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REVIEW

Microbial Degradation and Toxicity of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine

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In the present work, current knowledge on the potential fate, microbial degradation, and toxicity of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) was thoroughly reviewed, focusing on the toxicological assessment of a variety of potential RDX degradation pathways in bacteria and fungi. The present review on microbial degradation pathways and toxicities of degradation intermediates suggests that, among aerobic RDX degradation pathways, the one *via* denitration may be preferred in a toxicological perspective, and that among anaerobic pathways, those forming 4-nitro-2,4-diazabutanal (NDAB) *via* ring cleavage of 1-nitroso-3,5-dinitro-1,3,5-triazinane (MNX) may be toxicologically advantageous owing to its potential mineralization under partial or complete anoxic conditions. These findings provide important information on RDX-degrading microbial pathways, toxicologically most suitable to be stimulated in contaminated fields.

Keywords: RDX, microbial degradation, intermediate toxicity, biodegradation pathway, bioremediation

Energetic materials are nitrogen-containing organic compounds that decompose rapidly as a result of chemical or thermal shock, and produce a large amount of heat and gas [32]. Globally, millions of tons of energetic compounds have been produced for various industrial purposes and military applications. During these activities, the water, sediment, and soil may become contaminated, which can impact environmental and human health [32]. Among energetic compounds, recently, the most widely used explosive for military applications is hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) [22]. The concentrations of RDX in polluted soil are extremely heterogeneous, ranging from 0.7 to 74,000 mg/kg [4]. Recently RDX has replaced TNT as a primary explosive in various munitions formulations.

RDX, which is classified by the US Environmental Protection Agency (USEPA) as a potential carcinogen, is toxic to organisms, is comparatively mobile in the soil, with low rates of degradation in soils, and presents distinct problems for bioremediation [56]. Thus, the remediation of RDX-contaminated sites is important for the protection of human health and ecosystems. The conventional approach for the remediation of RDX is the incineration of soil, which is a costly, destructive process that results in the release of undesirable chemicals including greenhouse effect gases and dioxins [33]. Because environmental awareness has increased, alternative disposal methods must be developed, and the existing problem must be eliminated. Recently, the bioremediation (microbial degradation and transformation) of RDX has been considered as a cheap and environmentally friendly approach [15, 33, 51, 59].

Bioremediation is the use of biological strategies to return the polluted environment to its original state or to reduce the toxicity of the environment. In general, bioremediation includes the use of enzymes, growth stimulants, microbes, or plants to degrade, transform, sequester, mobilize, and contain contaminant organics, inorganics, and metals in the soil, water, and air [27]. The physicochemical and biological characteristics of a process must be understood to select the appropriate bioremediation strategy for a RDX-contaminated site. First, the potential fate of the target pollutant in the environment must be understood. Second, information on the biodegradability of RDX by natural microbes must be obtained. Third, information on the toxicity of intermediates produced during the microbial degradation of RDX is crucial for selecting the appropriate degradation pathway to stimulate in the remediation site. In the literature, knowledge on the fate and microbial degradation of RDX in the environment has been presented. In risk evaluations of RDX bioremediation strategies, one must determine if microbial RDX degradation pathways

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produce intermediates that are more toxic than the parent compound. Although several reviews on the potential fate and microbial degradation of RDX in the environment have been published [22, 26, 32, 33, 43, 56], the toxicity of intermediates of potential RDX microbial degradation pathways has not yet been reviewed, and any relationship between RDX degradation pathways and the toxicity of intermediates has not been established. In the present study, we reviewed current knowledge on the microbial degradation and toxicity of RDX and attempted to provide information on the linkage between microbial RDX degradation pathways and the toxicity of their intermediate products.

RDX and Its Potential Fate in the Environment

RDX is a highly explosive, white or gray powder that is often mixed with other explosives, oils, or waxes to make military munitions and other products [10]. RDX can be synthesized by treating hexamethylenetetramine with 98–100% nitric acid in the presence or absence of ammonium nitrate. The structure of RDX is unique because of the attachment of the nitro group to the central ring *via* nitrogen–nitrogen bonds (Fig. 1). To date, the nitrogen–nitrogen bond formation happens rarely in nature. This unusual feature makes RDX resistant to biological attack. Following introduction of RDX into the environment, both abiotic and biotic processes influence its fate [32]. The rate and extent of transport and transformation are governed by the physicochemical properties of RDX (solubility, K_{OW}, vapor pressure, Henry's law constant), environmental factors (weather conditions, soil properties, pH), and biological factors including the presence and/or absence of explosives-degrading microorganisms. Dissolution, adsorption, and volatilization processes influence the environmental fate, and hydrolysis, photolysis, reduction, and biodegradation processes influence the transformation of RDX and other explosive compounds [33].

The physical and chemical properties of RDX are shown in Table 1. The value of the octanolwater partition coefficient (log K_{OW} = 0.87) of RDX (Table 1) suggests that RDX may not be strongly sorbed to organic particles in soil and sediment [32]. Therefore, if water-swelling clays, such as montmorillonite, are not abundant in a subsurface environment, RDX is mobile *via* groundwater flow. Because of its mobility, plants may uptake and use dissolved RDX compounds in groundwater, which is an important implication for phytoremediation. RDX has a very low vapor pressure and Henry's law constant; thus, volatilization of RDX and other nitroaromatics associated with munitions from solid or aqueous phases is insignificant [33]. Therefore, pumping methods using air/gas as a fluid, such as soil vapor extraction and air sparging, are not options for the remediation of RDX-contaminated groundwater.

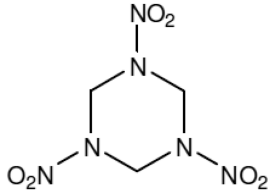

Fig. 1. Hexahydro-1,3,5-trinitro-1,3,5-triazine (adapted from Rylott *et al*. [56])

To conduct risk and exposure assessments for RDX-polluted water, sediment, and soil, the fate and effects of RDX and its transformation products on the environment must be known. Volatilization, dissolution, sorption, bioaccumulation, and biotic and abiotic degradation/transformation have a significant influence on the risk and fate of RDX in the environment [32]. Owing to its low vapor pressure, volatilization is not a significant environmental pathway for most common solid-phase RDX; therefore, direct mass transfer from the solid to vapor phase is negligible [32]. Solubility and dissolution into water are the primary mechanisms by which solid compounds are disseminated in the environment. As shown in Table 1, RDX is characterized by its low aqueous solubility (60 mg/l) [2]. Photolysis is the alteration of a compound by the direct or indirect effects of light [19]. RDX is also susceptible to photolysis. The resulting transformation products include azoxy-compounds, ammonia, formaldehyde, nitrate, nitrite, nitrous oxide, and *N*-nitroso-methylenediamine [19]. Photolysis half-lives of RDX have been estimated to range from 9 h to 14 d [66]. RDXs are susceptible to abiotic reduction and the rate of reduction is highly variable, and microbial processes can enhance reduction reactions. Abiotic reduction reactions require activation by solid catalysts, such as iron-containing compounds, organic macromolecules, or clay minerals [20]. The abiotic

Table 1. Physicochemical properties of RDX.

