

Biodegradation of RDX and MNX with *Rhodococcus* sp. Strain DN22: New Insights into the Degradation Pathway

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Previously we demonstrated that *Rhodococcus* sp. strain DN22 can degrade RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) aerobically via initial denitration. The present study describes the role of oxygen and water in the key denitration step leading to RDX decomposition using $^{18}\text{O}_2$ and H_2^{18}O labeling experiments. We also investigated degradation of MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine) with DN22 under similar conditions. DN22 degraded RDX and MNX giving NO_2^- , NO_3^- , NDAB (4-nitro-diazabutanal), NH_3 , N_2O , and HCHO with $\text{NO}_2^-/\text{NO}_3^-$ molar ratio reaching 17 and ca. 2, respectively. In the presence of $^{18}\text{O}_2$, DN22 degraded RDX and produced NO_2^- with m/z at 46 Da that subsequently oxidized to NO_3^- containing one ^{18}O atom, but in the presence of H_2^{18}O we detected NO_3^- without ^{18}O . A control containing NO_2^- , DN22, and $^{18}\text{O}_2$ gave NO_3^- with one ^{18}O , confirming biotic oxidation of NO_2^- to NO_3^- . Treatment of MNX with DN22 and $^{18}\text{O}_2$ produced NO_3^- with two mass ions, one (66 Da) incorporating two ^{18}O atoms and another (64 Da) incorporating only one ^{18}O atom and we attributed their formation to bio-oxidation of the initially formed NO and NO_2^- , respectively. In the presence of H_2^{18}O we detected NO_2^- with two different masses, one representing NO_2^- (46 Da) and another representing NO_2^- (48 Da) with the inclusion of one ^{18}O atom suggesting auto-oxidation of NO to NO_2^- . Results indicated that denitration of either RDX or MNX and denitrosation of MNX by DN22 did not involve direct participation of either oxygen or water, but both played major roles in subsequent secondary chemical and biochemical reactions of NO and NO_2^- .

Introduction

RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine, is a man-made cyclic nitroamine widely known for its powerful energetic properties. The chemical is extensively used by the military and in the construction industry (1). Large scale manufacturing and extensive global usage of RDX has led to the

contamination of vast areas of soil, sediment, and water. Environmental contamination by RDX raises serious concerns because the chemical is toxic, and is considered as a possible carcinogen and a potent convulsant (2). Extensive research has been conducted recently to understand biodegradation pathways of RDX under both aerobic and anaerobic conditions. Thus far we know that RDX can be degraded by two distinctive pathways, (1) anaerobic reductive pathway involving sequential reduction of the $-\text{N}-\text{NO}_2$ functional groups to give the corresponding nitroso derivatives MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine), DNx (hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine), and TNx (hexahydro-1,3,5-trinitroso-1,3,5-triazine) (3, 4), and (2) a denitration route involving the cleavage of the $-\text{N}-\text{NO}_2$ bond(s) leading to decomposition of the energetic chemical and the formation of benign products such as nitrite, ammonia, HCHO and HCOOH (5–9). The denitration route also leads to the formation of the two characteristic intermediates 4-nitro-2,4-diazabutanal (NDAB, $\text{NO}_2\text{NHCH}_2\text{NHCHO}$) and methylenedinitramine (MEDINA, $\text{NO}_2\text{NHCH}_2\text{NHNO}_2$) whose formation depends on the stoichiometry of the denitration step (Supporting Information (SI) Figure S1). RDX produces NDAB following the loss of two nitrite anions prior to RDX ring cleavage as we observed during aerobic treatment of RDX with the cytochrome P450 XplA/B system from *Rhodococcus* sp. (5, 7, 10).

On the other hand monodenitration of RDX often leads to the predominant formation of MEDINA as observed following RDX reduction with zerovalent iron, ZVI (11), and nano iron particles (12) or during anaerobic treatment with diaphorase (13), nitrate reductase (14), *Shewanella halifaxensis* (15) and *Geobacter metallireducens* (16). However NDAB was produced from monodenitrated RDX under mild alkaline hydrolysis (17). NDAB is stable in water under ambient conditions but MEDINA is unstable and decomposes to N_2O and HCHO (18, 19). Both MEDINA and NDAB are extremely soluble in water and only recently have been successfully detected because of their rapid elution during HPLC analysis.

The above discussion clearly demonstrates that an RDX degradation pathway triggered by denitration would be the preferred choice for the development of remediation technologies. To gain more insight into the initial denitration step involved in RDX degradation and the subsequent abiotic and biotic reactions that follow the denitration step we investigated biodegradation of RDX with *Rhodococcus* sp. strain DN22 in the presence of $^{18}\text{O}_2$ and H_2^{18}O and searched for ^{18}O -containing products to determine their role in the degradation process. We also investigated biodegradation of MNX, the most common nitroso derivative of RDX (20, 21), with DN22 to determine which of the two $\text{N}-\text{NO}$ and $\text{N}-\text{NO}_2$ bonds is preferentially cleaved. We hope that new knowledge gained on each of the initial denitration step(s), subsequent secondary reactions and products distributions would provide new insights into the degradation pathway of RDX and thus help in the development of effective in situ remediation technologies.

