6.14 Biodegradation and Bioremediation of TNT and Other Nitro Explosives☆

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Glossary

Coenzyme F_{420} A 7,8-didemethyl-8-hydroxy-5-deazariboflavin derivative ($E^{\circ\prime} = -360$ mV) involved in hydride transfer reactions similar to reduced pyridine nucleotides (NAD(P)H, $E^{\circ\prime} = -320$ mV). F_{420} is present in all methanogenic and certain nonmethanogenic archaea as well as in certain members of the *Actinobacteria* phylum (high G+C Gram-positive bacteria).

Cometabolism Fortuitous or gratuitous biotransformation of a pollutant catalyzed by broad-specificity biocatalyst(s) (broad substrate range) produced during the microbial metabolism of a primary substrate.

Cytochromes P450 A superfamily of hemoproteins (i.e., containing a heme cofactor) involved in the oxidation of a broad variety of organic compounds. Cytochromes P450 belong to external monooxygenases and so need an external electron donor (e.g., NAD(P)H) necessary for oxygen activation reactions and the subsequent substrate hydroxylation.

Electron-withdrawing Character of a chemical substituent, relative to hydrogen, that withdraws electron density due to its negative inductive effect (−I) and/or negative mesomeric effect (−M, resonance effect).

Explosive A high energy material, consisting commonly of elements of carbon, hydrogen, oxygen and nitrogen, which, when subjected to initiation by any stimuli, undergoes a very rapid, self-propagating and exothermic decomposition reaction resulting in the formation of more stable substances (e.g., CO_2, H_2O, N_2) and the development of a sudden and high pressure effect.

Explosophor A chemical substituent that is responsible for the explosiveness of a high energetic material.

Flavodoxin (Fldx) A family of small $1e^-$- or $2e^-$-transfer flavoproteins binding non-covalently the prosthetic group flavin mononucleotide (FMN).

Flavodoxin reductase (FdR) A family of flavoproteins binding the prosthetic group flavin adenine dinucleotide (FAD) that catalyzes the reversible transfer of electrons between the reduced pyridine nucleotide NADPH and flavodoxins or ferredoxins.

Old Yellow Enzyme (OYE) family A family of NAD(P)H-dependent flavoprotein reductases belonging to the class I flavin-dependent β/α barrel oxidoreductases.

Xenophor A chemical substituent that does not (or seldom) occur in nature, and as a consequence, causes biological persistence of a synthetic compound.

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6.14.1 Introduction

Before the emergence of a collective awareness of environmental deterioration and before establishing stringent regulations and being conscious of the high cost of remediation, humankind has developed a techno-industrial capacity and experienced intensive, far-reaching, and devastating military conflicts to bequeath an ever more threatening and polluted environment to future generations. Specifically, the natural resources and chemicals we use to prepare for wars and to conduct and support military conflicts, as well as the chemicals we just discard during postwar activities, have damaged ecosystems and imperiled human health. For a long time, explosives have attracted our attention as a fascinating tool for blasting and quarrying activities and as a misused destructive power in wartime.[1] During the second half of the 20th century, the scientific community made an issue of the toxicity and environmental fate of explosive compounds beyond their unique energetic properties. For instance, explosive remnants of war, such as, antipersonnel landmines or unexploded ordnance (UXO), are of particular concern since they trigger humanitarian disasters and release toxic explosive residues into soils and groundwater.[2] Therefore, the ever growing detection of explosive-contaminated environments has sounded an alarm since the 1970s,[3,4] showing why providing effective bioremediation processes is a priority, with environmental due diligence, adequate research for guidance to decision-making, and proper technical development and implementation.

The immediate and easy availability of nitrating agents since the discovery of the Haber-Bosch process and the unique chemical properties of the nitro ($-NO_2$) functional group have made nitro compounds pivotal intermediates in the organic synthesis of explosives. The nitro substituent, consisting of two atoms of oxygen and one atom of nitrogen, presents a coordinate covalent N–O bond, conferring a negative formal charge to the oxygen atom and a positive formal charge to the nitrogen atom. Furthermore, since oxygen ($\chi(O) = 3.5$) is more electronegative than the nitrogen atom ($\chi(N) = 3.0$),[5] the N–O bond is a polarized covalent bond ($\Delta\chi = 0.5$), conferring a positive partial charge to the nitrogen atom. As a consequence, the strong electrophilic nature of the N atom of the nitro group combined with its oxidation state of + III makes the nitro substituent very prone to reduction and an ideal candidate to act as an explosophor group that self-sustains the oxidation of nitro explosives. The sources of oxygen for the process of combustion can come from either the nitro or nitroester groups. Today, most extensively applied explosives commonly bear nitro group(s) and belong to the nitroaromatic, nitramine ($-N-NO_2$), nitroester ($-O-NO_2$), or nitroalkane family (Fig. 1). The homolytic reactions of the $C-NO_2$, $N-NO_2$, or $O-NO_2$ explosophor moieties are common primary fission processes undergone by nitro explosives after initiation by any stimuli.[6]

From an environmental point of view, almost all of nitro-bearing compounds present in nature are produced by anthropogenic activities so that the nitro substituent can be classified as a xenophor group. Particularly, the occurrence of multiple nitro groups with specific substitution patterns confers xenobiotic character to nitro explosives.[7] Because of the lack of naturally occurring analogs, they are not easily recognized by existing biodegradative machineries and, as a consequence, they accumulate in the environment. To our knowledge, only few organic compounds bearing nitro functional group(s) are produced by living organisms.[8,9] All the natural nitro compounds exhibit remarkable biological activities (e.g., antimicrobial), with the antibiotic chloramphenicol of the genus *Streptomyces* and the herbal carcinogen aristolochic acid of the plant genera *Aristolochia* and *Asarum* being the most emblematic members of this group. The toxicity of organic compounds bearing nitro substituent(s) is mainly due to reductive bioactivation and the formation of toxic nitroso ($-NO$), hydroxylamino ($-NHOH$), and amino ($-NH_2$) intermediates combined with redox cycling and the production of reactive oxygen and nitrogen species.[10,11]

In addition to the toxicity and the xenobiotic character of nitro explosives, which limit their utilization as a growth substrate by microorganisms, the nitro group is a strong electron-withdrawing substituent (e.g., Hammett constants: $\sigma_p = 0.81$; $\sigma_m = 0.71$; $\sigma_p^- = 1.27$; $\sigma_p^+ = 0.79$; $\sigma_m^+ = 0.73$[12]) deactivating aromatic compounds toward electrophilic substitution reactions. The electronegativity of the nitro group relative to H (= 2.176) is 3.421.[5] In trinitroaromatic compounds (Fig. 1), the aromatic ring is strongly electron deficient due to the symmetric localization of the three nitro groups. This prevents any electrophilic attacks of the aromatic π–e^- system catalyzed by oxygenases, a usual prerequisite for the mineralization of aromatic compounds. On the contrary, nitro moiety reduction of TNT is commonly observed and generates toxic, dead-end aminoaromatic or diaryl azoxy metabolites of TNT while preserving the highly stabilized aromatic ring structure.[13] In case of cyclic nitramines (Fig. 1), the process of cleavage of the saturated ring and further mineralization is enhanced due to the lack of a stable aromatic skeleton. Initial enzymatic reactions acting on RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine), or CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) can lead to the destabilization of the nitramine ring structure followed by spontaneous decomposition to nitrite, nitrous oxide, or C-1 compounds like formic acid or formaldehyde.[14] On the other hand, nitroester and nitroalkane explosives are considered more amenable to mineralization than nitroaromatics due to their sterically simpler chemical structure, even though the toxicity and recalcitrance of newly synthesized caged polynitrocycloalkane explosives, such as octanitrocubane (ONC) (Fig. 1), have not been investigated yet.

2,4,6-Trinitrotoluene (TNT), historically the most widely used explosive, is still being frequently detected at the sites of military and/or civilian applications in combination with other nitro explosives and multiple explosive derivatives. The US EPA's National Priorities List (final NPL active sites, US Environmental Protection Agency, July 25, 2018, http://cumulis.epa.gov/supercpad/cursites/srchsites.cfm) counts 13 TNT-contaminated groundwater sites, 32 RDX-contaminated sites and 15 HMX-contaminated sites that require monitoring and/or remediation. For instance, groundwater co-contamination by TNT, RDX, HMX and tetryl was detected at the Umatilla Chemical Depot (EPA ID: OR6213820917, Hermiston, Oregon) and in situ bioremediation strategies for this NPL site have been investigated for RDX and TNT removal using biostimulation, bioaugmentation, or both.[15–17] Because nitro explosives and their derivatives present different chemical structures and undergo dissimilar abiotic and/or biotic transformations, their transport in environmental systems must be assessed on a case-by-case basis. For instance, cyclic nitramines are generally

