Anaerobic bioremediation of RDX by ovine whole rumen fluid and pure culture isolates

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ABSTRACT

The ability of ruminal microbes to degrade the explosive compound hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in ovine whole rumen fluid (WRF) and as 24 bacterial isolates was examined under anaerobic conditions. Compound degradation was monitored by high performance liquid chromatography (HPLC) analysis, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) identification of metabolites. Organisms in WRF microcosms degraded 180 µM RDX within 4 h. Nitroso-intermediates 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) were present as early as 0.25 hours and were detected throughout the 24 h incubation period, representing one reductive pathway of ring cleavage. Following reduction to MNX, peaks consistent with *m/z* 193 and 174 were also produced which were unstable and resulted in rapid ring cleavage to a common metabolite consistent with an *m/z* of 149. These represent two additional reductive pathways for RDX degradation in ovine WRF which have not been previously reported. The 24 ruminal isolates degraded RDX with varying efficiencies (0-96%) over 120 hours. Of the most efficient degraders identified, *Clostridium polysaccharolyticum* and *Desulfovibrio desulfuricans* subsp. *desulfuricans* degraded RDX when medium was supplemented with both nitrogen and carbon, while *Anaerovibrio lipolyticus*, *Prevotella ruminicola* and *Streptococcus bovis* IFO utilized RDX as a sole source of nitrogen. This study showed that organisms in whole rumen fluid, as well as several ruminal isolates, have the ability to degrade RDX *in vitro* and, for the first time, delineated the metabolic pathway for its biodegradation.

Key words: Bioremediation, RDX, rumen, ovine, anaerobic

INTRODUCTION

Since World War II, the use of energetic compounds by military groups has resulted in the deposition of explosives residues at sites across the globe (Agency for Toxic Substances and Disease Registry 1995; Clausen et al. 2004). Munitions including TNT (2,4,6-trinitrotoluene), RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) and CL-20 currently contaminate the soil around manufacturing and storage facilities and bombing/training ranges at approximately 16,000 military bases in the United States alone (Environmental Protection Agency 1993; U.S. Environmental Protection Agency 2009). The U.S. Environmental Protection Agency classifies RDX as a group C possible human carcinogen and a priority pollutant (U.S. Environmental Protection Agency 2009). In humans, the target organ of toxicity for RDX exposure is the central nervous system (Agency for Toxic Substances and Disease Registry 1995; Brooks et al. 1997; Banerjee et al. 1999; U.S. Environmental Protection Agency 2009), where convulsions, loss of consciousness, vomiting, skin lesions, and even death have been observed (Hesselmann and Stenstrom 1994). Neurological impacts of RDX have also been observed in a variety of other species including birds (Gust et al. 2009), mice, rats, and rabbits (Agency for Toxic Substances and Disease Registry 1995), in addition to lethality in fish and reproductive effects in invertebrates (Turley and Burton 1995).

Cyclic nitramines such as RDX are resistant to degradation in soil and are highly mobile in the water column; thus, they threaten the health of humans and the surrounding ecosystem

when they leach into groundwater or offsite locations, such as farmland (Daniels and Knezovich 1994; Sunahara et al. 2009). The RDX molecule is a six-membered ring of alternating carbon and nitrogen atoms, with a nitro group attached to each nitrogen atom (Figure 1). Although RDX is not planar, all three nitro groups are directed towards the same side of the molecule (Qasim et al. 2007). This particular geometric configuration makes it possible for positive ions to electrostatically attract the oxygen atoms of the nitro assemblages, making them good leaving groups for nucleophilic reactions (Qasim et al. 2007; Sunahara et al. 2009), such as those catalyzed through enzymatic attacks by microorganisms (Prescott et al. 2005).

Recent research has demonstrated that cyclic nitramines can be degraded without oxygen; anaerobic electron acceptors, such as NO_3-, SO_4^2- and Fe(III) are used by bacteria to degrade these contaminants (Coleman et al. 1998; Hawari et al. 2000a; Hawari et al. 2000b; Crocker et al. 2006; Sunahara et al. 2009). Furthermore, anaerobic biodegradation appears to be the preferential pathway of converting cyclic nitramines since the nitro groups must be reduced prior to ring cleavage and mineralization to CO_2 under either anaerobic or aerobic conditions (Kwon and Finneran 2006). Aerobic biodegradation by indigenous microbes is simply not quick enough to prevent the movement of unadulterated cyclic nitramines through the aerobic portions of soil into the ground water and anaerobic sediment, where accumulation poses a threat to humans.

Of the anaerobic systems that have been studied, the most rapid remediation of RDX took place in ovine whole rumen fluid (WRF), with biotransformation of 30 μg mL^{-1} RDX occurring in 4 hours (Eaton et al. 2011). The rumen is a powerfully reductive, anaerobic and natural bioreactor (Hobson and Stewart 1997), which we hypothesize could be used in the bioremediation of energetic compounds. This remediation technique, termed *phyto-ruminal bioremediation*, would be cost-effective, in addition to providing a “green” alternative to the

current methods of soil removal and *ex situ* incineration, which release unburned explosive chemicals into the air, soil and water (Axtell et al. 2000; Qasim et al. 2007). This process would first utilize cool-season grasses to accumulate explosives residues from soil into the shoots (Duringer et al. 2010); the ruminant would then consume the munitions-laden plant material, which would be utilized by ruminal microbes for energy and thereby degraded to innocuous by-products (De Lorme and Craig 2008; Smith et al. 2008; Eaton et al. 2011). Over time, with successive pasture growth and grazing, the soil would be remediated.

Our previous study examining ovine WRF as a means to bioremediate RDX was successful in determining that microbial communities in WRF, and the resulting culturable consortia, could biotransform RDX, but the metabolite pathway of neither the WRF nor the cultured consortia were elucidated (Eaton et al. 2011). In this study, we determined the metabolic fate of RDX, in both WRF and in 24 commonly isolated individual bacteria from the rumen. We hypothesized from the results of our previous study that RDX would be degraded in rumen fluid faster and more completely than by the isolates, but that by examining isolates, we would gain insight into which organisms may be crucial in identifying genes responsible for RDX breakdown.

These objectives were accomplished by high performance liquid chromatography (HPLC) analysis of spent culture supernatants to identify possible degraders, followed by quantification and identification of metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Organisms in WRF microcosms degraded RDX to nitroso-intermediates and then to proposed ring cleavage metabolites. From these results, three pathways for RDX degradation by ruminal microbes were put forth. This study showed that organisms in WRF and several ruminal isolates have the ability to degrade RDX *in vitro* and, for the first time,

delineated the metabolic pathway for its biodegradation. This research supports application of our proposed *phyto-ruminal bioremediation* process to *in vivo* field trials using live grasses and sheep.

MATERIALS AND METHODS

Chemicals and reagents

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (99% purity) was purchased from ChemService (West Chester, PA). Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) (99% purity), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) (55% purity +17% MNX + 23% TNX), hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (>99% purity), 4-nitro-2,4-diazabutanal (4-NDAB) (98% purity) and methylenedinitramine (MEDINA) (98% purity) were provided by R.J. Spanggord from SRI International (Menlo Park, CA). Solvents were of HPLC and LC-MS/MS grade. Reagents were of analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO). An ELGA (Cary, NC) Ultra PureLab reverse osmosis water purification system was used to generate Milli-Q quality water (resistance >18.2 MΩ/cm) for all aqueous solutions.

