosms. A negative effect of nitrate during wastewater treatment was also observed by Ronen et al. (1998) and Brenner et al. (2000). Similar observations have also been reported for TNT, where nitrate served as a competing electron acceptor under anoxic conditions and was reported to halt

TNT respiration by *Pseudomonas* sp. strain JLR11 (Esteve-Núñez et al. 2000). Finally, nitrogen-bearing compounds are also potential inhibitors of enzyme expression, as presented by Nejidat et al. (2008) who showed that ammonium and nitrite repress cytochrome P450 expression.

Biodegradation rates are also influenced by the type of electron acceptors and electron donors. For example, the degradation of RDX, HMX and TNT was shown to be enhanced by additional hydrogen or hydrogen-producing electron donors under anaerobic conditions (Adrian et al. 2003; Adrian and Arnett 2007), and the degradation of HMX was enhanced by adding mixed electron acceptors to anaerobic microbial consortia (Boopathy 2001). Redox potential is another important factor that not only plays an important role in dictating the mechanism of degradation (as already described), but also influences the actual biotic degradation rate. Biodegradation is generally enhanced under reduced conditions, and in saturated soils (Price et al. 2001; Speitel et al. 2001; Ringelberg et al. 2003).

Toxicity and inhibitory effects of solution components also play an important role. At high concentrations, explosive compounds may be toxic to their potentially degrading bacteria as observed in TNT-degrading bacteria (Spiker et al. 1992), although these concentrations are often not of environmental relevance. Besides inhibition of their own degrading bacteria, explosive compounds may also inhibit the degradation of other coexisting explosive compounds. For example, TNT was shown to inhibit the activity of cytochrome P450 (Jackson et al. 2007) which is responsible for a key aerobic degradation step of RDX: denitration. This inhibitory effect was found reversible and disappeared with complete elimination of TNT from the medium (Nejidat et al. 2008). Another example is the inhibitory effect of high concentrations of RDX on the degradation of HMX by the purified xenobiotic reductase XenB. No inhibition was found at lower concentrations of RDX (Fuller et al. 2009).

Inhibitory effects and toxicity of the degradation products, rather than the parent compounds, are also of importance. Sagi-Ben Moshe et al. (2009) observed an inhibitory effect of the TNT metabolite tetranitroazoxytoluene on the degradation of RDX and HMX which was later on shown to be related to the metabolite's toxicity. In the absence of toxic metabolite, RDX and HMX degradation proceeded normally. The toxic effect of the azoxy dimers was found to be more pronounced in mixed-slurry experiments than in unsaturated soils and was related to the homogeneity of the microcosm (Sagi-Ben Moshe 2011).

The factors, that influence the rates at which explosive compounds degrade, should be considered in engineered bioremediation techniques in order to optimize the performance of the system. In such systems, it is relatively simple to control and manipulate these factors. On the other hand, in the natural heterogeneous environment, defining the conditions under which explosives degrade is itself a complicated task which can never be fully achieved. Moreover, manipulation of the conditions in the sub-surface is very limited and often even impossible.

5.4 Identifying and Quantifying In situ Biodegradation of Explosives

While identifying the potential of indigenous bacteria to biodegrade a compound is relatively simple, gaining evidence that biodegradation is actually occurring and further, quantifying its extent in complex environments is intrinsically difficult. Thus, studies on degradation potential, pathway identification, and rate quantification of explosives biodegradation under controlled laboratory conditions are frequently carried out. A few studies have been also aimed at characterizing the microbial degradation of explosives in the complex, poorly defined sub-surface environment.

The most conventional technique for identifying degradation potential in the environment is laboratory slurry experiments with contaminated sediments, in which the potential of the indigenous bacteria to degrade the target compound is tested. However, a positive response, showing that indigenous bacteria can indeed degrade the compound, does not imply that they actually perform this reaction in the sub-surface. Moreover, even if the reaction does take place in the sub-surface, the degradation rates at which it occurs in the laboratory, can vary by orders of magnitudes from the actual degradation rates in the field.