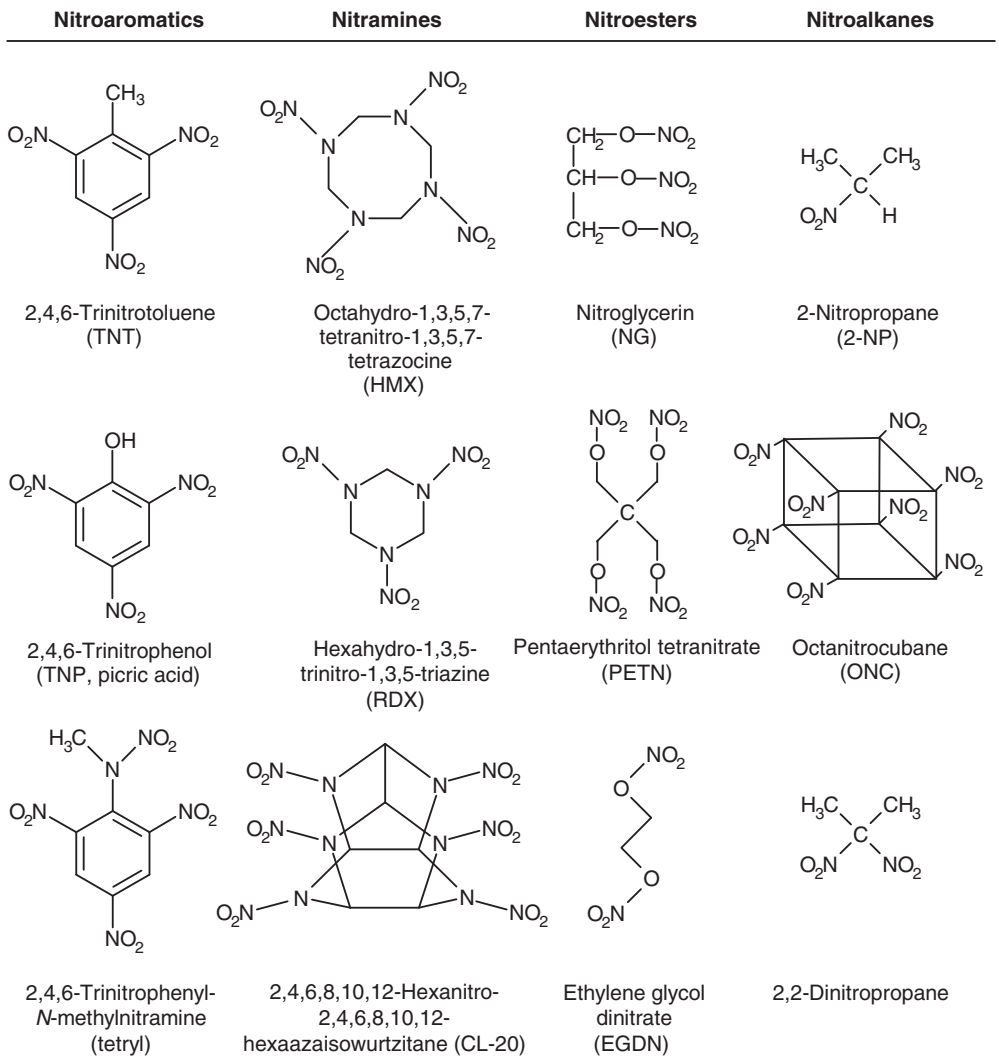

Figure 1 Chemical structures of some nitro explosives belonging to the nitroaromatic, nitramine, nitroester, or nitroalkane family.

considered far less sorptive to soils, and are thus more mobile, than nitroaromatics, so they may be a significant threat to recreational waters and drinking water sources.[18] In addition, the fate of nitro explosives in aquatic systems is expected to be divergent with regard to aqueous solubility values (Fig. 2).

Taking all the points together, the restoration of environmental sites contaminated by nitro explosives is very challenging and needs a multifaceted bioremediation approach due to their dissimilar biological degradation and transport processes. This article

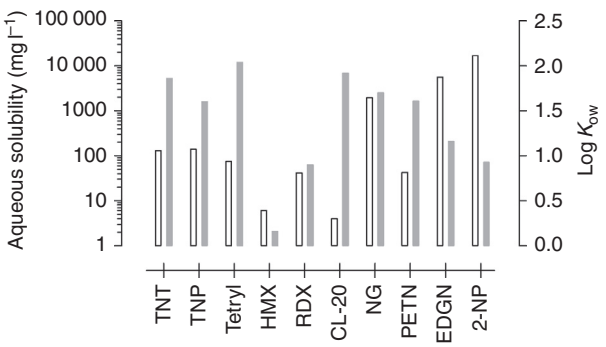

Figure 2 Aqueous solubility (open bars) and *n*-octanol-water partition coefficient, log K_{ow} (gray bars), of some nitro explosives.

highlights the recent discoveries of promising catabolic pathways of nitro explosives and the development of innovative biosensors and biomarkers to properly monitor bioremediation of contaminated sites. We finally present the most interesting approaches to integrate the required biological tools for the development of in situ bioremediation processes of sites contaminated by multiple nitro explosives.

6.14.2 Nitroaromatic Explosives

Because TNT has been the most widely used nitro explosive during the 20th century, this section focuses on this emblematic trinitroaromatic compound as well as the TNT analogs picric acid (2,4,6-trinitrophenol, TNP) and tetryl (Fig. 1). Although these nitro explosives are structurally very similar, their environmental fate is fairly different and so it is crucial to clearly distinguish their biodegradation and transport patterns to (1) assess the ecotoxicological impacts of nitroaromatic explosives and (2) select proper methods for bioremediation of contaminated sites.

6.14.2.1 Environmental Transport Processes of Nitroaromatic Explosives

The sorptive behavior of organic contaminants is commonly associated with hydrophobic partitioning resulting from both entropic and van der Waals forces (≤ -4 kJ mol^{-1}).[19] Natural organic matter (NOM) contains typical hydrophobic sorption domains and strongly influences the partitioning of aromatic compounds with a high *n*-octanol-water partition coefficient, K_{ow}, and a low water solubility, S_w. Accordingly, Karickhoff et al.[20] reported a linear relationship between the indicator of hydrophobicity (log K_{ow}) of a wide range of aromatic hydrocarbons and chlorinated hydrocarbons and the organic carbon partitioning coefficient in soils (log K_{oc}). Due to the symmetric localization of the three functional nitro groups, TNT is a weakly polar molecule with a molecular dipole moment of 1.37 D[21] and a log K_{ow} value (1.86) relatively high compared to other nitro explosives (Fig. 2). Therefore, TNT can bind to soil organic matter (SOM) through nonspecific hydrophobic partitioning reactions. Singh et al.[22] reported that the aliphatic (primarily alkyl) fraction of SOM mainly controls the nonspecific sorption of TNT in comparison to aromatic moieties. Accordingly, Ericksson et al.[23] showed that the hydrophobic partitioning is the major adsorption mechanism of TNT in particulate organic matter (POM) which is rich in aliphatic carbon.

On the other hand, transformation products of a defined contaminant (called metabolites, degradates, or derivatives) can show a sorption behavior as well as a toxicity very different from that of the parent compound, and thus present a divergent ecotoxicological risk. For instance, Boxall et al.[24] have indicated that about one-third of the metabolites derived from a range of pesticide types have a K_{oc} value of at least an order of magnitude lower than that of the corresponding parent compound. Thus, these derivatives may be more likely to be transported from soils to surface water and groundwater. On the contrary, the reduced TNT metabolites (e.g., nitroso, hydroxylamino, diaryl azoxy, and amino derivatives) are subject to both hydrophobic partitioning and specific (electrostatic and covalent) binding with SOM. As for the parent compound TNT, the association between TNT metabolites and POM was found to be dominated by hydrophobic partitioning processes (e.g., log K_{ow} value of the 6e$^-$-reduced TNT metabolite 2-amino-4,6-dinitrotoluene is 1.95).[25] In addition, reduced TNT metabolites present a preferential affinity to dissolved organic matter (DOM), rather than POM, through different types of specific interactions with DOM functional groups such as carboxyl and carbonyl groups.[23,26] Specifically, TNT degradation products bind to the smallest size fraction of DOM (<3.5 kDa), which has a substantially higher density of oxygen functional groups reactive toward amino substituents.[26] This fraction is not prone to flocculation mechanisms by major metals in soils and can be released into surface water and groundwater by leaching of DOM in contaminated soils. On the other hand, the colloidal water-dispersible clay fraction of the soil could significantly adsorb TNT and TNT metabolites and so favor colloid-facilitated transport of explosive residues in soils.[27] TNT transport by soil colloid mobilization was confirmed by Sharma et al.[28] but the exact mechanism of interactions between TNT and colloids was not elucidated.