Properties	Values	References
CAS number	00121-82-4	[33]
Chemical formula	$C_3H_6N_6O_6$	[33]
Color	White or gray	[33]
Physical state	Powder	[32]
Molecular weight	222.26	[2]
Melting point	204°C	[32]
Water solubility at 20°C	60 mg/l	[2]
Solubility in methanol	Slightly soluble	[2]
Solubility in ether	Slightly soluble	[2]
Vapor pressure at 20°C	1×10^{-9} mm Hg	[2]
log K_{OW}	0.87	[33]
log K_{OC}	1.80	[2]
Henry's law constant (25°C)	1.96×10^{-11} bar m^3/mol	[33]

Table 2. Known RDX-degrading bacterial and fungal isolates.

Organism	Degrading enzyme	Characterized degradation intermediates		Source
		Aerobic	Anaerobic	
Acetobacterium malicum	-	-	MNX, MEDINA, N_2O, HCHO	[1]
Clostridium bifermentans	-	-	MNX, DNX, N_2O, HCHO,CH_3OH	[80]
Clostridium sp. EDB2	-	-	NO_2., N_2O, HCHO, HCOOH,CO_2	[7]
Geobacter metallireducens strain GS-15	-	-	MNX, DNX, TNX, MEDINA, NO_2, HCHO	[36]
Gordonia TR4 /Williamsia KTR9	XplA	NDAB, NO_2., HCHO, CO_2	-	[69, 28]
Klebsiella pneumoniae strain SCZ-1	-	-	MEDINA, N_2O, HCHO,CH_3OH, CO_2	[79]
Phanerochaete chrysosporium	-	MNX, N_2O, CO_2	-	[61]
Pseudomonas fluorescens I-C, *Pseudomonas putida* II-B	XenA/ XenB	-	MNX, MEDINA, NDAB, HCHO	[15]
R. rhodochrous strain 11Y	XplA/XplB	NDAB, NO_2, HCHO	MEDINA, NO_2, HCHO	[30, 57, 60]
Rhizobium rhizogenes BL, *Burkholderia* sp. BL	-	NO_2, NO_3, N_2O, HCHO,CO_2	-	[38]
Rhodococcus sp. strain DN22	Cytochrome P450	NDAB, NO_2, NH4+, CO_2, NDAB, MEDINA, NO_2, NH_4^+,HCHO	NDAB, NO_2., HCHO, NH_4^+, MNX, MEDINA, N_2O	[12, 23]
Rhodococcus sp. strain YH1	-	aerobic	MNX, DNX, TNX, HCHO	[25, 46]
Shewanella halifaxensis HAW-EB4	-	-	MNX, DNX, TNX, MEDINA, NDAB, HCHO, N_2O	[81]
Shewanella oneidensis MR-1	-	-	MNX, DNX, TNX, MEDINA, NDAB, HCHO, N_2O	[51]
Acremonium sp. HAW-OCF3	-	MEDINA,MNX, DNX, TNX, HCHO,N_2O	-	[5]
Aspergillus niger	-	-	MNX, DNX, TNX, HCHO,N_2O	[6]
Bullera unica strain HAW-OCF2	-	-	-	[5]
Cladosporium cladosporioides	-	NO_2, NO_3, N_2O, HCHO,CO_2	-	[38]
Penicillium sp. HAW-OCF5	-	-	-	[5]
Phanerochaete chrysosporium	-	CO_2,N_2O	-	[13, 61, 67]
Rhodotorula mucilaginosa strain HAW-OCF1	-	-	-	[5]

reduction of RDX and related energetic compounds can also be achieved using zero valent iron [50]. Although the abiotic reduction of RDX may occur under environmental conditions, abiotic and biotic transformations are difficult to distinguish [32].

An abundant research has shown that microbial processes play an important role in the natural transformation of RDX in the environment (Table 2). Some bacteria and fungi are able to degrade RDX under aerobic and anaerobic conditions. Although microbial processes in nature can mineralize RDX into non-toxic compounds, such as CO_2, they often produce toxic intermediates or dead-end metabolites that can accumulate in the environment. Microbial degradation and transformation of RDX are further discussed below.

RDX-TRANSFORMING MICROBES AND ENZYMES

Over the last decade, numerous studies on the bacterial and fungal degradation of RDX have been conducted (Table 2). The degradation of RDX has been studied to identify and isolate enzymes involved in RDX degradation and to discover microorganisms that can be inoculated in a bio-reactor or *in situ* (bioaugmentation) to degrade the pollutant [32]. Thus, many isolated microbial strains that can degrade or mineralize RDX have been identified from contaminated soils and waters [33, 38].

The phylogenetic placement of known RDX-degrading bacterial 16S rRNA genes show that the RDX degraders are widely distributed in the phyla of Clostridia, Actinobacteria, and Proteobacteria (Fig. 2). The members

