Identification of Groundwater Microorganisms Capable of Assimilating RDX-Derived Nitrogen during *In-Situ* Bioremediation

Kun-Ching Cho¹,Mark E. Fuller², Paul B. Hatzinger², and Kung-Hui Chu^{1*}

¹Zachry Department of Civil Engineering,
Texas A&M University
College Station, TX 77843-3136, USA

²CB&I Federal Services, Lawrenceville, NJ.

Submit to Science of Total Environment

* Corresponding author: E-mail kchu@civil.tamu.edu

Phone 979-845-1403

Abstract

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a nitroamine explosive, is commonly detected in groundwater at military testing and training sites. The objective of this study was to characterize the microbial community capable of using nitrogen derived from the RDX or RDX intermediates during *in situ* bioremediation. Active groundwater microorganisms capable of utilizing nitro-, ring- or fully-labeled ^{15}N -RDX as a nitrogen source were identified using stable isotope probing (SIP) in groundwater microcosms prepared from two wells in an aquifer previously amended with cheese whey to promote RDX biodegradation. A total of fifteen 16S rRNA gene sequences, clustered in *Clostridia*, β -*Proteobacteria*, and *Spirochaetes*, were derived from the ^{15}N -labeled DNA fractions, suggesting the presence of metabolically active bacteria capable of using RDX and/or RDX intermediates as a nitrogen source. None of the derived sequences matched RDX-degrading cultures commonly studied in the laboratory, but some of these genera have previously been linked to RDX degradation in site groundwater via ^{13}C -SIP. When additional cheese whey was added to the groundwater samples, 28 sequences grouped into *Bacteroidia*, *Bacilli*, and α -, β -, and γ -*Proteobacteria* were identified. The data suggest that numerous bacteria are capable of incorporating N from ring- and nitro-groups in RDX during anaerobic bioremediation and that some genera may be involved in both C and N incorporation from RDX.

Keywords: RDX, SIP, Groundwater, Nitrogen, Bioremediation, Biodegradation.

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common military explosive that has been detected in soils and groundwater at many historical munitions and active testing and training sites. While RDX is only moderately soluble, it adsorbs only weakly to soil, resulting in the potential for RDX to form large groundwater plumes that impact local drinking water supplies. Although there is no federal Maximum Contaminant Level (MCL) for RDX in drinking water, it is considered a possible human carcinogen and has been listed on the Contaminant Candidate List 4 by the U.S. Environmental Protection Agency (EPA) ([USEPA, 2015](#)). The U.S. EPA has also issued a health advisory level of 2 µg/L for RDX in drinking water ([ATSDR 2012](#)).

Aerobic and anaerobic biodegradation of RDX have been previously documented ([Adrian and Arnett, 2004](#); [Bernstein et al., 2011](#); [Bhushan et al., 2006](#); [Boopathy et al., 1998](#); [Coleman et al., 1998](#); [Crocker et al., 2006](#); [Freedman and Sutherland, 1998](#); [Kwon and Finneran, 2006](#); [Kwon and Finneran, 2008](#); [Kwon et al., 2011](#); [Seth-Smith et al., 2002](#); [Thompson et al., 2005](#); [Zhao et al., 2002](#); [Zhao et al., 2004](#); [Zhao et al., 2003a](#)), and a number of RDX-degrading isolates have been reported, many of which are known to utilize RDX as a nitrogen and/or carbon source. While a wealth of knowledge about RDX biodegradation has been derived from these isolates, our understanding of RDX biodegradation *in situ*, including the dominant organisms involved in this process and the mechanism whereby they utilize RDX (e.g., as a C or N source, or electron acceptor) is less clear. Recently, stable isotope probing (SIP) has been utilized as a culture-independent method to track and identify functionally active bacteria in various

environmental samples([Andeer et al., 2012](#); [Dumont and Murrell, 2005](#); [Gallagher et al., 2005](#); [Gallagher et al., 2010](#); [Morris et al., 2002](#); [Radajewski et al., 2003](#); [Radajewski et al., 2002](#); [Roh et al., 2009](#); [Uhlík et al., 2009](#)), and has recently been applied to better understand their presence in natural or engineered systems during RDX degradation and their possible role in RDX-degrading microbial communities ([Andeer et al., 2013](#); [Andeer et al., 2012](#); [Cho et al., 2015](#); [Cho et al., 2013](#); [Jayamani and Cupples, 2015](#); [Roh et al., 2009](#)).

Applications of SIP with ^{13}C -labeled or ^{15}N -labeled RDX to laboratory and field samples have provided new insights into RDX-degrading microbial communities, and have identified bacteria that have not previously been associated with RDX biodegradation ([Andeer et al., 2013](#); [Cho et al., 2013](#); [Roh et al., 2009](#)). Given that RDX consists of a six-membered ring with alternating (CH_2 and N-NO_2) moieties, both the nitrogen (N) in the nitro (NO_2) group and in the ring structure may serve as a nitrogen source for bacterial growth. It is also likely that bacteria capable of using the nitro-N in RDX are different from those capable of using the ring-N in RDX.

For instance, a previous SIP study was conducted with ring-labeled ^{15}N -RDX and identified fifteen phylogenetically diverse bacteria in anaerobic samples derived from flow-through columns constructed from contaminated aquifer solids which incorporated nitrogen from RDX into their DNA ([Roh et al., 2009](#)). Similarly, Cho *et al.* recently reported identifying 30 groundwater microorganisms isolated from groundwater enrichment samples from the same site capable of incorporating carbon from RDX using ^{13}C -SIP analysis ([Cho et al., 2013](#); [Roh et al., 2009](#)).

The current study utilized the same general approach as the previous study ([Cho et al., 2013](#)) to characterize the RDX-degrading microbial community in groundwater from a site in Virginia with respect to the ability of individual organisms to assimilate nitrogen derived from RDX. We used ring-, nitro-, and fully-labeled ^{15}N -RDX to conduct SIP experiments in groundwater microcosms collected from the same location and wells used in a previous ^{13}C -SIP RDX study ([Cho et al., 2013](#)). In this study, the aquifer in New Jersey was previously amended with cheese whey to promote anaerobic degradation of RDX ([Hatzinger and Lippincott, 2012](#)). The identities of bacteria capable of using the different sources of nitrogen in the RDX were determined, both in the presence and absence of additional cheese whey as an alternate electron donor/carbon source, and these clones were then compared to results derived from the previous ^{13}C -SIP and ^{15}N -SIP RDX studies ([Andeer et al., 2013](#); [Cho et al., 2015](#); [Cho et al., 2013](#); [Roh et al., 2009](#)).

Materials and Methods

Chemicals and Bacterial Cultures

Ring-, nitro-, and fully-labeled ^{15}N -RDX (>99% chemically pure, 99% isotopically pure) were synthesized by Dr. Steve Fallis, U.S. Naval Air Weapons Station China Lake. 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), were obtained from SRI international (Menlo Park, CA). The cheese whey used in studies was an animal feed product provided by International Ingredient Corporation (St. Louis, MO) which consists of a 50:50 mix of powdered cheese product and spray dried whey.

