SUSTAINED AND COMPLETE HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX) DEGRADATION IN ZERO-VALENT IRON SIMULATED BARRIERS UNDER DIFFERENT MICROBIAL CONDITIONS

J. D. SHROUT[1,2*], P. LARESE-CASANOVA[1], M. M. SCHERER[1] AND P. J. ALVAREZ[1,3]

[1]Department of Civil and Environmental Engineering, The University of Iowa, Iowa City, Iowa 52242 USA
[2]Present address: Department of Microbiology, The University of Iowa, Iowa City, Iowa 52242 USA
[3]Present address: Civil and Environmental Engineering, Rice University, 6100 Main Street, MS 317, Houston, Texas 77005 USA

ABSTRACT

Flow-through columns packed with "aged" zero-valent iron (ZVI) between layers of soil and sand were constructed to mimic a one-dimensional permeable reactive iron barrier (PRB). The columns were continuously fed RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine, ca. 18 mg l⁻¹) for over one year. Two columns were bioaugmented with dissimilatory iron reducing bacteria (DIRB) *Shewanella algae* BrY or *Geobacter metallireducens* GS-15 to investigate their potential to enhance the reactivity of aged iron by reductive dissolution of passivating iron oxides or via production of biogenic reactive minerals. A third column was not bioaugmented to evaluate colonization by indigenous soil microorganisms. [¹⁴C]-RDX was completely removed in all columns at the start of the iron layer, and concentration profiles showed rapid and sustainable RDX removal over one year; however, a phylogenetic profile conducted after one year using DGGE analysis of recovered DNA did not detect *S. algae* BrY or *G. metallireducens* in their respective columns. Bacterial DNA was recovered from within the ZVI. Several unidentified ¹⁴C-labeled byproducts were present in the effluent of all columns. Dissolved ¹⁴C removal and the detection of dissolved inorganic ¹⁴C in these columns (but not in the sterile control) suggest microbial-mediated mineralization of RDX and sorption/precipitation of degradation products. Enhanced RDX mineralization in bioaugmented columns was temporary relative to the indigenously colonized column. However, shorter acclimation periods associated with bioaugmented PRBs may be desirable for rapid RDX mineralization, thereby preventing breakthrough of potentially undesirable byproducts. Overall, these results show that high RDX removal efficiency by ZVI-PRBs is achievable and sustainable and that the efficacy and start-up of ZVI-PRBs might be enhanced by bioaugmentation.

INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a recalcitrant and toxic contaminant present in the subsurface at many military installations. Because of its persistence, low tendency to volatilize (dimensionless Henry's constant, $H' = 2 \times 10^{-11}$), and high mobility in aquifers ($\log K_{ow} = 0.8$), clean-up of RDX-contaminated sites is a challenging problem. Several *ex situ* physical-chemical and biological practices to remove RDX from contaminated sites exist, but these are not cost-effective to treat large volumes of contaminated groundwater. In addition, complete destruction of RDX is not always achieved and toxic metabolites may be produced.

RDX is an oxidized pollutant that can be biodegraded under anaerobic conditions [1-7]. Anaerobic degradation of RDX with zero-valent iron (ZVI) under both abiotic and biotic conditions has been described previously [8-12]. Past studies showed that RDX could be extensively mineralized (to CO_2 and N_2O) in batch and flow-through column systems amended with ZVI and municipal anaerobic sludge [9,10]. Anaerobic RDX biodegradation is stimulated through the depletion of O_2 and the production of water-derived H_2 during ZVI corrosion (Equation i) [13-15]:

$$Fe^0 + 2H_2O \rightarrow Fe^{2+} + 2OH^- + H_2 \qquad (i)$$
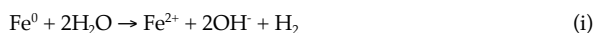
While past studies with ZVI have shown very high RDX removal efficiencies [8-12], passivation of iron and decreased removal efficiency over time are potential concerns. The oxidation of Fe^{2+} to Fe^{3+}, which is less soluble, may result in precipitation of secondary minerals including magnetite, green rust, siderite, ferrihydrite, hematite, goethite, and lepidocrocite [16]. It has recently been shown that RDX may be transformed by Fe^{2+} associated with some iron solids, such as green rust (e.g. $Fe^{II}_4Fe^{III}_2(OH)_{12}CO_3\text{-}yH_2O$) [17] and Fe^{2+} sorbed to magnetite (Fe_3O_4) [18]; however, Fe^{3+} oxides may inhibit reductive treatment with ZVI by forming a passivating oxide layer. Several PRB and column studies show secondary

mineral precipitation influences long-term performance [16,19-24]. Therefore, there is a clear need to understand the performance and sustainability of ZVI barriers during prolonged exposure to RDX and its transformation products.