Materials and Methods

Chemicals. RDX (99% pure) and ring-labeled [^{15}N]-RDX were provided by the Defense Research and Development Canada (DRDC), Valcartier, Canada. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX; 98% pure), ring-labeled [^{15}N]-MNX, 4-nitro-2,4-diazabutanal (NDAB; 99% pure), and methylenedinitramine (MEDINA) were purchased from SRI International. ^{18}O -labeled oxygen (minimum, 99 atom %) and ^{18}O -labeled water (97 atom %) were purchased from Isotec

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14. ABSTRACT Previously we demonstrated that Rhodococcus sp. strain DN22 can degrade RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) aerobically via initial denitration. The present study describes the role of oxygen and water in the key denitration step leading to RDX decomposition using ¹⁸O₂ and H₂¹⁸O labeling experiments. We also investigated degradation of MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine) with DN22 under similar conditions. DN22 degraded RDX and MNX giving NO₂ -, NO₃ -, NDAB (4-nitro-diazabutanal), NH₃, N₂O, and HCHO with NO₂ -/NO₃ - molar ratio reaching 17 and ca. 2, respectively. In the presence of ¹⁸O₂, DN22 degraded RDX and produced NO₂ - with m/z at 46 Da that subsequently oxidized to NO₃ - containing one ¹⁸O atom but in the presence of H₂¹⁸O we detected NO₃ - without ¹⁸O. A control containing NO₂ -, DN22, and ¹⁸O₂ gave NO₃ - with one ¹⁸O, confirming biotic oxidation of NO₂ - to NO₃ -. Treatment of MNX with DN22 and ¹⁸O₂ produced NO₃ - with two mass ions, one (66 Da) incorporating two ¹⁸O atoms and another (64 Da) incorporating only one ¹⁸O atom and we attributed their formation to bio-oxidation of the initially formed NO and NO₂ - respectively. In the presence of H₂¹⁸O we detected NO₂ - with two different masses, one representing NO₂ - (46 Da) and another representing NO₂ - (48 Da) with the inclusion of one ¹⁸O atom suggesting auto-oxidation of NO to NO₂ -. Results indicated that denitration of either RDX or MNX and denitrosation of MNX by DN22 did not involve direct participation of either oxygen or water, but both played major roles in subsequent secondary chemical and biochemical reactions of NO and NO₂ -.		
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Inc., Miamisburg, Ohio. All other chemicals, including NH_2CHO and sodium nitrite, were reagent grade.

Growth of *Rhodococcus* sp. Strain DN22 and Resting Cell Assays. *Rhodococcus* sp. strain DN22, provided by N. V. Coleman, (University of Sydney, Sydney, Australia) was grown in mineral salt medium (MSM) supplemented with succinate (2.4 mM) as the carbon source (5). RDX (220 μM) or MNX (220 μM) was added as the sole nitrogen source from an acetone stock solution. Acetone was evaporated followed by addition of the medium MSM (pH 7). Cultures were incubated at 25 °C with agitation (250 rpm) and growth was monitored at 530 nm as described by Fournier et al. (5).

Resting cell assays were conducted using midlog phase cultures ($A_{530} = 0.60\text{--}0.65$). Cells were harvested at 15 000g, 4 °C, for 15 min using a Sorvall RC6 Plus centrifuge (Thermo Electron Corporation, Milford, MA), then washed and resuspended in MSM to an absorbance of 1.0. No carbon or nitrogen sources were made available except for RDX or MNX (220 μM for both). In some cases NH_4Cl (1.0 mM) was added to prevent the uptake of NO_2^- . The assay bottles were then incubated at 25 °C with agitation (250 rpm) under aerobic conditions (5). Chemical controls containing either RDX or MNX or NO_2^- were prepared in MSM without DN22 cells. Products analyses were conducted as described below. When LC-MS analysis was required, cells were washed and resuspended in deionized water (pH 5.5) to an absorbance of 4.0. For certain cell suspensions, ring-labeled [^{15}N]-RDX or [^{15}N]-MNX (220 μM for both) was used in order to identify metabolites with nitrogen atoms using LC/MS (see below).