Even more complicated are attempts to *quantify* the extent of in situ biodegradation. This is normally done using techniques based on monitoring the change in the contaminant's concentration in time and space and fitting degradation rates using computational modeling. However, this strategy is often not sensitive enough, as the decrease in a contaminant's concentration may not be related only to biodegradation, but also to other processes such as dispersion, sorption or volatilization, the extents of which must also be quantified. Moreover, the temporal contaminant release to the aquifer is usually unknown, variable hydrogeological conditions are often not available, and only a limited number of monitoring wells generally exist in contaminated sites, which cannot produce exact knowledge of a plume's shape. All of these factors reduce the ability to generate precise calculations (Bockelmann et al. 2003; Wilson et al. 2004). An example of this problem is demonstrated by Pennington et al. (2001) who calibrated first-order decay constants for TNT and RDX concentrations measured in the field. The values obtained by these authors were 10^{-5} per day (half life of 190 years) and 8.13×10^{-6} per day (half life of 233 years) for TNT and RDX, respectively. However, their model was reported to be only moderately sensitive to degradation rate.

Analysis of the pollutant degradation products is an alternative strategy for quantifying degradation extent along the plume. However, this strategy may not always be conclusive, as products may not be detectable, may be non-conserved or can originate from different parent compounds. Therefore, the search for degradation products may not always be appropriate to gain proof of the biodegradation activity in the field. An example of this is demonstrated by Beller and Tiemeier (2002) who detected the anaerobic nitroso transformation products of RDX in

contaminated groundwater—an important evidence that the reductive transformation of RDX occurs in situ. Nevertheless, this evidence was not sufficient to estimate the degradation extent or rates of the compound, since the nitroso products may have been further attenuated and thus their monitored concentration might not reflect the true degradation extent. For similar reasons, absence of degradation product MEDINA in the groundwater in study of Beller and Tiemeier (2002) may not indicate that the MEDINA-producing ring-cleavage pathway is not occurring in situ.

A relatively new strategy for tackling the challenge of identifying and quantifying degradation of organic pollutants in general, and explosive compounds in particular, is the use of compound-specific stable isotope analysis (CSIA) (Meckenstock et al. 2004; Schmidt et al. 2004). This strategy is based on the preferential reaction of chemical bonds of lighter isotopes, implying that pollutants become enriched in the heavier isotopes as degradation proceeds. The extent of isotope enrichment depends on the extent of degradation, as well as on the isotopic enrichment factor which is typical of the rate-limiting reaction. Thus, the extent of isotopic enrichment along a contamination plume may be used to quantify the extent of biodegradation along the flow line using appropriate enrichment factors, as demonstrated in the last decade for various organic pollutants, mostly BTEX compounds (McKelvie et al. 2005; Spence et al. 2005; Mak et al. 2006), MTBE (Kuder et al. 2005; Spence et al. 2005; McKelvie et al. 2007), PAH (Moonkoo et al. 2006), and chlorinated aliphatics such as TCE and PCE (Hunkeler et al. 2004; Morrill et al. 2005). The application of this concept to other groups of contaminants, including explosives, is emerging.

The isotopic composition and isotope fractionation accompanying explosives biodegradation has so far received only scant attention, possibly due to analytical difficulties and because explosives are of less concern than the major pollutants, such as BTEX compounds and chlorinated ethylenes.

Some publications related to the isotopic composition of various explosives can be found in the forensics literature which aims to use these compounds' isotopic fingerprints to identify their origin (Nissenbaum 1975; McGuire et al. 1995; Diegor et al. 1999; Phillips et al. 2003). Others have studied the isotopic enrichment accompanying degradation processes of explosive compounds in a laboratory environment. Hartenbach et al. (2006) and Hofstetter et al. (2008) studied $\delta^{15}N$ isotope fractionation during the abiotic reduction of different nitroaromatic compounds, such as methyl- or chloro-substituted nitrobenzenes, or dinitrotoluenes. Beller et al. (2004) studied the isotopic signatures of nitrite and nitrate formed by RDX photolysis. Hoffsommer et al. (1977) studied the $\delta^{2}H$ isotope effects following RDX alkaline hydrolysis. Of more environmental relevance to the compounds that are the focus of this review is the work of Bernstein et al. (2008) who studied $\delta^{15}N$ and $\delta^{18}O$ enrichment during aerobic and anaerobic biodegradation of RDX, where the aerobic strain used for these experiments was *Rhodococcus* strain YH1, and for the anaerobic experiments, a slurry of sediments with an indigenous consortium excavated from a contaminated site was used.