Basic specifications indicated it contained (wt:wt) more than 60% lactose, 10 % protein, 10 % lactic acid, 5 % fat, and 10 % inorganic matter ([Prazeres et al., 2012](#)). The other chemicals used in the study was purchased from the vendors and described the detailed in supporting materials.

Escherichia coli, a non-RDX utilizer, was used to produce genomic ^{14}N -DNA and ^{15}N -DNA as ultracentrifugation reference standards. The detailed bacterial cultures and medium used in this study are described in supporting materials.

Sample Sites and Microcosm Setup

Groundwater samples were collected from two monitoring wells (MW-5 and MW-7D) at a U.S. Army facility in northern New Jersey. The site has been contaminated with explosives for several decades. Biodegradation of RDX and other explosives had been stimulated *in situ* at the site by repeated addition of cheese whey to the aquifer, which was observed to support RDX biodegradation in the site groundwater during prior treatability testing ([Roh et al., 2009](#)) and at field scale ([Hatzinger and Lippincott, 2012](#)). Groundwater from each well was used to prepare replicate microcosms in 2-L sterile glass bottles (1.6 L final volume). RDX (unlabeled, ring- $^{15}\text{N}_3$ -, nitro- $^{15}\text{N}_3$ -, or fully-labeled $^{15}\text{N}_6$ -RDX) was added to duplicate bottles to a final concentration of ~ 10 mg/L, and dry cheese whey powder was added to one replicate of each duplicate to a final concentration of 100 mg/L. Microcosm setup conditions are summarized in Table 1. Briefly, the microcosm ID (50-R, 50-N, 50-F, 5C-R, 5C-N, 5C-F, 70-R, 70-N, 70-F, 7C-R, 7C-N, 7C-F) indicated each microcosm's setup conditions. The coding for each microcosm ID was based on the groundwater sources (MW-5 or MW-7D), presence or

absence of cheese whey amendment, and the type of labeled RDX added (ring-, nitro-, fully-labeled ^{15}N -RDX). Additional site description and microcosm setup details are provided in supporting materials.

Separation of ^{14}N -DNA and ^{15}N -DNA

The DNA in the frozen Sterivex filters from the microcosms was extracted using the FastDNA spin kit for soil samples (MP Biomedical, Solon, OH). The extracted DNA was separated into ^{14}N -DNA and ^{15}N -DNA using equilibrium centrifugation in CsCl density gradients measurement with minor modifications as described in supporting materials. The DNA in the fractionated solution was extracted and purified as described previously ([Roh et al., 2009](#); [Yu and Chu, 2005](#)). The purified DNA was stored at $-20\text{ }^{\circ}\text{C}$.

Analysis of Microbial Community Structure

To characterize the overall and active RDX-degrading microbial community structure in each microcosm, real-time terminal restriction fragment length polymorphism (real-time-t-RFLP) assays were performed ([Yu and Chu, 2005](#)). Genomic DNA extracted from microcosms receiving unlabeled RDX was used as a template for determining overall microbial community structure. The ^{15}N -DNA fractions from ring, nitro, and fully labeled ^{15}N -RDX-amended microcosms were used as templates to determine the active RDX-degrading microbial community structure. The real-time PCR assays were performed using Bio-Rad iQ5 multicolor Real-Time PCR detection System (Hercules, CA).

PCR Cloning and Sequencing.

The identities of the active RDX-degrading microorganisms were determined based on the 16S rRNA gene sequences derived from ¹⁵N-DNA fractions of the microcosms receiving one of ring-, nitro- or fully-labeled ¹⁵N-RDX. The PCR reactions for 16S rRNA gene amplification were performed as described ([Roh et al., 2009](#)). Detailed descriptions are presented in the supporting materials. The 16S rRNA gene sequences were deposited in GenBank under accession numbers JX470441-JX470483.

Results and Discussion

Effect of Cheese Whey on RDX Biodegradation in Groundwater Microcosms.

Approximately 90 % of initial unlabeled RDX was degraded in microcosms without additional cheese whey amendment after 75 days of incubation (Microcosms 50-R, 50-N, 50-F, 70-R, 70-N, and 70-F) (**Error! Reference source not found.** in supporting information, and **Figure**). The aquifer had been amended with cheese whey four times prior to groundwater collection for these microcosms in order to promote in situ RDX biodegradation ([Hatzinger and Lippincott, 2012](#)). This biostimulation procedure was very effective, with RDX concentrations being < 0.2 µg/L (from baseline concentrations as high as 170 µg/L) throughout the treated area more than a year after the final cheese whey injection (when these groundwater samples were collected). In the unamended groundwater microcosms, the objective was to characterize the organisms incorporating N from RDX in the site groundwater during long-term in situ bioremediation. These organisms were most likely utilizing residual cheese whey, its degradation products, or dead biomass as electron donors for RDX degradation.

A five-fold longer lag period was observed in Microcosms 50-R, 50-N, and 50-F than in Microcosms 70-R, 70-N, and 70-F), i.e., 100 days vs. 20 days. Additionally, slower RDX biodegradation occurred in Microcosms 50-R, 50-N, and 50-F than in Microcosms 70-R, 70-N, and 70-F. At the time of groundwater collection, the TOC in well MW-5 was 4.2 mg/L, while that in well MW-7D was 14.9 mg/L ([Hatzinger and Lippincott, 2012](#)). This difference in carbon concentration (and possibly carbon lability) most likely accounts for the differing degradation kinetics between the groundwater microcosms derived from the two different wells.

In Microcosm 50-N (nitro-labeled ^{15}N -RDX), RDX started to degrade after ~ 100 days and reached 80% degradation on day 250. In Microcosm 50-F, (fully-labeled $^{15}\text{N}_6$ -RDX), a similar slow degradation pattern was observed, and three types of nitroso metabolites, included hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) were observed after 75 days of incubation (Figure S1 in supporting materials). In Microcosm 50-R (ring-labeled $^{15}\text{N}_3$ -RDX), less than 30% of initial RDX was degraded after 250 days of incubation, and therefore no sample was collected from this microcosm for SIP analysis. It is unclear why the ring-labeled RDX degraded to a lesser extent and more slowly than the other two isotopologues of RDX, but the low RDX degradation in this treatment did not allow comparisons between this treatment and those receiving nitro- or fully-labeled RDX.

RDX was degraded more rapidly in microcosms prepared from well MW-7D (70-R, 70-N, and 70-F, Figure 1(B)) than observed in microcosms from well MW-5 (50-N

and 50-F, Figure 1(A)), with approximately 50% of RDX degraded within 20 days. Also, rapid RDX degradation was observed after the second RDX addition (RDX degradation was completed in 5-10 days). Nitroso-metabolites (MNX, DNX and TNX) were detected in Microcosm 70-F at the concentrations between 0.1 and 0.5 mg/L (Figure S2 in supporting materials). The concentration of MNX in Microcosm 70-F was 0.5 mg/L, which was ~ five-fold lower than that observed in Microcosm 50-F.

