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The anaerobic and highly reductive conditions found in the ovine rumen are considered favorable for the degradation of the nitroaromatic explosives such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). In this study, we used stable-isotope-probing to determine the bacteria responsible for the degradation of RDX in the rumen. Results indicate 10 μg mL^{-1} (45 μM) of ^{13}C-RDX was degraded within 4 hours. 16S rRNA data implicate members of the genera *Brevundimonas* sp., *Methylobacterium* sp. and *Sphingomonas* sp. in RDX degradation from sheep rumen fluid. Members of the genus *Methylobacterium* have been known to degrade RDX previously whereas this is the first study implicating *Brevundimonas* sp. and *Sphingomonas* sp. in RDX degradation. Enzymes known to participate in RDX degradation, nitroreductase and XplA, were not detected in ^{13}C-DNA samples. This study indicates a previously unknown population of ovine ruminal bacteria is capable of degrading RDX via an as yet unidentified pathway.

Stable Isotope Probing of the Ovine Rumen for RDX Degrading Microorganisms

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Edward A. Mitchell, Author

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Introduction

Hexahydro-1,3,5-Trinitro-1,3,5-Triazine ($C_3H_6N_6O_6$, MW = 222.16) is a highly explosive nitroamine which also goes by the names Cyclonite, Hexogen, and most commonly Research Department composition X or RDX. The solubility of RDX is relatively low and has been measured to be approximately 38.4 mg L^{-1} at 20°C. Regular use of this compound dates back to World War II and it is still used in high explosives in the present day.

Residual contamination from live fire training exercises and ammunition manufacturing facilities exists at many sites across the US and Canada [1, 2] and in one case was detected in drinking water supplies within a military reservation [3]. Intoxication with RDX through accidental ingestion of contaminated plant matter or groundwater has yet to be documented. Direct accidental and intentional ingestion of RDX containing compounds by humans has occurred several times and was shown to induce rash, nausea, and neurological effects including headaches, dizziness and seizures [4]. For neurological effects in humans the lowest observable adverse effect level was determined to be 357 mg kg^{-1} body weight day^{-1}. RDX is classified as a possible human carcinogen [5]. In plants RDX has been shown to adversely affect growth; the chemical was transported into plant tissue and was correlated with the concentration in soil [6]. Agronomically important plants have been shown to take up RDX into aerial tissues and, upon accumulation over time, cause necrosis and bleaching of plant tissues [7]. Several animal studies have been conducted to establish

a toxicological profile for RDX. In juvenile deer mice the LD50 has been established as 135mg kg^{-1} [8]. RDX demonstrates higher lethal character in rats at a LD50 of ~100 mg kg^{-1} with the metabolite mononitroso form of RDX, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), proving almost half as lethal at a LD50 of 187 mg kg^{-1} [9].

The currently accepted method of contaminated site cleanup involves excavation and incineration of RDX containing soil at great monetary cost and further risk to the environment in the form of air pollution [2]. Additionally soil which has undergone thermal degradation is unable to sustain plant life whereas bioremediation has none of these side effects and may ultimately be a cheaper solution as well [10]. Phytoremediation has been looked at as a possible solution to munitions cleanup, however, it has been found that RDX is taken up by plants and concentrated in its parent form in aerial tissues potentially remaining toxic [6]. Microbial degradation of munitions is considered a promising form of bioremediation as bacteria have been known to naturally attenuate RDX *in situ* for some time [11, 12]. Several studies have been conducted with aerobic and anaerobic microbes and both have been shown to be effective at degrading RDX to some extent however anaerobic conditions have shown to be necessary to achieve complete degradation [13].

The ovine rumen is a self-contained obligately anaerobic environment and it is known that concentrations of microbes can reach in excess of 1 $x10^{10}$ cells g^{-1} with only the dominant species being well understood [14]. Estimations of the number of operational taxonomic units (OTUs) have reached as high as 341, or more than triple previous estimates, with only a fraction of the sampled population consisting of known

organisms [15]. Anaerobic conditions in the rumen are necessary to provide optimal conditions for fiber degradation and it has been known for some time that the resident microflora have a protective effect against known toxins. Previously it has been shown that the ovine rumen possesses microbes which degrade the toxic plant alkaloids thus protecting sheep from hepatocellular death whereas cattle do not possess these protective bacteria [16]. Breakdown of dihydroxypyridines, metabolic intermediates derived from mimosine, has been credited to ruminal microorganisms [17]. Nitropropionic acid, another plant toxin, is also degraded by ruminal microbes [18]. Given the presence of nitro groups present in nitropropionic acid and 1,3,5-trinitrotoluene (TNT) it was postulated that these same bacteria would be capable of detoxification of nitroaromatic explosives. Indeed it was shown that several ruminal bacteria were capable of individually degrading TNT in pure culture after relatively short incubation times [19]. Activity measured *in vitro* does not necessarily translate to how the microbe would act if it were used as soil inocula in bioremediation applications. Advanced molecular techniques will be needed to determine if bacteria are actively degrading nitroaromatic compounds *in situ*.

Stable-Isotope-Probing is a powerful technique and has shown the capacity to reveal uptake of labeled carbon substrates in complex bacterial communities. The technique has been applied in soils to detect acetate metabolism [20], in contaminated groundwater to detect benzene degrading bacteria [21], and to determine the fate of phenol in activated sludge [22]. Identification of microorganisms through SIP relies on the incorporation of ^{13}C labeled carbon into nucleic acids [23]. Eventual downstream differentiation and analysis is possible due to the separation of labeled

and unlabeled DNA in a self-forming cesium chloride (CsCl) gradient. Composition of the labeled substrate can vary widely from that seen in simple compounds such as ^{13}C-methanol [24] to complex carbon substrates as found in wheat [25]. The technique has been known for many years and was originally used to provide evidence of semi-conservative DNA replication [26].

RNA-SIP is a variant of the technique which depends on the extraction and analysis of the RNA molecule in a manner similar to DNA. In theory, sensitivity is increased with RNA as it is not dependent upon cell division for labeling to occur leading to much more rapid label incorporation [27]. This will reduce trophic crossfeeding effects which could otherwise obscure the actual microorganisms participating in degradation. RNA SIP has been used on conjunction with DNA to elucidate changes in complex microbial communities [28], however, the technique alone has never been used to find RDX degrading organisms.

Through leveraging SIP techniques we intend to probe whole rumen fluid (WRF) for the presence of RDX degrading microorganisms. Pilot studies conducted in the laboratory show that WRF is capable of degrading 10 μg ml^{-1} (45 μM) of RDX within 4 hours. It is believed that the cause is biological in origin due to previous studies on plant toxins being neutralized by ruminal microflora. Presently it is unknown to what extent RDX is mineralized in WRF. The goal of this study was to identify which microorganisms in WRF are degrading RDX through stable isotope probing. For technical considerations ^{13}C labeled RDX was used as the substrate with downstream analysis of RNA and DNA. Previously it has been found that firmicutes, specifically members of the *Clostridium* genus, were capable of RDX degradation

[29]. The rumen harbors several firmicutes and some members of the clostridiales clade [30]. It is expected that these organisms are participating in breakdown of RDX.

Literature review

Cleanup of RDX contamination has been an active research topic for many decades. Incineration is the commonly accepted form of cleanup though it is invasive, expensive, and generates air pollution. Abiotic means of cleaning up RDX spills have recently been pursued with investigations into electrochemical reduction [31], iron nanoparticles [32], boiling water [33], and ozone [34]. Biological degradation has been heavily pursued as well including the study of fungi [35], plants [36], constructed wetlands [37], and microbes. The most promising, and most heavily studied, route to remediation of contaminated water and soil involves the use of bacteria. In general the research can be divided into three areas; aerobic microbes, anaerobic microbes, and mixed cultures. The following review of literature involves the discussion of previously studied bacteria and archaea which are capable of degrading RDX and are divided into the three distinct areas of research. Major findings in each study are discussed with particular emphasis on quantity of RDX used, metabolites found, and potential degradation pathways.

RDX degrading aerobic bacteria

Aerobic bacteria were not originally thought to be capable of degrading RDX [11]. Subsequent studies have thoroughly disproven that and aerobes have become more intensively studied as a result. Soil has yielded most of the aerobes studied to date. Presence of RDX as a contaminant in the soil does not appear to determine if these bacteria are capable of degradation or not. A RDX degrading gene was identified in a handful of related aerobic bacteria; not all of aerobes possess this gene.

One of the more promising studies involves the degradation of RDX aerobically by the soil bacteria *Stenotrophomonas maltophilia* [38]. In less than 200 hours the bacteria was able to clear a concentration of about 44 μg mL^{-1} RDX while utilizing the explosive as a sole nitrogen source. This is one of the earliest reports of bacteria capable of relying on RDX as a growth substrate. This is also likely the first report describing aerobic degradation of RDX. Unfortunately metabolites were not very well characterized in this study and no further work has been carried out with this organism.

Rhodococcus rhodochrous DN22 is likely the most well studied RDX degrading microorganism to date. First isolated from RDX contaminated soil in 1997, the bacteria was shown to aerobically degrade a toxin concentration of 35 μg mL^{-1} in less than 20 hours [39]. Anaerobic conditions did not induce growth or degradation. DN22 is capable of utilizing RDX as a sole nitrogen source and degradation is inhibited by the presence of NH_4^+. The researchers went a step further and inoculated RDX contaminated soil with DN22 and degradation was observed raising the possibility that the bacteria would be active *in situ*. Metabolites were not characterized in this study, so the extent to which RDX had been degraded was unknown at the time. A separate study was conducted to identify the metabolites generated by *R. rhodochrous* [40]. Interestingly no nitroso intermediates were found, however, formaldehyde was a common intermediate. Much of the carbon was detected as CO_2 or a dead end metabolite of MW 119 suggesting the compound does not serve as a carbon source. Further studies located the gene responsible for degradation on a plasmid suggesting horizontal gene transfer may confer degrading

capabilities to other organisms [41, 42]. The gene was found to encode for a cytochrome P450 enzyme dubbed *xplA* and has a homolog named *xplB* [43, 44].