Beyond entropy-driven mechanisms, specific interactions have been proposed for the distribution of TNT in soil matrix because (1) the distribution coefficients (K_d; l kg^{-1}) of TNT in different types of soil are commonly not correlated with the total organic carbon content and (2) the surface area-normalized distribution coefficients (K_{ad}; l m^{-2}) of TNT to pure clays and other phyllosilicates are up to five orders of magnitude larger than adsorption expected for nonspecific interactions based on log K_{ow} values.[19] As mentioned above, the aromatic π–e$^-$ system of TNT is electron deficient and therefore has a positive molecular quadrupole moment, Θ_{zz}.[19] Such a chemical structure confers a π–e$^-$ acceptor character to TNT, which gives a substantial sorptive potential through electron donor–acceptor (EDA) interactions with nonbonding electrons or electron-rich π–e$^-$ systems. Therefore, TNT can bind specifically to negatively charged 2:1 clay mineral surfaces saturated by small and weakly hydrated cations such as K^+ (specific EDA interaction energy, $\pm$ 25–40 kJ mol^{-1}).[19] In the field, these interactions are mainly observed in soils having low organic carbon content and that are rich in highly negatively charged clays, like smectites.[27] Interestingly, the electron deficiency of the aromatic ring of TNT, and thus its specific adsorption to clay minerals (e.g., $K_d = 21{,}500$ L kg^{-1} for homoionic K^+-montmorillonite), will be lower than that of 2,4,6-trinitrobenzene (e.g., $K_d > 60{,}000$ L kg^{-1} for homoionic K^+-montmorillonite) due to the presence of (1) the electron-donating methyl group (positive inductive effect) and (2) the steric interactions due to the *o*-methyl substituent that prevent complete coplanarity and thus optimal resonance of the nitro substituents with the aromatic ring (*ortho* effect).[27] Similarly, due to the electron-donating character (+M, resonance effect) of the amino group, the aromatic ring of TNT-reduced metabolites such as aminodinitrotoluene (ADNT) and diaminonitrotoluene (DANT) isomers shows a higher electron

density than TNT and thus a lower affinity to phyllosilicates (e.g., K_d (2,4-DA-6-NT) = 3.5 L kg^{-1} for homoionic K^+-montmorillonite[27]). Since the production of tetryl was stopped in the early 1970s[29] and its use as military explosive was significantly lower in comparison to TNT, few data are available on the fate and interactions of tetryl with soil matrix. However, because tetryl is characterized by a similar log K_{ow} value as TNT (Fig. 2), its interactions with SOM are expected to be significant. In addition, tetryl is characterized by a very low specific adsorption to clay minerals (e.g., K_d = 5.8 L kg^{-1} for homoionic K^+-montmorillonite) due to a combination of both *ortho* and bulky (*N*-methylnitramine) substituent effects.[27] Conversely, *ortho*-nitrophenols may form an intramolecular hydrogen bridge between the hydroxyl group and the nitro group so that coplanarity between the nitro group and the aromatic ring is not disturbed by the *ortho* effect.[30] However, TNP is a very strong phenolic compound (pK_a = 0.38[31]) and is predominantly present in the environment as the dissociated picrate anion with a highly delocalized charge. Therefore, TNP does not sorb significantly to organic matter and its adsorption to phyllosilicates through EDA complex is significantly lower than TNT because of its decreased π–e^- acceptor property and the electrostatic repulsion of picrate anion by negatively charged siloxane oxygen donors. This relatively higher mobility of TNP in the subsurface, combined with its higher solubility than TNT (Fig. 2), is consistent with the fact that it did not accumulate in contaminated soils of military sites despite its worldwide use during World War II.[30]

6.14.2.2 (Bio)degradation Pathways of Nitroaromatic Explosives

TNT, which has a positive Θ_{zz}, mainly undergoes nucleophilic, reductive (bio)transformations due to the electrophilic character of the aromatic nucleus and the N atom of the nitro group.[13] C, N, and H isotope fractionation of nitroaromatic compounds in subsurface materials confirmed that TNT mainly undergoes reductive transformation, whereas the transformation of 2,4-DNT, which is characterized by a higher electron density than TNT, is mostly due to oxygenation.[32]

Consequently, TNT is easily reduced to nitroso, hydroxylamino, and ultimately amino aromatic derivatives through three successive transfers of an electron pair.[13] The initial four-electron nitroreduction step is catalyzed by typical NAD(P)H-dependent oxygen-insensitive (type I) nitroreductases[13] and type I and type II hydride transferases of the Old Yellow Enzyme (OYE) family.[33] It has been suggested that other enzymes can be further involved in the last two-electron nitroreduction step to form the aminoarene.[34]

In addition, TNT is not subject to the conventional electrophilic attack by oxygenases. Conversely, TNT is prone to superoxide-driven nucleophilic attack as it was observed with the biomimetic system containing reduced pyridine nucleotides and pyocyanin, a redox-active metabolite secreted by *Pseudomonas eruginosa*.[35,36] Shinkai et al.[37] have also reported a superoxide-mediated denitration of TNT catalyzed by a NADPH-cytochrome P450 reductase from rat liver with concomitant release of nitrite and formation of dimeric denitrated metabolites. In this reaction, a Janovsky complex was generated as an anionic intermediate resulting from the nucleophilic aromatic substitution of TNT by a C-nucleophile (4-ketooxime-DNT). Moreover, anionic σ-adducts of TNT, also termed TNT Meisenheimer complexes, are readily produced by covalent bond formation between nucleophiles, such as the negatively charged form of hydrogen atom, H^- (hydride ion), and the aromatic ring of TNT. So far, several enzymes from bacteria and plants have been characterized to catalyze the nucleophilic addition of hydride ions to the TNT aromatic ring, including specific NAD(P)H-dependent type II OYE hydride transferases (Fig. 3A)[38,39] and, to a much lesser extent, specific F_{420}-dependent actinomycetal hydride transferases.[40] Type II OYE hydride transferases, such as the pentaerythritol tetranitrate (PETN) reductase, catalyze two competing pathways for TNT biodegradation, i.e., the nitro moiety reduction of TNT and nucleophilic addition of hydride ions to the TNT aromatic ring. Following the transfer of hydride ions to the aromatic ring of TNT, hydride and dihydride Meisenheimer complexes of TNT ([H^-]-TNT and [$2H^-$]-TNT, respectively) are produced and can further yield nitrite and diverse denitrated metabolites of TNT that typically accumulate in the reaction medium. Parallel π-π stacking conformation between TNT and the FMN cofactor of the type II OYE hydride transferases is a prerequisite for TNT Meisenheimer complex formation, as demonstrated from computational docking studies on TNT-PETN reductase interactions.[41] The His181 and Tyr351 residues of the PETN reductase have been proposed to play a key role in the enzyme-substrate conformation, while the His184 residue controls the active center of the PETN reductase.[41] Even if nitrogen from TNT can be further assimilated via the activity of nitrite reductase and the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway, it has been commonly observed that denitrated metabolites of TNT (e.g., dimeric compounds) cannot be utilized as carbon source. The inherent problem of extensive chemical and metabolic misrouting precludes the emergence of a unique beneficial biodegradation pathway for TNT. For instance, condensation products of TNT metabolites can be produced between (1) nitroso- and hydroxylamino-dinitrotoluene isomers to form tetranitroazoxytoluene compounds like 4,4′,6,6′-tetranitro-2,2′-azoxytoluene and (2) hydroxylamino-dinitrotoluene isomers and protonated dihydride Meisenheimer complexes of TNT to form secondary diarylamines with the release of nitrite.[33] TNT metabolites derived from nitro moiety reduction can also undergo methyl moiety oxidation or amino moiety acetylation.[13] These diverse compounds are commonly referred to as dead-end metabolites because they accumulate. In addition, the formation of suicide metabolites due to the toxic effects of intermediates and secondary products that are generated by nitro moiety reduction seems to be a major barrier to a productive metabolism of TNT.[42]

However, some authors have recently reported beneficial TNT biodegradation pathways with (1) the production of the metabolizable compound 2,4-dinitrotoluene (2,4-DNT) from monohydride Meisenheimer complex of TNT[13,43,44] and (2) the evidence using stable isotope probing (SIP) of assimilation of ^{15}N and ^{13}C from TNT into bacterial DNA.[45]

Taking advantage of the catabolic capabilities of the yeasts *Yarrowia lipolytica* and *Geotrichum candidum* and the acidification of the culture medium through the production of organic acids, Ziganshin et al.[43,44] have reported the transformation under acidic

