Biodegradation of RDX nitroso products MNX and TNX by cytochrome P450 XplA

ARTICLE *in* ENVIRONMENTAL SCIENCE & TECHNOLOGY · JUNE 2012

Impact Factor: 5.33 · DOI: 10.1021/es3011964 · Source: PubMed

Environmental Science & Technology

Article

pubs.acs.org/est

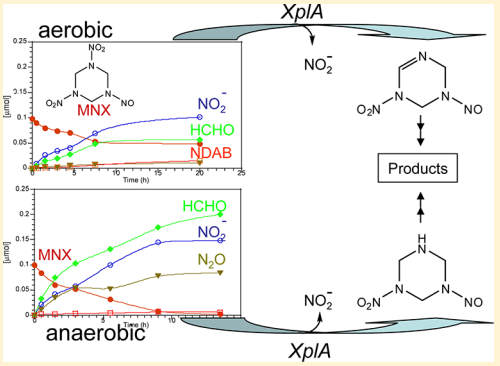
Biodegradation of RDX Nitroso Products MNX and TNX by Cytochrome P450 XplA

Annamaria Halasz,[†] Dominic Manno,[†] Nancy N. Perreault,[†] Federico Sabbadin,[‡] Neil C. Bruce,[‡] and Jalal Hawari[†,*]

[†]Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Ave. Montreal (PQ), Canada, H4P 2R2

[‡]CNAP, Department of Biology, University of York, York YO10 5DD United Kingdom

ABSTRACT: Anaerobic transformation of the explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by microorganisms involves sequential reduction of N−NO_2 to the corresponding N−NO groups resulting in the initial formation of MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine). MNX is further reduced to the dinitroso (DNX) and trinitroso (TNX) derivatives. In this paper, we describe the degradation of MNX and TNX by the unusual cytochrome P450 XplA that mediates metabolism of RDX in *Rhodococcus rhodochrous* strain 11Y. XplA is known to degrade RDX under aerobic and anaerobic conditions, and, in the present study, was found able to degrade MNX to give similar products distribution including NO_2^-, NO_3^-, N_2O, and HCHO but with varying stoichiometric ratio, that is, 2.06, 0.33, 0.33, 1.18, and 1.52, 0.15, 1.04, 2.06, respectively. In addition, the ring cleavage product 4-nitro-2,4,-diazabutanal (NDAB) and a trace amount of another intermediate with a $[M-H]^-$ at 102 Da, identified as $ONNHCH_2NHCHO$ (NO-NDAB), were detected mostly under aerobic conditions. Interestingly, degradation of TNX was observed only under anaerobic conditions in the presence of RDX and/or MNX. When we incubated RDX and its nitroso derivatives with XplA, we found that successive replacement of N−NO_2 by N−NO slowed the removal rate of the chemicals with degradation rates in the order RDX > MNX > DNX, suggesting that denitration was mainly responsible for initiating cyclic nitroamines degradation by XplA. This study revealed that XplA preferentially cleaved the N−NO_2 over the N−NO linkages, but could nevertheless degrade all three nitroso derivatives, demonstrating the potential for complete RDX removal in explosives-contaminated sites.



INTRODUCTION

The explosive RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine, is extensively used by the military and in the construction industry.[1] Presently, RDX is contaminating large areas of soil, sediment, and water. Since RDX is toxic, a possible carcinogen, and a potent convulsant,[2] extensive research has been conducted to understand its biodegradation pathways and to gain insight into its environmental fate and the mechanisms of its toxicity. Earlier research shows that RDX can be degraded by either sequential reduction under mostly reducing or anaerobic conditions[3−8] of the −N−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) or by initial cleavage of the −N−NO_2 bond(s) leading to decomposition of the chemical and the eventual formation of nitrite, ammonia, N_2O, HCHO, and HCOOH.[9−13] We also found that denitration of RDX leads to the formation of the two key intermediates 4-nitro-2,4-diazabutanal (NDAB, NO_2NHCH_2NHCHO) and methylenedinitramine (MEDINA, $NO_2NHCH_2NHNO_2$), whose formation depends on the stoichiometry of the denitration step.[11] The conditions under which NDAB and MEDINA are formed are best described in an earlier reference by Jackson et al.[11] A novel cytochrome P450 XplA and its partnering reductase XplB have been found to mediate the metabolism of RDX in bacteria isolated from RDX contaminated soils under aerobic conditions.[14] Recently, aerobic RDX-degrading *Rhodococcus* have been isolated from the microaerophilic zone (0.9 mg l^{-1} dissolved oxygen) of a RDX-contaminated aquifer[15] and slow RDX degradation by *Rhodococci* has been reported under extreme microaerophilic conditions (<0.04 mg L^{-1} dissolved oxygen).[13] The corresponding genes, *xplA* and *xplB*, were first identified in *Rhodococcus rhodochrous* 11Y,[16] but have since been found in many genera of the *Corynebacterineae* that can use RDX as the sole N source. The *xplA* gene is highly conserved among RDX degrading bacteria from geographically distinct regions including North America,[17] Australia,[16] the United Kingdom,[16] and Israel,[15] suggesting *XplA* evolved following the introduction of RDX into the environment and rapidly spread across the world by horizontal gene transfer. We established that XplA is able to degrade RDX via initial reductive denitration. Interestingly, the enzyme produces

Received: March 27, 2012
Revised: June 1, 2012
Accepted: June 13, 2012
Published: June 13, 2012

   dx.doi.org/10.1021/es3011964 | *Environ. Sci. Technol.* 2012, 46, 7245−7251

reaction products with different stoichiometries if the transformations are carried out under aerobic or anaerobic conditions. We found that under anaerobic conditions, one mole of nitrite anion is formed but under aerobic conditions, two moles of nitrite anions are produced for each mole of RDX degraded.[11]