Previous lab studies have suggested that ZVI barriers have the potential to effectively intercept and degrade RDX plumes *in situ*, and that process efficiency and robustness could be enhanced by bioaugmentation [9,10]. However, the long-term performance of such a system has not been evaluated. An integrated ZVI-microbial system should improve the longevity (via biogeochemical cycling) and the transformation capability of reactive barriers, leading to reduced RDX remediation costs. Yet, the benefits of ZVI bioaugmentation over the design life of the barrier are unknown. It is also poorly understood if colonizing bacteria participate directly to degrade pollutants or if they provide indirect benefit by maintaining or activating iron species.

Here we report on an investigation of lab-scale ZVI PRB flow-through columns inoculated with dissimilatory iron-reducing bacteria (DIRB). We hypothesized that DIRB would enhance the reactivity of this model barrier towards RDX and improve long-term performance. These columns were packed with "aged" iron obtained from columns that had been exposed to TCE, Cr(VI), sulfate, and nitrate mixtures for one year [25]. This iron was used to mimic iron that has been aged in a reactive barrier over time, which could be amenable for reactivation by DIRB [25]. These ZVI columns consistently degraded RDX under different conditions for greater than one year. Our objective was to characterize the RDX transformation end-products, microbial population, and mineral composition of these columns after sustained operation. Currently, there are few reports of bioaugmented ZVI performance over time; this study adds to the understanding of ZVI performance and its application for *in situ* degradation of RDX.

MATERIALS AND METHODS

Three columns (30-cm long, 2.5-cm ID) equipped with lateral sampling ports were packed with a 5-cm layer of soil followed by an 18-cm layer of aged iron and a 7-cm sand layer, as described previously [8,9]. Uncontaminated top soil was obtained from a field in Iowa City, IA. One of the columns was used to determine if soil bacteria colonize the ZVI layer, presumably to feed on cathodic H_2 produced by anaerobic ZVI corrosion. This "indigenous" column served as a baseline to evaluate the benefits of bioaugmentation. The second column was inoculated with the iron-reducing bacterium *Shewanella algae* BrY (10 ml of stock (52.6 mg protein l^{-1}) added at each port). The third column was inoculated with the iron-reducing bacterium *Geobacter metallireducens* GS-15 (10 ml of stock (36 mg protein l^{-1}) added at each port). Whereas *S. algae* can grow autotrophically on hydrogen and is a facultative anaerobe, *G. metallireducens* can only grow under heterotrophic conditions and is an obligate anaerobe [26]. A fourth column was prepared similarly and

influent contained a biocide, Kathon (1 ml l^{-1}, Rohm and Haas, Philadelphia, PA) to prevent growth of bacteria.

RDX, containing a fraction as [^{14}C]-RDX, was fed continuously at 18 mg l^{-1} (10 μCi l^{-1}) at 2.3 ml hr^{-1} (about 0.15 m day^{-1} superficial velocity) with bicarbonate-buffered synthetic groundwater medium (pH = 7.3) [27] using a peristaltic pump. The synthetic groundwater solution was stored in 2-liter Pyrex containers pressurized with N_2/CO_2 (95%/5%) to limit the influx of oxygen to the columns. RDX and [^{14}C]-RDX was synthesized in house using formaldehyde (Sigma Chemical, St. Louis, MO), ammonium hydroxide (Fisher Scientific, Pittsburgh, PA), and fuming nitric acid (Fisher Scientific) [28].

Analysis of aqueous samples was performed using high performance liquid chromatography (HPLC) analysis for RDX and its nitroso derivatives 1,3-dinitro-5-nitroso-1,3,5-triazacyclohexane (MNX), 1,3-dinitroso-5-nitro-1,3,5-triazacyclo hexane (DNX), and 1,3,5-trinitroso-1,3,5-triazacyclohexane (TNX) with a Hewlett Packard 1100 Series HPLC equipped with a 250 x 4.6 mm Supelcosil™ LC-18 column. The mobile phase consisted of deionized water and methanol (4:6, v/v) at a flow rate of 1.0 ml min^{-1}. UV detection was at 240 nm. [^{14}C]-RDX and its ^{14}C-metabolites were analyzed by HPLC using a radioactivity detector (Radiomatic, Series A-500, Packard Instrument Co., Downers Grove, IL). Additional analysis for soluble carbon products was performed using liquid chromatograph/mass spectrometry (LC/MS) with an eluent of acetonitrile and 1 g l^{-1} ammonium acetate in water (4:6, v/v). Ion scanning was performed in negative mode. Some samples were derivatized by addition of 2,4-dinitrophenylhydrazine after initial analyses to inspect for formaldehyde [18].