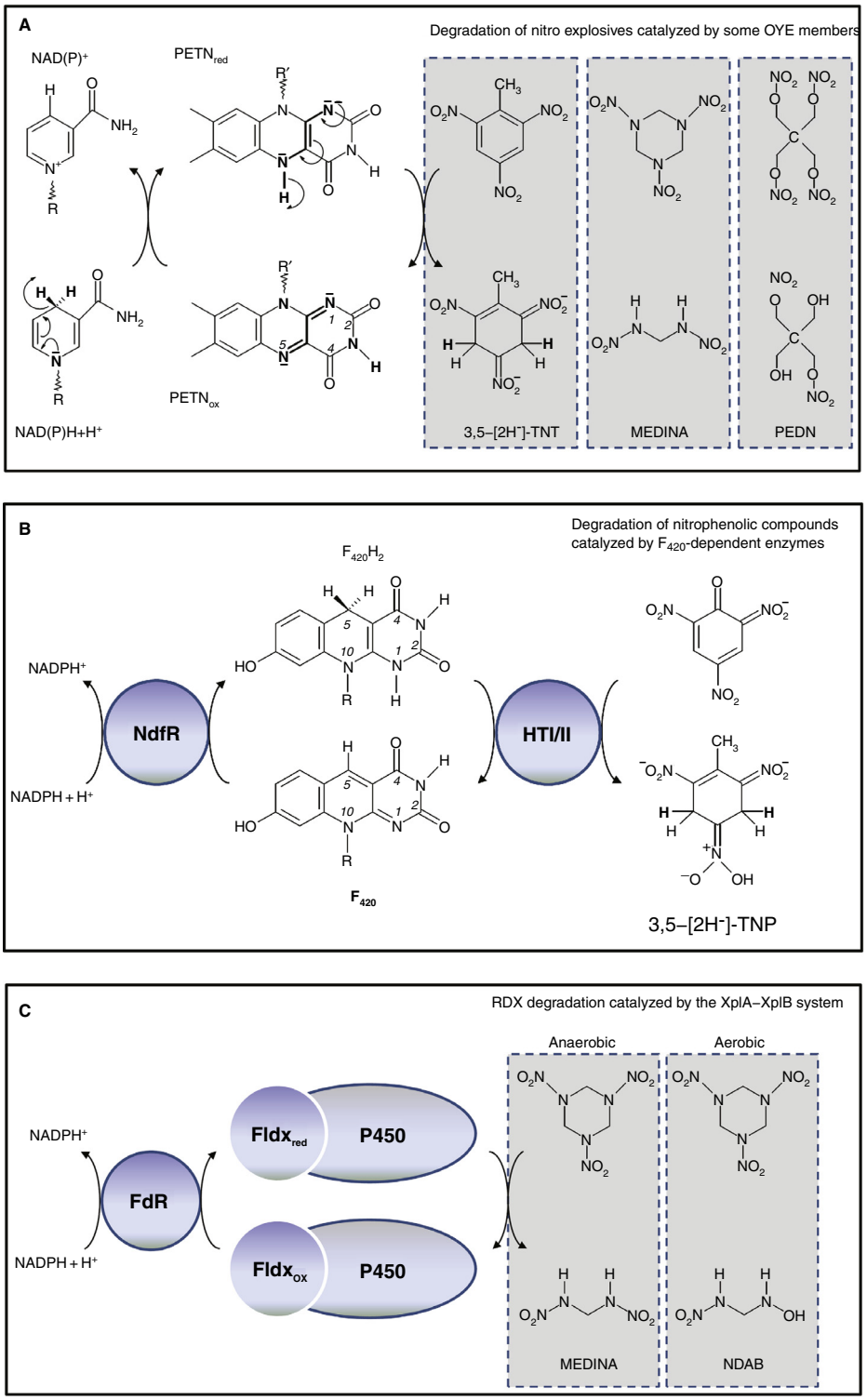

Figure 3 Biodegradation of nitro explosives catalyzed by (A) some members of the OYE family (e.g., the pentaerythritol tetranitrate (PETN) reductase (belonging to the type II OYE hydride transferases)), (B) the F420-dependent reductases of npd cluster, and (C) the XplA–XplB system.

conditions (i.e., at a pH < 4.2) of C_3-[H^-]-TNT to 2,4-DNT, a mineralizable TNT-denitrated metabolite,[46] with concomitant release of nitrite.

Using DNA-SIP and T-RFLP, the utilization of TNT as both carbon and nitrogen sources was reported under sulfogenic conditions by a *Lysobacter taiwanensis* strain initially present in anaerobic organic-rich estuarine sediments.[45] However, the incorporation

of TNT into *Lysobacter* cell biomass as either a primary substrate or a co-substrate (cometabolism) remains to be elucidated. Even though additional experiments in axenic cultures are necessary to decipher the complete biodegradation pathways catalyzed by *L. taiwanensis*, the unambiguous demonstration of anaerobic bacterial assimilation of both N and C from TNT holds new promising remediatory possibilities, such as bioaugmentation trials of anaerobic TNT-contaminated environments.

Although TNT biodegradation pathways reported in the literature are exclusively supported by cometabolism, specific bacteria of the order *Actinomycetales* have been reported to utilize TNP as the sole source of nitrogen, carbon, and energy without reduction of the nitro moiety (e.g., picramic acid, a monoamino derivative of TNP).[47] The major TNP catabolic pathway is the enzymatic hydride transfer to the aromatic nucleus followed by successive denitration reactions and funneling of cleaved metabolites into the tricarboxylic acid (TCA) cycle. The TNP degradation gene cluster (*npd* cluster) has been characterized, with the identification of different inducers and the transcriptional regulator NpdR.[48] The enzymes involved in the peripheral catabolic pathways of TNP in *Rhodococcus opacus* HL PM-1 are hydride transferase II (HTII), encoded by *npdI*, and hydride transferase I (HTI), encoded by *npdC*, which produce the monohydride Meisenheimer complex of TNP ([H^-]-TNP) and the dihydride Meisenheimer complex of TNP ([$2H^-$]-TNP),[48] respectively. The activity of HTII and HTI is dependent on an NADPH-dependent F_{420} reductase (NdfR), encoded by *npdG*, that acts as an electron shuttle between NADPH and F_{420}. As depicted in **Fig. 3**B, the transfer of hydride from reduced coenzyme $F_{420}H_2$ to the aromatic ring of TNP is catalyzed by HTII/HTI.[48] In *Nocardioides simplex* FJ2-1A, the same peripheral catabolic pathways of TNP occurs except that a unique hydride transferase (HT) catalyzes the production of [H^-]-TNP and [$2H^-$]-TNP.[47] Subsequently, the tautomerase NpdH catalyzes a proton-shift tautomerism of [$2H^-$]-TNP to produce the nitro form ($R_2C(-H)NO_2$) and the *aci*-nitro form ($R_2C{=}N^+(-O^-)OH$) in equilibrium. The latter undergoes an enzymatic denitration reaction catalyzed by cell-free extracts of *R. opacus* HL PM-1 or *N. simplex* FJ2-1A (containing an orphan denitrase) to form the monohydride Meisenheimer complex of 2,4-dinitrophenol (2,4-DNP).[47] Following a second hydrogenation by the HTI–NdfR (or HT–NdfR) system, the dihydride intermediate is protonated at pH 7.5 to form 2,4-dinitrocyclohexanone, which undergoes a hydrolase-catalyzed cleavage to 4,6-dinitrohexanoate. Because the latter can be finally channeled toward the TCA cycle, TNP biodegradation is self-sustaining and can provide, at sites contaminated by high TNP concentrations, selective advantages to the degrading microorganisms.

For the biodegradation of the TNT analog tetryl, it is recognized that its hydrolysis can produce TNP, which can undergo complete microbial biodegradation.[29] Enzymatic *N*-denitration of tetryl under anaerobic conditions has also been observed with the formation of *N*-methyl-2,4,6-trinitroaniline,[49] a trinitroaromatic compound for which biodegradability experiments need to be carried out.

In conclusion, no single microbe carries out enough reactions to derive a benefit from the biodegradation of TNT.[50] The emergence of a mineralization pathway may be more rapid when several consecutive reactions are found within a single microbe. Although a dioxygenase-catalyzed elimination of nitrite has been reported as the initial reaction for 2,6-DNP,[51] electrophilic attack of 2,4-DNP has not been described so far due to the negative inductive effect of the hydroxyl group and specific steric hindrance. Therefore, initial hydrogenation of the aromatic ring system is commonly observed for 2,4-DNP and TNP so that their biodegradation is catalyzed by the same enzymatic machinery in a single microbe. Conversely, dissimilar biodegradation pathways have been observed for TNT and DNT isomers. Indeed, 2,4-DNT and 2,6-DNT are completely degraded via an initial dioxygenase reaction.[46] Consequently, syntrophic processes, for example, reductive denitration of TNT to 2,4-DNT followed by oxidative biotransformation of 2,4-DNT, have been proposed as a suitable strategy to obtain TNT mineralization.[52] However, multiple competitive pathways occur during TNT denitration in both pure and mixed cultures and, as a consequence, they lead to dead-end denitrated metabolites rather than DNT isomers. In this context, experimental evidence of the production of 2,4-DNT from TNT biodegradation catalyzed by yeast strains[43,44] can be of primary importance to implement a mineralization-driven process of TNT.

6.14.3 Nitramine Explosives

Because of the economic burden of toxic wastewater disposal (e.g., red water) as well as the increasingly stringent environmental laws and the need for innovative green manufacturing, TNT production in the United States was stopped in the mid-1980s, whereas its import, mainly from Canada, continued. Today, RDX, which exhibits both high brisance and stability, has replaced TNT as the most important military high explosive in the US.[29] Although HMX exhibits higher performance than RDX due to its higher density, its higher cost of production has restricted its military use compared to RDX.[1] Similarly, because of its superior explosive properties (higher energy and moderate sensitivity), the next-generation nitramine explosive CL-20 is considered as a current candidate to replace the conventionally used nitramine explosives RDX and HMX.

Due to the increased use of nitramine explosives during the 20th century and the associated severe contamination of soils and groundwater, biodegradability studies on RDX, HMX, and, more recently, their analog CL-20 have been carried out. Due to different conformations, HMX (crown type) is chemically more stable and therefore less amenable to biodegradation than RDX (chair type).[53] Although the cyclic nitramine compound CL-20 is composed of a rigid and highly strained cage (**Fig. 1**), it is more susceptible to abiotic transformation than RDX and HMX, and so it is expected to be less persistent in the environment.[54] Since CL-20 is a newly synthesized explosive, the comprehensive understanding of its environmental fate before its use in military munitions could limit its adverse environmental impacts in a proactive and preventive pollution control way rather than a curative remediatory strategy. In addition, the vast literature on biodegradation of the CL-20 analogs RDX and HMX has allowed to predict biodegradation patterns of CL-20 and to direct scientific research.