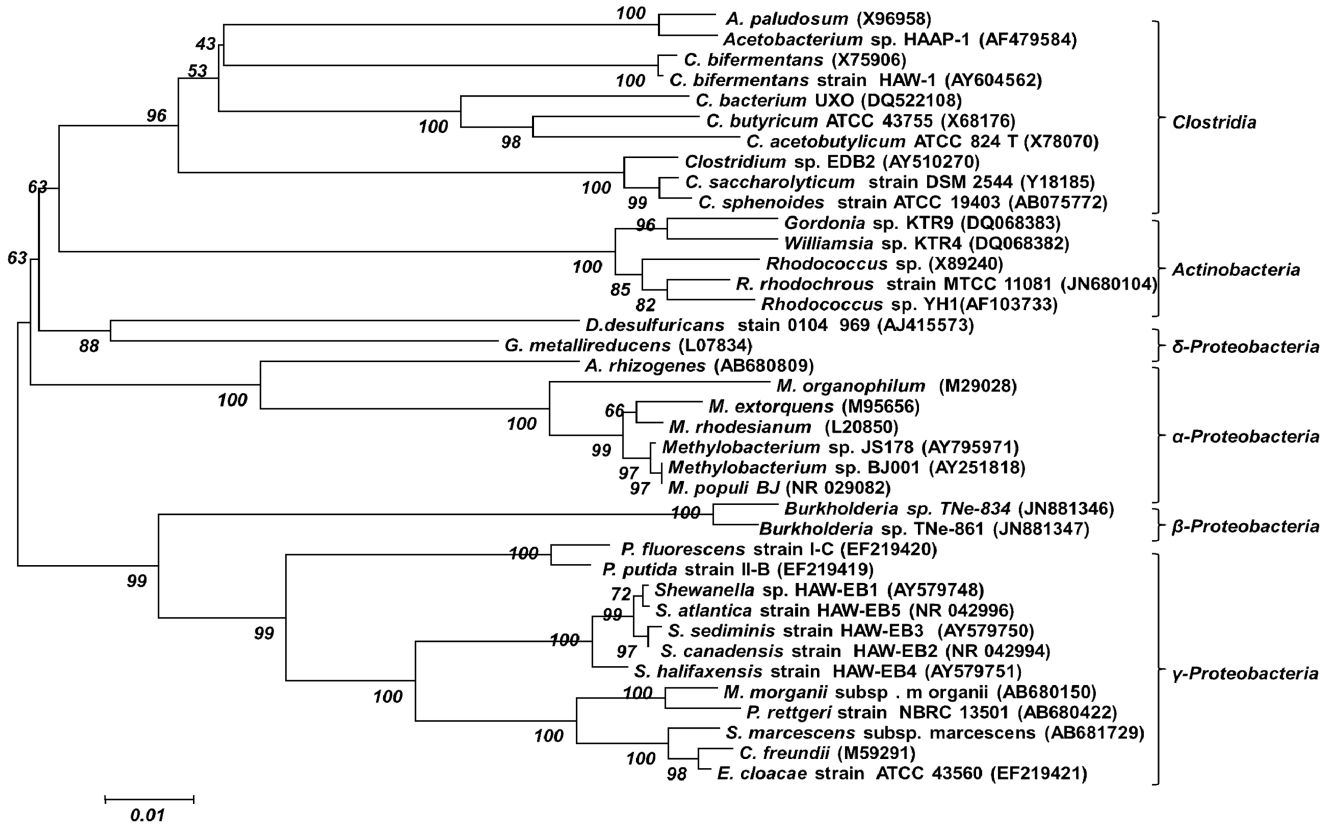

Fig. 2. Phylogenetic placement of known RDX-degrading bacterial 16S rRNA genes.
The scale bar represents a 16S rRNA sequence divergence of 1%.

of Clostridia have been known for their potential to transform a variety of nitro-containing pollutants [78]. Biotransformation of RDX has also been shown by a number of isolates belonging to Clostridia including *Clostridium bifermentans* strain HAW-1, *Clostridium acetobutylicum* (ATCC 824), and *Clostridium* sp. EDB2 [7, 78, 81]. Studies with *C. acetobutylicum* have shown that this strain has the ability to transform RDX in the presence of H_2 as the electron donor [78]. Likewise, EDB2 strain was shown to have the ability to degrade RDX. However, the most commonly found metabolites of RDX [1,3,5-trinitroso-1,3,5-triazinane (TNX), methylenedinitramine (MEDINA), and 4-nitro-2,4-diazabutanal (NDAB)] were not produced during reaction with this strain [7]. This suggests that these bacteria have a unique enzyme system that catalyzes the degradation reaction in a different way. So far, the only enzyme, diaphorase, has been identified in Clostridia [6]. However, other enzymes in Clostridia involved in RDX transformation have not been well characterized.

Biodegradation of RDX has also been reported by a number of isolates including *Rhodococcus rhodochrous* 11Y, *Rhodococcus* sp. strain DN22, *Rhodococcus* strain YH1, and *Gordonia* sp. KTR9, belonging to the Corynebacterineae suborder in the Actinobacteria [28, 46, 60]. *Rhodococcus* sp. strain DN22 is amongst the better characterized strains [12, 23] and under aerobic condition generated metabolites of NO_2^-, NO_3^-, methylenedinitramine (MEDINA), NDAB, N_2O, CO_2, NH_3, and formaldehyde (HCHO). *Rhodococcus rhodochrous* strain 11Y, isolated from explosive-contaminated land, is capable of degrading RDX when provided as the sole source of nitrogen for growth [60]. These bacteria have a unique cytochrome P450 system XplA/B [58] that can reductively denitrate RDX both under aerobic and anaerobic conditions [57]. The phylogenetic placement of known RDX-degrading enzymes and relatedness among the cytochrome P450 enzyme XplA, and reductase enzyme XplB is shown in Fig. 3. To date, *xpl*A and *xpl*B have been detected only in *Rhodococcus* and related bacteria isolated from contaminated soil (Fig. 3), and this highly conserved nature indicates for a single evolutionary origin [60]. XplA homologs have successfully been isolated [46] and lateral genes transfer maybe a possible route of global distribution [56].

Studies with bacterial isolates of *Citrobacter* sp., *Morganella* sp., *Klebsiella pneumoniae* strain SCZ-1, *P. fluorescens* I-C, *Pseudomonas putida* II-B, and *Shewanella oneidensis* MR-1, belonging to the Proteobacteria, showed that these isolates can transform RDX to 1-nitroso-3,5-dinitro-1,3,5-

triazinane (MNX), 1,3-dinitroso-nitro-1,3,5-triazinane (DNX), and 1,3,5-trinitroso-1,3,5-triazinane (TNX) [15, 35, 51, 79]. Strain SCZ-1, under anaerobic conditions, degraded RDX *via* both denitration and reduction pathways to produce methanol, nitrous oxide, carbon dioxide, and transient products of formaldehyde, MNX, and MEDINA [79]. A type I nitroreductase enzyme was considered to be involved in the transformation of RDX *via* a two-electron reductive pathway in sludge [25]. *Enterobacter cloacae* type I nitroreductase was expressed in *Escherichia coli* and whole cell assays showed significantly higher RDX nitroreductase activity in induced cells compared with uninduced cells. Later *in vitro* studies affirmed type I nitroreductase could transform RDX [34].