Organisms, media, and growth conditions

Pure cultures were maintained by our lab or obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) (Table 1). Some strains required species-specific media instead of a general complex medium for optimal growth. These included *Desulfovibrio* medium (DSMZ medium 63), *Clostridium polysaccharolyticum* (DSMZ medium 140), *Lactobacillus ruminus* (DSMZ medium 232), and *Wolinella succinogenes* (DSMZ medium 157). The remaining cultures were grown in a complex medium with 40% clarified rumen fluid (per liter: 400 mL clarified rumen fluid; 2.0 g trypticase; 1.0 g yeast extract; 4.0 g cellobiose; 4.0 g sodium carbonate; 1.0 mL 0.1% resazurin; 10.0 mL VFA solution [concentration, $\mu mol \cdot mL^{-1}$: 67.2 glacial acetic acid, 40.0 propionic acid, 20.0 butyric acid, 5.0 isobutyric acid, 5.0 2-methylbutyric acid, 5.0 valeric acid, 5.0 isovaleric acid]; 0.3 g potassium phosphate, dibasic; 0.6 g sodium chloride; 0.3 g ammonium sulfate; 0.3 g potassium phosphate, monobasic; 0.08 g calcium chloride, dihydrate; 0.12 g magnesium sulfate, heptahydrate; and 1.1 g sodium citrate, dihydrate). All media was prepared anaerobically by combining media components in a round bottom flask, adjusting to the appropriate pH, then boiling for 5 min and purging of oxygen by sparging with a N_2:CO_2 (75:25) gas mixture for 30 min. The media were then corked and immediately placed into an anaerobic glove box H_2:CO_2 (10:90). All media were dispensed as 9.7 mL aliquots into Balch tubes, which were sealed with butyl rubber stoppers and aluminum crimp caps. The sealed media was autoclaved for 35 min at 120° C and stored until use. Anaerobically prepared and sterilized reducing agent (1.25% cysteine sulfide) and B-vitamins solution (per 100 mL: 20 mg thiamin HCl, 20 mg *D*-pantothenic acid, 20 mg nicotinamide, 20 mg riboflavin, 20 mg pyridoxine HCl, 1.0 mg *p*-aminobenzoic acid, 0.25 mg biotin, 0.25 mg folic acid, and 0.1 mg cyanocobalamin) were added to media prior to inoculation. Cultures were grown at 39°C with shaking (150 rpm) for 18–24 h between transfers. Cultures

were transferred at least three times before degradation experiments.

Whole rumen fluid microcosms with RDX and TNX

Ovine WRF was collected from three cannulated male sheep maintained at the Oregon State University (OSU) Sheep Center (Corvallis, OR) in accordance with International Animal Care and Use Committee (IACUC) regulations. WRF was pooled in a sterile, pre-warmed thermos, which was filled to the top to maintain an anaerobic environment, followed by immediate transport to the lab. The thermos was then placed in an anaerobic glove box (Coy, Grass Lake, MI) with an atmosphere of CO_2:H_2 (9:1). The thermos was gently inverted and a 25 mL portion was poured into a sterile reagent basin and used for all inoculation experiments. The sheep were fed a high forage diet of alfalfa hay twice daily.

WRF (7 mL) was inoculated into sterile, anaerobically prepared screw-capped tubes. RDX was added to each tube for a final concentration of 180 µM, which is the lower limit of solubility (solubility is 180-270 µM, depending on the matrix (Hesselmann and Stenstrom 1994)). Tubes were incubated anaerobically in the dark at 39°C on a rotary shaker (150 rpm) for 24 hours. Autoclaved tubes of WRF were used as controls. This experiment was repeated with one of the RDX metabolites, TNX, at a concentration of 82 µM. All experiments were performed in triplicate. Samples were collected every hour for the first five hours, and then at 24 hours. Samples were frozen at -20˚C until prepared for LC-MS/MS analysis, as described below.

178 Rumen isolate incubations with RDX

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180 Each isolate (Table 1) was first incubated with 180 µM RDX in low nitrogen basal (LNB) and
181 low carbon basal (LCB) media (Eaton et al. 2011). This concentration of RDX was toxic to 22
182 organisms. We then determined the limit of toxicity for each organism by progressively
183 decreasing or increasing the amount of RDX until growth and elimination of RDX, as
184 determined by HPLC, was observed. The resulting starting RDX concentration defined for each
185 organism is listed in Table 2. Cultures were then incubated anaerobically, in the dark, at 39°C on
186 a rotary shaker (150 rpm) for 120 hours. A media control consisted of 256 µM RDX, as this was
187 the highest concentration used in both LNB and LCB, without the addition of test organism. A
188 solvent control consisted of both types of media with 1.0 mL of overnight culture of the test
189 organism and the addition of 0.1 mL acetonitrile. All controls and tests were repeated in
190 triplicate.

191

192 Sample preparation for chromatography

193

194 WRF samples were collected and then frozen at -20˚C until preparation for HPLC and LC-
195 MS/MS analyses through solid phase extraction using Waters Oasis HLB (3 mL/60 mg, 30 µm)
196 cartridges (Milford, MA) per the manufacturer's instructions. In brief, WRF samples were
197 treated 1:1 (*v/v*) with a basified water diluent consisting of 40 µL of concentrated ammonium
198 hydroxide in 1 mL of water. Samples were vortexed and then centrifuged at 8,000 x *g* for 10

minutes. Extraction was completed by evaporation of the final eluent in a Savant ISS 100 Integrated Speed Vac System (GMI, Inc., Ramsey, MN) at 43˚C, followed by reconstitution in methanol:water 55:45 (*v/v*). Isolate samples were not prepared via the solid phase extraction procedure outlined above, but were centrifuged at 16,000 x *g* at 4°C for 3 min. Supernatant was removed, filtered through a 0.2 µm PTFE membrane filter (VWR International, Brisbane, CA), and diluted 1:1 (*v/v*) with acetonitrile for immediate analysis by HPLC or LC-MS/MS.

HPLC and LC-MS/MS analyses

HPLC analyses were carried out using Environmental Protection Agency method 8330A (U. S. Environmental Protection Agency 2007). In brief, separations occurred on a Phenomenex Ultracarb ODS (20) column (250 mm x 4.6 mm i.d., 5 µm particle size) (Torrance, CA), eluting under isocratic conditions with water and methanol (55:45 *v/v*) at 28.3°C and a flow rate of 0.8 mL min^{-1}, with a total run time of 30 min. The HPLC system consisted of a Perkin–Elmer (Waltham, MA) Series 200 pump equipped with a Perkin–Elmer ISS 200 autosampler and Perkin–Elmer Series 200 UV/VIS detector, monitoring at 254 nm. TotalChrom software (Perkin–Elmer) was used to quantify HPLC data.

LC-MS/MS analyses were performed on an ABI/SCIEX 3200 QTRAP LC-MS/MS system (Applied Biosystems, Foster City CA) using atmospheric pressure chemical ionization in the negative ion mode, modified from Borton and Olson (2006). A Phenomenex Ultracarb ODS (20) column (250 mm x 4.6 mm i.d., 5 µm particle size) was used to separate RDX and its metabolites at a flow rate of 0.65 mL min^{-1} over 35 minutes using a mobile phase consisting of

0.6 mM ammonium acetate in water (A) and methanol (B) in a gradient program as follows: 10% B from 0-5 min, increasing to 20% B at 8 min, 25% B at 10 min, 30% B at 12 min, 35% B at 14 min, 40% B at 20 min, 45% B from 25-30 min, and 58% B at 35 min. Data was acquired using multiple reaction monitoring (MRM), using 281→46 and 281→59 (RDX + CH_3COO^-), 265→46 and 265→59 (MNX + CH_3COO^-), 249→46 and 249→59 (DNX + CH_3COO^-), 233→59 (TNX + CH_3COO^-), 135→61 (MEDINA) and 118→61 (4-NDAB) as transitions. Curtain gas was set at 10 psi, gas 1 at 35 psi, gas 2 at 0 psi, temperature at 275°C, nebulizer current at -8 µA, and dwell time to 75 msec. Declustering potential, entrance potential, collision entrance potential, collision energy and collision exit potential were as follows for each compound: RDX (-20, -2, -22, -14, -4) for the 281→46 transition and (-5, -2, -22 -48, -2) for the 281→59 transition; MNX (-5, -2, -14, -16, 0) for the 265→46 transition and (-5, -2, -14, -38, 0) for the 265→59 transition; DNX (-5, -2, -14, -16, 0) for the 249→46 transition and (-5, -2, -14, -38, 0) for the 249→59 transition; TNX (-5, -3, -12, -22, 0); MEDINA (-10, -2.5, -10, -20, 0); and 4-NDAB (-5, -3.5, -6, -10, 0). Quantitation was performed by establishing a calibration curve in Analyst 1.4.2 using a linear regression from WRF or media samples spiked with RDX at concentrations of 5-50 $ng \cdot mL^{-1}$. R^2 values for the transition 281/46 were 0.997, 0.993, and 0.973 for WRF, LCB and LNB medias, respectively.