To determine the role of oxygen in the denitration of RDX and MNX with DN22 the chemical(s) were incubated with the bacterium in the presence of either H_2^{18}O or $^{18}\text{O}_2$. For tests that required H_2^{18}O , RDX or MNX grown cells were harvested as described above except they were resuspended in H_2^{18}O in the presence of RDX, MNX, or NO_2^- (220 μM , 220 μM , or 430 μM , respectively). For tests that required $^{18}\text{O}_2$, RDX or MNX grown cells were harvested as described above and resuspended in deionized water. Cell suspensions along with solutions of RDX, MNX, or NO_2^- (220 μM , 220 μM , or 430 μM , respectively) in water were flushed with N_2 to remove air. Cell suspensions were then added to the substrate solutions in an anaerobic glovebox. $^{18}\text{O}_2$ was then added as described by Fournier et al. (5). Controls containing either RDX or MNX or nitrite were prepared in water in the presence of DN22 cells and air.

For experiments with NH_2CHO , RDX grown cells were harvested as described above and resuspended in deionized water to an absorbance of 1.28. No carbon or nitrogen sources were made available except for NH_2CHO (230 μM).

Chemical Analysis. Aliquots from the liquid culture media described above were centrifuged at 16 000g for 4 min using an Eppendorf Centrifuge 5415 D (Eppendorf AG, Hamburg) and the supernatant was used for chemical analysis. We determined RDX, MNX, and HCHO by HPLC (22) and NO_2^- , NO_3^- , and HCOOH by ion chromatography (23). Nitrous oxide was analyzed by GC/ECD (22). NH_2CHO was first derivatized by treatment with pentafluorobenzylhydroxylamine (24) and analyzed by LC-MS as its deprotonated molecular mass ion $[\text{M}-\text{H}]^-$. NDAB and MEDINA were determined by HPLC and by comparison with authentic materials (15). NH_4^+ was determined colorimetrically using EPA method 350.1 (25).

^{18}O -labeled NO_2^- , NO_3^- , NDAB coming from either MNX or RDX were analyzed using a mass spectrometer (MS) (Bruker MicroTOFQ mass analyzer) attached to an HPLC system (Hewlett-Packard 1200 Series) equipped with a DAD detector. Aliquots (20 μL) from the previous assays were injected into a 3.5 μm -pore size Zorbax SB-CN column (2.1 mm ID by 100 mm; Agilent, Mississauga, Canada) at 25 °C. The solvent system was composed of 10–80% (v/v) of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ gradient at a flow rate of 0.15 mL min^{-1} . A negative

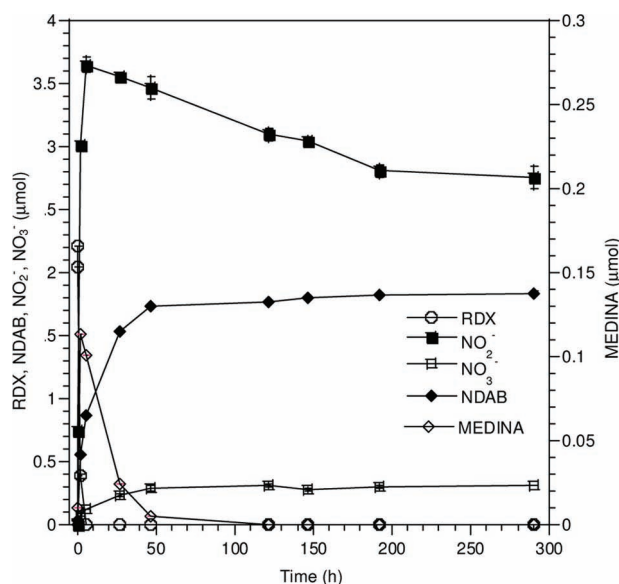


FIGURE 1. Time course of aerobic biodegradation of RDX with *Rhodococcus* sp. DN22

electrospray ionization mode was employed to generate the deprotonated molecular mass ions $[\text{M}-\text{H}]^-$ using a mass range from 30 to 400 Da.

Results and Discussion

RDX Decomposition by *Rhodococcus* sp. Strain DN22: Initial Denitration and Subsequent Secondary Reactions. Previously we found that RDX degradation with *Rhodococcus* sp. strain DN22 produced the key ring cleavage product 4-nitro-2,4-diazabutanal (NDAB, $\text{NO}_2\text{NHCH}_2\text{NHCHO}$) representing 60% of C-content in RDX, carbon dioxide (30% detected as $^{14}\text{CO}_2$), nitrous oxide (N_2O) together with nitrite and ammonia. The C mass balance was approximately 90% (5). In the present study we incubated RDX with DN22 under aerobic conditions and found that the missing 10% C was due to the formation of methylenedinitramine (MEDINA, $\text{NO}_2\text{NHCH}_2\text{NHNO}_2$) which decomposed to HCHO and $2\text{N}_2\text{O}$ (Figure 1).