Confirmation of [^{14}C]-RDX mineralization was investigated by analyzing for ^{14}C-labeled dissolved inorganic carbon (DIC) in the column effluent. Approximately 10 ml of column effluent was collected into sealed 30-ml serum bottles that were capped under a negative pressure. Each serum bottle contained a 1-ml test tube containing 0.75 ml of 0.5 M NaOH to trap any dissolved $^{14}CO_2$. The serum bottles were equilibrated on a reciprocating shaker (90 rpm) at 30 °C for 1.5 days. The partitioning of ^{14}C into each trap was determined by scintillation counting.

After 370 days, samples of solids from the sacrificed columns were collected and stored in an anoxic chamber for biological and mineralogical analyses. Samples of approximately 25 grams of column material were acquired from the soil zone (i.e., 2.5 cm from inlet), ZVI zone (i.e., 5 cm and 14 cm from inlet), and sand zone (i.e., 25 cm from inlet) for each column. Five samples: 1)indigenous column @ 2.5 cm; 2) BrY augmented column @ 2.5 cm; 3) GS-15 augmented column @ 2.5 cm; 4) GS-15 augmented column @ 14 cm; and 5) GS-15 augmented column @ 25 cm were analyzed by Mössbauer for iron speciation. Mössbauer samples were prepared by combining solids with acetone followed by brief agitation to remove residual soil or other organic particles. After the acetone was decanted, the solids were sonicated in

fresh acetone for two hours within an icewater bath. Sonication removed precipitates from the iron filing surface by abrasion against neighbor filings. The acetone-precipitate slurry was decanted and filtered using a 0.45-mm filter. The filtered precipitates were sealed between layers of impermeable tape and analyzed with Mössbauer spectroscopy at room temperature. To characterize microbial populations, Denaturing Gel Gradient Electrophoresis (DGGE) was performed using PCR amplification of DNA obtained from unamended, dried, solid material samples from the columns using a bead-beating method [29] by Microbial Insights (Rockford, TN). Protein was measured using a BioRad (Hercules, CA) Quick Start Bradford assay.

RESULTS AND DISCUSSION

Simulated Barrier Performance

RDX removal was monitored along the length of the columns for over one year. A profile that was taken after 65 days shows complete RDX removal in all columns (Figure 1). RDX removal was also observed in a similarly operated sterile control column [9]; however, no overall loss of soluble ^{14}C was observed in this column (Figure 1a). Comparatively, all biologically active columns showed significant removal of ^{14}C. The detection of dissolved inorganic ^{14}C in the effluent of non-sterile columns, but not in the sterile control column (data not shown) suggests microbial participation in the mineralization of RDX and/or its degradation byproducts. A fraction of inorganic ^{14}C produced should have precipitated as Fe^{2+}/Fe^{3+} carbonate species (as discussed below in **Mineralogical Characterization** with the recovery of siderite) that would not be measured in column effluent.