In situ RDX degradation to MNX, DNX, and TNX has been detected in groundwater and soil.[18,19] MNX is formed in the environment from RDX by anaerobes such as *Klebsiella*[20] and *Shewanella*,[21] which can use RDX as a terminal electron acceptor for growth, reducing N−NO_2 to N−NO. MNX can also be produced by nitroreductases,[22] zerovalent iron[5,23] and biogenic iron(II).[7,24] Despite the frequent formation of this key product during degradation of RDX, mostly under anaerobic conditions, little is known about the degradation pathways of its nitroso products, MNX, DNX and TNX. Recently we found that MNX could be degraded aerobically by *Rhodococcus* sp. strain DN22 via initial cleavage of N−NO_2 and N−NO.[25] The purpose of the present study was to determine whether XplA preferentially cleaves N−NO_2 or N−NO linkages in MNX. To do so, we investigated the kinetics of RDX, MNX, DNX, and TNX degradation incubated as a mixture or separately. The nitroso products are toxic[26] and the new knowledge gained on their degradation susceptibility should help in the development of effective in situ remediation technologies.

MATERIALS AND METHODS

Chemicals. RDX (99% pure) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX; 99% pure) 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), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX; 55% of DNX, 17% of MNX and 28% of TNX), 4-nitro-2,4-diazabutanal (NDAB; 99% pure), and methylenedinitramine (MEDINA) were purchased from SRI International (Menlo Park, CA). All other chemicals, including NH_2CHO, nicotinamide adenine dinucleotide phosphate (NADPH), and sodium nitrite, were reagent grade.

Enzymatic Assays. XplA and XplB were produced as described by Jackson et al.[11] Enzymatic assays using the XplA and XplB system were prepared to test the removal of MNX and to determine products distribution under aerobic and anaerobic conditions. Stock solutions of the substrates were prepared in 50 mM phosphate buffer (pH 6.9). Enzymes and NADPH were freshly prepared in water. In aerobic assays, the reaction mixtures contained 98 μM MNX, 700 μM NADPH, and 176 nM of XplA and XplB whereas in the anaerobic assays the reactions mixtures contained 99 μM MNX, 700 μM NADPH, and 220 nM XplA and XplB. The reactions were initiated by the addition of NADPH. The assays were conducted in 6 mL headspace vials, away from light, statically at 21 °C. Anaerobic reactions were prepared in an anaerobic chamber in the presence of nitrogen with up to 4% of hydrogen (Forma Anaerobic System, model 1025, Thermoscientific, Marietta, OH) using reagents that were made anaerobic by repetitive degassing (three times) and introducing O_2-free Argon to the reagent vials. Controls with boiled XplA and XplB, in addition to NADPH and MNX, were also prepared.

Other enzymatic assays were performed to determine the kinetics of the removal of RDX, MNX, and TNX incubated separately, as binary mixtures of RDX/TNX, MNX/TNX, and as a mixture of RDX, MNX, DNX, and TNX (approximately 123 μM each) with XplA/XplB under the same conditions as described above. In the case of the different mixtures, the concentrations of XplA and XplB were 220 nM under both aerobic and anaerobic conditions, and 1.6 mM NADPH was used.

Chemical Analysis. RDX, MNX, DNX, TNX, and HCHO were determined by HPLC[25] and NH_4^+, NO_2^-, NO_3^- and HCOOH by ion chromatography.[27] Nitrous oxide was analyzed by GC/ECD.[28] NH_2CHO was first derivatized by treatment with pentafluorobenzyl-hydroxylamine and analyzed by LC-MS as its deprotonated molecular mass ion $[M-H]^-$.[29] NDAB and MEDINA were determined by HPLC and by comparison with authentic materials.[21] NDAB and NO-NDAB were analyzed by LC-MS.[25] Because MEDINA is unstable, the samples were analyzed shortly after sampling.[30]

RESULTS AND DISCUSSION

Degradation of MNX with XplA. We found that, under aerobic and anaerobic conditions, XplA degraded MNX with the concurrent formation of NO_2^-, NO_3^-, N_2O, and HCHO (Figures 1 and 2) with varying stoichiometric ratio, 2.06, 0.33,

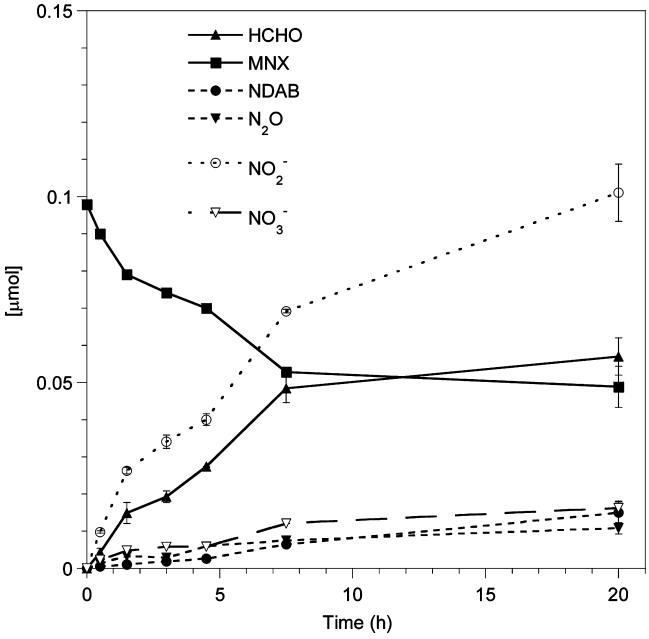


Figure 1. Time course of aerobic biodegradation of MNX by XplA.