Although NDAB was formed as a dead end product during RDX aerobic incubation with DN22, our previous studies demonstrated that the chemical can be degraded by *Methylobacterium* (26) and by *Phanerochaete chrysosporium* (27) and under strong alkaline conditions (17). In the present study we successfully hydrolyzed NDAB (20 mg L^{-1}) at pH 12.3 and room temperature and identified N_2O , HCHO, NH_3 , and HCOOH as degradation products. The rate of NDAB hydrolysis was much slower than that of RDX (data not shown). Products distribution suggested that hydrolysis of NDAB occurred via initial OH^- attack at the secondary C in $\text{O}_2\text{NNHCH}_2\text{NHCHO}$ to first produce the imine $\text{O}_2\text{NNHCH}=\text{NCHO}$ that upon reaction with water would initially give the α -hydroxynitroamine $\text{O}_2\text{NNHCH}(\text{OH})\text{NHCHO}$. The latter is unstable in water and should decompose readily to produce NH_2NO_2 (precursor to N_2O), hemiacetal $\text{CH}_2(\text{OH})_2$ (precursor to HCHO) and formamide (precursor to ammonia and HCOOH). We did not detect any nitrite or nitrate anions.

NDAB as we described earlier is recalcitrant to biodegradation by DN22 but NH_2CHO decomposed to NH_3 and HCHO (5). Indeed we found that in the presence of DN22 NH_2CHO biodegraded to first produce NH_3 and HCOOH that were later utilized by the bacteria (SI Figure S2). Abiotic controls containing NH_2CHO in the absence of DN22 did not produce any significant hydrolysis.

As for NDAB and MEDINA formation it is obvious that if RDX loses 2NO_2^- prior to ring cleavage and decomposition it produces NDAB ($\text{O}_2\text{NNHCH}_2\text{NHCHO}$) exclusively; how-

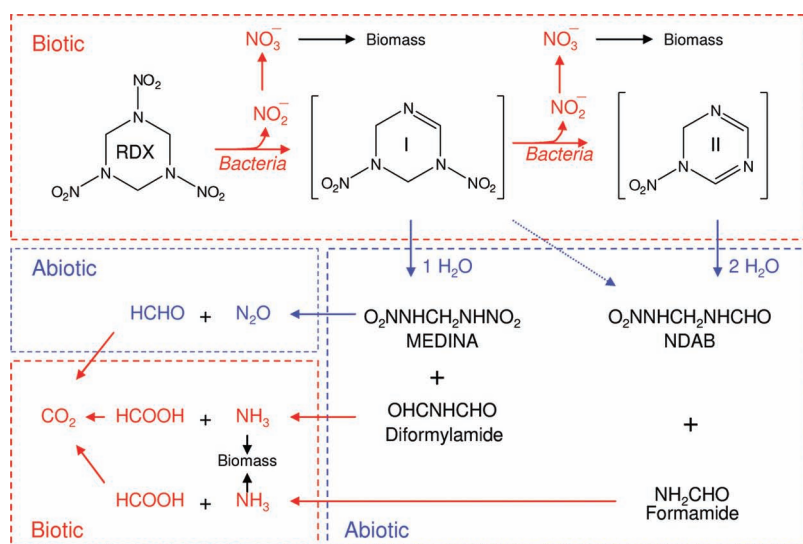


FIGURE 2. Constructed biotic (red) and abiotic (blue) degradation routes of RDX with *Rhodococcus* sp. DN22.

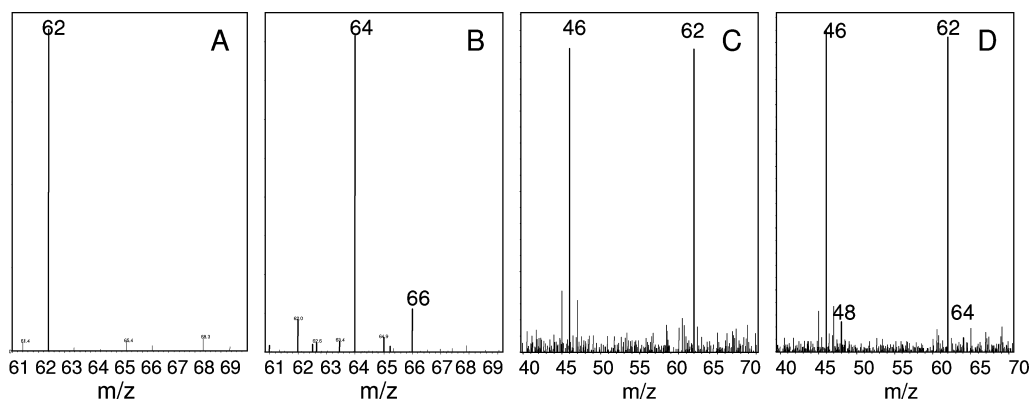


FIGURE 3. Mass spectra of nitrate anion produced from RDX during incubation in aerobic assays containing *Rhodococcus* sp. DN22 in the presence of unlabeled oxygen ($^{16}\text{O}_2$) (A) or $^{18}\text{O}_2$ (B), and mass spectra of nitrite and nitrate anions in the presence of ordinary water (H_2^{16}O) (C) or H_2^{18}O (D).