Addition of cheese whey to groundwater from each well resulted in faster RDX degradation; this was evident in Microcosms 5C-R, 5C-N, 5C-F, 7C-R, 7C-N, and 7C-F. After 5 days of incubation, almost 80% of initial RDX was degraded, and complete degradation of RDX was observed over a period of 30 days (Figure (A)-(B)). Similarly, a second spike of RDX in these microcosms was degraded in 5 days. The RDX breakdown products MNX, DNX and TNX were detected sporadically at low concentrations (ranging from 0.2 to 0.6 mg/L) in these microcosms (Figures S1 and S2 in supporting materials). These observations were similar to the finding of our previous SIP study with ¹³C-RDX that cheese whey addition enhanced RDX biotransformation/degradation ([Cho et al., 2013](#)). The enhanced RDX degradation was most likely due to an increased availability of electron donor or other nutrients, more strongly reducing conditions, and/or increased overall microbial biomass.

RDX-Degrading Microbial Communities in Groundwater Microcosms

The active RDX-degrading microbial community structure of microcosms in the presence and absence of cheese whey was examined using real-time-t-RFLP analysis with ¹⁵N-DNA fractions as templates. The microbial community profiles derived from

real-time-t-RFLP analyses are shown in Figures S3-S22 (see supporting materials). In Microcosms 50-N, 50-F, 70-R, 70-N, and 70-F (without cheese whey), the average 16S rRNA gene copies were 5×10^4 copies/ml. In contrast, in the presence of cheese whey, the average 16S rRNA gene copies were 3×10^6 copies/ml in Microcosms 5C-R, 5C-N, 5C-F, 7C-R, 7C-N, and 7C-F. The stimulative effect of cheese whey on the microbial community was expected, given that the groundwater at the site had been amended with whey several times prior to collection and use in these microcosms. It is also interesting that although cheese whey addition presumably added a relatively large amount of utilizable nitrogen (e.g., as protein), numerous bacteria still utilized nitrogen derived from RDX.

Microorganisms Capable of Using RDX or RDX Intermediates for Nitrogen

(i) *Clones potentially capable of assimilating RDX-derived nitrogen.* By using the ^{15}N -DNA fractions of five microcosms (50-Na, 50-Fa, 70-Na, 70-Ra and 70-Fa) as templates, a total of fifteen 16S rRNA gene sequences were derived. These sequences represented the metabolically active bacteria capable of using RDX and/or RDX metabolites as a nitrogen source. These sequences were phylogenetically diverse and clustered in three major phyla: *Clostridia* (2 sequences), β -*Proteobacteria* (7 sequences), and *Spirochaetes* (6 sequences) (Figure 3). None of the 15 sequences were an exact match to any of previously described RDX degrading isolates ([Adrian and Arnett, 2004](#); [Coleman et al., 1998](#); [Sherburne et al., 2005](#); [Thompson et al., 2005](#); [Zhang and Hughes, 2002](#); [Zhao et al., 2003b](#)).

241 Several clones derived from enrichment microcosms receiving ring-, nitro-, and
242 fully-labeled ^{15}N -RDX were identical. These identical clones were clustered within
243 *Clostridia*, β -*Proteobacteria* and *Spirochaetes*. For instance, two sequences 50-Na8
244 (nitro- $^{15}\text{N}_3$ -RDX) and 50-Fa19 (fully-labeled $^{15}\text{N}_6$ -RDX) clustered in *Clostridia* and were
245 identical, having the same T-RF=106 bp (predicted at 108 bp) in the microbial
246 community profile (See the Figures S2 and S4. in supporting materials). The nearest
247 relative for these clones was *Desulfosporosinus meridiei* (accession number: NR074129),
248 a soil isolate belonging to a family of sulfate-reducing bacteria. A previous study by our
249 group first identified the presence of *Desulfosporosinus* in enrichments degrading RDX
250 biodegradation under various electron-accepting conditions comprised of aquifer solids
251 and groundwater from a military range in Virginia ([Cho et al., 2015](#)). *Desulfosporosinus*
252 can utilize a wide spectrum of carbon sources, ranging from aromatic compounds to
253 short-chained fatty acids ([Pester et al., 2012](#)). The diverse metabolic ability of
254 *Desulfosporosinus* might offer a growth advantage over other bacteria and allow them to
255 thrive under various nutrient and redox conditions. This current study suggests that that
256 they can utilize nitro-N from RDX. This might also explain why several previous studies
257 have revealed that various sulfate-reducing bacteria, such as *Desulfovibrio* spp., are able
258 to utilize RDX as a nitrogen source ([Arnett and Adrian, 2009](#); [Boopathy et al., 1998](#);
259 [Zhao et al., 2003a](#)). The abundance, diversity, and significance of the role of
260 *Desulfosporosinus* and other sulfate-reducers in RDX degradation in response to different
261 conditions warrants future investigation.

Another sequence, 70-Ra22 (from ring-¹⁵N-RDX), had a high similarity to two iron-reducing strains: *Rhodoferrax ferrireducens* strain T118 (accession number: NR074760) and *Rhodoferrax fermentans* strain FR2 (accession number: NR025840) (Finneran et al., 2003). Increased numbers of *Rhodoferrax* were reported using pyrosequencing when acetate or lactate was added to an aquifer in Iowa to promote RDX biodegradation (Livermore et al., 2013). Another recent study also indicates the importance of Fe(III) reducers in RDX-degradation, showing dominant microorganisms in groundwater shifting from γ -*Proteobacteria* to β - and δ -*Proteobacteria* (e.g., *Geobacter* spp.) when acetate was added to stimulate RDX biodegradation (Kwon et al., 2011). Our current study suggests for the first time that this genus is capable of using ring-N from RDX.

(ii) Clones potentially capable of assimilating both RDX-derived nitrogen and carbon. We previously completed a study in which ring-labeled ¹⁵N-RDX was added to microcosms prepared from the NJ Army site years before the bioremediation effort was undertaken, and ¹⁵N-SIP was conducted with addition of yeast extract or cheese whey as electron donors (Roh et al., 2009). In addition, we previously used groundwater collected from MW-5 and MW-7D at the same time as for this study to conduct a ¹³C-SIP experiment (Cho et al., 2013). The clone libraries from the previous studies are compared with the current study in Table 2. The common clones identified from previous ¹³C- and ring-¹⁵N₃-RDX SIP studies that are of the same genus are shown in bold in in supporting information (Table S1).