The RDX concentration profiles after one year of operation showed complete removal of RDX, which shows the sustainability of this ZVI barrier treatment scheme (Figure 2). Significant RDX degradation occurred in soil and in the immediate vicinity of the ZVI layer (i.e, from samples collected at the first sampling port—2.5 cm from both the inlet and the second sampling port at the start of the ZVI layer
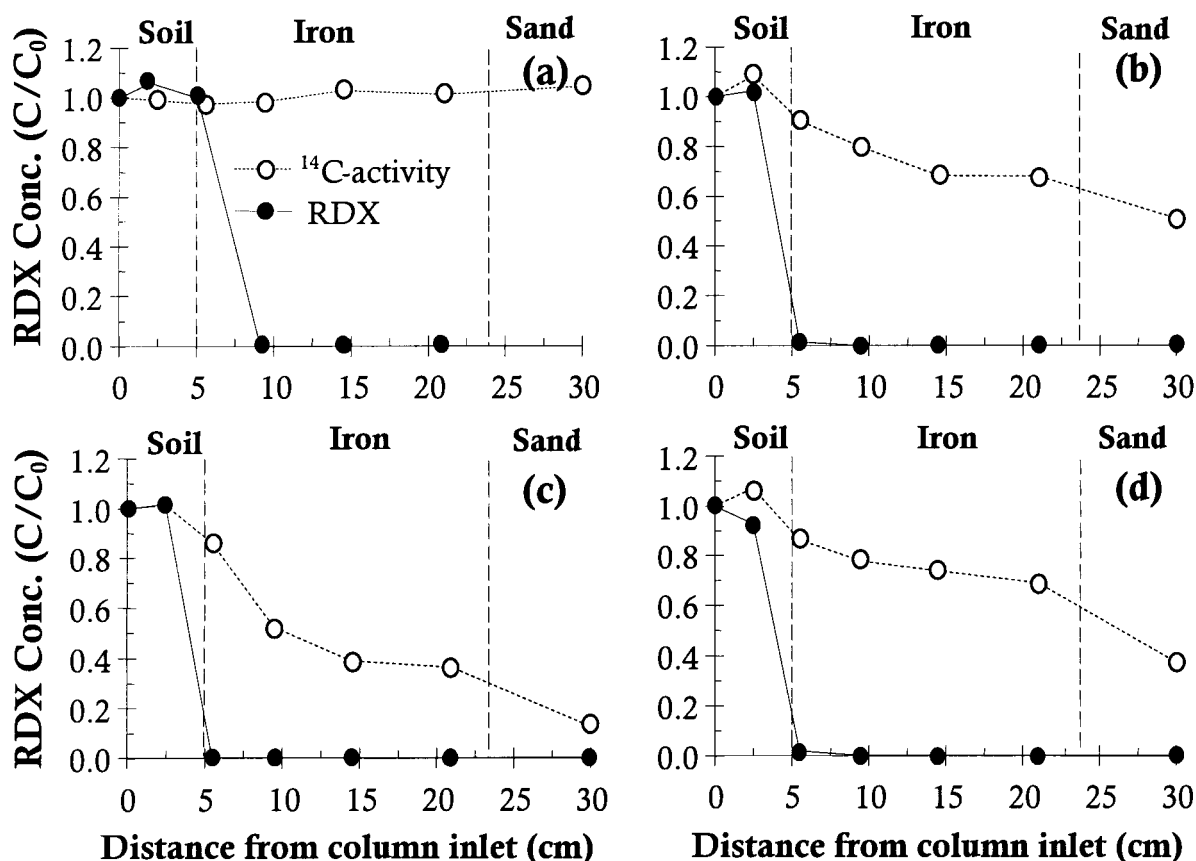

Figure 1.   Change of RDX concentration and soluble ^{14}C activity using rusted ZVI after 65 days of operation for (a) sterile control, (b) indigenously colonized column, (c) *Shewanella algae* BrY-bioaugmented column, and (d) *Geobacter metallireducens* GS-15-bioaugmented column. ($C_0 = 18$ mg l^{-1} and 10 µCi l^{-1}).
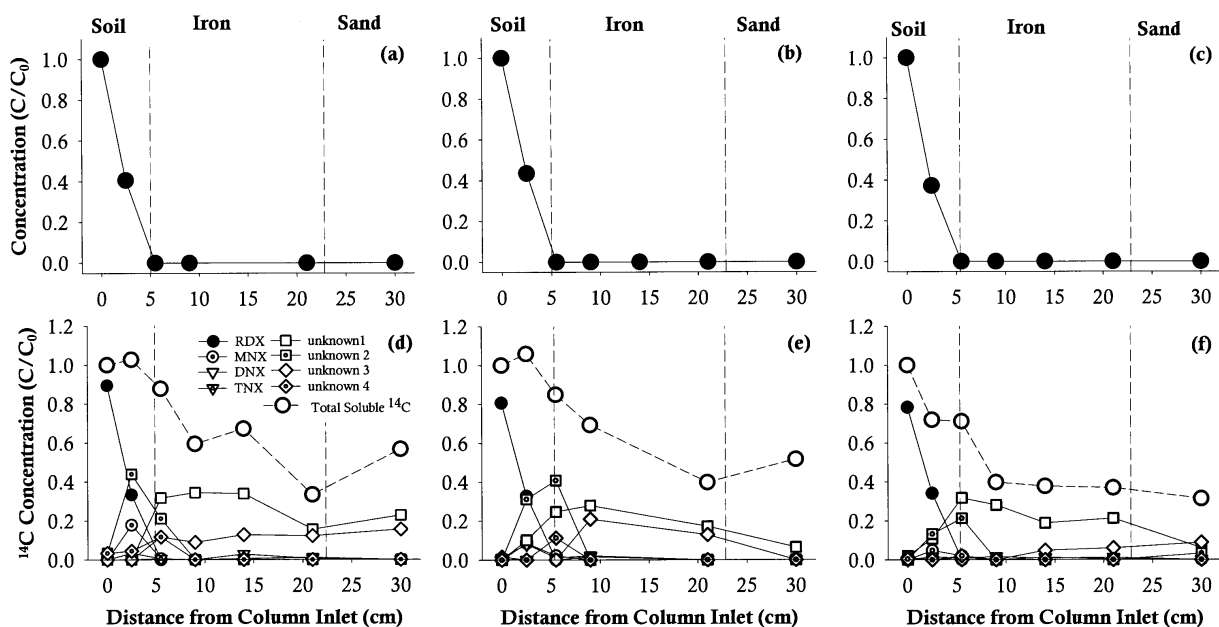
Figure 2. RDX concentration (a-c) and HPLC metabolites and total ^{14}C (d-f) versus for distance (a, d) *G. metallireducens* GS-15 (b, e) *S. algae* BrY, and (c, f) indigenously colonized flow-through columns operated for 370 days. (C_0 = 18 mg l^{-1} and 10 μCi l^{-1}).