Pseudomonas sp. HK6 is a soil microbe which was tested for ability to clear RDX from culture media [45]. The researchers approached the study from a different angle than previous works. Firstly they were able to determine the organism could completely degrade a 10 μg mL^{-1} (45μM) RDX concentration within 24 days. Determination of metabolic intermediates was not thoroughly carried out rather they chose to pursue identification of physiological damage and induction of stress shock proteins (SSPs) DnaK and GroEL in the organism. Scanning electron microscopy revealed physiological damage to cells in the form of pitting on the outer cell membrane. Induction of the two SSPs was also detected and appeared to correlate with concentration of RDX in the media. This is the first evidence of RDX possibly causing physiological damage to a microbe and characterization of the response.

Methylobacterium species are typically associated with the soil. In one instance, this organism was found to inhabit the leaves of poplar trees and displayed the ability to degrade RDX [46]. In approximately 40 days a RDX concentration of 20 μg mL^{-1} was cleared. The most interesting metabolic function of this organism is the ability to utilize formaldehyde, a degradation intermediate, as a carbon source. Intermediates methylene dinitramine (MEDINA) and MNX were detected but disappeared by the end of the incubation period, however, no degradative pathway was proposed. Another *Methylobacterium sp.* displayed ability to metabolize the RDX degradation intermediate 4-nitro-2,4-diazabutanal (NDAB) [47]. In spite of the slow rate of degradation, *Methylobacterium sp*. show promise as RDX bioremediators

as they appear to be effective at achieving near complete mineralization of the toxic explosive.

Closely related to the *Rhodococcus* species are two other soil microbes, *Gordonia sp*. KTR9 and *Willamsia sp*. KTR4, which were able to aerobically utilize RDX as a sole carbon and nitrogen source [48]. The soil from which they were isolated did not contain any measurable levels of RDX. Formaldehyde, nitrite, and nitrous oxide were detected as metabolites, however, no assay of the -nitroso intermediates was performed. Another missing piece of this work is the lack of identification of assay for the *xplA* gene or any insight into a possible degradative or metabolic pathway. Due to the organisms ability to utilize RDX as a carbon source, some assimilation of carbon is occurring as is mineralization evidenced by the detection of $^{14}CO_2$.

The most significant finding through the study of aerobic bacteria is the discovery of the *xplA* gene. Though it is unlikely that this gene evolved specifically to degrade RDX it does have a very high activity. Incubations with various *Rhodococcus sp*. that carry a plasmid with the *xplA* gene were carried out at RDX concentrations of 55.5 μg mL^{-1} (250μM) [44]. Five strains were able to remove over half of the starting concentration within 60 minutes with nearly all demonstrating degradation within 24 hours. These data suggests *xplA* has a high specificity for RDX and inter-strain variation is likely due to permeability differences. Strains of *Rhodococcus sp*. have not been tested as soil inocula, however, *xplA* has been successfully genetically engineered into *Arabidopsis thaliana* [49].

RDX degrading anaerobic bacteria

Morganella morganii, *Providencia rettgeri*, and *Citrobacter freundii* were all isolated from nitroaromatic contaminated soil [50]. Only *M. morganii* and *P. rettgeri* were able to fully degrade RDX, MNX, and the dinotroso form of RDX hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) intermediates at a concentration of 73.26 μg mL^{-1} (330 μM) over 45 days. *C. freundii* cleared about 90% of the initial RDX, however, MNX and DNX accumulated in the media suggesting the organism is unable to degrade these compounds. TNX accumulated in the media as well though in a much smaller portion relative to MNX and DNX. Interestingly, degradation of RDX would only occur under anaerobic conditions even though these organisms were facultative anaerobes. The study was conducted with radioactive RDX and $^{14}CO_2$ accumulation was only detected under anaerobic conditions indicating metabolism of the compound. Yeast extract was a media component so it is unknown if RDX is being utilized as a growth substrate.

Sludge isolate *Klebsiella pneumoniae* SCZ-1 was able to degrade 23.5 μg mL^{-1} (106 μM) of RDX in approximately 30 days [51]. Small amounts of MNX were detected in the media as a RDX intermediate, however, it was below detection limit at 30 days. MNX was degraded within 25 days when added to the media at a concentration of 100 μM. The researchers noted that DNX only accumulated in trace amounts. TNX was degraded at a rate six times slower than that of RDX or MNX. SCZ-1 was unable to grow when RDX was provided as the sole carbon or nitrogen source. Formaldehyde, MEDINA, and N_2O were all detected in the media suggesting

at least some of the toxicant was being mineralized. Degradative capabilities of this organism have not been investigated any further which likely results from the slow rate of RDX removal relative to other anaerobes.

Acetobacterium malicum HAAP-1 was isolated out of wastewater from an ammunition plant as an anaerobic mixed culture [52]. The culture was transferred serially into a mineral media under methane producing conditions until individual colonies were isolated by streak plate. Presumably the toxin would apply pressure to select for organisms which are at a minimum resistant to its toxic properties. Ultimately the organism *A. malicum* HAAP-1 was isolated and identified through 16S rDNA sequencing. This is the first report of an acetogen possessing the capability to degrade RDX. In minimal media, the isolate was able to degrade RDX at a beginning concentration of 6.66 μg mL^{-1} (30 μM) over 15 days with a headspace containing 80% hydrogen gas. Ultimately the researchers could not induce degradation if the headspace was hydrogen free or if the electron donor was changed to yeast extract. MNX, MEDINA, and formaldehyde were the only detected intermediates resulting in a cryptic degradation pathway. These intermediates are common amongst RDX degradation studies [29, 48, 53].

Clostridium bifermentans HAW-1 is another obligate anaerobe demonstrating less restrictive substrate requirements than the archaea *A. malicum*. Isolated from anaerobic sludge, an accepted treatment for RDX contaminated soil, the organism was able to degrade RDX at a concentration of 23.1 μg mL^{-1} (104 μM) over approximately 100 hours with yeast extract as the only other carbon source [29]. Brain-heart infusion broth had been used previously to characterize the RDX degrading capabilities of

another strain of *C. bifermentans* [54]. *C. bifermentans* HAW-1 produced metabolites MNX, MEDINA, and formaldehyde. Substantial quantities of methanol were also detected. Further, the isolate was able to grow on MNX which is likely the first evidence of an organism being able to grow on the toxic-nitroso intermediate. DNX and TNX were also produced though in relatively minor proportion. Attempts to grow the strain with TNX did not lead to compete degradation though common products, formaldehyde and methanol were detected. This isolate has shown promise as it does not require uncommon electron donors such as hydrogen gas to degrade RDX. It is not a single solution to bioremediation since it does not completely degrade toxic intermediates.

The most recently researched anaerobe *Desulfovibrio sp*. EFX-DES shows potential as a remediator [55]. It is capable of using RDX as the sole carbon and nitrogen source and had been tested at a concentration of 35.5 $\mu g\ mL^{-1}$ (160 μM) which was gone in 41 days. MNX and DNX were detected in low amounts, however TNX, MEDINA, and formaldehyde were not. Other *Desulfovibrio sp*. have been previously implicated in RDX degradation [56].

Efforts continue towards finding effective anaerobic RDX degrading isolates. *Clostridium bifermentans* HAW-1 and *Desulfovibrio sp.* EFX-DES show promise in reaching complete mineralization. These species may also serve as effective inocula for contaminated soil thus eliminating the need for expensive and time consuming excavation of contaminated sites however evidence suggests mixed cultures are more effective at removal of RDX and its nitroso intermediates.

Mixed culture degradation of RDX

The first insights into the microbial degradation of RDX occurred as early as 1973 with a more concerted effort to understand the process occurring in 1981 [11]. The major nitroso-intermediates were first detected in this work and a proposed pathway has served as the basis for most degradation studies to date. The researchers were able to detect degradation of 50 μg mL^{-1} RDX in approximately seven days in nutrient broth inoculated with sewage sludge with MNX, DNX and TNX disappearing by 18 days. The researchers wrongly concluded that degradation could only occur anaerobically as discussed later. Future studies produced sludge isolates *Klebsiella pneumoniae* SCZ-1 [51] and *Acetobacterium malicum* HAAP-1 [52]. Sewage sludge was revisited many years later with the further insights into the degradation pathway and production of intermediates MEDINA and bis(hydroxymethyl)nitramine [57].

Sulfate reducing and methanogenic conditions are considered favorable for complete RDX degradation. Several sulfate reducing bacteria, specifically *Desulfovibrio sp*., have been shown to utilize RDX as a sole nitrogen source with 25 μg mL^{-1} (112.6 μM) of the explosive being cleared in approximately 15 days [58]. Though it was shown that ammonia accumulated in the media, no further insights into intermediates or the pathway were achieved. Additionally, the researchers postulate such a consortia could be used in soil, however, no soil based experiments were conducted. Methanogenic mixed cultures show some promise, however, they require the addition of zero-valent iron (Fe^0) to achieve mineralization [59]. Fe^0 was revisited and found to select for methanogens which could mineralize RDX with methane being

the expected end product [60]. In both cases, the toxic nitroso intermediates were initially detected but later fell below detectable quantities. The mechanism of action relies on hydrogen gas serving as an electron donor in the eventual production of methane. It was shown later that a separate methanogenic mixed culture and an acetogen in pure culture were able to degrade RDX so long as H_2 was provided as the electron donor [52, 61]. In a longer term study where an anaerobic mixed culture was carried out to approximately one year, it was shown that an undefined methane producing mixed culture could rely on RDX as the sole source of carbon suggesting hydrogen gas is not a requirement [62]. Again, nitroso intermediates remained below detectable limits.