ever, if RDX loses only 1NO_2^- then both NDAB and MEDINA ($\text{O}_2\text{NNHCH}_2\text{NHNO}_2$) can potentially form (Figure 2). As the above discussion reveals the initial denitration step taking place during RDX incubation with DN22 is the most important step in the whole degradation process. Following denitration, the resulting monodenitrated intermediate I and didenitrated intermediate II follow a cascade of secondary abiotic and biotic reactions to finally give NO_2^- , NO_3^- , NH_2CHO , NH_3 , N_2O , HCHO , HCOOH , and NDAB (Figure 2). As for N_2O we suggest that MEDINA acted as a precursor for its formation.

Figure 1 shows that for each mole of RDX lost 1.7 and 0.1 mol equiv of NO_2^- and NO_3^- , respectively, a molar ratio reaching 17, were detected. Production of nitrate and nitrite was confirmed by LC-MS showing a mass ion at m/z 62 Da and m/z 46 Da, respectively (Figure 3A and 3C). When we incubated RDX with DN22 in the presence of $^{18}\text{O}_2$ we detected NO_2^- , with a mass ion at m/z 46 Da confirming that NO_2^- originated from RDX without the inclusion of any ^{18}O . Furthermore, we detected two mass ions, one with a major intensity at m/z 64 Da representing NO_3^- with the inclusion of 1^{18}O atom and a less intense ion with m/z at 66 Da representing NO_3^- with the inclusion of 2^{18}O atoms (Figure 3B). A control containing NO_2^- and DN22 in the presence of $^{18}\text{O}_2$ gave NO_3^- with two m/z values, one at 64 Da and another at 66 Da as was the case with NO_3^- detected during incubation of RDX with DN22. An abiotic control containing NO_2^- and oxygen in the absence of DN22 did not give NO_3^- . We concluded that the NO_3^- detected during RDX incubation

with DN22 resulted from bio-oxidation of the NO_2^- originally formed from N— NO_2 bond cleavage in RDX. For example, it has been reported that NO_2^- can biologically oxidize to NO_3^- (28, 29). We presumed that the trace amount of NO_3^- that we detected with two ^{18}O atoms originated from biotic oxidation of nitric oxide (NO) (30) produced from NO_2^- at pH 5.5 in sample prepared without phosphate buffer for LC-MS analysis. It has been shown that under slightly acidic conditions NO_2^- can produce NO through disproportionation (31).

Likewise when we incubated RDX with DN22 in the presence of H_2^{18}O we observed NO_2^- with m/z at 46 Da and another mass ion with m/z at 62 Da representing NO_3^- with no ^{18}O involvement (Figure 3D). However we detected a trace amount of the mass ions at m/z 48 and m/z 64 Da representing NO_2^- and NO_3^- with the inclusion of one ^{18}O atom. This experiment indicated that part of NO_2^- originated from RDX might also produce NO (31) which should subsequently auto-oxidize to revert back NO_2^- with one ^{18}O from water (32, 33) prior to biological oxidation to NO_3^- (28). Results from the above H_2^{18}O and $^{18}\text{O}_2$ labeling experiments indicated that RDX denitration does not involve direct participation of either oxygen or water but both water and oxygen play major roles in the subsequent secondary chemical and biochemical reactions of the nitrite anion once released from RDX. However similar to previous studies (5, 6) we found that degradation of RDX with DN22 in the presence of H_2^{18}O produces the ring cleavage product NDAB with the inclusion of one ^{18}O from water (data not shown).

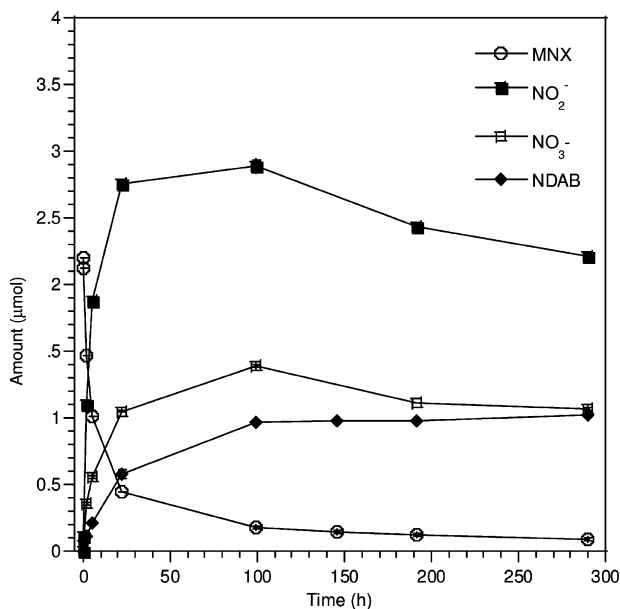


FIGURE 4. Time course of aerobic biodegradation of MNX with *Rhodococcus* sp. DN22.