—5 cm), possibly due to biostimulation by cathodic hydrogen (Equation i). RDX degradation in this soil zone increased with time (c.f., Figures 1 and 2). Even by day 65, the ZVI zone was primarily challenged with RDX metabolites generated in the soil zone rather than by the parent RDX compound. Thus, the long-term benefits of ZVI treatment appeared not to require the specifically added DIRB (to re-activate the iron surface), but rather required a diverse active microbial community capable of "polishing" (i.e., degrading further) RDX metabolites. This is in agreement with previous studies that reported greater mineralization of [^{14}C]-RDX to $^{14}CO_2$ by combined ZVI-bacterial systems compared to either ZVI or mixed-culture bacteria alone [10].

The ability to degrade RDX was consistent in all columns over a one-year period, and loss of ^{14}C-RDX and total organic ^{14}C generally increased over this period (data not shown). Activity within the soil layer upstream of the ZVI zone (i.e., the significant degradation of RDX observed on day 65 and nearly complete degradation of RDX and the loss of soluble ^{14}C on day 370) suggests considerable biological degradation capacity due to bacterial acclimation and growth. After the soil zone (> 5 cm from inlet), all RDX, all formed nitroso-degradation products MNX, DNX, and TNX, and much of the soluble ^{14}C was removed in all three columns, possibly due to a combination of mineralization (with some $^{14}CO_2$ escaping) and sorption/precipitation of metabolites. This has been shown by previous results which have also shown that radiolabel bound residue can be a significant sink

of dissolved ^{14}C [10]. It is unclear whether removal of ^{14}C observed by day 65 and afterward in bioaugmented columns was due to direct biotransformation by DIRB (e.g. refs. [30] and [31]) or to reductive dissolution of passivating oxides or their transformation to reactive species (such as green rust or Fe^{2+} bound to magnetite, e.g., refs. [17] and [18]) or to the role of other (soil) bacteria.

Due to anaerobic iron corrosion (Equation i), the pH increased in these columns from the inlet (pH ~ 7.2) to the outlet (pH ~9.5) (Figure 3). There was no significant difference, however, in the pH profile of the seeded columns compared to the indigenous column at the end of operation. It is likely that the elevated pH values present near the effluent of these columns resulted in a hostile habitat for bacteria that likely contributed to the low recovery of bacterial DNA from Ports 4 and 5 of the columns (discussed below). We did not investigate improvements to the reactive medium composition or water chemistry (e.g., buffers or nutrients) which could provide better environmental conditions for colonizing bacteria; as such amendments might compromise the maintenance-free attractiveness of a full-scale passive remediation system.