283 Two sequences (70-Ra22 and 70-Na4) in β -*Proteobacteria* showed high
284 homology (>97%) to one clone derived from background groundwater (GW clone 16,
285 EU907859), and one clone (^{13}C -RDX clone 50-a1, JX066666) identified from the
286 previous ^{13}C -RDX SIP study([Cho et al., 2013](#)). This data suggest that these clones are
287 able to use RDX and/or RDX metabolites as both a carbon and nitrogen source. These
288 two clones corresponded to the 105 bp T-RF in the microbial community profiles (See
289 the Figures S11 and S13 in supporting information).

290 Four sequences (50-Fa13, 70-Fa2, 70-Fa3, and 70-Fa12) in β -*Proteobacteria* also
291 showed similarity (>95%) to two ^{13}C -RDX clones, 70a28 and 70a92 ([Cho et al., 2013](#)).
292 These four sequences showed 92% homology to *Ferribacterium limneticum* (accession
293 number: NR026464.1), an Fe(III)-reducing bacterium isolated from mining-impacted
294 sediment ([Cumplings et al., 1999](#)). The high homology of these four sequences to Fe
295 (III)-reducing bacteria (such as *Albidiferax* sp. (basonym *Rhodoferox*) and
296 *Ferribacterium* sp.)) also suggests that Fe-reducers may be able to use RDX or RDX-
297 intermediates as both a carbon and nitrogen source. These data support recent studies
298 suggesting that iron-reducers are important in the degradation of RDX in the environment
299 ([Cumplings et al., 1999](#); [Finneran et al., 2003](#); [Kwon et al., 2011](#); [Livermore et al., 2013](#)).
300 These four sequences also corresponded to T-RF =106 bp (predicted at 108 bp) in the
301 microbial community profiles (See the Figures S4 and S15 in supporting materials).

302 Six clones (50-Na22, 50-Na28, 50-Na32, 50-Fa14, 50-Fa33, and 70-Fa15)
303 clustered in *Spirochaetes*, and were similar (>98%) to the ^{13}C -RDX clone 50a6
304 (accession number: JX066670), suggesting certain strains in *Spirochaetes* are capable of

using RDX and/or RDX metabolites as both a carbon and nitrogen source. These six sequences also have a theoretical T-RF of 108 bp and potentially corresponded to 106 bp T-RF in the microbial community profiles (See the Figures S3, S4 and S15 in supporting material).

Finally, one sequence, 70-Fa7, was related to *Herbaspirillum lusitanum* (accession number: NR028859), a nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris* (Valverde et al., 2003). We also detected this genus via ^{13}C - and ^{15}N -RDX SIP at a RDX-contaminated site in Virginia, suggesting nitrogen-fixing bacteria in this genus may have RDX degradation ability and, at a minimum, have the ability to assimilate C and N from RDX and/or RDX metabolites in situ (Cho et al., 2015).

In all, 7 of the 16 sequences detected in the current ^{15}N -RDX SIP study showed homology to previous clones from the ^{13}C -RDX SIP study, indicating that these genera are likely capable of assimilating both carbon and nitrogen from RDX and/or RDX breakdown products. It is somewhat surprising that so many different organisms may be capable of incorporating both C and N from RDX, particularly since only one previous anaerobic sulfate reducing isolate has been reported that can utilize both C and N from RDX for growth (Arnett and Adrian, 2009).

Effects of Cheese Whey Addition on the Community Profile of RDX Degradation.

Addition of cheese whey to the groundwater samples altered the types and distribution of RDX-degrading microbial populations compared to those observed in microcosms which received no additional cheese whey. A total of twenty-eight sequences were derived from the cheesewhey amended microcosms (5C-Na, 5C-Ra, 5C-

Fa, 7C-Na, 7C-Ra, and 7C-Fa). These sequences (n=28) were grouped into *Bacteroidia*, *Bacilli*, α -, β -, and γ -*Proteobacteria* (Figure 4), with the majority in α -, β -, and γ -*Proteobacteria*. Once again, none of the derived sequences were an exact match to any previously isolated RDX-degrading strains.

There were three overlapping groups in the clone libraries of ^{13}C -SIP and ^{15}N -SIP microcosms when cheese whey was added in the β - *Proteobacteria*, γ -*Proteobacteria*, and *Bacilli*. Two sequences (5C-Ra12 and 5C-Na12), derived from Microcosms 5C-Ra and 5C-Na, showed high similarity (95%) to two known RDX degraders, *Pseudomonas fluorescens* I-C and *Pseudomonas putida* II-B ([Blehert et al., 1999](#); [Pak et al., 2000](#)). Our results are consistent with previous studies identifying *Pseudomonas* sp. in groundwater or laboratory enrichments when biostimulation was performed to enhance RDX degradation ([Cho et al., 2013](#); [Fuller et al., 2010](#); [Roh et al., 2009](#)). *P. fluorescens* I-C and *P. putida* II-B strains can transform RDX using xenobiotic reductases XenA and XenB, respectively, under anoxic conditions ([Fuller et al., 2009](#)). Along with previous findings, these results suggest that *Pseudomonas* sp. play an important role in transforming RDX and other nitramine explosives in the environment.

Additionally, one sequence (5C-Fa3) was clustered in *Bacteroidia* under the genus as *Rikenella*; this finding is similar to our previous finding in a ^{13}C -SIP study conducted with samples from the same contaminated site ([Cho et al., 2013](#)). Consistent identification of this genus between the studies suggests that this taxonomic group is involved in the degradation of RDX and/or its metabolites at the site and that the genus may be able to utilize both C and N from the RDX molecule.

349 Interestingly, a total of 13 sequences from bottles receiving ring-, nitro- and fully
350 labeled RDX clustered in α -*Proteobacteria* (7C-Fa34) and β -*Proteobacteria* (5C-Ra9,
351 5C-Ra15, 5C-Ra25, 7C-Na1, 7C-Na3, 7C-Na 8, 7C-Ra8, 7C-Ra9, 7C-Ra11, 7C-Ra16,
352 7C-Fa3, and 7C-Fa10). One sequence (7C-Fa34) belonged in genus *Brevundimonas*. A
353 recent SIP study using Illumina sequencing also detected sequences in *Brevundimonas* in
354 soils in Michigan amended with glucose and RDX ([Jayamani and Cupples, 2015](#)). The
355 other twelve sequences were in the genus *Janthinobacterium*, for which there are no
356 closely related species reported in the literature on RDX biodegradation. A groundwater
357 isolate, *Janthinobacterium lividum*, capable of degrading herbicide terbuthylazine (N-
358 tert-butyl-6-chloro-N'-ethyl-1,3,5-triazine-2,4-diamine) has been recently reported
359 ([Caracciolo et al., 2010](#)). The strain contains genes encoding s-triazine-degrading
360 enzymes, *atzB* (hydroxyatrazine ethylaminohydrolase) and *atzC* (*N*-isopropylammelide
361 isopropylamidohydrolase), which hydrolyze side-groups on the triazine [43]. The
362 terbuthylazine molecule includes a heterocyclic ring with alternating C and N molecules
363 much like RDX, so it is possible that *atzB* and/or *atzC* have activity towards RDX,
364 possibly hydrolyzing one or more –NO₂ side groups. Further studies would be necessary
365 to determine if these or other enzymes in *Janthinobacterium* are responsible for RDX
366 degradation.