Environmental studies, that have adopted stable isotope tools to study the behavior of explosive compounds in the field, are rare. DiGnazio et al. (1998) related $\delta^{15}N$ values of nitrate in the groundwater to RDX degradation, but noted that they had no other isotopic data to confirm this correlation. Bordeleau et al. (2008) used both $\delta^{15}N$ and $\delta^{18}O$ values of nitrate to identify in situ degradation of RDX. However, the latter two studies focused on the possible RDX metabolite nitrate, rather than the parent compound itself, making the results less conclusive.

Pennington et al. (2001) explored the application of nitrogen and carbon stable isotope analysis in explosive compounds to track in situ attenuation processes. They suggested that measuring stable isotopic fractions of nitrogen in TNT is a promising strategy for monitoring the attenuation of TNT in the sub-surface.

CSIA was recently applied to RDX in a contaminated aquifer, demonstrating in situ degradation of RDX and enabling quantification of the degradation extent along the plume and an estimate of in situ degradation rates (Bernstein et al. 2010). This study revealed a decrease in degradation with depth. Stable isotope tools were also used in unsaturated soils, showing enhanced RDX degradation with increasing water content (Sagi-Ben Moshe et al. 2010). Both of these studies made use of analytical techniques which require rather laborious off-line purification procedures of the target compound, reducing the method's sensitivity.

Recently, an effort was made to improve analytical techniques for determining $\delta^{13}C$ and $\delta^{15}N$ in RDX and TNT (Gelman et al. 2011). The resultant new technique opens the way for convenient isotope analysis of compounds derived from the contaminated environments, thus shedding light on environmentally relevant processes that are difficult to detect with traditional methods. It is anticipated that the application of the new analytical method, as well as the development and optimization of other new methods, will significantly expand our knowledge of the behavior of explosives in the field.

Besides the possibility to characterize degradation products, stable isotope tools might also be useful in studying the indigenous microflora's ability to degrade explosives. A powerful new technique called stable isotope probing (SIP) has enabled researchers to identify metabolically active microorganisms in complex engineered and natural systems. Roh et al. (2009) used ^{15}N-labeled RDX to follow active RDX-degrading bacteria in a groundwater microcosm. They demonstrated that ^{15}N was incorporated into DNA sequences encoding XplA. This indicated that XplA-containing bacteria are actively taking nitrogen from the degraded RDX, an observation which enabled confirmation of activity without prior knowledge of the organisms involved.

5.5 Conclusions

In the last few years, significant efforts have been made in tracing possible degradation pathways of explosive compounds in the sub-surface. Increasing numbers of pathways have been postulated, involving mainly the nitro groups of the

aromatic or triazinic rings. While some of the documented pathways are environmentally undesirable, as they involve the accumulation of toxic products, others mineralize the compounds to simple and harmless molecules and are thus of greater interest.

Numerous microbial isolates have been identified as degraders of explosives, belonging to various families. Both facultative anaerobes (Enterobacteriaceae) as well as strict anaerobes (*Clostridia*) appear to play important roles in this process. *Pseudomonas* appears to be ubiquitous in their ability to attack explosives both aerobically as well as under anoxic conditions. A unique observation on the aerobic degradation of RDX indicates that the presence of this compound resulted in the evolution of a novel cytochrome P450 in different geographic locations. Interestingly, this evolution was restricted to a limited bacterial host range—*Rhodococcus* species from the family Nocardiaceae. Their degradation activity was shown to be controlled by various factors, including redox potential, nutrient availability, salinity and local toxicity.

Microbial strains isolated from the field imply that degradation may potentially occur in situ, even without human interference. Nevertheless, to reach remedial goals, proof must be provided of the actual occurrence of in situ degradation in a contaminated environment, and the extent of this process must be quantified. Using isotopic tools, it is anticipated that not only will these objectives will be fulfilled, but also a deeper understanding of the biodegradation processes, that actually occur in the sub-surface, will be gained, narrowing the gap between the knowledge accumulated from laboratory experiments and the uncertainties related to environmentally relevant processes.

Acknowledgments The work of A. Bernstein was supported by a generous contribution from Vera Barcza, Toronto, Canada Rosinger-Barcza Family Fund In support of Young Researchers at the Zuckerberg Institute for Water Research. This work was also supported in part by a grant 167/2008 from Israel Science Foundation.

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