Recently, the TNT detoxifying reductases XenA and XenB showed to have activity towards RDX. However, their activities are relatively low [56]. Fuller *et al*. [15] found that XenA and XenB, members of the Old Yellow Enzyme family (flavoprotein oxidoreductases), efficiently degrade RDX under both aerobic and anaerobic conditions, and degradation was faster in the absence of O_2 [79]. In addition, under anaerobic conditions, *P. fluorescens* I-C (with XenB) performed better than *P. putida* II-B (with XenA) [15]. To date, a little is known about the degradation potential of these genes as compared with *xpl*A and *xpl*B. Therefore, XenA and XenB, and other genes with less than 200 amino acid sequence, are not included in Fig. 3.

RDX transformations have also been observed in fungi [6, 13, 38, 67] (Table 2). The majority of investigations on the fungal degradation of RDX have focused on *P. chrysosporium* (Table 2). *P. chrysosporium* fungus has been shown to utilize the RDX as a sole nitrogen source [61]. In the study of Sheremata and Hawari [61], *P. chrysosporium* completely removed RDX from liquid medium containing glycerol as the predominant carbon source. Bayman *et al*. [3] attempted to use four species of

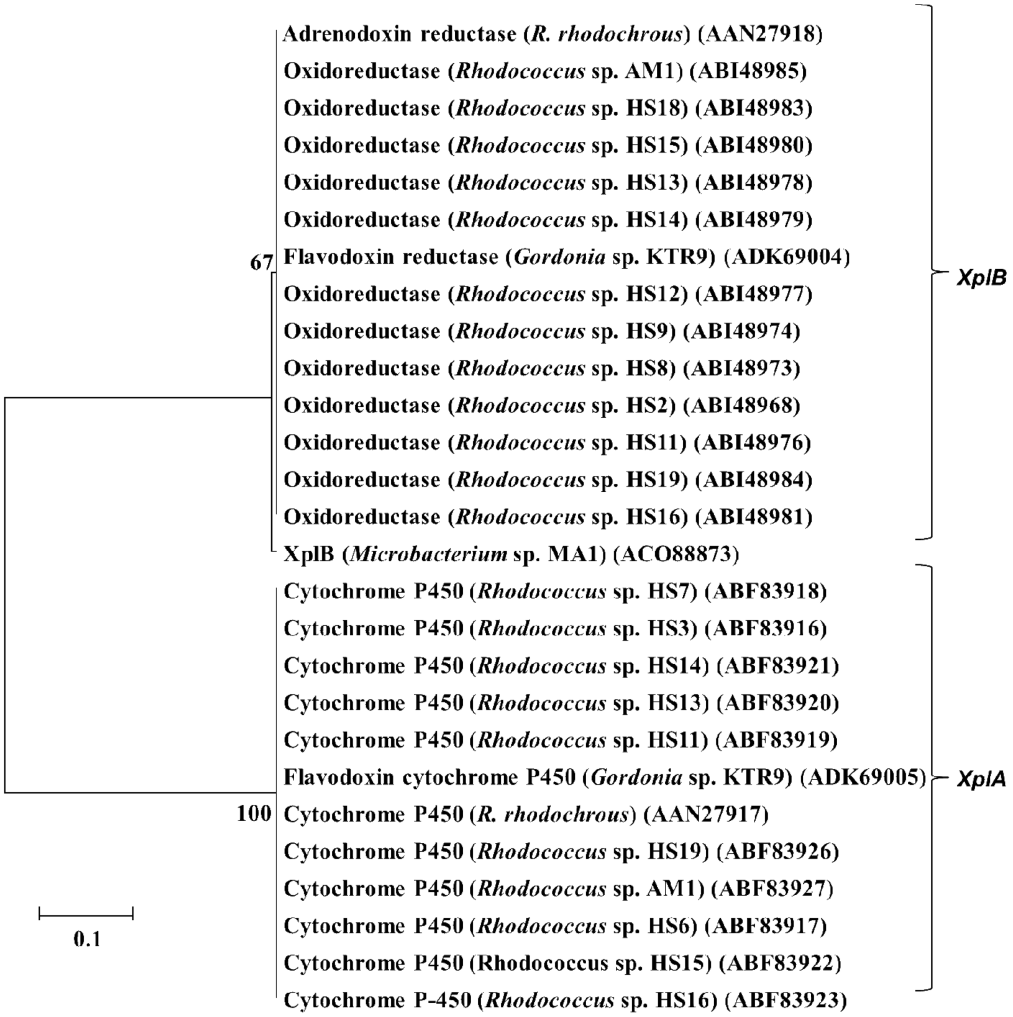

Fig. 3. Phylogenetic placement of known RDX-degrading enzymes and relatedness among the cytochrome P450 enzyme XplA, and reductase enzyme XplB.
The scale bar represents an amino acid sequence divergence of 10%.

fungi, including *C. echinulata*, *C. pallidus*, *P. chrysosporium*, and *C. resinae*, by using vegetable juice containing RDX as media. Unfortunately, although growth of the fungi was observed in the media, no evidence of RDX mineralization was observed for any of the four organisms. The fungus *C. cladosporioides* showed to have ability to aerobically degrade recalcitrant RDX in laboratory media, and several products from RDX biodegradation have been identified [38]. Recently, Bhatt *et al*. [5] found that fungi (*R. mucilaginosa* strain HAW-OCF1, *B. unica* strain HAW-OCF2, *Acremonium* sp. HAW-OCF3, and *Penicillium* sp. HAW-OCF5) isolated from Hawaii marine sediment are capable of aerobically biodegrading RDX to HCHO, CO_2, and N_2O through both direct ring cleavage and reduction to MNX prior to ring cleavage. This suggests that these fungi also have a specific enzymatic system that catalyzes the RDX degradation reaction. However, RDX-transforming fungi are less diverse than RDX-transforming bacteria and work only under limited and more specific environmental conditions.