Complete mineralization and lack of toxic intermediates are the hallmarks of mixed culture degradation of RDX. In general, they tend to act slower than most pure culture bacteria, aerobic and anaerobic, however, complete mineralization is the goal with regards to RDX cleanup. Work continues on identifying consortia which are capable of degrading RDX and serves as the basis for investigations into rumen fluid as a source of these microorganisms.

Materials and Methods

Rumen fluid collection

Equal volumes of WRF were collected from two cannulated wethers via a sterile suction probe and syringe. Samples were collected and mixed in sterile 60 mL screw cap test tubes and transported immediately back to the lab. The samples were passed into an anaerobic tent with an atmosphere of 92% CO_2:8% H_2. The WRF was blended, filtered through four layers of sterile cheesecloth to remove large particles, and collected into a sterile beaker.

Experimental protocol

Equal volumes of whole rumen fluid were added to sterile Balch tubes and sealed with butyl rubber stoppers. The first treatment consisted of rumen fluid with ^{13}C-RDX in acetonitrile (99% purity, Cambridge Isotopes, Andover MA) added to a concentration of 10 µg mL^{-1}. The second treatment consisted of rumen fluid with unlabeled RDX (^{12}C) (ChemService, West Chester, PA) added to a concentration of 10 µg mL^{-1}. The third treatment consisted of autoclaved rumen fluid with unlabeled RDX (^{12}C) added to a concentration of 10 µg mL^{-1}. The ^{12}C and autoclaved treatments were repeated in triplicate for the purpose of analyzing degradation profiles through High Performance Liquid Chromatography (HPLC). Samples were taken at zero, two, and four hours. Between sampling points, the tubes were placed in a shaking incubator at 39°C in the dark. After the four-hour time point was reached, the labeled and unlabeled tubes were amended with more RDX to bring the concentration up to 15 µg

mL^{-1}. Samples were taken again at zero, two, and four hours. An initial pulse of 15 µg mL^{-1} was used in autoclaved controls and no further additions of RDX were made.

HPLC and qualitative LC-MS analysis

Samples from each time point were spun in a microcentrifuge at 4°C for 10 minutes at 16,000 x *G*. The supernatant was then passed through a 2 µm PTFE syringe filter (VWR, Brisbane, CA) and stored at -20°C prior to analysis. Concentrations of RDX in each time point were analyzed on a Series 200 HPLC system (Perkin Elmer, Waltham, MA) coupled with an Acclaim Explosives E1 column (Dionex, Sunnyvale, CA). The analyte was eluted isocratically with a mobile phase of 43:57 MeOH:ddH_2O at a flow rate of 1 mL min^{-1}. Column temperature was maintained at 32°C with a column oven. Detection was performed on a Series 200 photodiode array (Perkin Elmer) at 243 nm.

LC-MS/MS was used to qualitatively assess the presence of the parent molecule and potential metabolites in the ^{12}C spiked samples. Analysis was performed on an ABI/SCIEX (Applied Biosystems, Foster City CA) QTRAP 3200 LC-MS/MS system using an atmospheric pressure chemical ionization (APCI) probe. Samples were separated on an HPLC system (Perkin Elmer Series 200 Micropump), based on a previous protocol [63]: a Phenomenex Ultracarb ODS(20), 250 x 4.6 mm, 5 µm particle size column (Phenomenex, Torrance CA) and mobile phase consisting of 0.5 mM ammonium acetate and methanol was run isocratically at 0.8 mL min^{-1} for 15 minutes. Data was acquired using enhanced MS (EMS) as the survey scan which triggered an enhanced product ion (EPI) scan using information dependent acquisition

experiments developed using Analyst 1.4.2 software (Applied Biosystems). Curtain gas (nitrogen) was set at 10 psi, ion source gas (nitrogen) at 30 psi, nebulizing current at -5μA, temperature at 350°C, declustering potential at -15V, entrance potential at -10V, collision energy at -10eV, and a scan range of 100 to 400 Da. Samples were prepared for LC-MS/MS analysis as described above with the additional step of diluting samples in a 1:1 ratio with 1% acetic acid. LightSight 2.0 software was used to capture and interpret data (Applied Biosystems).

DNA manipulation

DNA from WRF was collected from 1 mL of the four and eight-hour time points as described previously and resuspended in 10 mM Tris-Cl (pH 8.5). All DNA quantification was performed on a Nanodrop ND-1000 (Thermo Fisher, Waltham MA). Ten μg of WRF DNA was loaded into a 5.1 mL polyallomer centrifuge tube (Beckman Coulter, Carlsbad, CA) consisting of CsCl and 25 μL SYBR Safe DNA stain (Invitrogen, Carlsbad, CA) as described previously [64]. Density was adjusted to 1.725 g mL^{-1} through weighing 1000 μL volumes on an analytical balance and adding CsCl or TE accordingly.

A control tube of *E.coli* was used to visually differentiate the labeled and unlabelled DNA. To obtain ^{13}C-DNA, *E. coli* DH5α was grown on uniformly labeled ^{13}C-glucose (Sigma Aldrich, St. Louis MO) in M9 Minimal Media [65]. *E. coli* DNA was extracted with a Qiagen Puregene kit (Qiagen, Valencia, CA) following the manufacturer's protocol for gram-negative bacteria. Unlabeled DNA was collected in the same manner with analytical grade glucose. *E. coli* control tubes were prepared by

mixing 5 µg of ^{12}C-DNA and ^{13}C-DNA, as described above. Tubes were sealed and loaded into a VTi 80 rotor (Beckman Coulter) and centrifugation was performed in an Optima LE-80K ultracentrifuge (Beckman Coulter). Tubes were centrifuged at 60, 500 RPM (~248,000 x *g*) at 20°C for 24 hrs with maximum acceleration and no braking. After centrifugation, heavy and light fractions were visualized with a blue light transilluminator (Clare Chemical Research, Dolores, CO). Visible fractions were collected via puncturing the side of the tube with a sterile syringe and needle as described previously [23]. DNA was precipitated with three volumes of 100% ethanol, washed five times with one volume 70% ethanol and resuspended in 10 mM Tris-Cl (pH 8.5) buffer.

RNA manipulation

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad CA) following manufacturers protocol. RNA was resuspended in Tris-EDTA (TE) buffer and stored at -20° C before further downstream analysis. RNA was quantified on a Nanodrop ND1000 (Thermo Fisher). Preparation of cesium trifluoroacetate (CsTFA) gradients and centrifuge conditions were used as described previously [66]. Tubes were fractionated by cutting off the top with a sterile scalpel and pipetting 150 µL volumes from the top down [25]. RNA-CsTFA was transferred to individual wells of a 96-well block. RNA was precipitated by adding 0.6 volumes of isopropanol to each well, centrifuging at 4000 x g for 20 minutes, and washed twice with 2 volumes of 70% EtOH. RNA was resuspended in 20 µL TE buffer.

Nitroreductase primer design

17 nitroreductase protein sequences (*Bartonella henselae* str. Houston 1, *Burkholderia cepacia* AMMD, *Pseudomonas putida* PnbA, *Pseudomonas pseudoalcaligenes*, *Acidovorax* sp. JS42, *Enterobacter* sp. 638, *Shewanella* sp. MR 4, *Rhodopseudomonas palustris* BisA53, *Escherichia coli* NfsA, *Salmonella typhimurium*, *Vibrio harveyi*, *Pseudomonas putida* PnrA, *Clostridium perfringens* str. 13, *Streptococcus pyogenes* MGAS8232, *Enterobacter cloacae*, *Escherichia coli* NfsB, *Lactobacillus casei* ATCC 334) were imported from the Genbank database and used for primer development. The protein sequences were aligned using Clustal W v1.8 [67] using gap opening penalty of 10.0 and gap extension penalty of 0.01. To evaluate the best suitable protein model, protein alignments were first imported into a program called protTest [68] and analyzed. A Bayesian tree was made using a Bayes block of 500,000 generations with a sampling frequency of 10 and a burn in of 25%. CODEHOP program was used to design degenerate primers with varying degeneracies [69]. The potential primer pairs were blasted using the nucleotide BLAST tool [70] of GenBank and primers were ordered through IDT Corporation (IDT, San Diego, CA).

Polymerase chain reaction (PCR) and RT-PCR

PCR thermocycling was carried out using recombinant AmpliTaq® Gold polymerase (Applied Biosystems, Foster City, CA) in a PTC-200 thermocycler (MJ Research Inc., Watertown, MA). Each 50 µL PCR reaction contained: 75 ng of purified bacterial genomic DNA, 200 µmol of each dNTP, 5 µL of 10x PCR Buffer, 5 µL of 20 mM Mg^{2+}, 200 nM of each dNTP, 20 ng of bovine serum albumin (BSA),

primers at 25 pmoles each, 0.25 U polymerase and remaining made up with sterile water.

Universal PCR primers targeting the V3 region of the bacterial 16S rRNA gene for DGGE were used as described previously [71]. Two µL of resuspended DNA were used as a template for the PCR reaction for the heavy portion of ^{12}C-DNA and ^{13}C-DNA tubes at the four and eight hour time points. All reactions were done in triplicate and PCR products were pooled for downstream analysis. A multiplex PCR was used to amplify archaeal specific V3 products.

Three µL of template DNA was used in the first round with archaeal specific 16S rRNA primers A109F and A934R to exclude bacterial products [72]. DNA from untreated WRF was used as a control. For the second round of amplification, archaeal 16S rRNA V3-DGGE-specific primers were used for the amplification of the products [73].