Data used to identify possible new metabolites was acquired using enhanced mass spectra (EMS) and enhanced product ion (EPI) scans via information dependent acquisition (IDA) experiments developed in Analyst 1.4.2 software (Applied Biosystems). RDX and metabolites were separated using the same conditions as in the MRM method, except a different gradient was used: 0-5 minutes at 20% B, 5-30 minutes at 50% B, 30-50 minutes at 100% B, and 50-70 minutes at 100% B. Curtain gas was set to 10 psi, nebulizer gas to 35 psi, nebulizing current to -8

µA, temperature to 275ºC, declustering potential to -20 V, entrance potential to -10 V, collision energy to -10 V, scan rate to 1000 amu·s^{-1}, dynamic fill time and a mass range of 50 to 400 amu. Final EMS data was analyzed using LightSight 2.0 (Applied Biosystems) and ChemDraw Ultra 12.0 (CambridgeSoft, Cambridge, MA) software to capture and interpret data and possible metabolites.

Results

Degradation of RDX and TNX in ovine whole rumen fluid

Separation and fragmentation of RDX (*m/z* 281), nitroso intermediates MNX (*m/z* 265), DNX (*m/z* 248) and TNX (*m/z* 233), and ring cleavage products 4-NDAB (*m/z* 118) and MEDINA (*m/z* 135) by MRM analysis are illustrated in Fig. 1. This modified LC-MS/MS method (Borton and Olson 2006) was developed and optimized in order to detect RDX and its five commonly known metabolites in one injection. LC-MS/MS analysis of ovine WRF samples using this method showed anaerobic degradation from 180 µM RDX to 45 µM RDX within 4 h, with almost complete elimination of RDX (4.5 µM remaining) by 24 h (Fig. 2). Within 1 h, the reduction products MNX (28.5 µM) and DNX (0.37 µM) were visible. MNX increased in concentration until 3 h (35.8 µM), after which it slowly declined to 8.4 µM at the 24 h sampling point. DNX steadily increased to 4.2 µM at 24 h. The other nitroso-intermediate, TNX, appeared at 5 h (9 µM) and was present at about the same concentration through 24 h. The ring cleavage products MEDINA and 4-NDAB were not detected.

Not all of the initial RDX concentration could be accounted for by MRM analysis; hence we performed an enhanced mass spectrometry (EMS) scan to identify other possible metabolic products. EMS scans of RDX metabolism in WRF at 0.25 and 24 h are illustrated in Fig. 3A-B. Analysis showed a decrease in a peak consistent with an *m/z* of 193 (LC-MS Fig. 3C) and an increase in a peak consistent with an *m/z* of 149 (LC-MS Fig. 3D) over 24 hours which appear to be metabolites, since they were not visible in the autoclaved controls and contain a fragment of 59 amu, which is common to RDX and its five known metabolites (Fig. 1). The peak of *m/z* 193 is consistent with one of the bracketed molecules in the pathway described in Zhang (2003), yet we propose this second route of RDX degradation by ruminal microbes ends in a ring cleaved metabolite of *m/z* 149, as shown in Figure 4 (pathway 1). A peak consistent with an *m/z* of 175 was seen in the same spectra as the *m/z* 193 peak, which showed an increase over 24 hours. This may represent a daughter ion of the *m/z* 193 peak, forming from a loss of H_2O, or, alternatively, a third possible pathway which moves through the nitroso-intermediate MNX, ending in a ring cleaved metabolite of *m/z* 149 (Fig. 4, pathway 3). Figure 4 provides an overview for the pathways we propose for anaerobic bioremediation of RDX by WRF and how a metabolite of *m/z* 149 could result from ring cleavage of MNX via three different degradation routes. Lastly, peaks after 40 minutes were noted in the EMS scan (Fig. 3); however further method development is needed to separate metabolites eluting at this end of the chromatogram and should be the subject of future studies.

In the RDX incubations, TNX increased to 9 µM at 5 h, then plateaued until 24 hours (Fig. 2). To investigate if TNX was a dead-end metabolite, we performed ovine WRF incubations with 82 µM TNX for 24 h. When quantified via MRM analysis, autoclaved WRF controls showed an average of 36.8 µM TNX at 0.25 h, while live WRF samples showed an

289 average of 15.5 µM (coefficient of variation = 21.1%). Degradation was seen in one replicate
290 from 22.4 µM to 11.5 µM over 24 h, but this was not consistent with the other two replicates.
291 LightSight analysis of the 0.25 h live WRF samples showed metabolites consistent with
292 dehydrogenation and glycine conjugation (*m/z* 228.2); tridemethylation and glutamine
293 conjugation (*m/z* 259); taurine conjugation (*m/z* 280); loss of NO, oxidation and glutamine
294 conjugation (*m/z* 287); and loss of the cyclohexyl ring, trioxidation and possible conjugation with
295 glucose (*m/z* 300), which may explain the large discrepancy between the nominal and quantified
296 concentrations of TNX observed. Thus, overall, the TNX concentration did not decrease
297 significantly from 0.25 to 24 h in WRF, but the complication of conjugated metabolite formation
298 prevents any definitive conclusions from being made.
299
300 Twenty-four bacterial isolates from the rumen were tested for their ability to degrade RDX over
301 120 h (Table 1). Of these, two were identified as both being able to thrive in an environment with
302 a high concentration of RDX and being the most proficient at degrading it (Table 2).
303 *Desulfovibrio desulfuricans* subsp. *desulfuricans* was able to degrade 88% of the 256 µM RDX
304 supplied in low carbon basal medium; *C. polysachharolyticum* was able to degrade 65% of the
305 186 µM RDX in the low carbon basal medium (Fig. 5). Of the other three bacterial species that
306 demonstrated significant degradation of RDX, *A. lipolyticus* was able to degrade 70% of the 85
307 µM RDX supplied in low nitrogen basal medium (Fig. 5). Also capable of degradation of RDX
308 in low nitrogen basal medium were *S. bovis* IFO and *Prevotella ruminicola,* with high RDX-
309 degrading abilities of 86% and 96% respectively, but with a lower initial concentration of RDX
310 (34 µM, Fig. 5**)**. Nine more organisms exhibited low RDX-degrading ability (30-49%) in one or
311 both types of media (Table 2). Ten of the organisms appeared to be unable to grow when

incubated with RDX at a concentration of 34 µM in both types of media; no visible growth was observed after 24 h and RDX degradation was similar to that in reduced media with RDX, which was ≤ 25% (Table 2). In general, controls (reduced media without bacteria) resulted in a minor decrease in RDX concentration after 24 h (Table 2). Solvent controls did not appear to inhibit growth of any organism (data not shown). Trace amounts of MNX were detected at 25 minutes, but below the limit of quantitation for the five isolates discussed above. For the isolates *A. lipolyticus*, *P. ruminicola*, and *S. bovis* IFO in LNB media, MRM transitions were detected at 4.4 minutes for RDX, MNX, DNX, and TNX, with a second TNX peak detected at 3.0 minutes. These retention times do not correspond to the retention times established for these compounds using purified standards (Fig. 1). This data suggests that in source fragmentation of RDX conjugated metabolites is occurring and/or interactions with the LNB media are resulting in the formation of alternate, conjugated metabolic products, as these 4.4 and 3.0 minute peaks were not seen in WRF nor isolates studied in LCB media. In conclusion, the five bacterial isolates with moderate to high RDX-degrading ability processed the RDX molecule to trace amounts of the nitroso-intermediate MNX and then to unidentified metabolites over 120 hours.