0.33, 1.18, and 1.52, 0.15, 1.04, 2.06, respectively (Table 1). The degradation rate and products distributions under aerobic conditions were closely similar to those observed with *Rhodococcus* sp. strain DN22, confirming involvement of XplA during MNX degradation by DN22.[25] In addition, we detected the ring cleavage product 4-nitro-2,4,-diazabutanal (NDAB), identified by comparison with a reference material, but with a stoichiometric ratio higher under aerobic than under anaerobic conditions, 0.3 and 0.06, respectively. A trace amount of another intermediate with a $[M-H]^-$ at 102 Da, matching an empirical formula of $C_2H_5N_3O_2$, was detected under aerobic conditions, which was an intermediate detected in an earlier study with MNX and *Rhodococcus* sp. strain DN22.[25] In that study we used uniformly ring labeled ^{15}N-MNX and confirmed the intermediate to be the nitroso-derivative of NDAB, 4-nitroso-2,4-diaza-butanal (ONNHCH$_2$NHCHO, NO-NDAB). We presumed that the present intermediate shown at $[M-H]^-$

dx.doi.org/10.1021/es3011964 | *Environ. Sci. Technol.* 2012, 46, 7245−7251

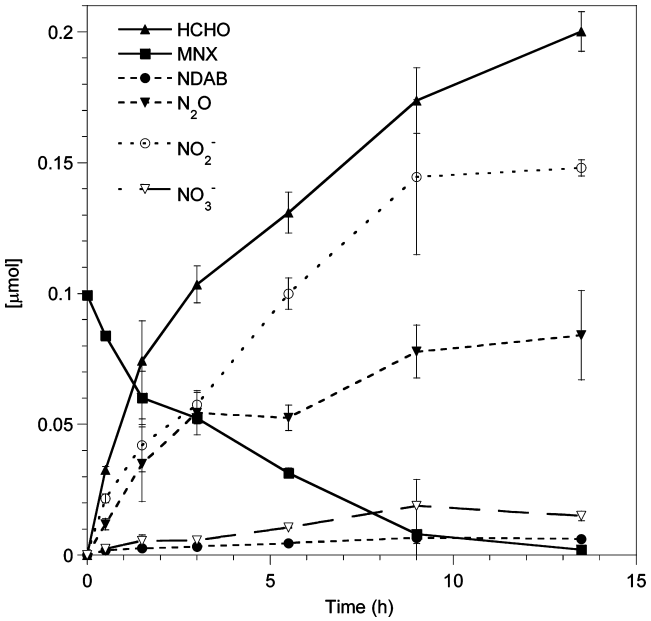


Figure 2. Time course of anaerobic biodegradation of MNX by XplA.

102 Da was also NO-NDAB. Trace amounts of formamide (NH_2CHO) were detected in the samples prepared under aerobic conditions. Ammonium ion (NH_4^+) was detected but could not be quantified because of the strong interference caused by NADPH during analysis. For the same reason, formate production was not determined. Thereby, carbon and nitrogen atoms recoveries reached only 60−73% and 63−66%, respectively, under aerobic and anaerobic conditions.

Although we were unable to observe methylenedinitramine, $O_2NNHCH_2NHNO_2$ (MEDINA), or its nitroso-derivative, O_2NNHCH_2NHNO (NO-MEDINA), the detection of HCHO and N_2O suggested that MEDINA and/or NO-MEDINA was formed but decomposed very rapidly in water under conditions used[30,31] (eqs 1 and 2).

$$\begin{aligned} &O_2NNHCH_2NHNO_2 + 2H_2O \\ &\rightarrow (OH)_2CH_2 + 2NH_2NO_2 \\ &\rightarrow HCHO + 2N_2O + 3H_2O \end{aligned} \quad (1)$$

$$\begin{aligned} &O_2NNHCH_2NHNO + 2H_2O \\ &\rightarrow (OH)_2CH_2 + HN{=}NOH + NH_2NO_2 \\ &\rightarrow HCHO + N_2 + N_2O + 3H_2O \end{aligned} \quad (2)$$

Both hemiacetal ($(OH)_2CH_2$) and nitramide (NH_2NO_2) are unstable in water and should decompose spontaneously to give HCHO and N_2O, respectively (eq 1). In eq 2, decomposition of O_2NNHCH_2NHNO should initially give hydroxydiazene (HN═NOH), in addition to NH_2NO_2 and hemiacetal. It is known that hydroxydiazene (HNNOH) decomposes in water to N_2.[32] The yield of N_2O in the anaerobic system was 3 fold higher than its yield under aerobic conditions, 1.04 and 0.33 nmol·nmol^{-1} MNX degraded, respectively, which accounted for only half of the calculated yield from $O_2NNHCH_2NHNO_2$. This observation suggests that one of the N−NO_2 (precursor of N_2O) in MEDINA was more likely N−NO (precursor to N_2). Also since we were able to detect MEDINA during RDX incubation with the same enzymatic system,[11] we suggest that NO-MEDINA, rather than MEDINA, was the MNX ring cleavage product whose decomposition in water should produce N_2, N_2O and HCHO (eq 2).

On the other hand, the relatively high stoichiometric ratio of nitrite produced under both aerobic and anaerobic conditions, 2.06 and 1.52, respectively, suggest that the suspected two nitroso intermediates of MNX, namely, NO-NDAB and NO-MEDINA can degrade further to produce nitrite. This is supported by the fact that hydroxydiazene (HN═NOH) in water exists as a nitroso-oxime tautomer with nitrosamide (NH_2NO) (eq 3), which is known to hydrolyze to HNO_2 and NH_3.[32]