Primers and PCR conditions for the known RDX degrading gene *xplA* were used as described previously [49, 74]. Amplification for nitroreductase PCR fragment was achieved with a touch down thermocycling program that consisted of a 9 min initial denaturation cycle at 95°C followed by subsequent denaturation for 20 sec. Initial 15 cycles were performed at 65°C with 0.5°C reduction of temperature at each cycle. The primer annealing time was 45 s for each cycle and the final touch down temperature was set at 57°C for an additional 15 cycles followed by a final extension time of 10 min at 72°C.

PCR products from the V3 universal primers were purified using a PCR cleanup kit (Qiagen). DNA concentration was determined on a Nanodrop ND-1000

(Thermo Fisher). Product sizes for the *xplA* and nitroreductase genes were determined through electrophoresis. Five µL of PCR product was loaded per well in a 1.5% agarose gel with a 100 bp ladder (New England Biolabs, Ipswich, MA) and electrophoresed at 125 V for 30 minutes.

RT-PCR was performed using a Superscript II RT-PCR kit (Invitrogen, Carlsbad CA) following manufacturers instructions. V3-DGGE primers were used as described above.

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE analysis was performed on a D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA) with an 8% polyacrylamide gel and 35% to 55% denaturing gradient. For the light fractions, 100 ng of purified PCR product were loaded per well. Only 30 ng of purified DNA was loaded for the heavy fractions due to low yields of PCR product. Electrophoresis was performed at 60 V for 16 hours. Gels were stained with a Silver Staining Kit (Bio-Rad) as per manufactures protocol. Bands of interest were excised from the gel with a sterile scalpel and DNA was extracted using a QIAEX II gel extraction kit (Qiagen). Ten µL of this solution was then used as template in a PCR with the same primers and conditions as described above.

Cloning, sequencing and phylogenetics

Three µL of DNA from selected gradient fractions and reamplified DGGE bands were cloned with a TOPO® TA kit (Invitrogen, Carlsbad CA). Colonies were selected and sequenced at the Center for Genome Research and Biocomputing

(CGRB) at Oregon State University using ABI v3.1 BigDye terminator sequencing technology. Taxonomic assignment and pairwise comparison was performed through the Ribosomal Database Project (RDPII) website [75]. Sequence data and BLAST [70] hits were used to construct a phylogenetic tree. Sequence alignment was performed through the MUSCLE 3.6 software package using default parameters [76]. A phylogenetic tree was constructed through Bayesian phylogenetics using the Mr. Bayes 3.1.4 software package [77]. The data was analyzed for five million generations with sampling every 1000 generations until significance dropped below 0.01. The mothur [78] software package was used to create a distance matrix and generate ACE, Chao1 non-parametric species richness and non-parametric Shannon diversity estimates. All sequences from this study were deposited to the GenBank data under accession numbers GU477781 - GU478313

Results

Disappearance of RDX in WRF

Disappearance of RDX was monitored through HPLC at two-hour intervals. When RDX was no longer present, the Balch tubes were amended with a second addition of RDX (four hours). The initial 10 μg mL^{-1} concentration of RDX was reduced to < 0.2 μg mL^{-1} by two hours and further reduced to < 0.1 μg mL^{-1} by four hours (Figure 1). The tubes were amended with an additional 15 μg mL^{-1} of RDX, and tubes were sampled again two and four hours after the amendment. HPLC based measurements showed the concentration of RDX was < 2 μg mL^{-1} after two hours and < 0.5 μg mL^{-1} four hours after the second amendment. A smaller pulse was used initially to allow for selection of RDX-degrading organisms. Pilot experiments in the laboratory did not consistently demonstrate degradation of RDX in concentrations greater than 15 μg mL^{-1} and so as a precaution the lower concentration was used to allow for adaptation of the native flora to RDX before reaching the maximum concentration (data not shown). A greater amount of RDX was necessary in the second pulse to ensure adequate labeling of microbial biomass. Autoclaved controls did not display disappearance of substrate suggesting that the mechanism is biological in origin (Figure 1).

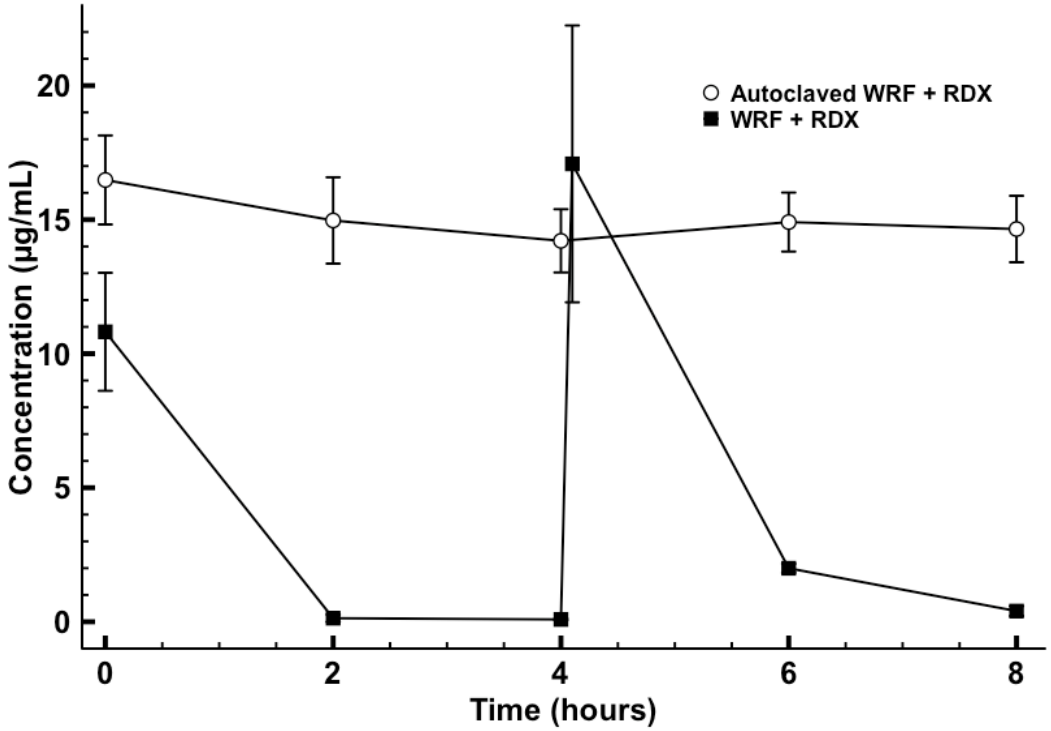

Figure 1 - HPLC plot of RDX degradation in WRF. Average of 3 replicates. Filled square had RDX added to a concentration of 10 µg mL^{-1} initially and reamended to 15 µg mL^{-1} at 4.1 hours. Open circle initially had a starting concentration of 15 µg mL^{-1} with no additions.

Qualitative LC-MS/MS analysis revealed an ion with a m/z of 195 Da [M + CH_3COO^-] consistent with ring breakage intermediate methylene dinitramine (MEDINA) (Figure 2). A second ion with a m/z of 121 Da [M - H] consistent with intermediate bis(hydroxymethyl)nitramine was also detected (Figure 3). Ions consistent with RDX or RDX + acetate adduct were not detected at the four hour time point. Nitroso intermediates hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-nitroso-5-dinitro-1,3,5-triazine (DNX) and hexahydro-1,3,5-nitroso-1,3,5-triazine (TNX) were not detected.

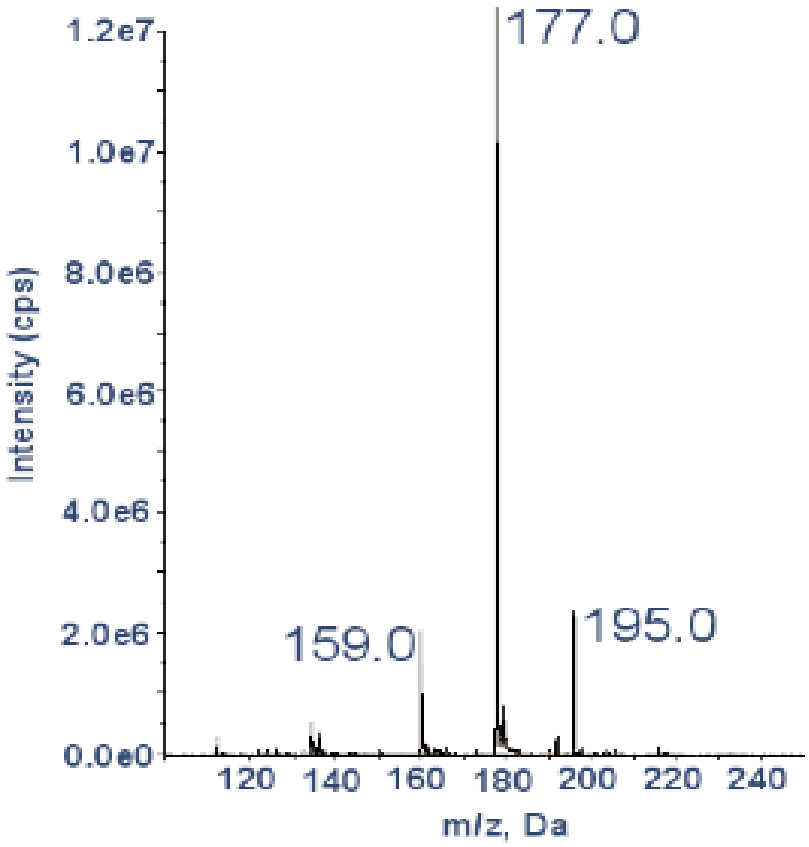

Figure 2 - Mass spectra of prominent ions detected through LC-MS analysis of WRF. Mass 195 is representative of MEDINA + Acetate with daughter fragments of 177 and 159 Da.