6.14.3.1 Environmental Transport Processes of Nitramine Explosives

Because of the higher log K_{ow} value of CL-20 (1.92) (Fig. 2), and thus its greater hydrophobicity compared to RDX and HMX (0.90 and 0.16, respectively), the mobility and bioavailability of CL-20 are expected to be limited in soils relative to its nitramine analogs. Accordingly, Balakrishnan et al.[55] have reported that CL-20 is highly immobilized by soils having high organic carbon content (e.g., $K_d = 311$ L kg^{-1} for slightly acidic soils with 20% total organic content). On the contrary, CL-20 presents a very low affinity for mineral surfaces (e.g., $K_d = 0.6$ L kg^{-1} for montmorillonite K10).[55] Indeed, non-aromatic nitramine explosives cannot adsorb specifically to clay minerals through the EDA complex formation like trinitroaromatic compounds. In addition, the particular caged structure of CL-20 generated a bulky effect, which prevented its interactions with the interlayer nanopores of clay minerals.

Conversely, RDX and HMX show a very low adsorption on soil matrix and present a higher affinity for the clay fraction (e.g., K_d (HMX) = 15.6 L kg^{-1} for montmorillonite K10[55]; K_d (HMX) = 1.2 L kg^{-1} for homoionic K^+-montmorillonite[54]) rather than the organic carbon content of soil. However, the tendency of nonaromatic RDX and HMX to adsorb to clay minerals is very low in comparison to TNT, which acts as a strong π-acceptor in EDA complex. Specific NO_2–cation complexation, that is, formation of direct inner sphere complexes of NO_2 substituents with exchangeable surface cations, has been proposed as the adsorption mechanism of RDX and HMX on clay minerals.[7] Because of both its low sorptive potential to soil particles and its significant aqueous solubility (Fig. 2), RDX is more mobile in soils than its nitramine analogs HMX and CL-20. This was demonstrated by the observation of RDX contamination in a sole-source drinking water aquifer located close to Camp Edwards (Massachusetts Military Reservation (MMR), Cape Cod, MA[56]), where RDX has migrated farthest from the source followed by HMX and TNT. Therefore, the MMR has been extensively studied and is the subject of an Impact Area Groundwater Study Program (http://jbcc-iagwsp.org/). Particularly, the site-specific transport mechanisms of the high explosives RDX and TNT have been assessed in sandy soils sampled from the MMR site,[57] confirming the higher mobility of RDX than TNT and the higher mobility of nitro explosives, in general, in sandy soils with low organic matter.

6.14.3.2 (Bio)degradation Pathways of Nitramine Explosives

The biodegradation of nitramine explosives has been recently reviewed,[14,54] and the evidence of ring cleavage and mineralization of cyclic nitramines under both aerobic and anaerobic conditions has been reported. Initial reductive enzymatic attacks or abiotic reactions toward RDX, HMX, or CL-20 can lead to the destabilization of the nitramine ring structure followed by spontaneous water-driven decomposition of the newly synthesized derivatives to produce inorganic nitrogen species (nitrite, nitrous oxide, ammonia) and formaldehyde (RDX and HMX) or formic acid and glyoxal (CL-20) as common end products.[14,54] As highlighted by Crocker et al.,[54] different mechanistic processes have been reported to destabilize the nitramine cycle of RDX, HMX, and CL-20, which form similar endpoint degradation products. The initial transformations are (i) 2e$^-$-reduction of nitro functional groups to form nitroso intermediates, (ii) denitration reaction from 1e$^-$-reductive pathway and free radical formation, (iii) denitration reaction from hydride ion transfer, (iv) enzymatic α-hydroxylation of methylene C–H bonds, and (v) direct enzymatic cleavage of inner C–N and N–NO_2 bonds.[54]

Under aerobic conditions, the bacterial isolates *Williamsia* sp. strain KTR4 and *Gordonia*sp. strain KTR9 are able to utilize RDX as the sole source of nitrogen, carbon, and energy,[58] giving selective advantages to the degrading microorganisms at sites highly contaminated by RDX. This is particularly relevant in the field since soil microorganisms are commonly limited by inorganic nitrogen, even on relatively fertile sites.[59] The denitration pathway releases two nitro groups per mole of RDX via two successive 1e$^-$-transfer reactions and free radical formation. Subsequently, the di-denitrated, unstable imine intermediate undergoes hydrolytic ring cleavage.[54] It has been further suggested that this pathway is catalyzed by a novel fused flavodoxin (Fldx)—cytochrome P450 (XplA) in conjunction with a flavodoxin reductase (FdR) (XplB). Halasz et al.[60] also reported that the XplA-XplB system is able to degrade the nitroso derivatives of RDX. The XplA and its reductase partner XplB are both encoded by plasmid-borne genes (*xplA* and *xplB*, respectively) initially identified by Seth-Smith et al. in *Rhodococcus rhodochrous* 11Y.[61–63] Indest et al.[64] have characterized a 182-kb plasmid, pGKT2, from *Gordonia* sp. KTR9 containing *xplA*, *glnA-xplB* (encoding a glutamine synthetase (GS)-XplB fusion protein) and *xplR* (encoding a GntR-type transcriptional regulator). The XplA (CYP177A1), in which the cytochrome P450 is fused to a Fldx domain at its N-terminus, is the first example of the novel class VI cytochrome P450 system.[65] The recombinant glutathione *S*-transferase (GST)-tagged XplB is able to transfer electrons from NADPH to XplA and 1.3 equivalents of NADPH are required to degrade 1 equivalent of RDX.[66] However, even if the rate of RDX degradation is lower, XplB is not necessary for the activity of XplA.[67] This partly explained why the RDX biodegradation rate of strain KTR9 is lower than that observed with strain 11Y, since one single mutation in *xplB* of strain KTR9 was shown to inactivate XplB and the fusion of GS and XplB detected in strain KTR9 could result in increased inhibition of reductase activity.[68].The XplA–XplB degrading system is widely distributed within the order *Actinomycetales* (e.g., *Rhodococcus*, *Gordonia*, and *Williamsia* sp.). Interestingly, these genes have been found to be plasmid encoded, suggesting recent horizontal gene transfer.[69] The highly conserved nature of *xplA–xplB* argues for a single evolutionary origin of this operon prior to its global distribution.[62] The XplA–XplB enzymatic system catalyzes the reductive denitration of RDX under both aerobic and anaerobic conditions to generate, respectively, (1) the aliphatic nitramine 4-nitro-2,4-diazabutanal (NDAB), two nitrite ions, and one equivalent of formaldehyde; and (2) the RDX breakdown product methylenedinitramine (MEDINA), one nitrite ion, and two equivalents of formaldehyde[69,70] (Fig. 3C). The discovery of a cytochrome P450 that catalyzes reductive denitration of RDX is not expected since the activity of cytochromes P450 is by definition associated with monooxygenase

attack. The possibility of a direct hydroxylation of RDX catalyzed by a rabbit liver cytochrome P450 has been excluded using radiolabeled $^{18}O_2$, suggesting similar catalytic behavior of XplA.[71] XplA-heme shows a highly unusual active site environment for substrate binding in comparison to other P450 heme domains.[69] Whereas the structure of common cytochrome P450 harbors specific, conserved active site amino acid residues necessary for oxygen activation and substrate oxygenation,[69] the specific heme environment of XplA (e.g., Met-394 and Ala-395 in place of the well-conserved Asp/Glu and Thr/Ser) suggests an oxygen-independent electron transfer for reductive denitration of RDX. Bui et al.[72] have investigated the spectroscopic and thermodynamic properties of XplA and reported unusual structural and ligand-binding properties (e.g., high P450 affinity for imidazole and weak flavodoxin FMN binding) which can explain the adaptation of XplA as a specialized RDX reductase catalyst. Because of the RDX ring cleavage, the release of a microbial nitrogen source and the degradation of RDX nitroso products, this enzymatic system holds great promises for the bioremediation of different RDX-contaminated sites. However, Indest et al.[64] have reported that the expression of the *xplAB* genes is repressed in the presence of competing inorganic nitrogen sources which could require the monitoring of nitrogen species during RDX bioremediation trials. Transcriptomic studies using custom *Gordonia* sp. strain KTR9 microarrays confirmed that nitrogen limitation induces RDX degradation catalyzed by the XplA-XplB system.[73]

In an XplA-independent biodegradative pathway, Zhao et al.[74] have reported the anaerobic growth of the psychrophile isolate *Shewanella halifaxensis* HAW-EB4 on RDX as the sole terminal electron acceptor through the NADH-dependent RDX reductive denitration catalyzed by an RDX-inducible *c*-type cytochrome. Product analysis showed that RDX was degraded by a mono-denitration pathway to give MEDINA and formaldehyde.[74] However, in contrast to the aerobic degradation of RDX catalyzed by *xplA*-bearing microorganisms (e.g., *R. rhodochrous* 11Y) which is exclusively supported by reductive denitration,[63] *S. halifaxensis*HAW-EB4 utilizes both the nitro group $2e^-$-reduction pathway and the denitration pathway for initial metabolism of RDX. Since *S. halifaxensis* HAW-EB4 was isolated at a UXO-contaminated deep cold marine sediment site and harbors a novel, specific *c*-type cytochrome involved in anaerobic RDX metabolism, this discovery is of great interest not only because it extends the catalog of RDX-degrading enzymes, but also because *in* situ bioremediation using microorganisms that colonize different niches can be envisaged. Perrault et al.[75] also reported anaerobic RDX degradation catalyzed by the cytochrome *c* CymA of *Shewanella oneidensis* MR-1 using two different pathways, i.e., (1) sequential reduction of the nitro groups to the nitroso groups or (2) denitration followed by ring cleavage.