The use of bacteria in biological remediation techniques for RDX is advantageous because the types of enzymatic reactions present in bacteria are diverse, and bacterial enzymes can catalyze a wide array of biochemical reactions [71]. Therefore, future research on the discovery, identification, and isolation of suitable bacterial strains must be conducted to enhance the bioremediation process. However, at present, the main technical hindrance for the discovery of explosive-degrading microorganisms is the inability to study unculturable soil microorganisms [56]. Most recently, stable isotope probing (SIP) has been used to identify explosive-degrading bacteria in contaminated environments [17]. The use of this technique combined with advances in functional metagenomics technologies should aid in the discovery of RDX-degrading activity.

Owing to the high microbial diversity of soil/sediment, full sequencing of the soil metagenome would be inappropriate for RDX degradation research. Instead, a metagenome of explosive-metabolizing populations could be isolated using SIP [17]. In addition, gene-targeted metagenomic techniques combined with next-generation sequencing could be employed to detect specific biodegradative genes in the environment [29, 39]. The combination of gene-targeted metagenomics and RDX-SIP is a promising method for the selection of an appropriate bioremediation strategy. The current weakness of gene-targeted metagenomics is that a universal functional (biodegradative) gene for detecting RDX-degrading bacteria in the environment has not yet been identified. To circumvent this weakness, we propose that the novel cytochrome P450 enzyme XplA, and reductase enzyme XplB, could be used as biomarkers for monitoring RDX-degrading bacteria *in situ*. Specifically, XplA and XplB enzymes are distributed in diverse organisms, particularly bacteria, whereas *Rhodococcus* bacteria that are predominant in RDX-contaminated soils contain the highly conserved cytochrome P450 enzyme XplA, and reductase enzyme XplB system. In addition, the predominance of *Rhodococcus* and *Clostridium* is often observed in RDX-contaminated soils [33], which suggests that *Rhodococcus* and *Clostridium* may be of interest for RDX bioremediation.

Possible Pathways of Microbial Degradation of RDX

As the supply of oxygen is one of the most expensive processes in the field, one must know whether a certain biodegradation event occurs under aerobic or anaerobic conditions. In addition, such information can be used to select the appropriate respiration conditions for the isolation of RDX-degrading organisms. From this perspective, RDX microbial degradation pathways under aerobic and anaerobic conditions have been reviewed below. In addition, we attempt to construct all of the possible pathways of RDX microbial degradation. To this end, we performed a wide range of literature search in a less restricted manner than that of previous review studies, which were focused solely on directly observed pathways.

RDX Degradation Pathways Under Aerobic Conditions

Bacterial mineralization of RDX has been shown to occur under aerobic conditions following utilization of the compound as a nitrogen source [7, 55, 69]. A potential aerobic RDX degradation pathway is shown in Fig. 4 [22]. The microbial transformation of RDX is usually initiated by a denitration–hydration step, resulting in ring cleavage and production of formaldehyde and NDAB with the loss of two nitrite anions prior to RDX ring cleavage, whereas the loss of only one nitrite anion prior to ring cleavage yields MEDINA and/or NDAB, as aerobic transformation products of RDX [12, 16]. Several other studies have reported the formation of NDAB and MEDINA together under both aerobic and anaerobic conditions, including photodenitration [25], denitration by *Shewanella* [80], biogenic Fe(II) [37], and mutants from *Shewanella oneidensis* MR-1 [51]. The ring cleavage product NDAB is further mineralized to nitrate and carbon dioxide in *Methylobacterium* sp. JS178 [14] and *Phanerochaete chrysosporium* [13]. In RDX degradation using the purified XplA cytochrome P450 system isolated from *Rhodococcus rhodochrous* strain 11Y, Jackson *et al*. [30] obtained two nitrite anions and only NDAB under aerobic conditions, and one nitrite anion and MEDINA under anaerobic conditions. MEDINA is unstable in water and decomposes further to N_2O and HCHO through the formation of NH_2NO_2 [22].

Studies with xenobiotic reductases and other related enzymes have shown that the presence of O_2 can affect the transformation of explosive compounds [15]. Pak *et al*.

(2000) [48] observed that although TNT was degraded by XenB both aerobically and anaerobically, the presence of O_2 altered the product distribution. In another study investigating degradation of RDX by three Enterobacteriaceae isolates, O_2 showed a central role in the final outcome, as RDX was degraded only under oxygen-depleted conditions [35]. Similarly, Zhao *et al*. [79] found that RDX degradation by *Klebsiella pneumoniae* strain SCZ-1 was completely quenched in the presence of O_2. In addition, several compounds that were not degraded (or degraded very slowly) under aerobic conditions were degraded anaerobically, and the rates of transformation reported were also higher under anaerobic compared with aerobic conditions [15].

RDX Degradation Pathways Under Anaerobic Conditions

The majority of research into RDX biodegradability has been undertaken using anaerobic conditions. A potential anaerobic RDX degradation pathway is shown in Fig. 5 [22]. The biodegradation of RDX in anaerobic sludge shows that at least two degradation routes are involved. Under anaerobic conditions, degradation of RDX may proceed *via* reduction and ring cleavage or *via* direct ring cleavage, as shown in Fig. 5. In one route, sequential reduction of the nitro groups results in the formation of MNX, DNX, and TNX [51]. It is proposed that further transformation of MNX, DNX, and TNX results in the formation of hydroxylamine derivatives, although these products are yet to be isolated [33]. Ring cleavage of the hydroxylamine derivatives results in the formation of a number of low-molecular-weight products, including formaldehyde and methanol. In the second route, MEDINA and bis(hydroxymethyl)nitramine (BHNA) are formed [12, 26]. These products undergo further transformation, resulting in the formation of nitramine and formaldehyde. Nitramine may be abiotically converted *via* hydrolysis to N_2O, whereas the actions of methanogenic and acetogenic bacteria convert HCHO to CO_2 [33]. Transformation of formaldehyde to CO_2 is also important, as formaldehyde is also known for its toxicity and as a possible human carcinogen [24, 44].