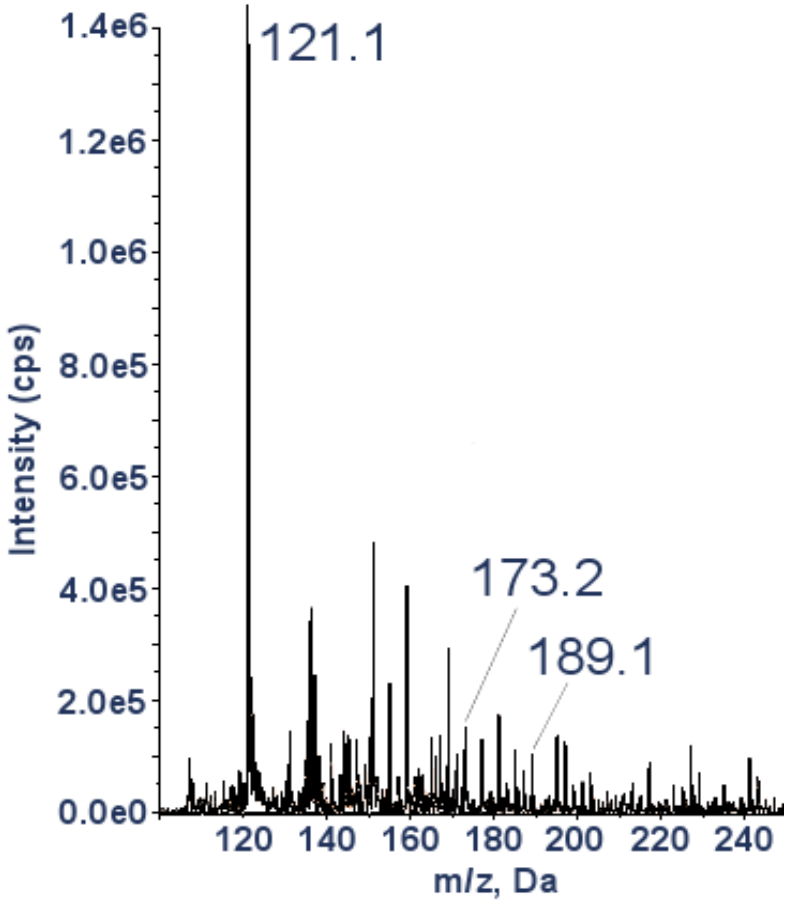

Figure 3 - Mass spectra of other potential metabolite through LC-MS analysis of RDX in WRF. Mass 121.1 is representative of bis(hydroxymethyl)nitramine.

Separation and analysis of 16S-RNA

RNA could not be visualized in the polyallomer tubes with a stain therefore we had to rely on DGGE images to determine if heavy-RNA was separated from light-RNA. Upon staining, which represent density increasing down the tube, none of the DGGE fractions displayed any unique banding profiles (Figure 4) therefore no bands were excised or prepared for sequencing.

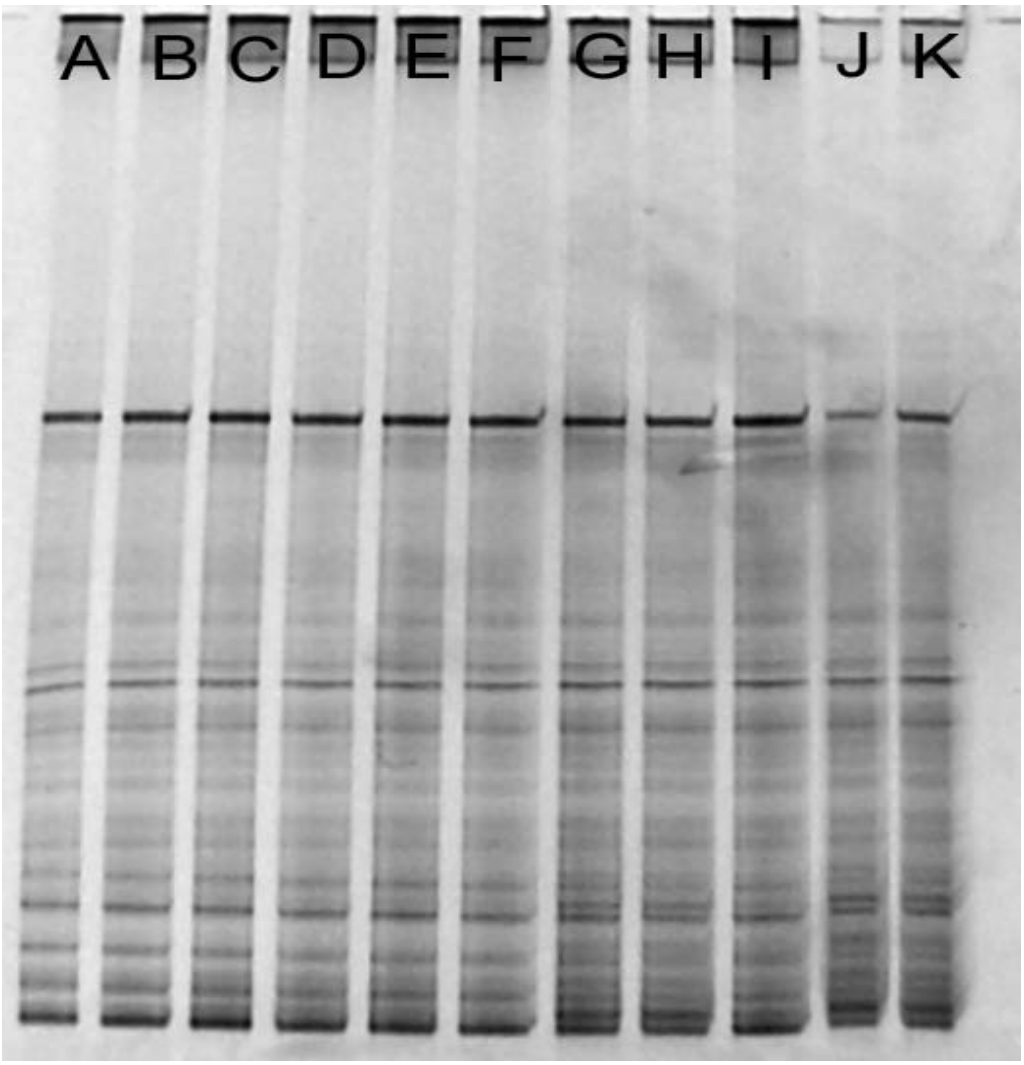

Figure 4 - RNA-SIP DGGE image of V3 regions amplified from ^{13}C spiked WRF. All fractions were loaded with 100 ng total cDNA. All bands represent fractions represent RNA/cDNA fractioned from the same tube and increasing in density from A (lightest) to K (heaviest).

Separation and analysis of 16S-DNA

Due to the heavy ^{13}C-DNA band being invisible in the ultracentrifuge tubes, the density and position of 13C-labeled WRF DNA was approximated relative to an *E. coli* control, which contained an equal mixture of ^{12}C and ^{13}C-genomic DNA. The buoyant density of unlabeled *E. coli* DNA is approximately 1.70 g mL $^{-1}$ while ^{13}C-

labeled *E. coli* DNA is 1.74 g mL^{-1}(Figure 5). Using the V3-DGGE primers, PCR products from the light fractions of the CsCl gradients produced a bright band. The heavy fractions all generated a product; however, the bands were much less intense, likely owing to a much smaller amount of starting template. Due to the low yields, several reactions had to be performed and pooled for the heavy DNA fractions. DGGE analysis of the bacterial portions revealed a distinct banding pattern in the heavy portion of the ^{13}C-DNA eight-hour time point as compared to all other fractions (Figure 6, Lane H). These bands were not present in heavy or light portion of the ^{12}C-labeled DNA lanes or in the heavy portion of the four-hour ^{13}C-labeled DNA lane (Figure 6, Lanes A – G). All light fractions exhibited similar banding patterns (Figure 6, Lanes A – D).

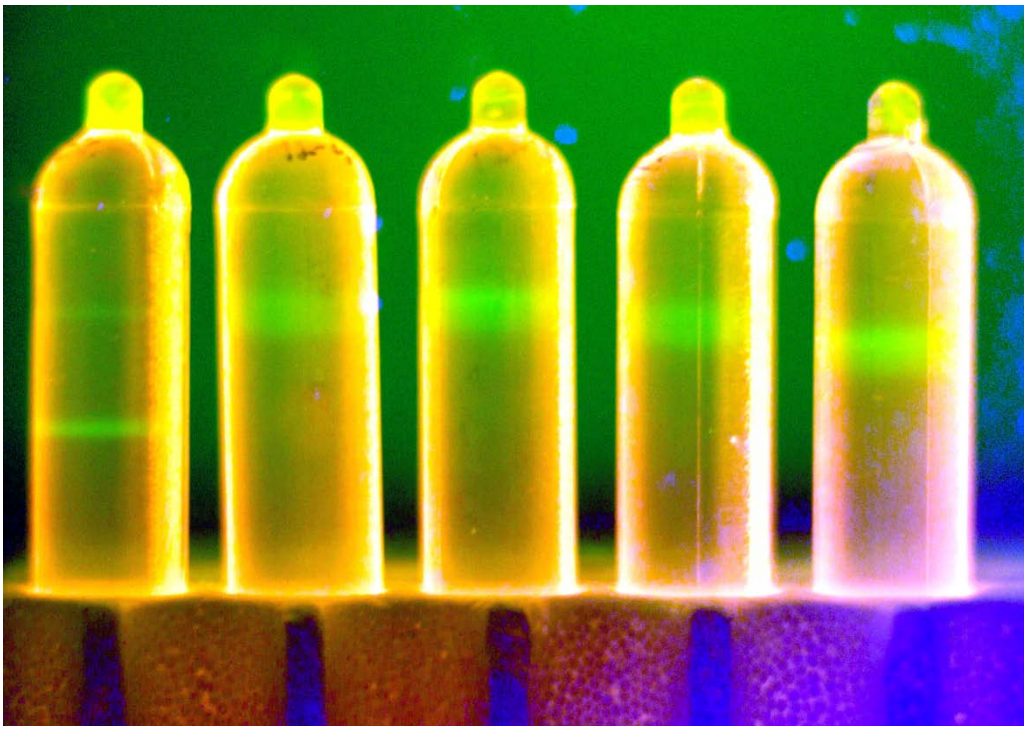

Figure 5 - Image of DNA in CsCl tubes under blue light transillumination with SYBR Safe DNA stain. Far left tube contains approximately 10 µg of ^{13}C E. coli DNA and 10 µg of ^{12}C E. coli DNA. Next is the ^{12}C-WRF DNA sampled @ four hours, ^{13}C-WRF DNA sampled @ four hours, ^{12}C-WRF DNA sampled @ eight hours, and ^{13}C-WRF DNA sampled @ eight hours.