$$HN{=}NOH \leftrightarrow NH_2N{=}O \quad (3)$$

We detected NO_3^- under both aerobic and anaerobic conditions but with higher stoichiometric ratio in the former case, 0.33, and 0.15, respectively. The formation of NO_3^- can originate from NO formed by the cleavage of N−NO linkage in MNX following its initial denitration. Nitric oxide, NO, is known to undergo aerobic bio-oxidation to NO_3^-[25,33] and autoxidation to NO_2^-.[34] In an earlier study, during incubation of RDX with *Rhodococcus* sp. strain DN22 in the presence of $^{18}O_2$, we observed both NO_2^- and NO_3^- and attributed the small formation of the latter ion to bio-oxidation of NO_2^- to NO_3^-.[25] In addition, it is known that in the P450 catalytic cycle, autoxidation of the unstable oxy-ferrous intermediate ($Fe^{2+}.O_2$) can generate a superoxide radical anion ($O_2^{\cdot-}$).[35] Reportedly, nitric oxide can react with $O_2^{\cdot-}$ to yield $ONOO^-$, which upon protonation gives the unstable peroxynitrous acid (ONOOH) whose spontaneous decomposition in water would yield nitrate (NO_3^-).[36] On the other hand, homolytic decomposition of ONOOH can produce nitrogen dioxide free radical ($\cdot NO_2$), which upon dimerization gives dinitrogen tetroxide, N_2O_4. Decomposition of N_2O_4 in water would produce nitrite and nitrate.[37] Previously we proposed the formation of $\cdot NO_2$ during denitration of RDX and MNX with *Rhodococcus* sp. strain DN22[25] and we will explain its formation and role in the degradation process later on in the present study.

Relative Reactivities of RDX and its Nitroso Products MNX, DNX, and TNX with XplA. When we incubated RDX, MNX, and TNX separately with XplA under aerobic and anaerobic conditions we found that the enzyme degraded both RDX and MNX but not TNX. The initial removal rates of RDX and MNX were 357 and 72 nmol·h^{-1}·nmol^{-1} protein under aerobic conditions and 472 and 118 nmol·h^{-1}·nmol^{-1} protein under anaerobic conditions, respectively. The present data showed that the anaerobic degradation rates of both chemicals were higher than the aerobic rates, suggesting that molecular

Table 1. Stoichiometric Ratio of the Products to MNX Degraded with XplA under Aerobic (49 nmol of MNX, 20h) and Anaerobic (97 nmol MNX, 13.5 h) Conditions

products	NO_2^- (RSD)	NO_3^- (RSD)	N_2O (RSD)	HCHO (RSD)	NDAB (RSD)
aerobic conditions	2.06 (3.1)	0.33 (0.0)	0.33 (22.9)	1.18 (2.6)	0.30 (8.2)
anaerobic conditions	1.52 (0.6)	0.15 (10.7)	1.04 (15.7)	2.06 (4.3)	0.06 (4.4)

oxygen might not be needed to initiate degradation. Also the data clearly showed that under both aerobic and anaerobic conditions, MNX degradation was slower than RDX. This is consistent with our previous finding that the dissociation constant (K_D) of XplA heme domain with MNX is about twice the value of the one calculated with RDX (~24.2 μM and 11.4 μM, respectively). High K_D with MNX would imply higher K_M (Michaelis–Menten constant) and, likely, lower k_{cat} (maximum turnover rate) values and, in general, lower activity.[38]

In other experiments, RDX, together with its three nitroso derivatives, was incubated with XplA under aerobic and anaerobic conditions (Figure 3). In aerobic assays, RDX and

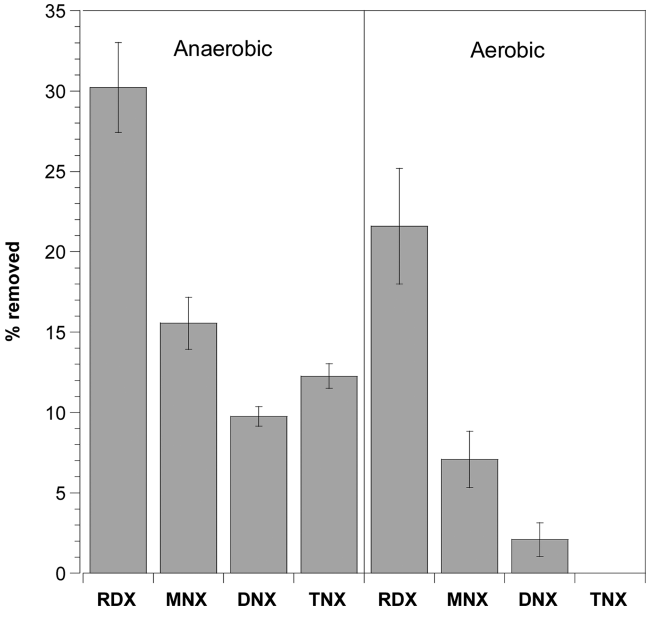


Figure 3. RDX and its nitroso derivatives removal from mixture incubated with XplA for 1 h under anaerobic and aerobic conditions.

two of its nitroso derivatives MNX and DNX degraded with initial rates equaled to 126, 40, and 11 nmol·h^{-1}·nmol^{-1} of protein, respectively, lower than their removal rates when incubated separately with the enzyme. TNX $(CH_2NNO)_3$, which does not contain N–NO_2, did not degrade aerobically with XplA. In anaerobic assays, under otherwise identical conditions, the four chemicals RDX, MNX, DNX, and TNX degraded with initial rates of 180, 91, 51, and 69 nmol·h^{-1}·nmol^{-1} of protein, respectively (higher than those observed under aerobic conditions). This showed that the successive replacement of N–NO_2 by N–NO in RDX reduced the removal rate of the chemical. We also found that TNX can only degrade with XplA under anaerobic conditions and in the presence of a cosubstrate containing N–NO_2 functionality (Table 2). A reactive intermediate possibly coming from degradation of an –N–NO_2 containing substrate, for example, RDX or MNX might have initiated TNX degradation (discussed below).