Finally, Fuller et al.[76] have investigated the catalytic rates and substrate specificities of two xenobiotic reductases (XenA of *Pseudomonas putida* II-B (type I OYE hydride transferase) and XenB of *Pseudomonas fluorescens* I–C (type II OYE hydride transferase)) of the OYE family, previously characterized to transform TNT.[77] XenA exhibits a five times lower activity toward TNT than XenB and catalyzes only reduction of the nitro groups of TNT, whereas XenB is able to reduce TNT via two reductive reactions in two competing pathways within the oxidative half-reaction of the enzyme, that is, the ubiquitous nitro moiety reduction or the specific nucleophilic addition of hydride ions to the TNT aromatic ring.[77] As observed for the biodegradation of RDX catalyzed by the purified XplA-XplB system, the endpoint TNT biodegradation products produced by XenB are mainly governed by the redox state of the reaction medium. For instance, the release of nitrite ions from TNT and the production of denitrated dimeric isomers are observed only in the presence of oxygen or under anaerobic conditions in the presence of an oxidizing agent such as $NADP^+$.[77] Therefore, Fuller et al.[76] have investigated the biodegradation of RDX by XenA and XenB and the effects of different initial oxygen tensions. Under anaerobic conditions, XenA and XenB are able to degrade RDX with the formation of MEDINA and formaldehyde as major derivatives. Although XenB catalyzes the transformation of RDX $\sim$ 30-fold faster than XenA, both enzymes exhibit a similar O_2 concentration–dependent inhibition of RDX transformation. As RDX transformation occurs under different redox conditions (anoxic to anaerobic), in situ bioremediation of RDX-contaminated sites with different redox niches can be envisaged. The reductase activity of the PETN reductase toward RDX and HMX has also been reported using purified enzymes.[78] For instance, the reduction of RDX by PETN reductase leads to the release of one equivalent of nitrite per one equivalent of NADH oxidized. Although PETN reductase is able to catalyze both $1e^-$ and $2e^-$-reduction of oxygen with $\sim$ 33% formation of superoxide, Nivinskas et al.[78] have reported that the reductive denitration of RDX by PETN reductase occurs without the significant formation of free radicals, which could be reoxidized by molecular oxygen with the formation of superoxide (futile cycle). Even if these reports expand the range of substrates of the OYE family, these reactions proceed at slow rates in comparison to the XplA–XplB system and the identification of the endpoint degradation products needs to be thoroughly evaluated.

For the cyclic nitramine HMX, very low specific activities have been detected with XenB and XenA.[76] In addition, no activity of XplA was detected under aerobic conditions in the presence of HMX as the substrate.[70] The inherent differences in chemical structures and physicochemical properties of HMX and RDX (**Figs. 1** and **2**) can explain the dissimilar biodegradation patterns and the plausible lower interactions of HMX with the active sites of the enzymes. Due to its lower water solubility and its chemical stability, HMX is less amenable to biodegradation than RDX, and no specific purified enzyme involved in the biodegradation of HMX has been detected so far. However, the different biodegradation pathways of HMX reported in the literature can lead to the formation of formic acid, formaldehyde, and inorganic nitrogen species. For instance, under anaerobic conditions, HMX is mainly transformed to nitroso derivatives before ring cleavage.[54] The denitration reaction of HMX from $1e^-$-reductive pathway and free radical formation has also been reported using a xanthine oxidase in the presence of xanthine or NADH.[79] Under aerobic conditions, HMX denitration activity catalyzed by xanthine oxidase is lower than that observed under anaerobic conditions, suggesting the involvement of an initial oxygen-sensitive process.

For the cyclic nitramine CL-20, common nitroso derivatives can also be formed, which destabilize the CL-20 structure and further promote ring cleavage to produce formic acid, glyoxal, and inorganic nitrogen species.[54] Evidence for the $1e^-$-reduction/denitration of CL-20 has been reported in the presence of NADH and salicylate 1-monooxygenase.[80] Similar to the transformation of HMX by xanthine oxidase,[79] molecular oxygen can inhibit CL-20 biotransformation through (1) reaction with the CL-20 anion

radical to form a superoxide radical and the original nitro group and (2) competition with CL-20 at the FAD site of the monooxygenase enzyme where O_2 can be reduced.[80] In another report, Bhushan et al.[81] have used a diaphorase from *Clostridium kluyveri* to demonstrate hydride transfer mechanism from NAD(P)H to CL-20, followed by denitration and further ring cleavage.

In conclusion, although several reports have demonstrated the mineralization of RDX, HMX, and CL-20 by pure or mixed cultures with the identification of various intermediates and endpoint degradation products,[14,54] very limited information is available on the biochemistry and genetics of the biodegradation of cyclic nitramines. As an initial attack of the cycle is sufficient to disrupt the nitramine compound with subsequent water-driven decomposition of intermediates, the discovery of new specific enzymes that initiate degradation of RDX, HMX, and CL-20 could enhance the implementation of bioremediation processes.

6.14.4 Nitroester and Nitroalkane Explosives

Since the discovery of nitroglycerin (NG), also called glycerol trinitrate (GTN), by Ascanio Sobrero in 1846, the extensive development of nitroester explosives has led to their widespread use for military applications, primarily PETN, the only solid nitroester explosive besides nitrocellulose.[82] In addition, nitroester compounds, such as NG and PETN, have also been widely used as vasodilators for the treatment of acute and chronic angina and congestive heart failure. Indeed, when employed at clinically relevant concentrations (<1 μM), their bioactivation leads to effective vasodilation.[83] At higher levels, nitroesters are toxic and remediatory action is required when they are released into the environment because of their ecotoxicological risk profiles.[84,85]

Nitroalkane explosives have not been used as widely as other classes of nitro explosives. Nevertheless, many polynitroaliphatic compounds are powerful explosives like the explosive 2,2-dinitropropane, which is characterized by an energetic power higher than that of TNT.[1] However, a major drawback of polynitroaliphatic explosives is the cost and availability of raw materials. In comparison, nitroaromatic, nitramine, and nitroester explosives are synthesized in the presence of cheap and readily available reagents like mixed acid (sulfuric and nitric acids mixture).[1] With the demand for more powerful explosives of high thermal and chemical stability, the next-generation nitroalkane explosives belonging to polynitrocycloalkanes, such as ONC, have been developed.[1] In contrast to the environmental fate of nitroester explosives, which has already been reviewed,[85] limited information is available on the biodegradation and toxicity of nitroalkane explosives.

6.14.4.1 Environmental Transport Processes of Nitroester and Nitroalkane Explosives

Because of the low soil retention capacity of nitroester explosives, they are mobile in the subsurface and may threaten groundwater and drinking water facilities. Particularly, NG and ethylene glycol dinitrate (EGDN) are highly soluble in water in comparison to other nitro explosives, and thus they can easily contaminate surface water and groundwater. Due to its lower log K_{ow} value, EGDN is expected to weakly interact with the natural subsurface constituents, which makes it even more mobile in the subsurface than NG and PETN. Finally, the derivatives of nitroester explosives can be more soluble than the parent compound (e.g., 8000 mg L^{-1} for glycerol-1,2-dinitrate and 1950 mg L^{-1} for NG).

Nitroalkane explosives are also expected to have a very high leaching potential, even though no reliable data on sorption of nitroalkanes to natural subsurface constituents are available. For example, 2-nitropropane (2-NP) is susceptible to contaminate surface water and groundwater because of its high aqueous solubility and low log K_{ow} value (**Fig. 2**).