Sequence analysis of partial 16S clone library and excised DGGE bands

Of the ^{12}C heavy-DNA and ^{13}C heavy-DNA, 253 and 267 clones were sequenced successfully. Short reads were used in order to perform a comparison between DNA extracted and sequenced from DGGE bands of interest. Clone sequence data was processed using the Classifer tool of the RDPII [75] at a significance of 50%. Approximately 65% of each clone library consisted of members of the phyla Bacteroidetes. As the next largest phyla in the community, Firmicutes comprised 14-15% and Proteobacteria comprised 8-9% (Figure 7). A pairwise

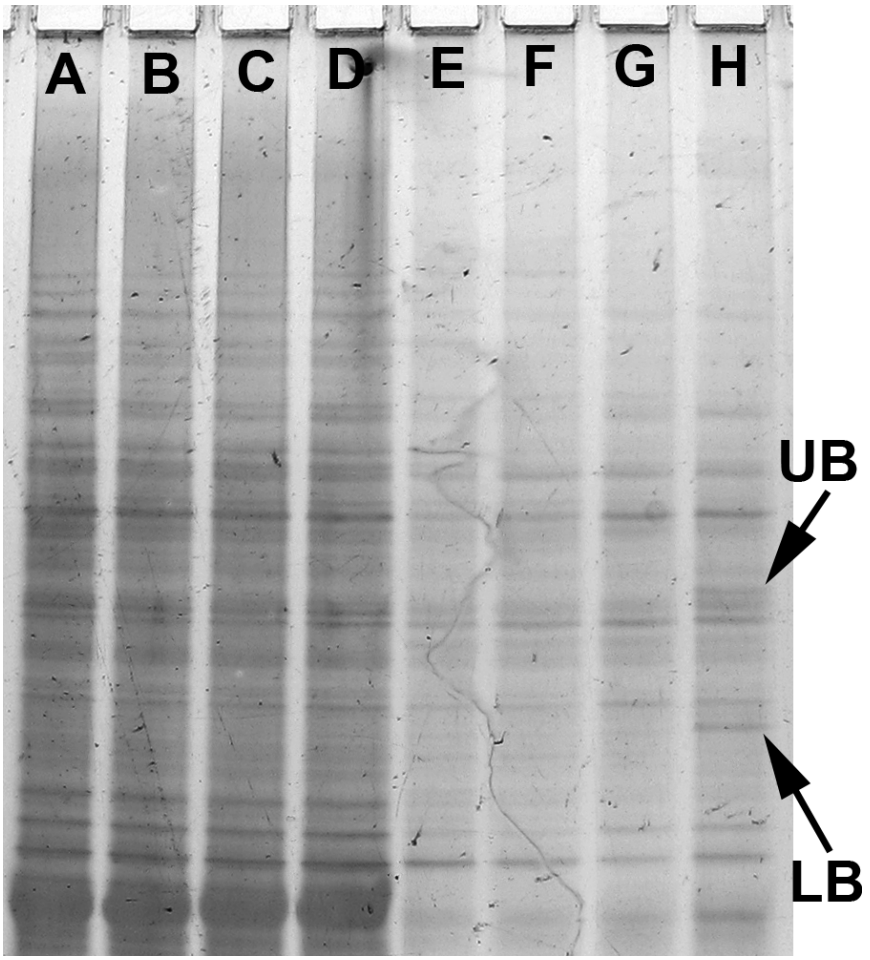

Figure 6 - DNA-SIP DGGE image of V3 regions amplified from heavy and light fractions. Light fractions were loaded with 100 ng total DNA, Heavy fractions loaded with 30 ng total DNA due to low amplification. A, ^{12}C-light at 4 hours; B, ^{13}C-light at 4 hours; C, ^{12}C-light at 8 hours; D, ^{13}C-light at 8 hours; E, ^{12}C-heavy at 4 hours; F, ^{13}C-heavy at 4 hours; G, ^{12}C-heavy at 8 hours; H, ^{13}C-heavy at 8 hours. Two bands stood out in this gel and were labeled upper band (UB) and lower band (LB) as indicated by arrows.

comparison of the two sequenced clone libraries using the Libcompare tool [75] revealed a significant enrichment of members from the order α-proteobacteria in the ^{13}C treatment (p<0.01). No other orders or levels of taxonomy displayed significant differences as compared through the Libcompare tool (data not shown).

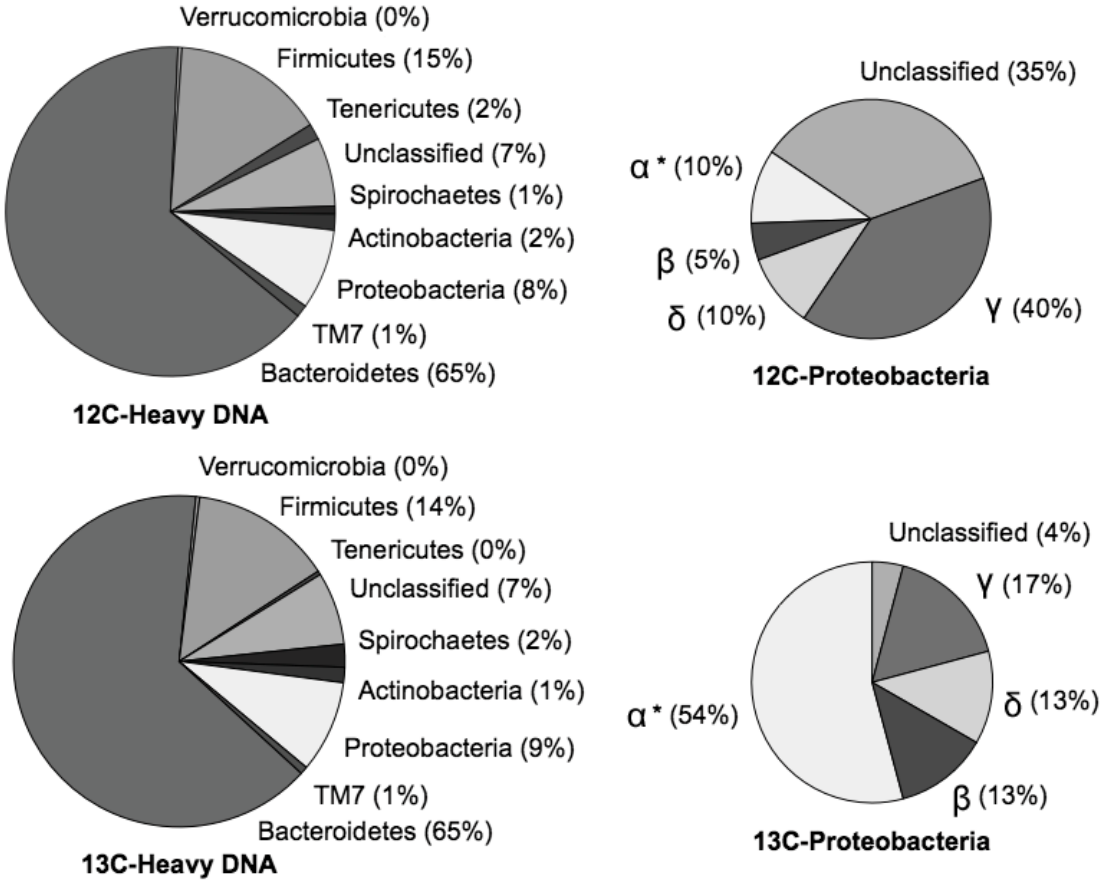

Figure 7 - Chart of Phylum level and Proteobacteria specific classification of ^{12}C and ^{13}C heavy-DNA fractions. Number of clones are 253 and 267 for ^{12}C and ^{13}C respectively. Asterisks denote signifant ($p<0.01$) difference as determined through the statistical tools available through the Ribosomal Database Project.

At the 95% sequence similarity cutoff, Chao1 species richness estimates indicate 164 and 199 OTUs whereas ACE estimates with the same criteria estimate 184 and 309 OTUs in the ^{12}C and ^{13}C heavy fractions (Table 1). With the exception of the Chao1 97% sequence similarity cutoff, all species richness estimates were greater for the ^{13}C heavy fraction. The non-parametric Shannon diversity estimate for the ^{12}C fraction was 4.59 whereas the ^{13}C estimate was 4.80 at the 95% similarity cutoff. A higher value indicates greater diversity.