Experimental data gathered thus far prove that with XplA (1) the heterocyclic compound with the highest number of –NO_2 groups reacts faster, that is, rates decreased in the order RDX > MNX > DNX and (2) denitration, N–NO_2 bond cleavage, is favored over denitrosation, N–NO bond cleavage, under both aerobic and anaerobic conditions. Conversely to biologic degradation with XplA, abiotic degradation of RDX and its nitroso products with highly reactive organically complexed

Table 2. Percent of RDX, MNX, and TNX Removed after 1h Incubation with XplA under Anaerobic Conditions[a]

mixture	RDX (%)	MNX (%)	TNX (%)
RDX and TNX	47	na[b]	20
MNX and TNX	na[b]	36	8

[a]Initial concentrations of RDX, MNX and TNX were 147, 135, and 127 μM, respectively. [b]Not applicable.

iron(II) species showed reaction rates in the order TNX > DNX > MNX ≫ RDX.[6] As we mentioned earlier, XplA degraded RDX via initial denitration and its nitroso derivatives via denitration and/or denitrosation, which is different from what was proposed for the Fe(II) reaction. In the latter case, RDX is suggested to degrade via sequential reduction of the N–NO_2 groups to the corresponding N–NO functionalities. Interestingly, in a much earlier study using *Clostridium bifermentans* HAW-1, we found the following degradation rates for RDX and its nitroso derivatives MNX and TNX: 28.1, 16.9, and 7.5 μmol h^{-1} g(dry wt) cells^{-1}, respectively.[39] MNX denitration as major route and continuous reduction to DNX and TNX as minor degradation route have been proposed.[39]

Insights into the Initial Steps Involved in the Degradation of MNX—Denitration versus Denitrosation. *Aerobic Degradation.* Earlier we investigated MNX biodegradation with *Rhodococcus* sp. strain DN22[25] using conditions similar to those employed with RDX[9] and demonstrated that both denitration (N–NO_2 bond cleavage) and denitrosation (N–NO bond cleavage) can occur before ring-opening. In the present study, we investigated the degradation of MNX by the rhodococcal P450 XplA. During the aerobic incubation with XplA, we detected nitrite and nitrate ions in stoichiometric ratio equaled to 2.06 and 0.33, respectively, for each disappearing mole of MNX. The comparatively high yield of nitrite (2.06) suggested that the first step in the MNX degradation was possibly denitration caused by hydrogen atom abstraction followed by $\cdot NO_2$ elimination as was the case with DN22[25] (Figure 4). Following this key initial denitration step, MNX can either undergo

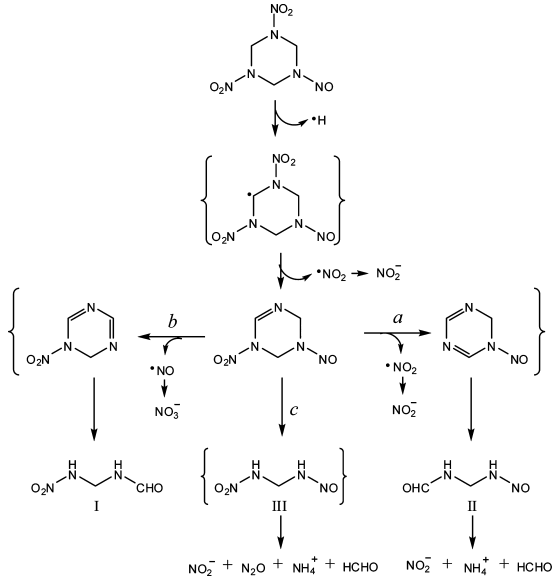


Figure 4. Proposed aerobic degradation routes of MNX by XplA. Compounds in brackets were not detected.

dx.doi.org/10.1021/es3011964 | Environ. Sci. Technol. 2012, 46, 7245–7251

further denitration (Figure 4, path *a*) or denitrosation (Figure 4, path *b*) before ring cleavage. In line with this explanation, we detected nitrate anion and the key ring cleavage product NDAB (I) and its nitroso-derivative NO-NDAB (II) (Figure 4, paths *a* and *b*). We also detected N_2O. From earlier studies, we know that N_2O can be formed from the decomposition of MEDINA ($O_2NNHCH_2NHNO_2$, eq 1), an RDX ring cleavage product.[31] We speculate here that in the case of MNX, it is more likely that N_2O originated from the nitroso-derivative of MEDINA (O_2NNHCH_2NHNO, III), which should undergo spontaneous decomposition in water to give N_2O and HCHO that were both detected (Figure 4. path *c*).

Anaerobic Degradation. Previously we found that degradation of RDX by XplA under anaerobic conditions is initiated by monodenitration followed by ring cleavage to produce MEDINA.[11] In a subsequent review article,[40] we elaborated further on the anaerobic formation of MEDINA from RDX and suggested the occurrence of denitrohydrogenation followed by ring cleavage to give MEDINA, which, being unstable in water, decomposes to HCHO and N_2O (eq 1). In the present study, for each mol of MNX reacted anaerobically with XplA we detected 1.52 and 0.15 mols of nitrite and nitrate, respectively. In addition, we detected HCHO and N_2O in stoichiometric ratio equaled to 2.06 and 1.04, respectively. The formation of one mole of N_2O tends to suggest that MNX, after initial denitration, underwent ring cleavage to give NO-MEDINA ($ONNHCH_2NHNO_2$, III) as the key ring cleavage product whose degradation would give N_2O, N_2, NH_3 and HCHO (Figure 5A). As we explained in eq 3, intermediate III can exist