RDX-Degradation Products

The reduction (nitroso) byproducts MNX, DNX, and TNX were observed in the inlet soil zone (0-5 cm) and ZVI zone (5-23 cm). MNX, DNX, plus TNX accounted for up to
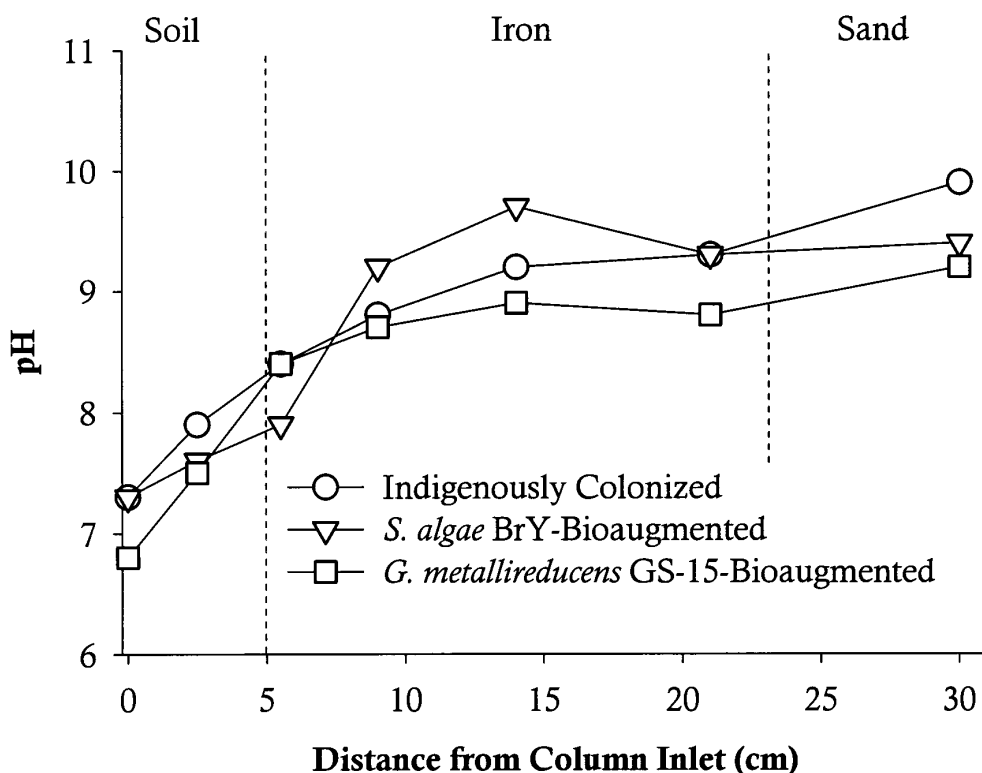
Figure 3.   Change in pH for flow-through ZVI columns after 370 days.

16%, 9%, and 21% of the soluble ^{14}C within the soil layer (2.5 cm from inlet) for the BrY-bioaugmented, GS-15-bioaugmented, and indigenously colonized columns, respectively (Figure 2). These products were degraded in the iron layer, accounting for 5% (BrY column), 4% (GS-15 column), and 0% (indigenously-colonized column) of total ^{14}C at the 8-cm sampling port (within the iron layer). No MNX, DNX or TNX was detectable in the column effluents.

At least four unidentified ^{14}C metabolites were detected in effluent and other samples, using HPLC-radiochemical analysis. Additional analyses using LC/MS suggested that these HPLC-RC peaks (Unknowns 1-4) may have contained multiple compounds each because multiple molecular masses were detected at the different HPLC retention times. Unknown 1 may have contained methylenedinitramine (MDNA) as one constituent that has been previously detected in microbial-iron systems [10]. LC/MS results suggested a mass consistent with MDNA (i.e., MDNA + formate; m/z = 181) was present in Unknown 1; however, this was not conclusive as multiple constituents can yield a mass/charge signal equivalent to that of MDNA by this analysis. No RDX metabolites in unknowns 2, 3, and 4 could be identified within LC / MS spectra. In addition to LC/MS analysis, the presence of formaldehyde was investigated within each unknown as this is a potential RDX degradation product [32]. Our analysis showed that Unknown 2 contained formaldehyde but accounted for less than 10% of ^{14}C in this unknown. The loss

of ^{14}C in analyses conducted over time to identify these unknowns suggested the volatility or solid-matrix sequestration of one or more formed metabolites [10]. We cannot rule out also that the unidentified ^{14}C could exist as small C-compounds with molecular masses less than our instrument detection limit of 50 Da.

Microbial Characterization

It was unclear what roles *Shewanella algae* BrY and *Geobacter metallireducens* GS-15 played in these columns over time. The loss of soluble ^{14}C from solution measured on day 65 compared to the sterile control column suggests that some bacteria (although not necessarily the added DIRB) contributed to the degradation of RDX during the early days of operation. It is possible that both biotransformation and chemical reaction with biogenic iron surfaces could account for some loss of soluble ^{14}C (e.g., through volatilization of $^{14}CO_2$, precipitation of ^{14}C-labeled carbonates, or the formation of bound residue). However, it is unknown to what extent, if any, these bacteria influenced the chemistry or reactivity iron surfaces at that time. Others have shown that DIRB may enhance the reactivity of rusted ZVI through bacterial reduction of Fe^{3+} surfaces [33].