367 Further, 12 sequences derived from cheese wheyamended microcosms clustered
368 in *Bacilli*. The same SIP study using Illumina sequencing cited above also detected
369 unclassified *Bacteroidetes* ([Jayamani and Cupples, 2015](#)). Among the 12 sequences,
370 seven sequences (5C-Ra27, 5C-Ra29, 5C-Na2, 5C-Na3, 5C-Na4, 5C-Fa7 and 5C-Na9)

were derived from Microcosms 5C-Na, 5C-Ra and 5C-Fa. The other five sequences (7C-Ra2, 7C-Na22, 7C-Fa1, 7C-Fa2, and 7C-Fa9) were derived from Microcosms 7C-Na, 7C-Ra, and 7C-Fa. Most of these sequences were highly homologous (>97%) and were repetitively observed in microcosms receiving ring-, nitro-, or fully-labeled ¹⁵N-RDX. These results were similar to the previous ¹³C-RDX SIP study(Cho et al., 2013) in that a high numbers of sequences in *Bacilli* (nine sequences in ¹³C-SIP study, 5Ca7, 5Ca28, 5Ca33, 5Ca60, 5Cb4, 5Cb13, 5Cb17, 7Ca13, and 7Cb43) were associated with the cheese whey addition. These 12 sequences exhibited high identity with *Trichococcus flocculiformis* (accession number: NR042060), a low G+C content strain capable of utilizing various carbohydrates for acid production (Liu et al., 2002). Although no studies have reported the RDX degradation ability of *Trichococcus* spp., our results clearly indicated that the *Bacilli* were stimulated by cheese whey addition and that they could assimilate ¹⁵N from ¹⁵N-labeled RDX or RDX breakdown products.

Summary and Conclusions

In this study, we identified microorganisms capable of using nitro-, ring- or fully-labeled ¹⁵N-RDX and/or its metabolites as a nitrogen source in the presence or absence of fresh cheese whey addition. Our results indicate that there are a diversity of active microorganisms associated with RDX biodegradation and N assimilation in groundwater, and that the microorganisms involved are largely different than those that have been studied in the laboratory to date and from which the degradative genes and pathways for RDX have been derived. A total of fifteen clones identified in the absence of added cheese whey grouped into *Clostridia*, *β-Proteobacteria*, and *Spirochaetes*, suggesting

that these clones were able to incorporate nitrogen from RDX and/or its metabolites. Significantly, some genera we identified were homologous with genera detected previously at other sites where carbon sources were added to promote anaerobic RDX biodegradation, including *Desulfosporosinus* from a site in Virginia and *Rhodoferrax* spp. from an aquifer in Iowa ([Cho et al., 2013](#); [Livermore et al., 2013](#)). This suggests that some specific groups of organisms may play an important role in RDX anaerobic biodegradation at many sites during bioremediation efforts (i.e., when electron donors are added). Conversely, some of the genera detected that incorporated N from RDX, such as *Janthinobacterium*, were reported for the first time in this study.

With the addition of cheese whey to groundwater samples, significant shifts in microbial communities were observed. While more clones (a total of 28 clones) were isolated, these clones were less diverse and resided in *Bacteroidia*, *Bacilli*, α -, β -, and γ -*Proteobacteria*. A similar loss in community diversity was observed in a companion study in which ^{13}C -SIP was conducted with samples from the same wells at this site at a much earlier time ([Cho et al., 2013](#)). A comparison of the clones identified in the current study and the previous study is provided in Figure 5, where the increase in clone number and loss in diversity is apparent with cheese whey addition, particularly in well MW5. Moreover, some, (but not all), clones in the two studies were similar suggesting that there is potentially some consistency in the microbial communities that develop during RDX biodegradation at this site. For example, *Spirochaetes* were observed in both ^{13}C and ^{15}N SIP studies in both wells, but only in microcosms without additional added cheese whey, suggesting that these organisms can possibly derive carbon and nitrogen from RDX or

RDX metabolites, and that they might be important for intrinsic RDX biodegradation. *Clostridia* and *β-Proteobacteria* were also detected in both studies, but only in MW-5, and only without added whey. Conversely, *Bacilli* were only observed in microcosms in the presence of added cheese whey, appearing to thrive in environments with high labile organic carbon, while *β-Proteobacteria* were found in all but one microcosm. The data clearly show that the RDX-degrading members of groundwater microbial communities are much more complex than suggested from studies with isolated pure cultures, and they also indicate some recurring genera both in time and at differing locations. Additional studies at a broader range of sites are required to develop a better overall understanding of these communities and the roles of different microbial species during in situ RDX biodegradation.

Acknowledgements

This project was supported by the US Strategic Environmental Research and Development Program (SERDP) under contract W912HQ-08-C-0031 and the Environmental Security Technology Certification Program (ESTCP) under contract W912HQ-04-C-0041. Views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of Defense position or decision unless so designated by other official documentation. We wish to acknowledge Charles Condee at CB&I for his excellent technical assistance with this project.