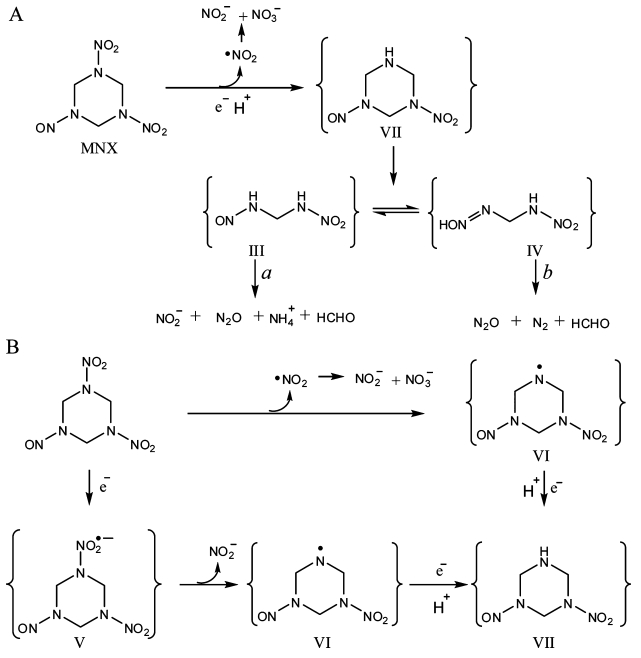


Figure 5. Proposed anaerobic degradation route of MNX by XplA. Compounds in brackets were not detected.

in equilibrium with its hydroxydiazenyl tautomer (HON-NCH_2NHNO_2, IV) (Figure 5A). Decomposition of tautomer IV should produce N_2 but we were unable to analyze the gas because of interference from the nitrogen gas in the air. However, we detected nitrite with stoichiometric ratio of 1.52, which was approximately 50% higher than the yield calculated based on the cleavage of only one N−NO_2 bond in MNX. These experimental findings suggested that about half of NO-MEDINA was present as a nitrosamide tautomer (III) that decomposed to NO_2^- and NH_4^+ (Figure 5A).

Considering all of the above experimental evidence we suggest that, under anaerobic conditions, XplA reduced MNX to initially form the unstable intermediate 1-nitroso-3-nitro-perhydro-1,3,5-triazine (VII), which should decompose to give NO-MEDINA (III) and its tautomer IV (Figure 5A and B). Indeed, McHughes et al.[41] reported that reduction of RDX with Na(Hg) process via denitrohydrogenation leads to the initial formation of the unstable denitrohydrogenated intermediate 1,3-dinitro-perhydro-1,3,5-triazine whose decomposition gives MEDINA.

The remaining challenge here is to know how initial denitration of MNX (and other substrates investigated in this study) took place. Based on our earlier discussion, we propose two routes for the possible formation of 1-nitroso-3-nitro-perhydro-1,3,5-triazine (VII) (Figure 5B). One route involved direct homolysis of −N−NO_2 resulting in the formation of the $\cdot NO_2$ and aminyl radical (VI). The other route involved the formation of MNX anion radical (V), which upon loss of nitrite would also produce the aminyl radical (VI) (Figure 5B). Hydrogen atom abstraction by VI would lead to the formation of 1-nitroso-3-nitro-perhydro-1,3,5-triazine (VII).

Finally, the potential involvement of $\cdot NO_2$ and the aminyl radical (VI) in the degradation pathways of MNX (or RDX[42]) can help explain the degradation of TNX when the latter is incubated with XplA in the presence of a N−NO_2 containing substrate. A free radical generated from degradation of MNX (and either RDX or DNX) might thus be responsible for initiating TNX degradation. For example hydrogen abstraction by either $\cdot NO_2$ or aminyl radical from the $-CH_2-$ in TNX, $(CH_2NNO)_3$, would destabilize the molecule causing it to decompose.

Environmental Significance. The nitroso derivatives of RDX are toxic and need to be removed from the environment.[26] Understanding their transformation pathways should help to develop efficient remediation strategies that completely remove RDX and its nitroso products from contaminated sites. Aerobic XplA-containing *Rhodococcus* have been isolated from RDX-contaminated soils[16] and from a microaerophilic aquifer.[15] Because RDX degradation by *Rhodococcus* strains was shown to be possible under extremely microaerophilic conditions,[13] it is reasonable to presume that *Rhodococcus* can degrade the anaerobically formed nitroso in micro-oxic environments. They would also degrade the nitroso compounds that may migrate to the oxic zone.[19]

The cytochrome P450 XplA was shown to degrade MNX and DNX, and also to degrade TNX in the presence of RDX and MNX but only under anaerobic conditions. MNX is a key degradation product of RDX and is detected in most groundwater and soil showing the presence of RDX.[18,19,30] We established that XplA degraded MNX by two distinctive pathways, one pathway occurring under aerobic (Figure 4) and another under anaerobic (Figure 5) conditions, preferentially cleaving the N−NO_2 bonds over N−NO. These pathways are complementary to the pathways we recently reported for RDX.[25,40] Knowledge of the type and order of bond cleavage would help in the development and application of stable isotope fractionation and stable isotope probing tools recently considered important in the understanding of the fate of N-containing explosives in the environment.[19,43−45] In a recent study we successfully detected RDX transformation products,

including MNX and the ring cleavage products NDAB, NO_2^-, NO_3^-, and N_2O in contaminated groundwaters, as a sign of past RDX contamination and in situ natural attenuation.[30] In the present MNX study, we also detected NDAB and some characteristic products such as NO_2^-, NO_3^-, and N_2O. Since the three N-oxide compounds can also originate from other processes in the field, we can apply ^{15}N and ^{18}O stable isotope analyses as a sensing tool to differentiate between compounds derived from the cyclic nitramines (RDX and MNX) and those formed via other processes and use data to monitor and enhance in situ remediation and natural attenuation.

AUTHOR INFORMATION

Corresponding Author

*Phone: 514 496 6267; fax: 514 496 6265; e-mail: Jalal.Hawari@nrc.ca.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was funded by Strategic Environmental Research and Development Program (SERDP) of the US Department of Defense.

REFERENCES

(1) Jenkins, T. F.; Hewitt, A. D.; Grant, C. L.; Thiboutot, S.; Ampleman, G.; Walsh, M. E.; Ranney, T. A.; Ramsey, C. A.; Palazzo, A. J.; Pennington, J. P. Identity and distribution of residues of energetic compounds at army live-fire training ranges. *Chemosphere* **2006**, *63*, 1280−1290.