Degradation of MNX with *Rhodococcus* sp. strain DN22—Denitration versus Denitrosation. MNX has been frequently observed with RDX as a contaminant (20, 21) or as an early degradation product of RDX. For example we detected MNX aerobically during RDX incubation with the fungus *P. chrysosporium* (34) and anaerobically with *Shewanella halifaxensis* (15). To determine the fate of MNX biodegradation with DN22, we incubated MNX with resting cells of DN22 using conditions similar to those employed with RDX. We found that DN22 degraded MNX but at a slower rate than that of RDX, indicating that if MNX had been a degradation product of RDX by DN22, we would have been able to observe it. Also when we incubated MNX with DN22 we did not observe RDX as an oxidized MNX product. This is in line with our earlier observation showing that DN22 is unable to degrade TNX (5).

The disappearance of MNX with resting cells of DN22 was accompanied with the formation of NO₂⁻ and NO₃⁻ (Figure 4) in addition to NH₃, N₂O, HCHO, and HCOOH (data not shown). We also detected NDAB (23%) with approximately one-third its yield from RDX (Figure 4) and a trace amount of an intermediate with a [M-H]⁻ at *m/z* 102 Da matching an empirical formula of C₂H₅N₃O₂. Using ring labeled ¹⁵N-MNX the [M-H]⁻ was observed at *m/z* 104 Da, suggesting the inclusion of two ¹⁵N atoms from the inner aza Ns in MNX. We tentatively assigned the metabolite as the nitroso equivalent of NDAB, that is, 4-nitroso-2,4-diazabutanal (4-NO-DAB, ONNHCH₂NHCHO). Although we were unable to observe MEDINA we detected its suspected decomposition products HCHO that disappeared later and N₂O that persisted (8%). However, both HCHO and N₂O can also be produced from the decomposition of the nitroso derivative of MEDINA, that is, O₂NNHCH₂NHNO (SI Figure S3).

The formation of NDAB requires the initial loss of NO whereas the formation of 4-NO-DAB requires the initial loss of NO₂ before MNX decomposition. In support of our hypothesis we detected 1.3 mol equiv of nitrite and 0.6 mol equiv of nitrate ions for each disappearing mole of MNX (Figure 4). In contrast, in the case of RDX, the stoichiometry of denitration was almost two molar equivalents of nitrite and only trace amounts of nitrate formed for each disappearing mole of RDX (Figure 1). The relatively high nitrate production from MNX was attributed to MNX denitrosation and subsequent oxidation of the resulting NO to nitrate. It

has been reported that some microbial flavohemoglobin (flavoHb) can exhibit nitric oxide dioxygenase activity (30) and oxidize NO to NO₃⁻ using ¹⁸O₂-labeled oxyheme (flavoHb(Fe^{III}-¹⁸O₂)) prepared from *Escherichia coli* (35). In this respect, when we incubated MNX with DN22 in the presence of ¹⁸O₂ we detected NO₃⁻ with two mass ions: a major one at *m/z* 66 Da incorporating two ¹⁸O atoms and another minor one at *m/z* 64 Da incorporating only one ¹⁸O (Figure 5A). We attributed the formation NO₃⁻ with one ¹⁸O to the bio-oxidation of the initially formed NO₂⁻ from MNX to NO₃⁻ and the formation of NO₃⁻ with two ¹⁸O atoms to bio-oxidation of the initially formed NO (N-NO cleavage) to NO₃⁻. Experimental evidence gathered thus far suggested that besides initial denitration N-NO bond cleavage might also take place during MNX incubation with DN22 (SI Figure S3).

When we incubated MNX with DN22 in the presence of H₂¹⁸O we detected NO₃⁻ with two different masses: one with major intensity at *m/z* 62 Da representing NO₃⁻ without ¹⁸O incorporation and another with much lower intensity at *m/z* 64 Da (Figure 5C) representing NO₃⁻ with the inclusion of one ¹⁸O atom. Like for RDX, we concluded that part of NO coming from MNX first auto-oxidized to NO₂⁻ prior to being biotically oxidized to NO₃⁻. Also we detected NO₂⁻ with two different masses, one at *m/z* 46 Da representing NO₂⁻ coming directly from MNX and another at *m/z* 48 Da representing nitrite with the inclusion of one ¹⁸O confirming the potential auto-oxidation of NO in water to NO₂⁻.

Results from the above H₂¹⁸O and ¹⁸O₂ labeling experiments indicated that MNX can undergo both denitration and denitrosation and both water and oxygen can play major roles in the subsequent secondary chemical and biochemical reactions of both nitrogen species.