Discussion

The rumen is the first of four chambers in the stomach of a ruminant, which includes species such as deer, goats, cows, and sheep. Continuous fermentation and mixing occur in the rumen through contractions every few minutes, which would benefit the remediation of xenobiotic compounds by maximizing bioavailability to the fungi, protozoa, bacteria, and archaea inhabiting this niche. As an example, previous research has shown ruminal

bioremediation of TNT to be quite effective (Fleischmann et al. 2004; De Lorme and Craig 2008; Smith et al. 2008). This study examined the possibility of using the rumen as a bioreactor for the remediation of the energetic compound RDX. We utilized whole rumen fluid, the matrix in which the remediation would occur, to define the fate of RDX and ensure it would be degraded in the rumen within 16-20 hours, which would prevent absorption into the bloodstream of the animal and resultant toxicity. We also examined the capability of 24 commonly isolated ruminal bacteria to utilize RDX in low carbon and low nitrogen basal media to gain an understanding as to which bacteria may be responsible for RDX degradation in the rumen, with the idea that successful isolates could be used in future studies to examine the genes involved in RDX metabolism.

Our data showed that ruminal microorganisms, as a community under anaerobic conditions, readily transformed the nitro groups of RDX into the corresponding nitroso groups of the commonly observed metabolites MNX, DNX and TNX (Fig. 2). MEDINA and 4-NDAB were not detected using MRM analysis, suggesting that these metabolites are not the end products of degradation; it is still unclear if these are possible intermediate metabolites that are degraded further, as seen in Hawari et al., (2000). Based on previously defined anaerobic pathways, summarized in Fuller et al., (2009), we were not expecting to see 4-NDAB as a metabolite since it has only been detected in aerobic degradation pathways to date. The concentrations of all nitroso intermediates amounted to approximately 20 µM at 24 hours (Fig. 2), accounting for only 11% of the total initial concentration of RDX. The pathway seen in Hawari et al., (2000) suggests an additional pathway to explain the *m/z* 149 peak shown in Figure 4. A peak representing *m/z* 174 was not detected in that study, but we are proposing that degradation of MNX through *m/z* 174 to MEDINA and finally to *m/z* 149 is a major pathway of

RDX breakdown in WRF. We acknowledge that MEDINA was not identified in our study; however, we suggest that the transformation from MNX to the *m/z* 149 peak is occurring at such a rate that the MEDINA intermediate is not produced in amounts significant enough to be detected. Additional experiments, similar to those elucidating the degradation of RDX in WRF need to be conducted with MNX and MEDINA to investigate if these metabolites are further degraded to *m/z* 149 by utilizing high resolution time of flight (TOF) mass spectrometry to identify accurate masses and obtain an accurate chemical formula of *m/z* 149, the proposed end product of RDX degradation by WRF. Nonetheless, the two additional pathways described in Figure 4 would account for the dramatic increase of *m/z* 149 from 0.25 to 24 hours, with three possible routes for the same *m/z* 149 value. Our data indicates that most of the RDX was degraded via pathways 1 and 3 (Fig. 4), with only small amounts being transformed to the nitroso-intermediates (pathway 2), but further chemical analyses with heavy or radioisotopes are needed to confirm this hypothesis.

The rate of disappearance of 180 µM RDX in whole rumen fluid within 4 h (Fig. 2) is considerably faster than microbial species currently used to degrade RDX via composting, which takes several weeks (Williams et al. 1992), and treatment by municipal sludge, in which degradation of 45 µM RDX took 48 hours (Hawari et al. 2000b). When compared to bioremediation techniques by organisms in contaminated soil or sludge digesters (Bayman et al. 1995; Boopathy et al. 1998; Axtell et al. 2000; Hawari et al. 2000b; Crocker et al. 2005; Fuller et al. 2005), the rumen not only increases access to the compound through continuous mixing, but the environment is more reductive (Hobson and Stewart 1997), which could aid in faster degradation of RDX.

We also examined the ability of whole rumen fluid to degrade TNX, the last nitroso-intermediate in published anaerobic pathways of RDX metabolism (Zhao et al. 2003; Crocker et al. 2006). The amount of TNX we quantified in our RDX incubations with WRF was very small, and previous research has hypothesized that TNX, if further reduced, becomes a highly unstable molecule that spontaneously reacts or breaks down to ring cleavage products (McCormick et al. 1976; Zhao et al. 2003; Fuller et al. 2009). Perhaps the reductive environment in the WRF microcosms was powerful enough to reduce TNX to the point where it degraded abiotically. While it is possible that TNX breaks down in WRF because of the metabolite seen with the loss of the cyclohexyl ring, its ability to form conjugates with various amino acids and phase two metabolites has not been fully investigated. Our initial loss of 45% of the starting concentration of TNX, even in the 0.25 hour autoclaved control, may be explained by the WRF conjugating a certain amount of the TNX (65 µM) immediately, which induced a saturation limit and/or was toxic to the microbes in the 24 hour time period we examined. In the TNX experiment, we used a significantly higher level of TNX than was produced in the 24 hour degradation of RDX (82 µM compared to 9 µM), so toxicity seems plausible.

In the TNX experiment, the main conjugates formed were with glutamine. Glutamine is a compound of central importance in nitrogen metabolism (Krishnan et al. 1986). Glutamine synthetase is an essential enzyme present in all bacteria that is responsible for modulating the overall flow of NH_4^+ to organic nitrogen by assimilating ammonia and synthesizing glutamine (Kumada et al. 1993). The glutamine conjugate formed from WRF incubations with TNX could represent metabolism of the TNX by bacteria in WRF, but further studies would be necessary to determine if this enzyme was responsible for the degradation of TNX; glutamine may be present simply as a by-product of the metabolism of other nitrogen sources in the rumen. Other

403 conjugates formed with TNX were with glycine and taurine. Glycine is naturally present in
404 rumen fluid and metabolized very efficiently by ruminal microbes, while taurine is an essential
405 amino acid provided to ruminants in their diet because they are not able to synthesize adequate
406 amounts (Hobson and Stewart 1997; Sejrsen et al. 2006). Conjugates of TNX with glycine and
407 taurine were not seen with the low levels of TNX produced from RDX degradation, but only in
408 the rumen fluid samples with high amounts of TNX (82 µM). Therefore, we speculate that these
409 conjugates are the result of saturation and not metabolism of TNX. Because the concentration of
410 TNX in the WRF incubations quantified by LC-MS/MS was less than half of that initially added,
411 our study concurs with published literature that TNX lacks stability and reacts abiotically with its
412 surroundings (Zhao et al. 2003; Crocker et al. 2006; Fuller et al. 2009).
413 Of the four ruminal isolates that demonstrated significant RDX degradation ability (Table
414 2), the two most adept were *Clostridium* sp. and *Desulfovibrio* sp. which are both sulfate-
415 reducers found in low concentrations in the rumen (Hobson and Stewart 1997) that have
416 demonstrated RDX degradation previously (Zhao et al. 2003; Arnett and Adrian 2009). Third,
417 *Streptococcus bovis* IFO has been identified before as a member in a ruminal consortia in an
418 enrichment for RDX-degraders (Eaton et al. 2011) and has been classified as one of the highest
419 proteolytically active ruminal bacteria in digesting substrates for amino acids (Hobson and
420 Stewart 1997). Fourth, *Anaerovibrio lipolyticus,* a lipolytic organism, has been previously shown
421 to degrade TNT (De Lorme and Craig 2008), but not RDX. Future studies will involve
422 examining the active genes used to degrade RDX in these bacteria.
423 Ruminal isolates were able to degrade RDX between a range of 34 to 256 µM in 120
424 hours, compared to near complete degradation of 180 µM RDX within 24 hours in whole rumen
425 fluid. This data indicates that, while several rumen bacterial strains may have the individual

426 ability to degrade RDX, bioremediation in whole rumen fluid is more efficient. This is
427 undoubtedly due to the volume of culturable and unculturable organisms within the rumen fluid
428 that had access to RDX and its metabolites, as well as an abundance of other substrates, which
429 organisms could utilize in co-metabolism. Since the parent compound RDX was nearly
430 eliminated within 24 hours in whole rumen fluid, we propose this compound would be safe for
431 sheep to ingest since the passage time in the rumen is 16-20 hours (Hobson and Stewart 1997), as
432 long as the concentration of nitroso-intermediates generated are not toxic; this will require
433 further investigation through feeding trials to determine if and where the nitroso-intermediates
434 are translocated (i.e. fat, muscle, brain, liver, feces, etc.).