The formation of MNX establishes an important point in the reduction of RDX; it can either continue reduction to produce DNX and TNX, denitrate, or denitrosate, leading to ring cleavage and decomposition (Fig. 5). Only a few

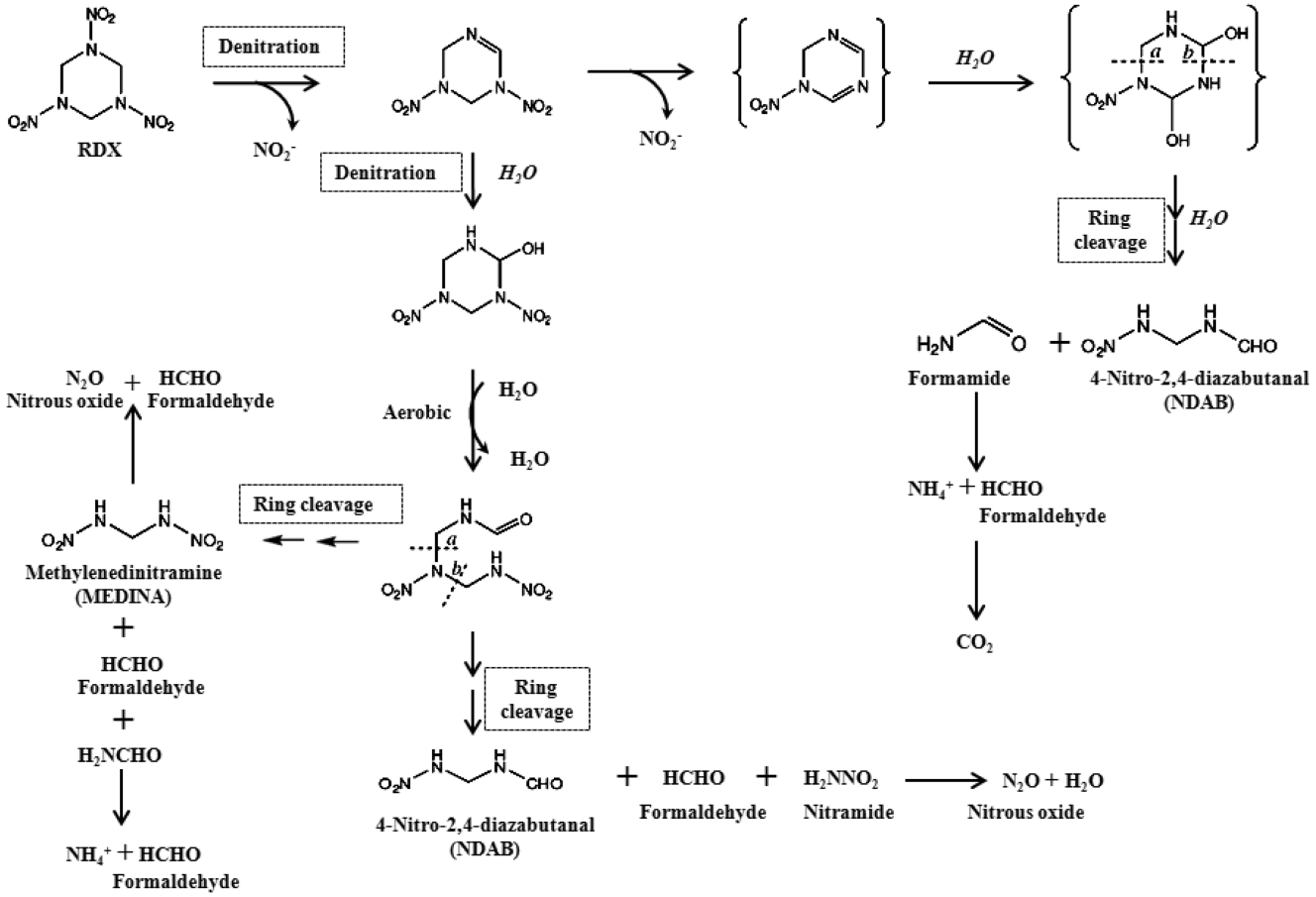

Fig. 4. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) aerobic degradation pathway (adapted from Halasz and Hawari [22]).

studies have been done on MNX for its potential degradation with *Shewanella* isolates from Halifax harbor [79, 81] and with the soil isolate *Rhodococcus* sp. DN22 [23]. MNX degradation with *Rhodococcus* sp. DN22 resulted in the formation of nitrite, nitrate, and ring cleavage products including NH_3, N_2O, HCHO, and HCOOH, similar to those observed following RDX denitration. The formation of NDAB and 4-nitoso-2,4-diazabutanal (NO-NDAB) clearly shows that MNX degraded *via* initial cleavage of the N-NO and N-NO_2 bonds, respectively [23].

Promising results on the mineralization of RDX have only been obtained with a few bacterial strains (Table 2). However, further knowledge on RDX degradation pathways in these organisms is required before they can be used to remediate RDX-polluted soil and water.