DNA encoding 16S rRNA was recovered from the columns and separated by DGGE at several sampling points (Figure 4). This DGGE profiling suggested a significant
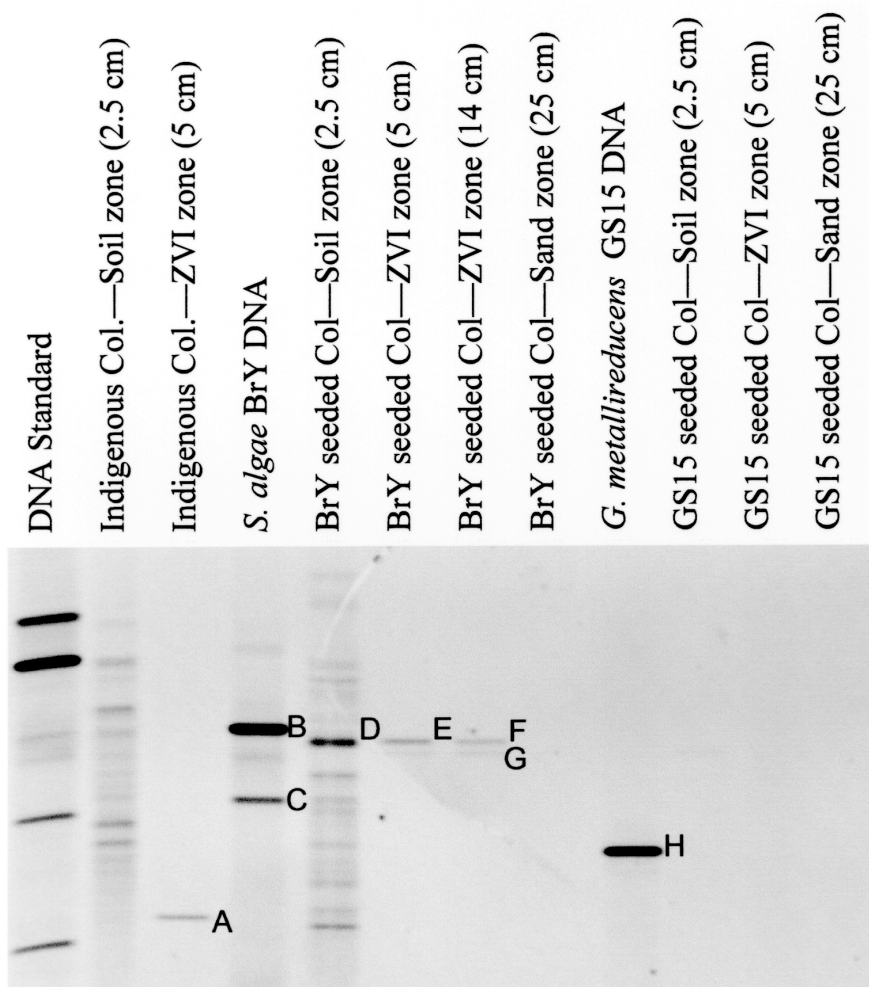
Figure 4. DGGE profile of amplified DNA from a portion of the 16S rRNA gene. Banding patterns and relative intensities of the recovered bands provide a means of comparing the communities. Bacteria must constitute at least 1-2% of the total bacterial community to form a visible band. Labeled bands were excised and sequenced. Results from sequencing can be found in Table 1.

bacterial presence in the inlet soil zone (2.5 cm) that was not observed in the ZVI (5 cm and 14 cm) and sand (25 cm) layers. 16S rRNA sequences were all associated with common soil microbes and showed similarity at the genus level compared to 16S rRNA of bacteria contained in the GenBank Database (Table 1). Sequenced DNA from Band A was less specific, however, many Actinobacteria are soil microbes [34]. The presence of multiple bands (including those identified as Actinobacteria and *Sphingomonas*) suggests the development of a diverse bacterial community with broad biodegradation capabilities. The Actinobacteria class is diverse and contains both aerobic and strictly anaerobic organisms that have been shown to be predominant in soil [35]; some bacteria from this class are capable of autotrophic growth, fermentation, oxidation of Fe^{2+}, reduction of Fe^{3+}, reduction of pesticides, and degradation of RDX [34,36,37]. The presence of *Sphingomonas*, which are aerobic bacteria, suggests that the synthetic groundwater feed solution contained some traces of oxygen, or that O_2 leaked in through the lateral sampling ports.