435 The reduction and mineralization of cyclic nitramines by microorganisms under
436 anaerobic conditions (Boopathy et al. 1998; Hawari et al. 2000a; Adrian et al. 2003; Fournier et
437 al. 2005; Crocker et al. 2006; Arnett and Adrian 2009) generally involves reductive or
438 substitutive removal of the nitro group from the ring (Sunahara et al. 2009). Results from this
439 study have led us to propose a pathway for the degradation of RDX by ovine ruminal microbes
440 under anaerobic conditions (Fig. 4). First, RDX is reduced to MNX. Trace amounts of MNX are
441 further reduced to DNX and TNX. However, the majority of MNX is further degraded via two
442 other pathways: (1) pentahydro-1,3-dinitro-1,3,5-triazine, which is an unstable molecule that
443 results in rapid ring cleavage and degradation to *m/z* 149 (Fuller et al. 2009) and (2) via *m/z* 193
444 (Zhang and Hughes 2003) to an end product of *m/z* 149 .

445 Environmental contamination by explosive compounds such as TNT and RDX is an
446 international concern; many countries lack the money and technology to remediate dangerous
447 sites. The bioremediation technique discussed in this paper, “phytoruminal-bioremediation,”
448 would provide a low maintenance, affordable, and “green” way to rid unwanted contamination,

provided grass can be grown to pull compounds out of the soil for grazing sheep (Duringer et al. 2010). RDX degradation is affected by environmental conditions, which must be considered when proposing an effective remediation strategy. These include adsorption, binding capacity and complexation of media; the presence of other explosives; reactivities of proliferated transformation products (Qasim et al. 2007; Sunahara et al. 2009); air and light exposure; and the ability of RDX to move throughout the water column (Best et al. 1999). Many techniques succeed in the laboratory, but fail in larger field trials because the expected outcome is thwarted by factors impossible to control, including weather, climate and geology (Talley and Sleeper 1997). The benefit of the rumen is that it is a natural, transportable bioreactor, which is contained and unaffected by weather; it is the ultimate combination of *in situ* and *ex situ* treatments. However, variation in the bioremediation of RDX as affected by the factors outlined above still need to be determined for phytoruminal-bioremediation to be a realistic mitigation approach. Further, if grass will translocate multiple explosive residues out of the soil for grazing sheep, research aimed at understanding if degradation of one compound such as RDX, is affected by the presence of others, such as TNT, HMX and various forms of dinitrotoluene (DNT), needs to be carried out. Lastly, the efficiency of the ruminal microbes to extract munitions from the ingested explosive-laden plant material as a delivery vehicle (Stenuit and Agathos 2010) needs to be investigated.

Acknowledgements

The authors would like to thank Michael Wiens for his technical assistance. This research was supported in part by a gift from Ruminant Solutions, LLC (New Mexico), the Oregon

Agricultural Experiment Station project #ORE00871 and the United States Department of Agriculture, Agriculture Research Service project #50-1265-6-076. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the United States Department of Agriculture.

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591 1,3,5-triazine through initial reduction to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine followed
592 by denitration in *Clostridium bifermentans* HAW-1. Appl Microbiol Biotechnol **63**, 187–193.

593

594

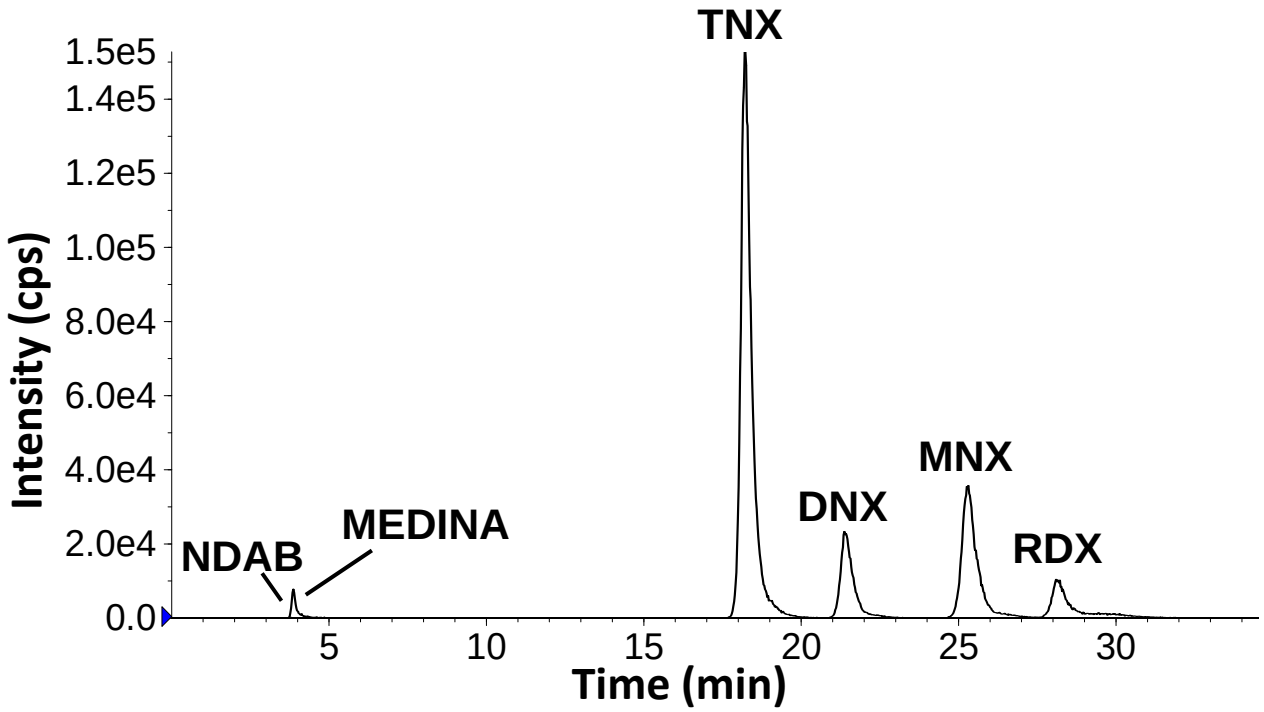
Fig. 1. A: Extracted ion chromatogram overlay of RDX and metabolite standards (50 ng/mL) obtained via LC-MS/MS (APCI-) (top). Extracted ions include RDX (46.0→281.0), MNX (46.0→265), DNX (46.0→249.0), TNX (59.0→233.0), NDAB (61.1→117.9) and MEDINA (60.9→134.9). Fragmentation was obtained using a Q1 scan, scanning from 50-600 amu.

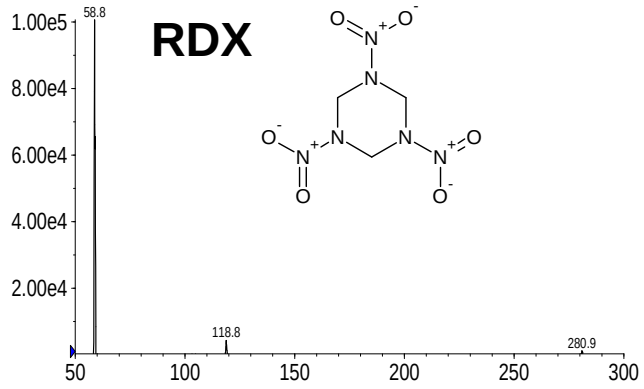
Fig. 2. RDX and metabolite concentrations as determined by MRM quantification via LC-MS/MS over 24 hours in whole rumen fluid microcosms. Error bars represent the standard deviation of three replicate samples per time point.