Learning More of the Initial Denitration Step Leading to RDX and MNX Decomposition. We know from the alkaline hydrolysis of RDX that denitration is caused by an OH⁻ attack on the acidic -CH₂- group of RDX leading to the formation of the unstable 3,5-dinitro-3-cyclohexene whose subsequent decomposition in water gives products distribution similar to that obtained during RDX incubation with *Rhodococcus* sp. DN22, that is, NO₂⁻, NH₃, N₂O, HCHO, and HCOOH (17, 36). The chemical insight gained from RDX hydrolysis under alkaline conditions might thus help us understand the initial enzymatic denitration step(s) leading to RDX decomposition during its incubation with DN22 and P450 system XplA/B. In this context one might presume that biotic denitration of RDX might also have been triggered by an enzymatic H⁻-containing species that first abstracted a proton (H⁺) from one of the CH₂ groups in RDX leading to the formation of a carbenyl anion whose denitration would produce the imine intermediate (-HC=N-) (**I**, SI Figure S1, *a*). **I** might undergo a second denitration to produce intermediate **II**. Likewise decomposition of **I** would then give both NDAB and MEDINA depending on what type of C-N bond in RDX is hydrolyzed first but decomposition of **II** can only give NDAB (SI Figure S1). However we do not know if DN22/XplA can carry out specific hydride anion (H⁻) reaction with RDX. If RDX truly involved initial reaction with H⁻ one might expect to see hydrogen gas as a product which we did not detect. Initial denitration might also take place through an e-transfer process leading to the formation of a radical anion which upon denitration would give the aminyl radical **III** (SI Figure S1, *b*). H⁻-atom abstraction from a reducing radical by the aminyl radical **III**, denitrohydrogenation, would give 1,3-dinitro-perhydro-1,3,5-triazine **IV** (SI Figure S1, *b*). McHugh et al. (37) reported the synthesis of **IV** by reducing RDX with Na(Hg)/THF/H₂O. **IV** is unstable and thus far has been reported as its nitrated salt that decomposes to MEDINA, HCHO, and NH₃ (19, 38).

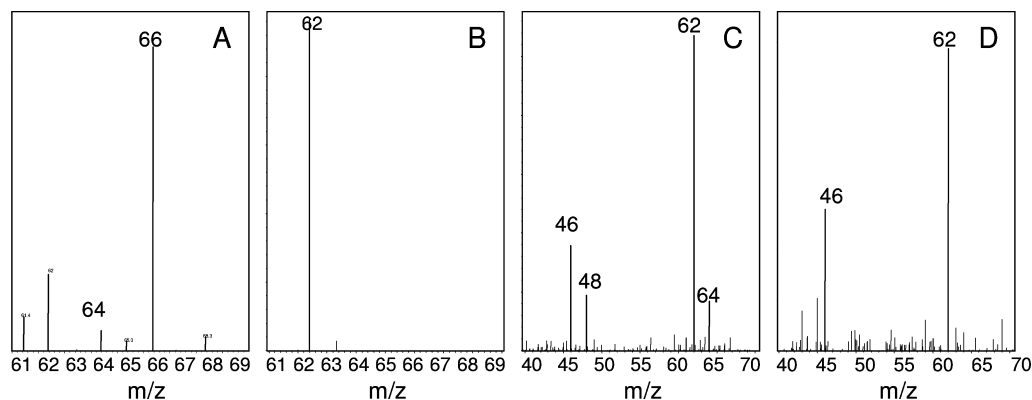


FIGURE 5. Mass spectra of nitrate anion produced from MNX during incubation in aerobic assays containing *Rhodococcus* sp. DN22 in the presence of labeled $^{18}\text{O}_2$ (A) or $^{16}\text{O}_2$ (B), and mass spectra of nitrite and nitrate anions in the presence of H_2^{18}O (C) or H_2^{16}O (D).