Table 1 – Species richness and diversity estimates for sampled heavy fractions. Values for ACE and Chao1 estimates represent operational taxonomic units. NP (Non parametric) Shannon index is dimensionless. Percentages represent cutoffs for sequence similarity with 97% representing species level identification, 95% representing genus level identification, and 90% representing family level identification.

	ACE				Chao1				NP Shannon index	
	Average		CI		Average		CI			
ID%	*12C*	*13C*	*12C*	*13C*	*12C*	*13C*	*12C*	*13C*	*12C*	*13C*
97%	314	491	242-436	403-611	347	325	251-522	250-455	5.07	5.23
95%	184	309	149-246	255-386	164	199	134-223	162-268	4.59	4.80
90%	70	140	64-88	115-179	65	122	61-77	92-159	3.67	3.95

Two sequences from the ^{12}C treatment were classified as α-proteobacteria, however, these sequences were not identified beyond the order level of taxonomy. In contrast, all of the ^{13}C α-proteobacteria clones were identified to the genus level. One clone displayed 100% pairwise identity (169 bp) to known sequences from the genus *Methylobacterium* with another displaying 99.4% identity (168/169 bps). Five clones displayed 100% identity with *Brevundimonas* sp. (169/169 bps). One clone displayed 100% sequence identity with the *Sphingomonas* sp. genus. Another clone demonstrated 99.4 % identity to *Sphingomonas* sp. with a third showing 98.2% (166/169 bps) identity (Table 2). Only two bands were excised from the polyacrylamide gel (Figure 6, Lane H). These bands were named upper band (UB) and lower band (LB).

Table 2 – Closest BLAST hit for all heavy fraction α-proteobacteria clones
All heavy fraction clones which the RDP identified as α-proteobacteria. Cloeset BLAST hit and % ID between the hit and the clone sequence. If the sequence demonstrated 100% homology to multiple sequences the one identified to the genus or species level was chosen.

Clone ID	Nearest BLAST hit	% Identity
13CV3 (Clone 111)	*Brevundimonas* sp. (AB526328)	100%
13CV3 (Clone 251)	*Brevundimonas* sp. (AB526328)	100%
13CV3 (Clone 144)	*Brevundimonas* sp. (AB526328)	100%
13CV3 (Clone 156)	*Brevundimonas* sp. (AB526328)	100%
13CV3 (Clone 260)	*Brevundimonas* sp. (AB526328)	100%
13CV3 (Clone 261)	*Brevundimonas* sp. (AB526328)	99.40%
13CV3 (Clone 87)	*Brevundimonas* sp. (AB526328)	99.40%
13CV3 (Clone 1)	*S. alaskensis* (AB526329)	100%
13CV3 (Clone 73)	*S. alaskensis* (AB526329)	99.40%
13CV3 (Clone 93)	*S. alaskensis* (AB526329)	98.20%
13CV3 (Clone 115)	*Methylobacterium* sp. (FP103142)	100%
13CV3 (Clone 252)	*Methylobacterium* sp. (FP103142)	99.40%
12CV3 (Clone 42)	Uncultured rumen bacterium (AF001764)	100%
12CV3 (Clone 8)	Uncultured bacterium (EU775169)	99.40%

Eight sequences were successfully amplified from UB and five were amplified from LB. Of the sequences from the upper band four showed identity with the class Bacteroidetes and the other four were affiliated with the class α-proteobacteria. One band sequence, UB Clone 9, demonstrated 100% identity with a clone from the metagenomic data set most closely related to the genus *Brevundimonas* (Figure 8). The remaining three α-proteobacteria clones from the upper band grouped together and away from sequences from the metagenomic data sets. From the lower band, three sequences showed affiliation with the Bacteroidetes. The other two, LB Clones 3 and 4, showed affiliation with the α-proteobacteria and were 100% identical to UB Clone 9 and several sequences from the ^{13}C metagenomic data set (Figure 8).

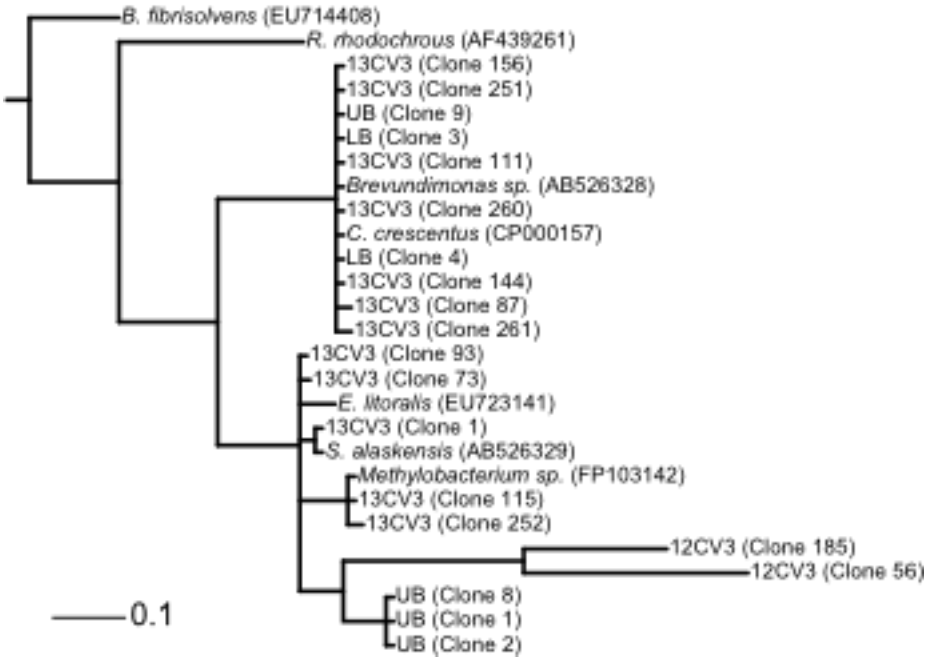

Figure 8 - Phylogenetic tree of all α-proteobacteria identified in this study with selected reference species. Reference sequences were included for all clones identified in 13C-labeled fractions. Known RDX degrading organism *R. rhodochrous* included for reference. Rumen organism *B. fibrisolvens* included as an outgroup.

DGGE analysis of labeled-RNA did not produce any noticeable banding differences therefore no bands were excised and sequenced. Sequence analysis of heavy-DNA corresponding to band H of the DGGE image (Figure 4) for the ^{13}C-RNA and the equivalent density in the ^{12}C-RNA tube did not reveal any significant differences between the two samples. The number of clones sequenced for the ^{13}C-RNA and ^{12}C-RNA samples were 229 and 238 respectively. Due to banding profiles being the same and lack of population differences as found through sequence comparison, no further analysis was performed on RNA samples.

Assay for Nitroreductase and xplA genes

Currently, only two genes are known to produce enzymes which break down the RDX molecule: *xplA* and nitroreductase [12]. Attempts to amplify PCR products using primer sets for either of these genes were unsuccessful. No new primer sets for the *xplA* gene were created for this study. Reaction conditions and primers designed by Roh et al. (2009) were the first to attempt amplification of the *xplA* gene. No PCR products of the expected 549 base pair size were amplified when analyzed through agarose gel electrophoresis. Next, we attempted to amplify DNA with primers designed by Rylott et al. (2006) for amplification of the *xplA* gene from a sampling of *Rhodococcus* species. No PCR products were generated with this primer set. Lastly, the nitroreductase-specific primers were used on all fractions. Products of the expected size (400 bp) were amplified in all light fractions; however, no products were generated for any of the heavy fractions. Attempts to optimize PCR conditions in all instances did not result in amplification of products of the expected size or did not generate products at all.

Discussion

SIP is a powerful technique that has proven capable of identifying organisms capable of degrading a variety of substrates. Probing the rumen system with ^{13}C-RDX has the potential to reveal bacteria and archaea capable of degrading the chemical. Coupling SIP with analytical techniques such as HPLC and LC-MS/MS analysis will provide evidence of degradation and information regarding degradation intermediates respectively.

Whole rumen fluid has demonstrated rapid degradation of RDX with 10 μg mL^{-1} (45 μM) being degraded within four hours and a total of 25 μg mL^{-1} (112 μM) within eight hours. WRF demonstrated this capability over technical replicates (n=3) and experiments conducted on different days suggesting that the microbes responsible for breakdown are not transient to the system being studied. The time needed to remove these quantities of RDX compare favorably with several previous studies. An artificial anaerobic consortia consisting only of members from the *Desulfovibrio* genus required 15 days to degrade 25 μg mL^{-1} (112 μM) of RDX [58]. Seven days were required to degrade 50 μg mL^{-1} (224 μM) of RDX in a mixed culture experiment with sewage sludge as an inoculum [11]. Both the *Desulfovibrio* sp. consortia and the sewage sludge were maintained with artificial culture media raising the possibility of external electron donors contributing to the breakdown RDX in these experiments. Degradation with WRF did not require any artificial additions due to the absence of media components. Large particles were filtered out and it is quite likely that partially degraded plant material carried over in the rumen digesta could have provided some

measure of unknown electron donors. Concentrations greater than 15 μg mL^{-1} did not consistently demonstrate RDX degradation over periods greater than eight hours. This may be due to cytotoxic effects on the resident bacteria. Cytotoxic effects on bacteria have been demonstrated previously with a *Pseudomonas* sp. which was capable of degrading an equivalent concentration of RDX in pure culture but required 24 days to do so with induction of stress shock proteins being demonstrated [45]. To account for any potential adaptive period, we started with a lower initial pulse of 10 μg mL^{-1} to ensure survival of RDX degrading organisms and later increased the concentration to 15 μg mL^{-1} to ensure sufficient labeling of degrading bacteria.