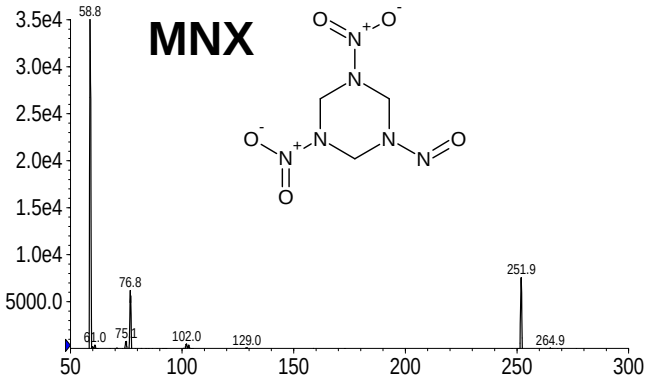
Fig. 3. Total ion chromatograms (TIC) of WRF incubated with 180 µM RDX at 0.25 (A) and 24 hours (B), obtained using an enhanced mass spectra scan. C and D represent LC-MS (APCI-) scans of metabolite peaks consistent with *m/z* of 193 and 149, respectively.

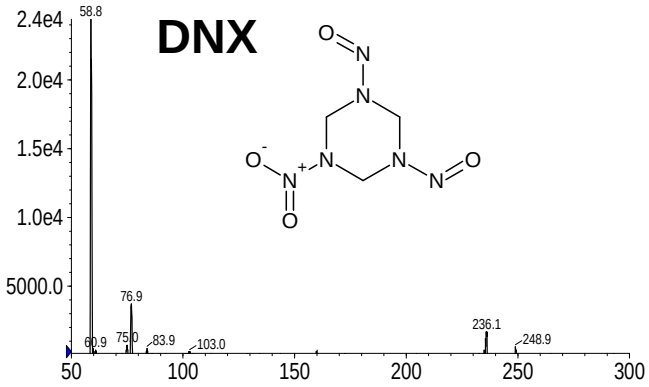
Fig. 4. Proposed biodegradation pathway for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by ruminal microbes under anaerobic conditions as determined by enhanced mass spectra and LightSight analysis. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX); hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX); hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX).

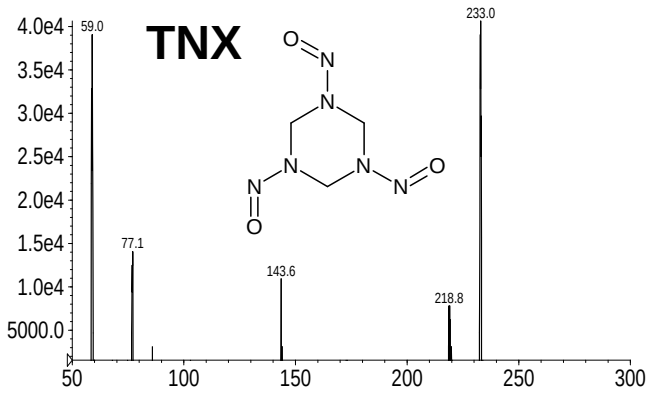
Fig. 5. RDX concentration as determined by MRM quantification via LC-MS/MS over 120 hours from incubations with ruminal isolates identified to be most efficient at RDX degradation.

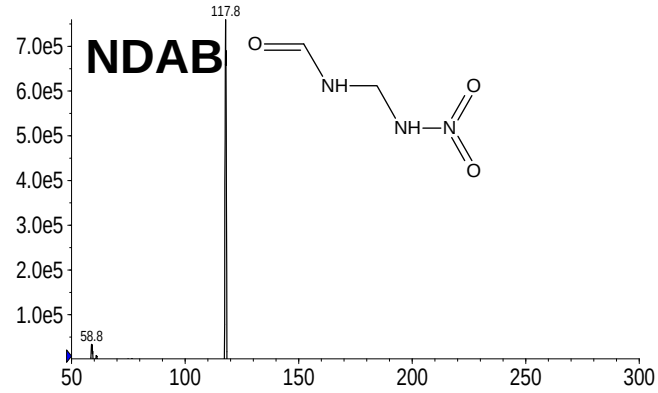

TNX
MNX
DNX
MEDINA
NDAB
RDX
Intensity (cps)
Time (min)