6.14.4.2 (Bio)degradation Pathways of Nitroester and Nitroalkane Explosives

In contrast to nitro moiety reduction of nitroaromatic and nitramine explosives, which can generate nitroso, hydroxylamino, and amino derivatives, the reduction of the nitroester C–O–NO_2 moiety leads to the production of the corresponding organic alcohol (R–OH) product with the release of nitrite ions.[84]

Even though NG has been discovered more than 150 years ago, Husserl et al.[84] are the first authors to report, in 2010, the growth of a pure bacterial culture in the presence of NG as the sole source of carbon and nitrogen. Using classic liquid culture enrichments inoculated with a NG-contaminated soil sample, the authors isolated *Arthrobacter* sp. strain JBH1 for its capacity to convert NG to glycerol via 1,2-dinitroglycerin and 1-mononitroglycerin. Because this strain exhibits reductive denitration activity in the pH range of 5.1–7.3 and maintains biodegradation activity at high concentrations of NG (1.2 mM), this isolate could be relevant for further bioremediation endeavors of NG-contaminated environments. Subsequently, Husserl et al.[86] identified a NADPH-dependent NG oxidoreductase, PfvC, from the OYE family that selectively produces 1-MNG from NG and an ATP-dependent mononitroglycerol kinase (MngP) that transforms 1-MNG to glycerol which can be used by *Arthrobacter* sp. strain JBH1 as the sole source of carbon. Bioaugmentation with *Arthrobacter* sp. strain JBH1 was then investigated in saturated porous media and NG mineralization was detected in the absence of other sources of carbon and nitrogen.[87] On the contrary, a number of authors have reported the bacterial cometabolic denitration of nitroester explosives utilized as the sole source of nitrogen with the production of partially denitrated metabolites as dead-end products.[88] The enzymes involved in this cometabolic denitration have been thoroughly characterized, such as glycerol trinitrate reductase (NerA) from *Agrobacterium radiobacter*[88] or PETN reductase,[78] from the broad-specificity OYE family. The broad substrate range was confirmed for PETN reductase by its ability to denitrate 15 different nitroester compounds.[78] However, complete denitration of nitroester explosives to the corresponding alcohol compound, such as glycerol from NG, has never been reported for (1) the purified members of the OYE family or (2) the corresponding microbes expressing them.

Finally, limited information about the biodegradation of nitroalkane explosives is available in the literature, so they deserve careful consideration.[89] Nishino et al.[8] have recently reported the utilization of the naturally-occurring toxin 3-nitropropionic acid (NPA) as the sole source of carbon, nitrogen, and energy by *Cupriavidus* sp. strain JS190 and *Pseudomonas* sp. strain JS189. Even if NPA is not an explosive, the deciphering of the biodegradation pathways of this compound is useful to investigate the complete biodegradation of its structural analogs. The NPA mineralization occurs through *aci*-nitro tautomerism followed by the oxidative attack of proprionate-3-nitronate (P3N) catalyzed by a P3N monooxygenase (P3NO). The newly formed malonic semialdehyde (MSA) metabolite is then decarboxylated by an orphan oxidative decarboxylase enzyme to acetyl-CoA, which enters the TCA cycle for complete oxidation to CO_2 and H_2O. In addition, Nishino et al.[8] have shown that P3NO and closely related enzymes belong to a separate cluster of COG2070, formerly referred to as 2-NP dioxygenase-like proteins.

Such recent knowledge has allowed classification of the nitroalkane-oxidizing flavoproteins into three types: (1) the family of nitroalkane oxidase (NAO) (EC 1.7.3.1) from the fungus *Fusarium oxysporum*; (2) the family of nitronate monooxygenase (NMO) (EC 1.13.12.16, COG2070) from *Pseudomonas aeruginosa* (PA1024, http://www.pseudomonas.com) and the yeasts *Neurospora crassa* or *Williopsis saturnus var. mraki*[90]; and (3) the family of P3NO of *Cupriavidus* sp. strain JS190, *Pseudomonas* sp. strain JS189, or *P. aeruginosa* (PA4202).[8,91] In contrast to P3NO, NAO and NMO have broad substrate ranges and preferentially oxidize nitroalkanes over NPA. The NAO and NMO members are active on a broad range of substrates containing primary and secondary nitro groups. However, the NAO members oxidize exclusively neutral nitroalkanes, whereas the NMO members can effectively oxidize anionic alkyl nitronates.[8,91] The reader is referred to recent articles on the NAO and NMO families for additional mechanistic and structural properties.[8,91,92]

Although the oxidation of mononitroaliphatic explosive compounds, such as 2-NP, has been known for a long time through the catalytic activity of the aforementioned nitroalkane-oxidizing flavoproteins,[91] the enzymatic degradation of polynitroaliphatic compounds, such as 2,2-nitropropane, has never been reported. In this context, the screening of nitroalkane-oxidizing enzymes for the degradation of different nitroalkane explosives could be promising. Similarly, the environmental fate of ONC, a caged cubane which has not yet been used as an explosive, needs to be assessed before its potential use as a military explosive.

6.14.5 Bioremediation of Environments Contaminated by Nitro Explosives

Although several bacteria have been isolated to utilize TNP, RDX, or NG as the sole source of nitrogen and carbon, no in situ microbial mineralization-based technologies have been implemented for any nitro explosives. The biodegradation pathways beneficial for microorganisms in the laboratory have commonly failed at the field scale and did not generate a high enough biomass to maintain a prolonged catabolic activity and support an effective removal of nitro explosives. On the other hand, with the exception of powerful ligninolytic peroxidases secreted by white-rot fungi (but unable to tolerate high concentrations of TNT and to outcompete indigenous microbial populations),[93] no significant mineralization of TNT has been detected in biological systems. Conversely, the widespread capacity of microorganisms to transform TNT into reduced metabolites has deviated investigations toward *ex situ* microbial immobilization technologies, such as bioslurry, windrow composting, and landfarming (e.g., DARAMEND) processes. Although these processes are simple and cost-effective and the covalent binding of the reduced TNT metabolites from nitro moiety reduction has been demonstrated using ^{15}N-TNT- and ^{13}C-enriched organic matter, the immobilization technologies designed for soil matrix led to substantial scientific debate. For instance, significant transport of potentially toxic TNT derivatives in the subsurface can take place by leaching of DOM (including the so-called colloidal organic matter (COM)).[26] Therefore, whether immobilization-driven remediation of TNT-contaminated soils can be considered as an acceptable solution or not remains an open-ended question.

The remediation of contaminated sites can benefit from a few enzymatic systems that have emerged as capable of catalyzing the degradation of different classes of nitro explosives, including the OYE family, the F_{420}-dependent hydride transferases of the *npd* gene cluster, or the XplA–XplB system. However, bioremediation endeavors that exploit microorganisms harboring these biological activities in the field (i.e., natural attenuation, biostimulation, or bioaugmentation) have been scarce and commonly led to failures in comparison to the results obtained at the laboratory scale. Beyond the field conditions characterized by a multiscale physicochemical and biological complexity, the long-term persistence of TNT in a contaminated site is mainly due to the inability of microorganisms to use it as a growth substrate. In addition, community-based analysis of TNT-contaminated environments shows a very low microbial and plant species richness and evenness,[94] which directly affects the functionality and stability of a given ecosystem. The TNT-degrading isolates are commonly copiotrophic, fast-growing bacteria (typically *r*-strategists, such as *Pseudomonas* sp.), whereas oligotrophic, slow-growing bacteria (*k*-strategists), such as the *Acidobacteria* (the second most abundant phylum in soils), are severely affected by TNT.[95] Furthermore, as TNT biotransformation pathways are governed by cometabolism, the addition of primary substrate(s) is required for the induction of TNT biodegradation. Multiple competitive pathways with the cometabolic reactions and the cometabolic energy demands can make the microorganisms inefficient to detoxify a TNT-contaminated environment. Therefore, in spite of the predominance of *Pseudomonadaceae* (which harbors a pool of highly conserved type II OYE homologues) in TNT-contaminated sites, the biodegradation of TNT is misrouted toward products of nitro moiety reduction, with a similar or greater toxicity levels than the parent molecule, followed by multiple interactions with the environmental matrix.

Because TNP is highly mobile in soils, it is not persistent in the subsurface and is commonly found as a surface water and groundwater contaminant. Although TNP mineralization has been demonstrated for a few actinomycetal strains and homologous *npd* genes were identified in other TNP-degrading strains of *Rhodococcus*,[96] only one report demonstrates the successful application

of bioaugmentation for TNP-contaminated wastewater or groundwater in aerobic sequencing batch reactors.[97] Using *Rhodococcus opacus* strain JW01, efficient TNP removal below drinking water standards and significant release of nitrogen from the parent compound were accomplished.

Similar to TNP, RDX frequently leaches out in the soil profile and is a common groundwater contaminant. In spite of the frequent detection of the XplA–XplB system in RDX-contaminated military sites,[62] biodegradation processes catalyzed by the indigenous microbial community seem to be carried out at rates not fast enough to protect groundwater contamination. In addition, TNT is a potent inhibitor of RDX biodegradation catalyzed by the XplA–XplB system.[70] Since TNT and RDX are commonly detected as co-contaminants, bioremediation of explosive-polluted sites is very challenging. Sagi-Ben Moshe et al.[98] reported the inhibition of anaerobic RDX/HMX biodegradation in contaminated soil samples in the presence of TNT. Indeed, TNT and its metabolites are toxic for the native RDX- and HMX-degrading bacteria, which prevent simultaneous biodegradation of TNT, RDX, and HMX in a polluted soil.[98] Recent evaluation studies of bioaugmentation of RDX-contaminated groundwater have been carried using microorganisms bearing the XplA (cytochrome P450 (Class VI)) and the XenB (Type II OYE hydride transferase).[15,17,99] First, Crocker et al.[17] demonstrated the viability and effective transport of RDX-degrading strains within the aquifer of the Umatilla Chemical Depot NPL site. Subsequently, a field demonstration at the same contaminated site validated the bioaugmentation strategy for different injection wells with the strain *Gordonia* sp. KTR9 and provided guidance to strategies for in situ RDX biodegradation supported by *xplA*-bearing strains.[15] This approach was further tested in reactor columns using RDX-contaminated aquifer materials from a US Navy site.[99] Bioaugmentation with *Gordonia* sp. KTR9 (bearing XplA) and *Pseudomonas fluorescens* I–C (bearing XenB) combined to biostimulation with fructose showed effective RDX biodegradation at different dissolved oxygen levels, which holds great promise for in situ bioaugmentation strategies of RDX-contaminated sites under fluctuating redox conditions.[99]