Conflict of interest

437 The authors declare that there are no conflict of interest.

438 **Supporting Information Available**

439 The supporting materials are available free of charge via the Internet at

440 <http://www.xxxx>

References

- Toxicological profile for RDX. U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Atlanta, GA, 2012.
- Adrian NR, Arnett CM. Anaerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Acetobacterium malicum* strain HAAP-1 isolated from a methanogenic mixed culture. *Current Microbiology* 2004; 48: 332-340.
- Andeer P, Stahl DA, Lillis L, Strand SE. Identification of microbial populations assimilating nitrogen from RDX in munitions contaminated military training range soils by high sensitivity stable isotope probing. *Environmental Science & Technology* 2013.
- Andeer P, Strand SE, Stahl DA. High-sensitivity stable-isotope probing by a quantitative terminal restriction fragment length polymorphism protocol. *Applied and Environmental Microbiology* 2012; 78: 163-169.
- Arnett C, Adrian N. Cosubstrate independent mineralization of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a *Desulfovibrio* species under anaerobic conditions. *Biodegradation* 2009; 20: 15-26.
- Bernstein A, Adar E, Nejdat A, Ronen Z. Isolation and characterization of RDX-degrading *Rhodococcus* species from a contaminated aquifer. *Biodegradation* 2011; 22: 997-1005.
- Bhushan B, Halasz A, Hawari J. Effect of iron(III), humic acids and anthraquinone-2,6-disulfonate on biodegradation of cyclic nitramines by *Clostridium* sp. EDB2. *J. Appl. Microbiol.* 2006; 100: 555-563.
- Bleher DS, Fox BG, Chambliss GH. Cloning and sequence analysis of two *Pseudomonas flavoprotein* xenobiotic reductases. *J. Bacteriol.* 1999; 181: 6254-6263.
- Boopathy R, Gurgas M, Ullian J, Manning JF. Metabolism of explosive compounds by sulfate-reducing bacteria. *Current Microbiology* 1998; 37: 127-131.
- Caracciolo AB, Fajardo C, Grenni P, Sacca ML, Amalfitano S, Ciccoli R, et al. The role of a groundwater bacterial community in the degradation of the herbicide terbuthylazine. *FEMS Microbiol Ecol* 2010; 71: 127-36.
- Cho K-C, Lee DG, Fuller ME, Hatzinger PB, Condee CW, Chu K-H. Application of ¹³C and ¹⁵N stable isotope probing to characterize RDX degrading microbial communities under different electron-accepting conditions. *Journal of Hazardous Materials* 2015; 297: 42-51.
- Cho K-C, Lee DG, Roh H, Fuller ME, Hatzinger PB, Chu K-H. Application of ¹³C-stable isotope probing to identify RDX-degrading microorganisms in groundwater. *Environmental Pollution* 2013; 178: 350-360.
- Coleman NV, Nelson DR, Duxbury T. Aerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source by a *Rhodococcus* sp., strain DN22. *Soil Biol. Biochem.* 1998; 30: 1159-1167.
- Crocker FH, Indest KJ, Fredrickson HL. Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20. *Appl. Microbiol. Biotechnol.* 2006; 73: 274-290.

- Cummings DE, Caccavo Jr F, Spring S, Rosenzweig RF. *Ferribacterium limneticum*, gen. nov., sp. nov., an Fe(III)-reducing microorganism isolated from mining-impacted freshwater lake sediments. Archives of Microbiology 1999; 171: 183-188.
- Dumont MG, Murrell JC. Stable isotope probing - linking microbial identity to function. Nat. Rev. Microbiol. 2005; 3: 499-504.
- Finneran KT, Johnsen CV, Lovley DR. *Rhodoferrax ferrireducens* sp. nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). International Journal of Systematic and Evolutionary Microbiology 2003; 53: 669-673.
- Freedman DL, Sutherland KW. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) under nitrate-reducing conditions. Water Sci. Technol. 1998; 38: 33-40.
- Fuller ME, McClay K, Hawari J, Paquet L, Malone TE, Fox BG, et al. Transformation of RDX and other energetic compounds by xenobiotic reductases XenA and XenB. Appl. Microbiol. Biotechnol. 2009; 84: 535-544.
- Fuller ME, McClay K, Higham M, Hatzinger PB, Steffan RJ. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) bioremediation in groundwater: Are known RDX-degrading bacteria the dominant players? Bioremediation Journal 2010; 14: 121-134.
- Gallagher E, McGuinness L, Phelps C, Young LY, Kerkhof LJ. ¹³C-carrier DNA shortens the incubation time needed to detect benzoate-utilizing denitrifying bacteria by stable-isotope probing. Appl. Environ. Microbiol. 2005; 71: 5192-5196.
- Gallagher EM, Young LY, McGuinness LM, Kerkhof LJ. Detection of 2,4,6-trinitrotoluene-utilizing anaerobic bacteria by ¹⁵N and ¹³C incorporation. Applied and Environmental Microbiology 2010; 76: 1695-1698.
- Hatzinger P, Lippincott D. In Situ bioremediation of energetic compounds in groundwater. Project ER-200425 Final Report. Environmental Security Technology Certification Program. Available at: <https://www.serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/ER-200425>, 2012.
- Jayamani I, Cupples AM. Stable isotope probing reveals the importance of Comamonas and Pseudomonadaceae in RDX degradation in samples from a Navy detonation site. Environ Sci Pollut Res Int 2015.
- Kwon MJ, Finneran KT. Microbially mediated biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by extracellular electron shuttling compounds. Applied and Environmental Microbiology 2006; 72: 5933-5941.
- Kwon MJ, Finneran KT. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) biodegradation kinetics amongst several Fe(III)-reducing genera. Soil Sediment Contamination 2008; 17: 189-203.
- Kwon MJ, O'Loughlin EJ, Antonopoulos DA, Finneran KT. Geochemical and microbiological processes contributing to the transformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in contaminated aquifer material. Chemosphere 2011; 84: 1223-1230.
- Liu J-R, Tanner RS, Schumann P, Weiss N, McKenzie CA, Janssen PH, et al. Emended description of the genus *Trichococcus*, description of *Trichococcus collinsii* sp. nov., and reclassification of *Lactosphaera pasteurii* as *Trichococcus pasteurii*

comb. nov. and of *Ruminococcus palustris* as *Trichococcus palustris* comb. nov. in the low-G+C gram-positive bacteria. International Journal of Systematic and Evolutionary Microbiology 2002; 52: 1113-26.

Livermore JA, Jin YO, Arnseth RW, LePuil M, Mattes TE. Microbial community dynamics during acetate biostimulation of RDX-contaminated groundwater. Environmental Science & Technology 2013; 47: 7672-7678.

Morris SA, Radajewski S, Willison TW, Murrell JC. Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. Appl. Environ. Microbiol. 2002; 68: 1446-1453.

Pak JW, Knoke KL, Noguera DR, Fox BG, Chambliss GH. Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. Appl. Environ. Microbiol. 2000; 66: 4742-4750.

Pester M, Brambilla E, Alazard D, Rattei T, Weinmaier T, Han J, et al. Complete genome sequences of *Desulfosporosinus orientis* DSM765T, *Desulfosporosinus youngiae* DSM17734T, *Desulfosporosinus meridiei* DSM13257T, and *Desulfosporosinus acidiphilus* DSM22704T. Journal of Bacteriology 2012; 194: 6300-6301.

Prazeres AR, Carvalho F, Rivas J. Cheese whey management: a review. Journal of Environmental Management 2012; 110: 48-68.

Radajewski S, McDonald IR, Murrell JC. Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. Curr. Opin. Biotechnol. 2003; 14: 296-302.

Radajewski S, Webster G, Reay DS, Morris SA, Ineson P, Nedwell DB, et al. Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing. Microbiology 2002; 148: 2331-2342.

Roh H, Yu C-P, Fuller ME, Chu K-H. Identification of hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading microorganisms via ¹⁵N-stable isotope probing. Environ. Sci. Technol. 2009; 43: 2505-2511.

Seth-Smith HMB, Rosser SJ, Basran A, Travis ER, Dabbs ER, Nicklin S, et al. Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from *Rhodococcus rhodochrous*. Appl. Environ. Microbiol. 2002; 68: 4764-4771.

Sherburne LA, Shrout JD, Alvarez PJJ. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation by *Acetobacterium paludosum*. Biodegradation 2005; 16: 539-547.