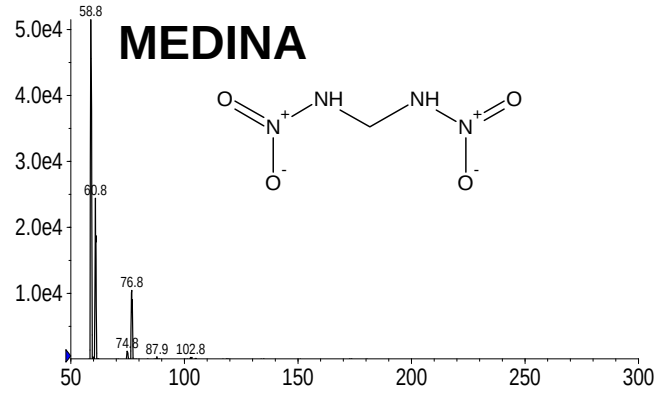

RDX
58.8
118.8
280.9

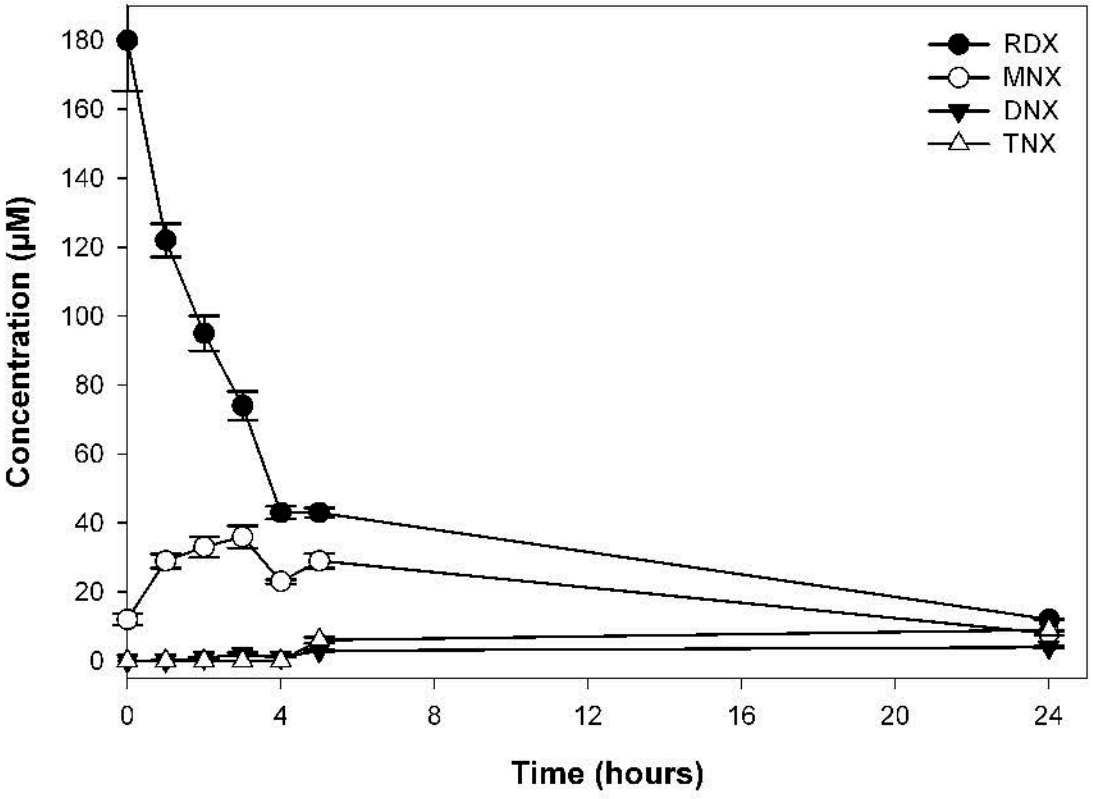

MNX
58.8
76.8
251.9

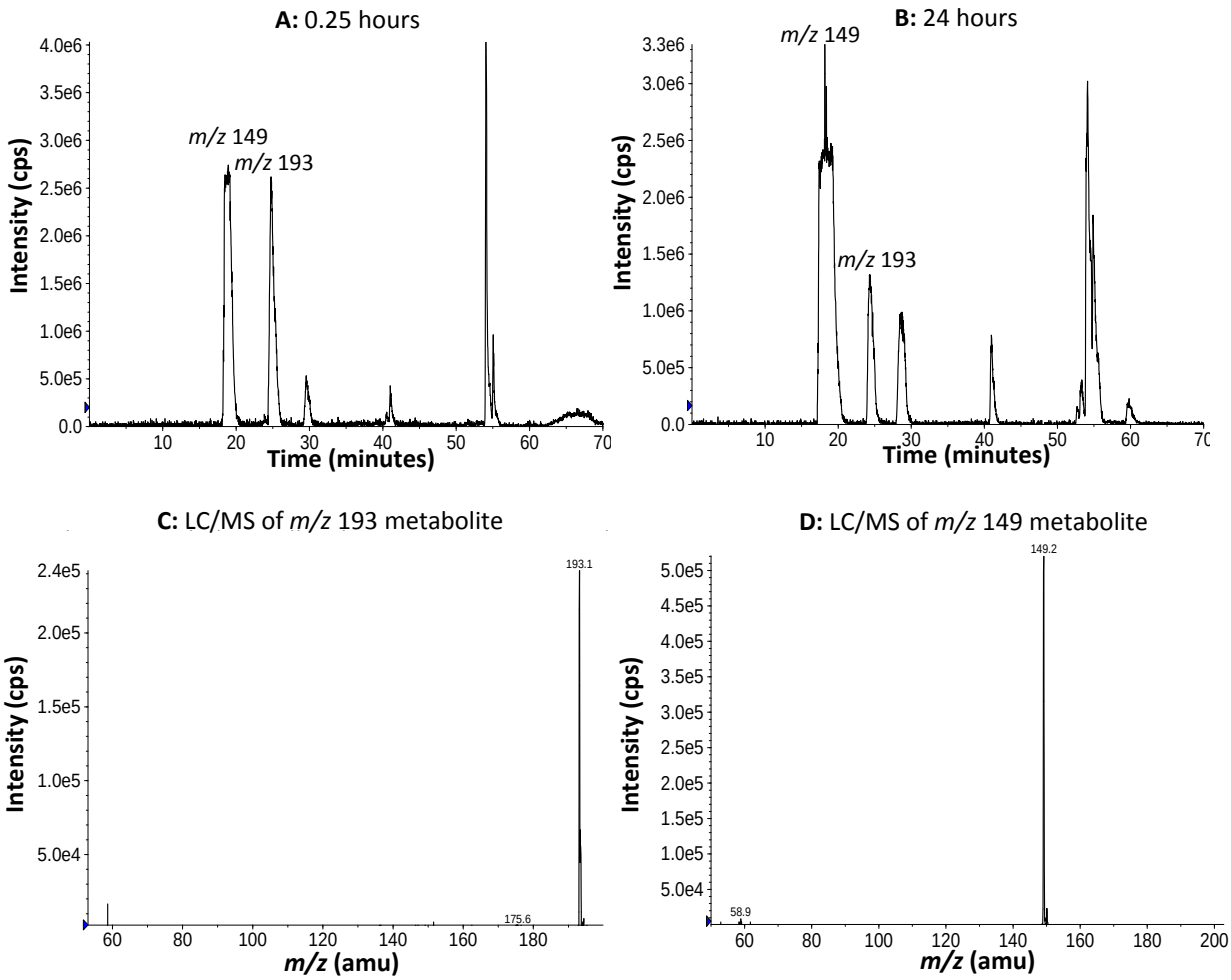

DNX
58.8
76.9
236.1

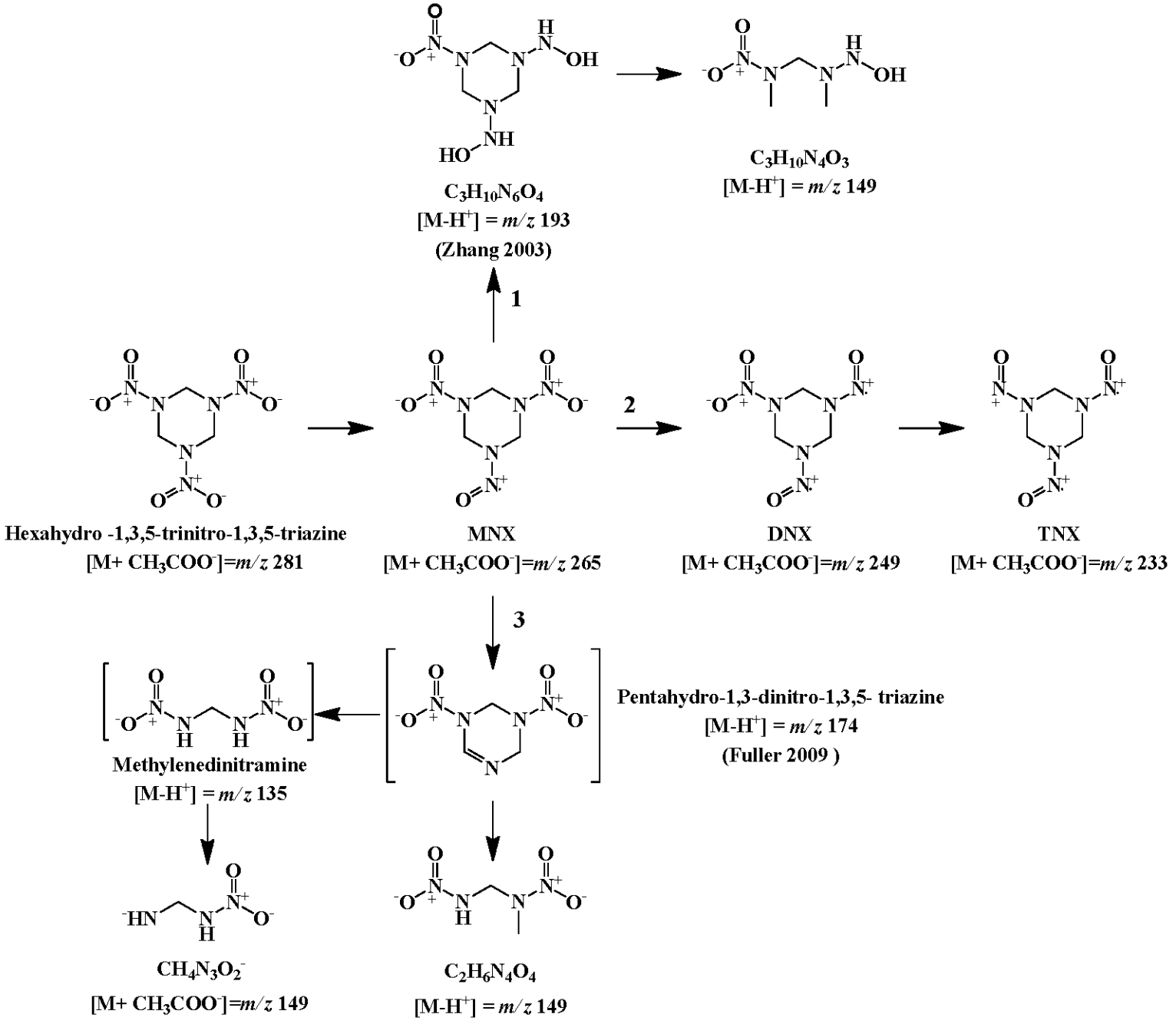

TNX
59.0
77.1
143.6
218.8
233.0


NDAB
117.8
58.8


MEDINA
58.8
60.8
76.8


RDX
MNX
DNX
TNX
Concentration (µM)
180
160
140
120
100
80
60
40
20
0
0
4
8
12
16
20
24
Time (hours)