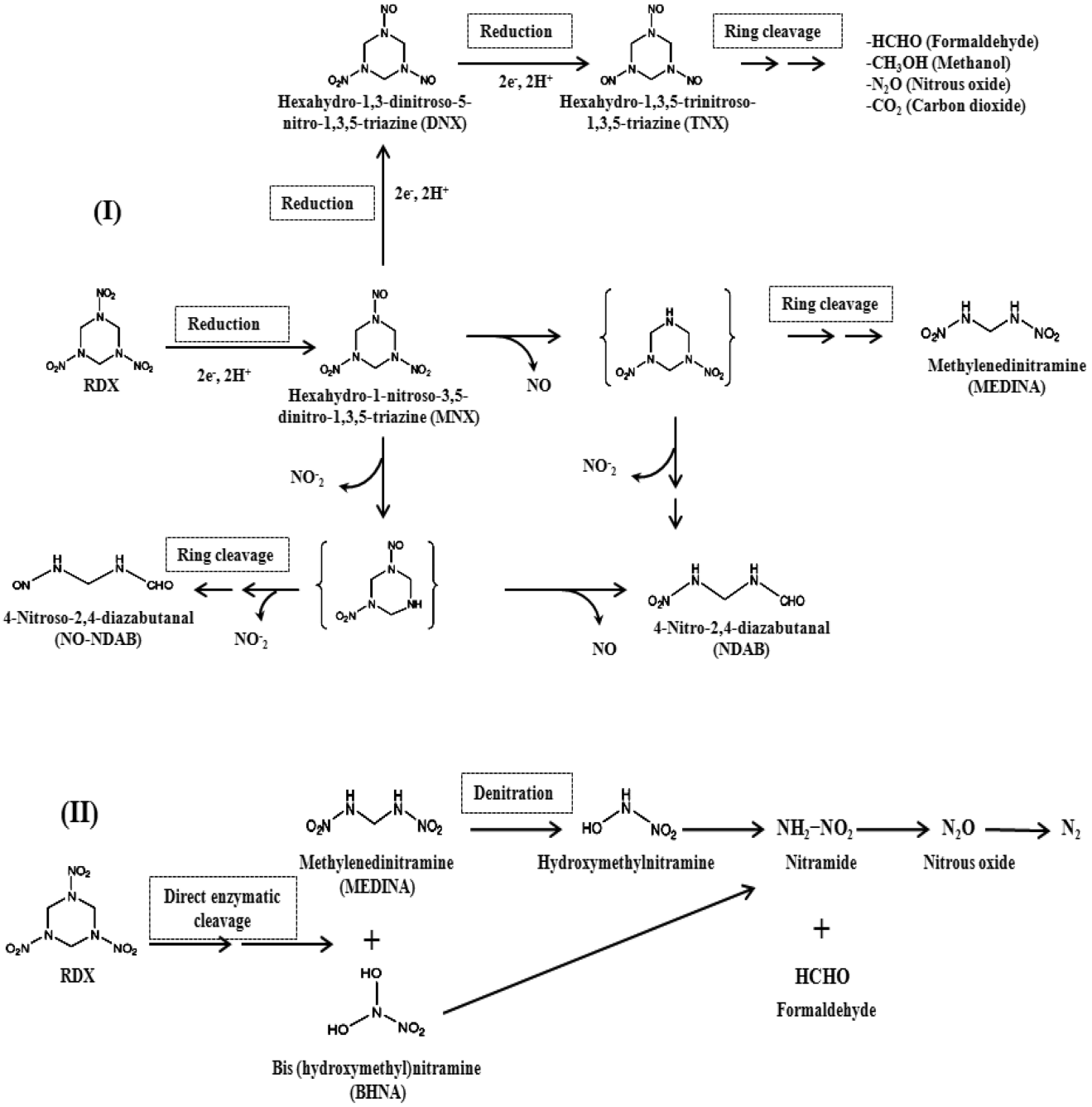

Fig. 5. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) microbial degradation pathways (I and II) under anaerobic conditions (adapted from Halasz and Hawari [22]).

POTENTIAL TOXICITIES OF RDX AND ITS INTERMEDIATES

The estimated risk of explosive compounds is directly related to its toxicity and potential for exposure. The toxicity of RDX has been extensively studied using a variety of reporter organisms, such as plants, algae, invertebrates, vertebrates, and microorganisms (Table 3). The diversity of toxicity values obtained from different bioassays shows the diversity of the sensitivity of different organisms. Observed effects of RDX exposure in the ecotoxicological model species include lethality, impaired growth, and reduced reproduction [21]. RDX readily crosses the blood brain barrier, alters the expression of multiple brain genes, and evokes pronounced seizure-like responses in a wide range of species [9, 21, 52, 72, 77]. Evidence that the central nervous system is also the primary toxicological target for RDX include observations of RDX-induced convulsions in rats [42], Northern bobwhite [31, 52], fish [21, 72], and humans [9, 73]. Toxic doses can also vary depending on the exposure time and conditions; however, a reference dose for human exposure can be determined from these data. The health recommendation of the USEPA is 2.0 μg of RDX per liter of drinking water [11]. Occupational Safety and Health Administration (OSHA) set a construction industry permissible exposure limit (PEL) for RDX of 1.5 mg/m^3 of workplace air (mg/m^3) for an 8 h workday for a 40 h workweek [47]. The EPA has not established an ambient air level or a cleanup standard for RDX in soil [11].

RDX transformation to *N*-nitroso metabolites has been shown to occur *in vivo*, as Pan *et al.* [49] showed, by formation of RDX *N*-nitroso metabolites in the gut of deer mice (*Peromyscus maniculatus*) following RDX exposure *via* food. Therefore, with the presence of RDX and its *N*-nitroso metabolites in the environment, the potential risk for exposure to RDX as well as *N*-nitroso metabolites exists for both humans and wildlife species [64]. Extensive literature review reveals that only a few researches have been conducted to evaluate the toxicity of RDX degradation products (Table 4). In these studies, microorganisms, invertebrates, and vertebrates have been used as reporter organisms. The relative toxicity of RDX degradation intermediates compared with the parental compound is dependent on the individual intermediate (Table 4). It has been shown that acute doses of MNX and TNX induced seizures and even death in Sprague–Dawley rats and deer mice [42, 63]. However, according to Smith *et al*. [63], these compounds are acutely less toxic as compared with RDX [MNX lethal dose (LD_{50}) values: 181–574 mg/kg; TNX LD_{50} values: 338–999 mg/kg]. Smith *et al*. [65] chronically exposed deer mice to TNX under controlled conditions, and results showed an increase in offspring mortality, dose-dependent bioaccumulation in liver, and decreased body and kidney weight from birth to ablactation in developing deer mice. Recently, Smith *et al*. [64] also

Table 3. RDX toxicity.

Toxicity	Value	Exposure time	Organism tested	RDX concentrations	References
No observed adverse effect concentration (NOAEC)	<95 mg/kg	28 d	*Eisenia andrei*	95–1,671 mg/kg	[53]
	13.3 mg/l	96 d	*Danio rerio*	0–40 mg/l	[45]
	9586 mg/kg	21 d	*Lolium perenne*	0–10,000 mg/kg	[54]
	3 mg/kg/d	14 d	*Colinus virginianus*	0.5–17 mg/kg	[52]
	2.5 mg/kg/d	14 d	*Sceloporus occidentalis*	0–60 mg /kg	[41]
Lowest observed adverse effect concentration (LOAEC)	95 mg/kg	28 d	*Eisenia andrei*	95–1,671 mg/kg	[53]
	16.5 mg/l	96 d	*Danio rerio*	0–40 mg/l	[45]
	100 mg/kg	21 d	*Oryza sativa*	0–10,000 mg/kg	[70]
	-	-	*Homo sapiens*	-	[9]
	20 mg/kg	3 d	*Colinus virginianus*	20–180 mg/kg	[31]
	5 mg/kg/d	14 d	*Sceloporus occidentalis*	0–60 mg /kg	[41]
	8 mg/kg	14 d	*Colinus virginianus*	0.5–17 mg/kg	[52]
	0.625 mg/l	10 d	*Pimephales promelas*	0.625–10 mg/l	[21]
	22 mg/l	16 h	*Danio rerio*	22–222 mg/l	[72]
Lethal dose (LD50)/ Inhibitory concentration (IC50)	40.2 mg/l	15 min	*Vibrio fischeri*	10–60 mg/l	[68]
	23 mg/l	96 h	*Danio rerio*	0–40 mg/l	[45]
	71–118	14 d	Sprague–Dawley rat	50 mg/kg	[8]
	136 mg/kg	14 d	*Peromyscus maniculatus*	136 mg/kg	[63]
	12 mg/kg/d	14 d	*Colinus virginianus*	0.5–17 mg/kg	[52]
	72 mg/kg/d	14 d	*Sceloporus occidentalis*	25–200 mg /kg	[41]
	9.9 mg/l	10 d	*Cyprinodon variegates*	3–30 mg/l	[40]

reported reduction in litter size of deer mice due to exposure to TNX at 1,000 µg/l and increased postpartum mortality of deer mice offspring at the highest exposure levels.