(2) Lachance, B.; Robidoux, P. Y.; Hawari, J.; Ampleman, G.; Thiboutot, S.; Sunahara, G. I. Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro. *Mutat. Res.* **1999**, *444*, 25−39.

(3) McCormick, N. G.; Cornell, J. H.; Kaplan, A. M. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. *Appl. Environ. Microbiol.* **1981**, *42*, 817−823.

(4) Gregory, K. B.; Larese-Casanova, P.; Parkin, G. F.; Scherer, M. M. Abiotic transformation of hexahydro-1,3,5-trinitro-1,3,5-triazine by Fe^{II} bound to magnetite. *Environ. Sci. Technol.* **2004**, *38*, 1408−1414.

(5) Oh, B.-T.; Craig, L. J.; Alvarez, P. J. J. Hexahydro-1,3,5-trinitro-1,3,5-triazine mineralization by zerovalent iron and mixed anaerobic cultures. *Environ. Sci. Technol.* **2001**, *35*, 4341−4346.

(6) Kim, D.; Strathmann, T. J. Role of organically complexed iron(II) species in the reductive transformation of RDX in anoxic environments. *Environ. Sci. Technol.* **2007**, *41*, 1257−1264.

(7) Kwon, M. J.; Finneran, K. T. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) reduction is currently mediated by direct electron transfer from hydroquinones and resulting biogenic Fe(II) formed during electron shuttle-amended biodegradation. *Environ. Eng. Sci.* **2009**, *26*, 961−971.

(8) Williams, A. G. B.; Gregory, K. B.; Parkin, G. F.; Scherer, M. M. Hexahydro-1,3,5-trinitro-1,3,5-triazine transformation by biologically reduced ferrihydrite: Evolution of Fe mineralogy, surface area, and reaction rates. *Environ. Sci. Technol.* **2005**, *39*, 5183−5189.

(9) Fournier, D.; Halasz, A.; Spain, J.; Fiurasek, P.; Hawari, J. Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with *Rhodococcus* sp. strain DN22. *Appl. Environ. Microbiol.* **2002**, *68*, 166−172.

(10) Bhushan, B.; Trott, S.; Spain, J. C.; Halasz, A.; Hawari, J. Biotransformation of RDX by cytochrome P450 2B4: Insights into the mechanisms of RDX biodegradation by *Rhodococcus* sp strain DN22. *Appl. Environ. Microbiol.* **2003**, *69*, 1347−1351.

(11) Jackson, R. G.; Rylot, E.; Fournier, D.; Hawari, J.; Bruce, N. C. Exploring the biochemical properties and remediation applications of the unusual explosive-degrading P450 system XplA/B. *Proc. Natl. Acad. Sci.* **2007**, *104*, 16822−16827.

(12) Kwon, M. J.; Finneran, K. T. Electron shuttle-stimulated RDX mineralization and biological production of 4-nitro-2,4-diazabutanal (NDAB) in RDX-contaminated aquifer material. *Biodegradation* **2010**, *21*, 923−937.

(13) Fuller, M. K.; Perreault, N.; Hawari, J. Microaerophilic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by three *Rhodococcus* strains. *Lett. Appl. Microbiol.* **2010**, *51*, 313−318.

(14) Seth-Smith, H. M. B.; Edwards, J.; Rosser, S. J.; Rathbone, D. A.; Bruce, N. C. The explosive-degrading cytochrome P450 system is highly conserved among strains of *Rhodococcus* spp. *Appl. Environ. Microbiol.* **2008**, *74*, 4550−4552.

(15) Bernstein, A.; Adar, E.; Nejidat, A.; Ronen, Z. Isolation and characterization of RDX-degrading *Rhodococcus* species from a contaminated aquifer. *Biodegradation* **2011**, *22*, 997−1005.

(16) Seth-Smith, H. M. B.; Rosser, S. J.; Basran, A.; Travis, E. R.; Dabbs, E. R.; Nicklin, S.; Bruce, N. C. Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from *Rhodococcus rhodochrous*. *Appl. Environ. Microbiol.* **2002**, *68*, 4764−4771.

(17) Indest, K. J.; Crocker, F. H.; Athow, R. A TaqMan polymerase chain reaction method for monitoring RDX-degrading bacteria based on the *XplA* functional gene. *J. Microbiol. Methods* **2007**, *68*, 267−274.

(18) Beller, H. R.; Tiemeier, K. Use of liquid chromatography/tandem mass spectrometry to detect distinctive indicators of in situ RDX transformation in contaminated groundwater. *Environ. Sci. Technol.* **2002**, *36*, 2060−2066.

(19) Sagi-Ben Moshe, S.; Ronen, Z.; Dahan, O.; Bernstein, A.; Weisbrod, N.; Gelman, F.; Adar, E. Isotopic evidence and quantification assessment of in situ RDX biodegradation in deep unsaturated zone. *Soil. Biol. Biochem.* **2010**, *42*, 1253−1262.

(20) Zhao, J.-S.; Halasz, A.; Paquet, L.; Beaulieu, C.; Hawari, J. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine and its mononitroso derivative hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine by *Klesiella pneumoniae* strain SCZ-1 isolated from an anaerobic sludge. *App. Environ. Microbiol.* **2002**, *68*, 5336−5341.

(21) Zhao, J.-S.; Manno, D.; Hawari, J. Regulation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) metabolism in *Shewanella halifaxensis* HAW-EB4 by terminal electron acceptors and involvement of c-type cytochrome. *Microbiology* **2008**, *154*, 1026−1037.

(22) Bhushan, B.; Halasz, A.; Spain, J. C.; Thiboutot, S.; Ampleman, G.; Hawari, J. Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) catalyzed by a NAD(P)H: nitrate oxidoreductase from *Aspergillus niger*. *Environ. Sci. Technol.* **2002**, *36*, 3104−3108.