A: 0.25 hours
m/z 149
m/z 193
Intensity (cps)
Time (minutes)
B: 24 hours
m/z 149
m/z 193
Intensity (cps)
Time (minutes)
C: LC/MS of m/z 193 metabolite
193.1
175.6
Intensity (cps)
m/z (amu)
D: LC/MS of m/z 149 metabolite
149.2
58.9
Intensity (cps)
m/z (amu)


C3H10N6O4
[M-H+] = m/z 193
(Zhang 2003)
C3H10N4O3
[M-H+] = m/z 149
1
2
3
Hexahydro -1,3,5-trinitro-1,3,5-triazine
[M+ CH3COO-]=m/z 281
MNX
[M+ CH3COO-]=m/z 265
DNX
[M+ CH3COO-]=m/z 249
TNX
[M+ CH3COO-]=m/z 233
Pentahydro-1,3-dinitro-1,3,5- triazine
[M-H+] = m/z 174
(Fuller 2009)
Methylenedinitramine
[M-H+] = m/z 135
CH4N3O2-
[M+ CH3COO-]=m/z 149
C2H6N4O4
[M-H+] = m/z 149

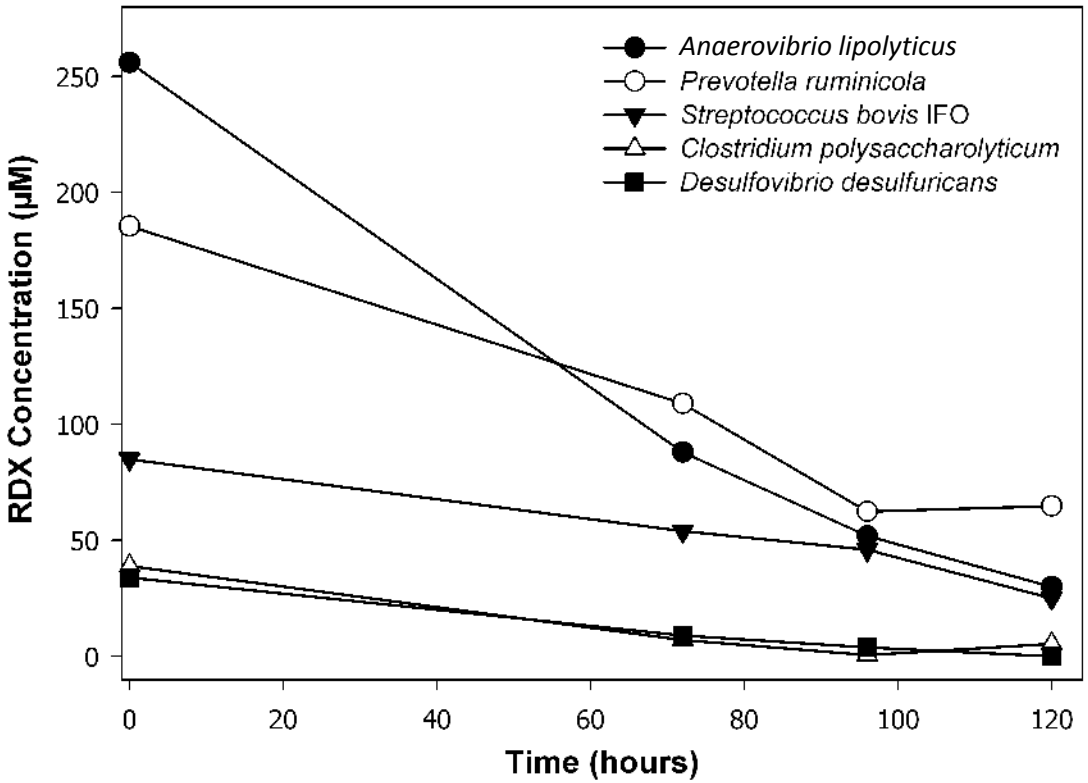

Anaerovibrio lipolyticus
Prevotella ruminicola
Streptococcus bovis IFO
Clostridium polysaccharolyticum
Desulfovibrio desulfuricans
RDX Concentration (µM)
250
200
150
100
50
0
0
20
40
60
80
100
120
Time (hours)

Table 1. Strains and sources of ruminal bacteria tested for RDX degradation ability.

Organism	Strain	Source[1]
Anaerovibrio lipolyticus		ATCC 33276
Butyrivibrio fibriosolvens	D1	ATCC 19171
	nyx	ATCC 51255
Clostridium bifermentans		ATCC 17836
Clostridium pasteurianum	5	ATCC 6013
Clostridium polysaccharolyticum	B	ATCC 33142
Desulfovibrio desulfuricans subsp. *desulfuricans*	MB	ATCC 27774
Eubacterium ruminantium	GA 195	ATCC 17233
Fibrobacter succinogenes	S85	ATCC 19169
Lactobacillus ruminus	RF1	ATCC 27780
Lactobacillus vitulinus	T185	ATCC 27783
Megasphaera elsdenii	T-81	ATCC 17753
Peptococcus heliotrinreducens		ATCC 29202
Prevotella albensis	M384	DSMZ 13370
Prevotella bryantii	B14	DSMZ 11371
Prevotella ruminicola		ATCC 19189
Selenomonas ruminantium	HD4	ATCC 27209
	PC18	ATCC 19205
Streptococcus bovis	IFO	ATCC 15351
	JB1	ATCC 700410
Streptococcus caprinus	2.2	ATCC 700065
Succinovibrio dextrinosolvens	0554	ATCC 19716
Veillonella parvula	TE3	ATCC 10790
Wolinella succinogenes	FDC 602 W	ATCC 29543

[1]ATCC = American Type Culture Collection; DSMZ = Leibniz-Institut DSMZ-German Collection of Microorganisms and Cell Cultures

Table 2. Percent degradation of RDX by ruminal isolates after 120 hours, where RDX was supplemented as either the sole source of nitrogen (LNB) or carbon (LCB).[a]

Organism	Initial RDX concentration (μM)	%RDX degradation in LNB	%RDX degradation in LCB
Anaerovibrio lipolyticus	85	70	5
Butyrivibrio fibriosolvens D1	34	37	15
Butyrivibrio fibriosolvens nxy	34	7	18
Clostridium bifermentans	34	19	29
Clostridium pasteurianum	34	3	49
Clostridium polysaccharolyticum	186	40	65
Desulfovibrio desulfuricans subsp. *desulfuricans*	256	3	88
Eubacterium ruminantium	34	32	6
Fibrobacter succinogenes	34	0	3
Lactobacillus ruminus	34	6	0
Lactobacillus vitulinus	34	39	0
Megasphaera elsdenii	34	33	27
Peptococcus heliotrinreducens	34	0	14
Prevotella albensis	34	19	7
Prevotella bryantii	34	25	4
Prevotella ruminicola	34	96	3
Selenomonas ruminantium HD4	34	33	34
Selenomonas ruminantium PC18	34	41	25
Streptococcus bovis IFO	39	86	18
Streptococcus bovis JB1	34	12	20
Streptococcus caprinus	34	49	18
Succinovibrio dextrinosolvens	34	0	18
Veillonella parvula	34	2	21
Wolinella succinogenes	34	14	7
Control[b]	256	19	20

[a]*Data are expressed as average percent degradation of initial RDX concentration during 120 h incubations in low nitrogen basal (LNB) and low carbon basal (LCB) media, using three replicates for each organism.*

[b]*Control consisted of anaerobically reduced medium with RDX and no bacteria.*