The LC-MS data is generally representative of RDX breakdown. Qualitative LC-MS analysis of RDX (m/z of 281which represents RDX + CH_3COO^-), were not detected at four hours. The substrate fell below the limit of detection (0.05 ng/mL) at four hours, which is consistent with the results retrieved through HPLC analysis (Figure 1). Two putative RDX degradation intermediates were detected through LC-MS analysis. One of the more prominent peaks in the spectrogram was m/z of 195 Da [M + CH_3COO^-] and is representative of degradation intermediate MEDINA (Figure 2). Another ion, m/z of 121.1 Da, is consistent with the presence of degradation intermediate bis(hydroxymethyl)nitramine. Together these two molecules have been putatively identified as direct ring cleavage products in an anaerobic sludge RDX degradation pathway [57]. No molecules representative of nitroso or denitration intermediates were detected reinforcing the conclusion that this pathway is representative of breakdown within WRF [12]. A mass balance study will be necessary to better understand the intermediates and the mineralization components of

this degradation. Inclusion of standards and isotopic analysis through LC-MS/MS would allow for confirmation of intermediates and determination of the definitive pathway in the WRF matrix.

Community profiling through DGGE is one common method to determine differences between labeled and unlabeled fractions. DGGE analysis of the ^{13}C-RNA did not reveal any banding differences across the CsTFA gradient (Figure 4, lanes A-K). This is likely due to the fraction collection procedure used and is not representative of ^{13}C incorporation. Though pipette extraction of gradient fractions has been used previously, the preferred method requires a peristaltic or HPLC pump, both of which were unavailable. Syringe and needle extraction through the side of the centrifuge tube is valid for ^{13}C-DNA extraction and DGGE results reflect the validity of this technique through displaying unique banding patterns between the ^{12}C and ^{13}C treatments (Figure 6).

Members of the α-proteobacteria class have been known previously to be capable of degrading RDX in groundwater [74] and pure culture. Partial 16S rRNA analysis of the V3 region revealed that all of the sequences identified in this study are members of the α-proteobacteria class. *Methylobacterium* sp. BJ001, an α-proteobacteria, had been implicated previously in aerobic degradation of RDX in pure culture [46]. Originally affiliated with poplar leaves and naive to RDX, the bacteria were able to degrade a starting concentration of 20 μg mL^{-1} in approximately 40 days. It is interesting to note that *Methylobacterium* sp. in general are considered strict aerobes capable of utilizing simple carbon compounds [79] with no known anaerobic members. In general, members of this genus are primarily found on the surface of

leaves and in the soil and have never been detected in the rumen. Closely related bacteria *Methylobacterium* sp. JS178 had shown unable to metabolize RDX. It was, however, able to aerobically utilize NDAB as a sole nitrogen source [47]. It is also important to note that previous work did not indicate either isolate was capable of utilizing RDX or NDAB as a carbon source. These results suggest a putative member of the genus *Methylobacterium* is capable of degrading RDX. However, it is not clear whether it is able to degrade the NDAB intermediate.

Other sequences were identified as members of the genera *Brevundimonas* and *Sphingomonas*. Three of the DGGE bands matched the *Brevundimonas* sp. clones sequenced in the clone library, suggesting that their enrichment in the ^{13}C fraction is contributing to the appearance of these two bands. Members of the genus *Brevundimonas* have not been previously implicated in RDX breakdown. However, a previous study found that members of this genus were enriched in TNT contaminated soil, signifying tolerance to explosives contamination [80]. In addition, members of this genus have been found in RDX contaminated soil across a wide range of sampling depths [81, 82] although the organism was not directly implicated in RDX degradation. In general, all of the sequences implicated in this study are generally considered to be soil microorganisms and not previously found in the rumen.

Bacteroidetes have been shown to dominate counts of bacteria within WRF [83] which is consistent with these findings (Figure 7). On the other hand, Proteobacteria typically make up a small percentage of sequenced rumen clones. One explanation may be that these organisms are simply transient within the animals sampled since all three genera are typically associated with soil. Secondly, primer bias

may account for lower representation of these organisms in previous metagenomic surveys [30]. Third, it is possible that these microbes are simply present at very low numbers, which would escape a normal survey of rumen diversity. When rarefaction curves were constructed for the ^{12}C and ^{13}C heavy clone libraries, they did not attain an asymptotic shape, suggesting an unknown quantity of unsequenced diversity. With 95% being defined as the sequence similarity at the genus level, Chao1 and ACE estimations predict a greater number of OTUs in the ^{13}C-heavy DNA sample over the ^{12}C-heavy DNA sample (Table 1). ACE estimations at the 95% cutoffs also suggest that the treatments were undersampled with 309 predicted OTUs (CI 255-356), which if sequenced, potentially would reveal additional RDX degrading organisms.

Archaea have been implicated in RDX degradation. *Acetobacterium malicum* HAAP-1, an acetogen isolated from a methanogenic mixed culture, was able to degrade RDX with H_2 supplied as an electron donor [52]. In this study, the atmosphere contained about 8% hydrogen, much lower than the 80% used in the previous study. We attempted to assay for archaeons with no success. It is unlikely that methanogens nor acetogens are participating in degradation under the conditions tested as it was shown previously [52] that either additional hydrogen gas or a syntrophic H_2-producing substrate was needed to stimulate degradation. It is possible that RDX-degrading archaea are present in the rumen; however an enrichment culture would be necessary for their isolation.

Genes known to degrade RDX were not detected within labeled DNA. Attempts to amplify the cytochrome P450 gene *xplA* using known primer sets gave differing results. With the primer pair designed by Roh *et. al*. (2009), we generated PCR

products shorter than the required 549 base pair expected size. The primer pair designed by Rylott *et. al*. (2009), did not generate a product at all. These results are not surprising, even if the rumen organisms were utilizing RDX as a carbon source because the *xplA* gene has only been isolated from organisms within the Actinomycetales order. For example, two soil organisms, *Gordonia* sp. KTR9 and *Williamsia* sp. KTR4, were able to utilize RDX as a sole carbon source while utilizing the *xplA* cytochrome to break down the compound [48]. *Rhodococcus rhodochrous* 11Y, the organism from which *xplA* was first isolated, cannot use RDX as a sole carbon source [43]. These results suggest *xpl*A is not playing a role in RDX breakdown by ruminal microorganisms. Nitroreductase genes have also been previously implicated in RDX degradation [84]. In this study, using a new set of primers, we did not detect the nitroreductase gene in heavy DNA fractions. Light fractions and positive control reactions all generated products of the expected size. These results suggest the nitroreductase gene is present within the rumen but is not responsible for RDX degradation in this system.

Conclusion

We have shown the degradation of RDX within WRF through the labeling of organisms with ^{13}C ring-labeled RDX. The inclusion of autoclaved controls reinforces the finding that this process is biological in origin. Through the application of the SIP technique, it is clear that these organisms are within the α-proteobacteria clade with the genera responsible being putatively identified as *Methylobacterium sp*., *Brevundimonas sp*., and *Sphingomonas sp*. Members of these genera are typically thought to be soil organisms. This work is the first to identify them within the rumen. Identification of a putative Methylobacterium is important for explaining the degradation of RDX within the rumen because a member of that genus has been previously identified as degrading RDX in pure culture. For DNA-SIP approximately 260 clones were picked per treatment to establish a metagenomic library. Statistical analysis through the RDP II website was used to ascertain differences between the treatments. We have also linked a metagenomic library to sequenced bands from a denaturing gradient gel to reinforce the finding that the significant fraction from the library is contributing to unique banding patterns within the labeled community. No significance was seen through RNA-SIP even though approximately 229 clones were sequenced per treatment.

Preliminary metabolite analysis through qualitative LC-MS suggests the intermediates MNX, DNX, and TNX are not produced in the degradation of RDX within WRF. Data suggests the metabolites MEDINA and bis(hydroxymethyl)nitramine are present within the rumen fluid. The nitroso

intermediates have shown to be toxic, therefore, it is important to establish their presence or absence in WRF. This metabolite data coupled with the incorporation of labeled carbon into bacterial nucleic acids suggests complete mineralization may be occurring.

Stable isotope probing with labeled RDX continues to hold a great deal of potential within the rumen ecosystem. Obtaining the full-length 16S sequences from these organisms will be necessary for identification down to the strain level. This can be obtained through the type of analysis performed here, however, universal primers will be necessary as will a substantial amount of sequencing data. As newer sequencing technologies emerge, the prohibitive cost of full-length sequencing should come down making this type of analysis much easier to achieve. One potential way to circumvent the need for large metagenomic libraries would be to create enrichment cultures of these organisms prior to labeling. Culture conditions will select for RDX degrading organisms if RDX is provided as the sole carbon source over an extended period of time. Introduction of labeled RDX after a predetermined amount of time, application of stable isotope fractionation, and sequencing will establish the identity of degrading organisms while at the same time eliminating a great deal of the background biodiversity. Introduction of ^{15}N-labeled RDX as a separate analysis will go one step further and establish which organisms in the rumen can utilize RDX as a N source.

Greater understanding of the metabolites generated in the degradation process will be necessary to establishing a pathway. Analysis as performed in this work is not sufficient to establish such a pathway, however, it does show the promise of the system to fully remove RDX toxicity from the supplied matrix. This could occur

through more robust LC-MS analysis or use of ^{14}C-RDX as has been done previously [11].

Plant tissues have been known to take RDX up into aerial tissues though it remains in parent form. This raises the possibility of harvesting RDX contaminated plant matter and providing it to ruminants as a feedstock. The benefits to such a system are enormous for several reasons. Firstly, assuming complete mineralization is achieved, toxicity will be completely neutralized. Second, it is a pollution free alternative to the currently accepted method of *ex situ* incineration of contaminated soil. Finally, this method would potentially be far cheaper than other methods as the major costs would only encompass plant seed, a flock, and periodic monitoring of the contaminated site.

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