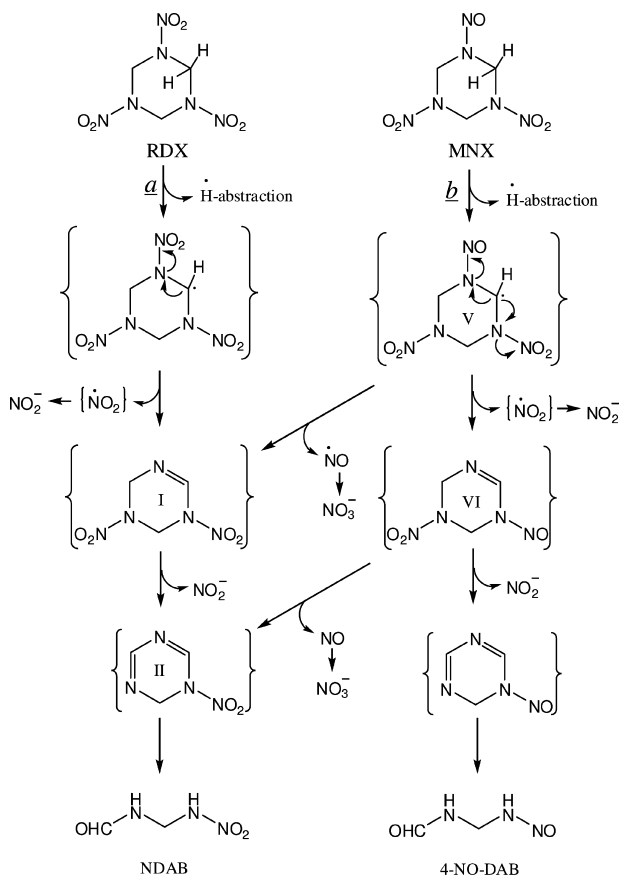


FIGURE 6. Proposed denitration route of RDX (a) and denitrosation and denitration routes of MNX (b) with *Rhodococcus* sp. DN22 under aerobic conditions.

Alternatively, RDX intermediates **I** and **II** could be generated by H[•]-abstraction from one of the CH₂ groups in RDX by an XplA enzymatic system in the form of peroxy radical leading first to the formation of a carbenyl centered radical whose denitration would produce intermediate **I** (Figure 6, a). A second denitration of RDX by the same mechanism would give intermediate **II** (Figure 6, a). Reaction of **I** with water would produce the unstable α-hydroxylamine —NH—CH(OH)— that should trigger ring cleavage and autodecomposition in water. Recently Guengerich explained a classical enzymatic carbon hydroxylation route of alkyl amines through H[•]-abstraction/oxygen rebound mechanism wherein the resulting α-hydroxyl amine decomposes to produce aldehydes and an amine (39). The H[•]-abstraction/oxygen rebound mechanism resembles the α-hydroxylation of the —CH₂— group in RDX leading to its decomposition

but in our case the oxygen originated from water and not from air. We thus speculate that a superoxide radical formed from O_{2(g)} by a redox Cytochrome P450 system was involved in hydrogen atom abstraction from one of the CH₂ groups in RDX leading to its decomposition. Strobel and Coon describe a hydroxylation reaction catalyzed by cytochrome P450 coupled with a superoxide generating system (40).

As for MNX denitrosation of the carbenyl intermediate (**V**) would give intermediate **I** which in turn should decompose in water to give NDAB (Figure 6, b). If MNX carbenyl intermediate undergoes denitration intermediate **VI** would be formed whose decomposition in water should produce the nitroso derivative of NDAB, ONNHCH₂NHCHO (Figure 6, b). Indeed we detected both NDAB and 4-NO-DAB during MNX incubation with DN22.

Based on experimental evidence gathered thus far from ¹⁸O-labeling experiments and products distribution we suggest that the H[•]-abstraction mechanism (39) is the most likely route to initiate denitration and/or denitrosation of RDX and MNX.

Environmental Significance of the Denitration Pathway of Cyclic Nitroamines. The denitration pathway in Figure 2, summarizing most significant biotic and abiotic reactions involved in RDX degradation with DN22, provides valuable information on how much of the N and C content originally present in RDX can actually be utilized by the microorganism. For example, formation of the dead end product NDAB seizes two Cs out of a total of three in RDX leaving only one C for mineralization as demonstrated by the detection of only 30% mineralization of disappearing RDX. In the MEDINA pathway most of the C in RDX is liberated as HCHO (from MEDINA decomposition) and HCOOH (from diformylamide) that are subsequently mineralized by DN22 (5). The MEDINA pathway mostly occurs under anaerobic conditions and is supported by the fact that high mineralization (60–70%) is frequently observed (41, 42). Obviously RDX degradation by denitration is of great environmental significance because it can lead to the development of an effective RDX remediation technology.

The denitration pathway should also serve as a template to help in the development of molecular tools to monitor and enhance in situ bioremediation. For example, the pathway clearly shows the actual chemical bonds, that is, —N—N—, —N—C—, —N—O— that are being attacked or broken during RDX degradation. Such information on the type and order of bond cleavage would be useful in the understanding of stable isotope fractionation (43) and stable isotope probing (SIP) (44, 45).

Finally, products from RDX denitration pathway such as NDAB and nitrite can be employed as useful markers to detect and monitor fate of RDX in the environment. Indeed we detected NDAB in several soils and groundwater samples taken from sites contaminated with RDX, for example, in soil

sample collected from an ammunition plant in Valleyfield, Quebec, Canada (27).

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Supporting Information Available

Figure S1 summarizes RDX denitration routes under aerobic (a) and anaerobic (b) conditions. Figure S2 shows biodegradation of formamide with resting cells of *Rhodococcus* sp. DN22. Figure S3 shows biotic and abiotic degradation routes of MNX with *Rhodococcus* sp. DN22. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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