As an alternative to in situ bioaugmentation strategy,[100] Bruce and colleagues have proposed to combine the catabolic versatility of microorganisms with the high biomass and stability of plants in order to enhance the rehabilitation of sites contaminated by nitro explosives.[101] In this context, seeds from transgenic tobacco (*Nicotiana tabacum*) plants expressing *onr* coding for PETN reductase were able to germinate and grow in the presence of inhibitory concentrations of GTN (1 mM) and TNT (0.05 mM) for the wild-type seeds.[101] In addition, transgenic *Arabidopsis thaliana* plants expressing *xplA* remove and detoxify RDX from liquid media and can tolerate an RDX concentration in soils eight times higher than that tolerated by untransformed plants.[102] Subsequently, Jackson et al.[70] have generated transgenic *A. thaliana* plants expressing both *xplA* and *xplB* that remove RDX at saturating concentrations from soil leachate at a higher rate than wild-type plants and *xplA*-expressing transgenic lines. Finally, Bruce and colleagues have proposed to engineer transgenic plants expressing genes coding for both TNT-detoxifying enzymes and RDX-degrading enzymes to rehabilitate sites contaminated by explosive mixtures.[103] To date, this strategy remains the single, viable remedial process that has been proposed to simultaneously degrade nitro explosives present in a contaminated field site. Phytoremediation of TNT-contaminated sites could also benefit from the recent discovery of Johnston et al.[104] who identified the pro-oxidant activity of monodehydroascorbate reductase 6 (MDHAR6) of *Arabidopsis thaliana* in the presence of TNT with the generation of reactive oxygen species responsible of the acute phytotoxicity of TNT.[104] *A. thaliana* mutants (*mdhar6-1*) impaired in monodehydroascorbate reductase exhibited enhanced TNT tolerance which can be a promising research direction for phytoremediation of TNT-contaminated sites using plants with MDHAR6 deficiency.

Furthermore, the development of selective and sensitive biomonitoring tools to evaluate the performance of a bioremediation process is of equal importance, including (1) strain- and function-specific biomarkers and (2) biosensing circuits for environmental monitoring of specific analytes. For instance, since almost all of phylogenetically and geographically distinct bacteria that use RDX as a nitrogen source harbor the highly conserved *xplA–xplB* RDX degradation system, the *xplA* functional gene could be used as a key biomarker (1) to assess the biodegradative capability of a given RDX-contaminated environment, (2) to link the catabolic activity of the target RDX-degrading organisms with process performance and (3) to monitor the viability and transport of the bioaugmentation strains during in situ bioremediation. The integration of innovative biosensors in monitoring programs of bioremediation is also of high importance. Taking advantage of the promiscuity and broad effector (relaxed) specificity of transcriptional regulators such as XylR,[105] NtdR[106] and DntR,[107] 2,4-DNT- and TNT-responding variants of transcriptional regulators can be generated in order to design specific bioreporter genes (e.g., TNT-responding XylR variant and binding onto the *xylR* promoter (*Pu*) fused with *gfp*). In this way, the effector profile of the LysR-type transcriptional regulator NtdR can be altered (amino acid substitution(s)) to efficiently respond to various nitroaromatic compounds including TNT.[106] Lönneborg et al.[107] also reported the production of a DntR variant with greatly improved response toward 2,4-DNT. Similarly, reintroduction of XylR variants responsive to 2,4-DNT into *P. putida* strains bearing a transcriptional fusion between the *Pu* promoter and a reporter gene has also been reported.[105] This work, in combination with implantation of orthogonal sensor circuits in *P. putida*,[108] was successfully used for the detection of 2,4-DNT residues in a soil microcosm. These effector-responsive biosensing circuits are well suited for optical output detection because the most common reporter proteins are either luminescent (e.g., luciferase) or fluorescent (e.g., green fluorescent protein (GFP)).[109] However, since this detection mechanism relies on transcriptional activation, the maintenance of engineered cells in a growth state is necessary for biomonitoring, in addition to the consideration of potential ecological impacts from the environmental release of such genetic constructs. Therefore, de las Heras et al.[108] have developed an orthogonal genetic platform for (1) the stable chromosomal incorporation of sensing circuits in whole-cell bioreporters and (2) the safe environmental release of the constructed whole-cell bioreporters by deleting antibiotic resistance genes. This innovative strategy, in combination with techniques to increase the long-term maintenance and the viability of the whole-cell bioreporters,[110] could be valuable for biomonitoring programs during bioremediation of TNT-contaminated soils.

6.14.6 Conclusion and Perspectives

Because biodegradation data of nitro explosives have been obtained under laboratory incubations, mostly with pure cultures, the crucial issue of the in situ bioremediation process is to scale-up, control, and anticipate key biodegradative processes from the laboratory (e.g., reactions operating at volumes from that of the microbial cell (0.1–2 μm^3) to that of the spinner flask (500 cm^3)) to open large-scale ecosystems (e.g., >50 m^3 of contaminated soils). Therefore, the bioremediation of contaminated sites in the field is challenging in terms of accessibility, heterogeneity, predictability, dynamics of catabolic microbial populations, and process monitoring. Specifically, military-related sites are commonly contaminated by different classes of nitro explosives with dissimilar transport/distribution into the environment. For instance, nonspecific hydrophobic and/or specific interactions make TNT as well as its reduced metabolites significant sorbents on environmental matrices and thus contaminants of soils and sediments. Furthermore, strong adsorption of reduced TNT metabolites to DOM leads to enhanced transport in the subsurface and substantial contamination of water ecosystems. Other nitro explosives such as RDX and TNP are highly mobile in soils and are therefore common groundwater contaminants. In this context, the development of transgenic plants harboring higher catabolic capabilities than wild-type plants and the use of durable and robust root systems have emerged as the most relevant strategy to control contamination of explosives.

Few bacterial isolates can utilize nitro explosives (i.e., TNP,[47] NG[84] and RDX[58]) as the sole source of nitrogen, carbon, and energy, and the responsible enzymes have been characterized for some catabolic reactions. Particularly, bacteria belonging to the order *Actinomycetales* (e.g., *Rhodococcus* or *Gordonia* sp.) are the most promising nitro explosives-degrading microorganisms and could prove suitable for the simultaneous bacterial mineralization/assimilation of TNP (F_{420}-dependent HTI/II) and RDX (XplA–XplB system). On the other hand, members of the OYE family have particularly drawn our attention for their broad substrate range and diverse categories of transformations they catalyze, specially the reduction of nitroaromatic, nitramine, as well as nitroester explosives. However, OYE members catalyze only partial degradation of TNT and nitroester compounds to produce dead-end metabolites or their activities are very low as observed for RDX and HMX. Consequently, OYE members will be preferentially used to generate transgenic plants with higher tolerance to nitroaromatic or nitroester explosives than wild-type plants.

Whereas one-electron transfer is necessary and sufficient to cause N-denitration of RDX and HMX and further spontaneous decomposition to C-1 compounds,[71,80] the highly stabilized aromatic ring of TNT as well as the very diverse chemical and metabolic misrouting (with production of suicide and/or dead-end metabolites) prevent its mineralization. In addition, *xplB* and *xplA* encoding RDX-degrading enzymes are plasmid-borne and can lead to bacterial evolutionary innovation and horizontal gene transfer. Because TNT is highly toxic for RDX-degrading bacteria,[98] bioaugmentation with genes rather than exogenous microorganisms can be a suitable strategy to overcome the inhibitory activity and toxicity of TNT in explosives-contaminated sites and therefore stays independent from the survival and propagation of the donor strain(s). Improved performance can be expected by the transfer of appropriately packaged *xplA* and *xplB* catabolic genes from one or more donor strain(s) to the already fit indigenous microflora.[100] Therefore, plasmid-mediated bioaugmentation in a habitat that allows a higher frequency of catabolic gene transfer as well as higher metabolic activity compared with bulk soils, such as the rhizosphere, holds interesting perspectives for in situ biodegradation of RDX in rhizoremediation or rhizophytoremediation trials.[111] To the best of our knowledge, genes encoding TNT-degrading enzymes discovered so far are only chromosome-borne, such as the chromosomally encoded members of the OYE family. Therefore, the prospecting of the metaplasmidome, referred here as the collective plasmids extracted from a given environmental site, can be a valuable strategy to find new TNT-degrading genes and potential new catabolic reactions toward TNT.

Finally, the characterization of the biodegradative patterns and cytotoxicity mechanisms of nitroaliphatic explosives need to be assessed in order to anticipate their environmental fate as well as their ecotoxicological safety endpoints.[112] In the same way, the synthesis of new nitro explosives with improved energetic performance is still under way, with the recent emergence of ONC or the tetranitrate ester Hisk75.[82] The environmental fate and biodegradability of all these next-generation nitro explosive compounds have to be evaluated.

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