Thompson KT, Crocker FH, Fredrickson HL. Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by *Gordonia* and *Williamsia* spp. Appl. Environ. Microbiol. 2005; 71: 8265-8272.

Uhlík O, Jecná K, Leigh MB, Macková M, Macek T. DNA-based stable isotope probing: a link between community structure and function. Science of The Total Environment 2009; 407: 3611-3619.

USEPA. Contaminant Candidate List (CCL) and Regulatory Determination, Chemical Contaminants-CCL4. 2015, 2015.

Valverde A, Velázquez E, Gutiérrez C, Cervantes E, Ventosa A, Igual J-M. *Herbaspirillum lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*. International Journal of Systematic and Evolutionary Microbiology 2003; 53: 1979-1983.

- Yu C-P, Chu K-H. A quantitative assay for linking microbial community function and structure of a naphthalene-degrading microbial consortium. *Environ. Sci. Technol.* 2005; 39: 9611-9619.
- Zhang C, Hughes JB. Biodegradation pathways of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Clostridium acetobutylicum* cell-free extract. *Chemosphere* 2002; 50: 665-671.
- Zhao J-S, Halasz A, Paquet L, Beaulieu C, Hawari J. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine and its mononitroso derivative hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine by *Klebsiella pneumoniae* strain SCZ-1 isolated from an anaerobic sludge. *Appl. Environ. Microbiol.* 2002; 68: 5336-5341.
- Zhao J-S, Paquet L, Halasz A, Manno D, Hawari J. Metabolism of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by *Clostridium bifermentans* strain HAW-1 and several other H₂-producing fermentative anaerobic bacteria. *FEMS Microbiol. Lett.* 2004; 237: 65-72.
- Zhao J-S, Spain J, Hawari J. Phylogenetic and metabolic diversity of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-transforming bacteria in strictly anaerobic mixed cultures enriched on RDX as nitrogen source. *FEMS Microbiol. Ecol.* 2003a; 46: 189-196.
- Zhao JS, Paquet L, Halasz A, Hawari J. Metabolism of hexahydro-1,3,5-trinitro-1,3,5-triazine through initial reduction to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine followed by denitration in *Clostridium bifermentans* HAW-1. *Appl. Microbiol. Biotechnol.* 2003b; 63: 187-193.

Table Legends

Table 1. Groundwater microcosm setup and conditions. The microcosm ID (50-R, 50-N, 50-F, 5C-R, 5C-N, 5C-F, 70-R, 70-N, 70-F, 7C-R, 7C-N, 7C-F) indicated each microcosm setup conditions. The rule of abbreviation each microcosm ID was based on the groundwater sources (MW-5 or MW-7D), cheese whey amendment (with or without cheese whey addition), and the RDX treatment (ring-, nitro-, fully-labeled ^{15}N -RDX), respectively.

Table 2. Comparison of clones derived from this ^{15}N -RDX (ring-, nitro- and fully-labeled ^{15}N -RDX) SIP study with those identified from previous studies with ring- $^{15}\text{N}_3$ -RDX and ^{13}C -RDX SIP studies and other known strains ([Cho et al., 2013](#); [Roh et al., 2009](#)).

Table 1. Groundwater microcosm setup and conditions. The microcosm ID (50-R, 50-N, 50-F, 5C-R, 5C-N, 5C-F, 70-R, 70-N, 70-F, 7C-R, 7C-N, 7C-F) indicated each microcosm setup conditions. The rule of abbreviation each microcosm ID was based on the groundwater sources (MW-5 or MW-7D), cheese whey amendment (with or without cheese whey addition), and the RDX treatment (ring-, nitro-, fully-labeled ^{15}N -RDX), respectively.

Microcosm setup condition and coding				
Groundwater source	Cheese whey addition	RDX addition	Microcosm ID	Sample ID*
MW-5	No	^{15}N -ring labeled	50-R	Not available (no degradation)
		^{15}N -nitro labeled	50-N	50-Na
		^{15}N -fully labeled	50-F	50-Fa
MW-5	Yes	^{15}N -ring labeled	5C-R	5C-Ra & 5C-Rb
		^{15}N -nitro labeled	5C-N	5C-Na & 5C-Nb
		^{15}N -fully labeled	5C-F	5C-Fa & 5C-Fb
MW-7D	No	^{15}N -ring labeled	70-R	70-Ra & 70-Rb
		^{15}N -nitro labeled	70-N	70-Na & 70-Nb
		^{15}N -fully labeled	70-F	70-Fa & 70-Fb
MW-7D	Yes	^{15}N -ring labeled	7C-R	7C-Ra & 7C-Rb
		^{15}N -nitro labeled	7C-N	7C-Na & 7C-Nb
		^{15}N -fully labeled	7C-F	7C-Fa & 7C-Fb

Table 2. Comparison of clones derived from this ¹⁵N-RDX (ring-, nitro- and fully-labeled -¹⁵N-RDX) SIP study with those identified from previous studies with ring-¹⁵N₃-RDX and ¹³C-RDX SIP studies and other known strains (Cho et al., 2013; Roh et al., 2009).

Sequences from this ¹⁵ N-RDX SIP study Sequence name (access number)	Sequences from previous ¹³ C-RDX and ¹⁵ N-RDX SIP studies Sequence name (access number)	Homology
70-Ra22 (JX470449) 70-Na4 (JX470450)	GW clone 16 (EU907859) and ¹³ C-RDX clone 50-a1 (JX066666)(Cho et al., 2013)	>97%
50-Fa13(JX470445) 70-Fa2 (JX470451) 70-Fa3 (JX470452) 70-Fa12(JX470454)	¹³ C-RDX clones 70a28 (JX066676) and 70a92 (JX066677)(Cho et al., 2013) <i>Ferribacterium limneticum</i> (NR026464.1) (Cummings et al., 1999)	>95% >92%
70-Ra22 (JX470449)	<i>Rhodeferax ferrireducens</i> strain T118 (NR074760) and <i>Rhodeferax fermentans</i> strain FR2 (NR025840) (Finneran et al., 2003)	97-99%
50-Na22 (JX470441) 50-Na28 (JX470442) 50-Na32 (JX470443) 50-Fa14 (JX470446) 50-Fa33 (JX470448) 70-Fa15 (JX470455)	¹³ C-RDX clone 50a6 (JX066670) (Cho et al., 2013)	>98%
70-Fa7 (JX470449)	<i>Herbaspirillum lusitanum</i> (NR028859) (Valverde et al., 2003)	91%
5C-Ra27(JX470460) 5C-Ra29 (JX470461) 5C-Na2(JX470462) 5C-Na3 (JX470463) 5C-Na4 (JX470464) 5C-Fa7 (JX470467) 5C-Fa9 (JX470468) 7C-Ra2(JX470469) 7C-Na22(JX470467) 7C-Fa1(JX470478) 7C-Fa2(JX470479) 7C-Fa9(JX470481)	<i>Trichococcus flocculiformis</i> (NR042060) (Liu et al., 2002) ¹³ C-RDX clone (Cho et al., 2013) 50a6 (JX066670) 5Ca7 (JX066679) 5Ca28 (JX066682) 5Ca33 (JX066684) 5Ca60(JX066685) 5Cb4 (JX066687) 5Cb13 (JX066690) 5Cb17 (JX066691) 7Ca13(JX066694) 7Cb43(JX066696)	97-99%
5C-Ra12 (JX470457) 5C-Na12 (JX470465)	<i>Pseudomonas fluorescens</i> I-C (EF219420.2) and <i>Pseudomonas putida</i> II-B (EF219419) (Bleher et al., 1999; Pak et al., 2000)	>95%