(23) Naja, G.; Halasz, A.; Thiboutot, S.; Ampleman, G.; Hawari, J. RDX degradation using zero-valent iron nanoparticles. *Environ. Sci. Technol.* **2008**, *42*, 4364−4370.

(24) Bhushan, B.; Halasz, A.; Hawari, J. Effect of iron(III), humic acids and anthraquinone-2,6-disulfonate on biodegradation of cyclic nitramines by *Clostridium* sp. EDB2. *J. Appl. Microbiol.* **2006**, *100*, 555−563.

(25) Halasz, A.; Manno, D.; Strand, S. E.; Bruce, N. C.; Hawari, J. Biodegradation of RDX and MNX with *Rhodococcus* sp. strain DN22: New insight into the degradation pathway. *Environ. Sci. Technol.* **2010**, *44*, 9330−9336.

(26) Zhang, B.; Cox, S. B.; McMurry, S. T.; Jackson, W. A.; Cobb, G. P.; Anderson, T. A. Effect of two major *N*-nitroso hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) metabolites on earthworm reproductive success. *Environ. Pollut.* **2008**, *153*, 658−667.

(27) Balakrishnan, V. K.; Monteil-Rivera, F.; Halasz, A.; Corbeanu, A.; Hawari, J. Decomposition of the polycyclic nitramine explosive, CL-20, by Fe^0. *Environ. Sci. Technol.* **2004**, *38*, 6861−6866.

(28) Bhatt, M.; Zhao, J.-S.; Halasz, A.; Hawari, J. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by novel fungi isolated from unexploded ordnance contaminated marine sediment. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 850−858.

(29) Hawari, J.; Halasz, A.; Groom, C.; Deschamps, S.; Paquet, L.; Beaulieu, C.; Corriveau, A. Photodegradation of RDX in aqueous

solution: A mechanistic probe for biodegradation with *Rhodococcus* sp. *Environ. Sci. Technol.* **2002**, *36*, 5117−5123.
(30) Paquet, L.; Monteil-Rivera, F.; Hatzinger, P. B.; Fuller, M. E.; Hawari, J. Analysis of the key intermediates of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) in groundwater: occurrence, stability and preservation. *J. Environ. Monit.* **2011**, *13*, 2304−2311.
(31) Halasz, A.; Spain, J.; Paquet, L.; Beaulieu, C.; Hawari, J. Insights into the formation and degradation mechanisms of methylenedinitramine during the incubation of RDX with anaerobic sludge. *Environ. Sci. Technol.* **2002**, *36*, 633−638.
(32) Casewit, C. J.; Goddard, W. A., III Energetics and mechanisms for reactions involving nitrosamide, hydroxydiazenes, and diimide *N*-oxides. *J. Am. Chem. Soc.* **1982**, *104*, 3280−3287.
(33) Ignarro, L. J.; Fukuto, J. M.; Griscavage, J. M.; Rogers, N. E.; Byrns, R. E. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: Comparison with enzymatically formed nitric oxide from L-arginine. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8103−8107.
(34) Awad, H. H.; Stanbury, D. M. Autoxidation of NO in aqueous solution. *Int. J. Chem. Kinet.* **1993**, *25*, 375−381.
(35) Guengerich, F. P. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem. Res. Toxicol.* **2001**, *14*, 611−650.
(36) Jourd'heuil, D.; Kang, D.; Grisham, M. B. Interactions between superoxide and nitric oxide: Implications in DNA damage and mutagenesis. *Front. Biosci.* **1997**, *2*, d189−196.
(37) Augusto, O.; Bonini, M. G.; Amanso, A. M.; Linares, E.; Santos, C. X.; De Menezes, S. L. Nitrogen dioxide and carbonate radical anion: Two emerging radicals in biology. *Free Radicals Biol. Med.* **2002**, *32*, 841−859.
(38) Sabbadin, F. Engineering cytochromes P450 for biocatalysis and bioremediation, Ph.D. Thesis, University of York, UK, October 2010.
(39) Zhao, J.-S.; Paquet, L.; Halasz, A.; Hawari, J. Metabolism of hexahydro-1,3,5-trinitro-1,3,5-triazine through initial reduction to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine followed by denitration in *Clostridium bifermentans* HAW-1. *Appl. Microbiol. Biotechnol.* **2003**, *63*, 187−193.
(40) Halasz, A.; Hawari, J. Degradation routes of RDX in various redox systems. In *Aquatic Redox Chemistry*, ACS Symposium Series; Tratnyek, P., Grundl, T.J., Haderlein, S., Eds.; American Chemical Society: Washington, DC, 2011; Vol. *1071*, pp 441-462.
(41) McHugh, C. J.; Smith, W. E.; Lacey, R.; Graham, D. The first controlled reduction of the high explosive RDX. *Chem. Commun.* **2002**, *21*, 2514−2515.
(42) Bhushan, B.; Halasz, A.; Spain, J. C.; Hawari, J. Diaphorase catalyzed biotransformation of RDX via N-denitration mechanism. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 779−784.
(43) Bernstein, A.; Ronen, Z.; Adar, E.; Nativ, R.; Lowag, H.; Stichler, W.; Meckenstock, R. U. Compound-specific isotope analysis of RDX and stable isotope fractionation during aerobic and anaerobic biodegradation. *Environ. Sci. Technol.* **2008**, *42*, 7772−7777.
(44) Schmidt, T. C.; Zwank, L.; Elsner, M.; Berg, M.; Meckenstock, R. U.; Haderlein, S. B. Compound-specific stable isotope analysis of organic contaminants in natural environments: a critical review of the state of the art, prospects, and future challenges. *Anal. Bioanal. Chem.* **2004**, *378*, 283−300.
(45) Roh, H.; Yu, C.-P.; Fuller, M. E.; Chu, K. H. Identification of hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading microorganisms via ^{15}N-stable isotope probing. *Environ. Sci. Technol.* **2009**, *43*, 2502−2511.