With respect to acute oral toxicity to Sprague–Dawley rats, MNX was found as the most potent toxicant of the RDX *N*-nitroso metabolites (as the incidence of lethality was 100%, 67%, and 67% at 400 mg/kg for MNX, DNX, TNX, respectively) and an estimate of 187 mg/kg for its LD_{50} was established and found to be equivalent to that of RDX ascertained with the same protocol [42]. In an earthworms (*Eisenia fetida*) study, Simini *et al*. [62] showed that the cocoon production EC_{20} value for RDX was 19 mg/kg (soil dry weight) in aged Sassafras sandy loam (SSL) soil. In 2008, Zhang *et al*. [76] exposed *Eisenia fetida* to soil with similar properties to SSL and demonstrated that the cocoon production EC_{20} values for MNX and TNX were 8.7 and 9.2 mg/kg (soil dry weight). These results show that the *N*-nitroso metabolites are more toxic than the parent compound (RDX). In addition, the estimated reproductive lowest observed adverse effect concentration (LOAEC) of TNX in the mouse was lower than the previously determined no observed adverse effect concentration (NOAEL) for RDX in rats [64]; thus, remediation options that rely on reductive RDX transformation may not provide adequate environmental security until RDX transformational *N*-nitroso compounds have sufficiently degraded to further non- or less toxic compounds.

According to these studies, the degradation of toxic RDX metabolites (MNX, DNX, and TNX) is as important as the degradation of the parent compound (RDX). Hence, complete mineralization/degradation or transformation of RDX to non-toxic final products must be the goal of any bioremediation strategy. Most of the other intermediates produced during RDX degradation pathways (Fig. 4 and 5) can be classified as uncharacterized, because their relative toxicities are not well reported in the literature.

The toxicological classification of RDX degradative intermediates was used in the present study to discuss the risk of potential RDX microbial degradation pathways (Fig. 4 and 5). Owing to the absence of RDX *N*-nitroso metabolites, aerobic pathways of RDX degradation *via* denitration (Fig. 4) are preferred over anaerobic pathways. However, the supply of oxygen, which is a very expensive process, is the disadvantage of aerobic RDX degradation (Fig. 4). In the anaerobic RDX degradation pathway (Fig. 5), the subpathway (I) to MNX, DNX, and TNX by nitroreductases may provide compounds with greater toxicities than that of the other anaerobic subpathway (II). From a toxicological point of view, all of the intermediates are *N*-nitroso compounds, which are typically more toxic than the parent compound (RDX). The subpathway (I) of anaerobic degradation leading to the formation of NDAB *via* ring cleavage of MNX may be toxicologically preferred owing to the further mineralization of NDAB to nitrate and carbon dioxide, which are relatively less toxic and more stable end products. The aerobic subpathway to NDAB formation *via* denitration (with the loss of two nitrite anions prior to RDX ring cleavage) may be the most suitable choice in a toxicological perspective for the remediation of RDX in a system with sufficient supply of oxygen.

In summary, we thoroughly reviewed the current knowledge on the potential fate, microbial degradation, and toxicity of

Table 4. Toxicity of RDX metabolites.

Metabolites	Toxicity	Exposure time	Organism tested	Relative toxicity	References
Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX)	Survival	14 d	Sprague–Dawley rats	≥ RDX; >DNX and TNX	[42]
	Survival, growth	30 d	*Acheta domesticus*	<TNX	[74]
	Survival, growth	14 d	*Eisenia fetida*	<TNX	[75]
	Survival	14 d	*Peromyscus maniculatus*	>RDX and TNX	[63]
	Reproduction	30 d	*Eisenia fetida*	≥TNX; >RDX	[76]
Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX)	Mutation	-	*Salmonella* Typhimurium	<TNX	[18]
	Survival	14 d	Sprague–Dawley rats	<MNX	[42]
Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)	Mutation	-	*Salmonella* Typhimurium	>RDX, MNX and DNX	[18]
	Survival	14 d	Sprague–Dawley rats	<MNX and RDX	[42]
	Survival	30 d	*Acheta domesticus*	>MNX	[74]
	Survival, growth	14 d	*Eisenia fetida*	>MNX	[75]
	Survival	14d	*Peromyscus maniculatus*	<RDX and MNX	[63, 65]
	Reproduction	30 d	*Eisenia fetida*	≤ MNX; >RDX	[76]
	Offspring survival	45 d	*Peromyscus maniculatus*	Toxic	[64]
4-Nitro-2,4-diazabutanal (NDAB)	Luminescence	15 min	*Vibrio fischeri*	<RDX	[13]

RDX, and examined the linkage between RDX microbial degradation pathways and intermediate toxicities. The results of the present review on the environmental fate of RDX suggest that the pollutant is fairly mobile through groundwater in most soil/sediment environments, especially when water-swelling clay particles are not abundant. In addition, owing to the relatively high hydrophilicity of RDX, the pollutant may be available for uptake by plants, supporting the feasibility of phytoremediation as a bioremediation option. Our review on RDX biodegradation pathways and their potential intermediates suggests that aerobic stimulation of RDX microbial degradation that involves the formation of NDAB *via* denitration would be the preferred choice for the development of remediation technologies in a toxicological perspective, whereas anaerobic stimulation of RDX microbial degradation leading to the formation of NDAB *via* ring cleavage of MNX may be toxicologically preferred owing to the further mineralization of NDAB to the less toxic and stable compounds. Thus, microbial populations that can efficiently mineralize toxic intermediates that appear in the aforementioned pathways under both aerobic and anaerobic conditions must be identified. Functional metagenomics combined with next-generation sequencing would be a powerful investigation tool for this emerging area of biodegradation research.

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