Figure Legends

Figure 1. RDX degradation over time in microcosms receiving one of ring-, nitro-, fully-labeled ^{15}N -RDX. Microcosms constructed from MW-5 groundwater (A) with ring-labeled ^{15}N -RDX (50-R), nitro-labeled ^{15}N -RDX (50-N) or fully-labeled ^{15}N RDX (50-F). Microcosms constructed from MW-7D groundwater (B) with ring-labeled ^{15}N -RDX (70-R) or nitro-labeled ^{15}N -RDX (70-N) or fully-labeled ^{15}N -RDX (70-F). Arrows indicate the addition of RDX. Stars (*) indicate time of sampling for SIP and other molecular analyses. Initial concentration of RDX in microcosms was 10 mg/L. No samples were taken from Microcosms 5-0-R for further analysis. The microcosms were respiked with RDX on Day 35.

Figure 2. RDX degradation over time in microcosms receiving cheese whey and one of ring-, nitro-, fully-labeled ^{15}N -RDX. Microcosms constructed from MW-5 groundwater (A) with ring-labeled ^{15}N -RDX (5C-R) or nitro-labeled ^{15}N -RDX (5C-N) or fully-labeled ^{15}N -RDX (5C-F). Microcosms constructed from MW-7D groundwater (B) with ring-labeled ^{15}N -RDX (7C-R) or nitro-labeled ^{15}N -RDX (7C-N) or fully-labeled ^{15}N -RDX (7C-F). Arrows indicate the addition of RDX. Stars (*) indicate time of sampling for SIP and other molecular analyses. Initial concentration of RDX in microcosms was 10 mg/L. The microcosms were respiked with RDX on Day 28.

Figure 3. Phylogenetic tree representing 16S rRNA gene sequences derived from ^{15}N -DNA fractions of groundwater microcosms from MW-5 and MW-7D receiving ring-, nitro-, fully- ^{15}N -labeled RDX. The tree was rooted with *Methanococcus thermolithotrophicus* and was constructed using the neighbor-joining algorithm. The number of clone sequences and the GenBank accession numbers are in parentheses. Only bootstrap values above 85% are shown (1,000 replications). Bar, 10% estimated sequence divergence. An asterisk (*) indicates a known RDX degrader.

Figure 4. Phylogenetic tree representing 16S rRNA gene sequences derived from ^{15}N -DNA fractions of groundwater microcosms receiving either MW-5 or MW-7D groundwater, cheese whey, and receiving ring, nitro, fully ^{15}N -labeled RDX. The tree was rooted with *Methanococcus thermolithotrophicus* and was constructed using the neighbor-joining algorithm. The number of clone sequences and the GenBank accession numbers are in parentheses. Only bootstrap values above 85% are shown (1,000 replications). Bar, 10% estimated sequence divergence. An asterisk (*) indicates a known RDX degrader.

Figure 5. Comparison of the distribution of order of 16S rRNA gene sequences of ^{15}N - or ^{13}C -labeled clones derived from the microcosms in the presence and absence of cheese whey.

671
672

(A)

(B)

Figure 1. RDX degradation over time in microcosms receiving one of ring-, nitro-, fully-labeled ^{15}N -RDX. Microcosms constructed from MW-5 groundwater (A) with ring-labeled ^{15}N -RDX (50-R), nitro-labeled ^{15}N -RDX (50-N) or fully-labeled ^{15}N RDX (50-F). Microcosms constructed from MW-7D groundwater (B) with ring-labeled ^{15}N -RDX (70-R) or nitro-labeled ^{15}N -RDX (70-N) or fully-labeled ^{15}N -RDX (70-F). Arrows indicate the addition of RDX. Stars (*) indicate time of sampling for SIP and other molecular analyses. Initial concentration of RDX in microcosms was 10 mg/L. No samples were

taken from Microcosms 50-R for further analysis. The microcosms were respiked with RDX on Day 35.

(A)

(B)

Figure 2. RDX degradation over time in microcosms receiving cheese whey and one of ring-, nitro-, fully-labeled ^{15}N -RDX. Microcosms constructed from MW-5 groundwater (A) with ring-labeled ^{15}N -RDX (5C-R) or nitro-labeled ^{15}N -RDX (5C-N) or fully-labeled

¹⁵N-RDX (5C-F). Microcosms constructed from MW-7D groundwater (B) with ring-labeled ¹⁵N-RDX (7C-R) or nitro-labeled ¹⁵N-RDX (7C-N) or fully-labeled ¹⁵N-RDX (7C-F). Arrows indicate the addition of RDX. Stars (*) indicate time of sampling for SIP and other molecular analyses. The microcosms were respiked with RDX on Day 28.

Figure 3. Phylogenetic tree representing 16S rRNA gene sequences derived from ¹⁵N-DNA fractions of groundwater microcosms from MW-5 and MW-7D receiving ring-, nitro-, fully- ¹⁵N-labeled RDX. The tree was rooted with *Methanococcus thermolithotrophicus* and was constructed using the neighbor-joining algorithm. The number of clone sequences and the GenBank accession numbers are in parentheses. Only bootstrap values above 85% are shown (1,000 replications). Bar, 10% estimated sequence divergence. An asterisk (*) indicates a known RDX degrader.

Figure 4. Phylogenetic tree representing 16S rRNA gene sequences derived from ^{15}N -DNA fractions of groundwater microcosms receiving either MW-5 or MW-7D groundwater, cheese whey, and receiving ring, nitro, fully ^{15}N -labeled RDX. The tree was rooted with *Methanococcus thermolithotrophicus* and was constructed using the neighbor-joining algorithm. The number of clone sequences and the GBank accession numbers are in parentheses. Only bootstrap values above 85% are shown (1,000 replications). Bar, 10% estimated sequence divergence. An asterisk (*) indicates a known RDX degrader.

Figure 5. Comparison of the distribution of order of 16S rRNA gene sequences of ¹⁵N- or ¹³C-labeled clones (Cho et al., 2013) derived from the microcosms in the presence and absence of cheese whey. Source of groundwater: MW-5 or MW-7D.