Comparisons along column depth were generally impossible due to lack of amplification of DNA product. In the indigenous column, the Port 1 sample (i.e., 2.5 cm—soil layer) produced several faint bands, yet none of these aligned well with band A (the only band seen in the port 2 sample). In the BrY-seeded column, bands D, E, and F (seen in port 1, port 2, and port 4 samples respectively), appeared to align and may indicate the presence of the same organism (identified as *Sphingomonas* in band D) in all samples. DNA from bands E and F could not be sequenced due to low recovery. Analyses to identify the bacterial species present in these columns at the end of operation did not show the presence of *Geobacter metallireducens* GS-15 or *Shewanella algae* BrY.

Table 1. Sequence results from bands excised from Figure 4. Identifications are based on DNA sequences in the Ribosomal Database Project (RDP). Similarity indices above .900 are considered excellent, .700-.800 are good, and below .600 are considered to be unique sequences.

Band	Similar genus/ genera	Similarity index	Known electron Donors	Known electron acceptors	Natural environment	GenBank accession number
A	Actinobacteria (class)	0.841	Organics, Fe^{2+}, H_2	O_2, NO_3^-, CO_2	Soil	AJ229243
B	*Shewanella* spp.	0.966	organics	Fe^{3+}, Mn^{4+}, O_2, NO_3^-	Aquatic, aerobe	U91547
C	*Serratia* spp.	1.00	organics	O_2, NO_3^-	Freshwater, soil, rhizosphere, nitrogen fixer, weak human pathogen	AJ297950
D	*Sphingomonas* spp.	0.953	many organics, recalcitrant compounds	O_2	Freshwater & soil aerobe	AF235997
H	*Geobacter* spp.	0.934	short chain alcohols & acids, monaromatics	Fe^{3+}, Mn^{4+}, U^{6+}, NO_3^-	soil, anaerobe	L07834
E,F,G	Unsequenceable					

The inability to recover evidence of DIRB survival is most likely due to sub-optimal (if not adverse) environmental conditions for DIRB growth. We primarily attribute the recovery of few species (including DIRB) from these columns, particularly within and downstream of the ZVI zone, to the relatively hostile environment that the iron layer presents to colonizing bacteria (e.g., high pH and lack of organic substrates). For example, while the optimum pH of most DIRB remains uncharacterized, growth of *Geobacter bremensis*, *G. pelophilus*, and *G. hydroenphilus* is generally restricted to a pH range of 5.5 to 7.0 [26,38-41]. Therefore, the elevated pH resulting from ZVI corrosion (effluent pH 9.2-9.9) likely selected against bacteria with a circumneutral pH requirement. In addition to the fact that no organic carbon was added to support growth of GS-15 (an obligate heterotroph), *Geobacter* species are known to be strict anaerobes [26,41], and it is possible that some oxygen leaked into the columns over the one year operation. Apparently, bacteria that colonize the ZVI layer must be tolerant to high pH and able to grow autotrophically (e.g., on cathodic H_2) or in partnership with autotrophs as hydrogen and inorganic carbon might be the dominant electron donor and carbon source, respectively.

We attribute the lack of total DNA (i.e., not just a lack of *G. metallireducens* GS-15) recovered from all GS-15-column zones to limitations of the sampling and analysis rather than a lack of colonized bacteria, particularly for the soil zone. The similar ^{14}C profiles of all three columns after 375 days including the complete degradation of RDX observed in the soil layer of the GS-15 column (i.e., Figure 3c) support this idea that an active microbial population was present even though none was detected. Furthermore, while it was expected that a high-pH environment would provide a poor growth environment for bacteria, there are many alkaline tolerant microorganisms and the lack of recovered species by this DGGE analysis is also likely to be a result of limitations to the recovery and amplication of bacterial DNA from the collected samples. Iron can limit or prevent recovery of desired products from the utilized protocols including PCR used to amplify DNA for DGGE [42]. It is possible such interferences masked the presence of bacteria present in the tested samples from all columns. Nevertheless, the recovery of Actinobacteria from the ZVI layer of indigenously colonized column and the presence of unsequenceable bands E, F, and G from the ZVI layer of the BrY-augmented column (Table 1) shows that bacteria did colonize the ZVI zone (and not just the upstream soil zone) within the first year of operation. The class Actinobacteria is comprised of bacteria possessing interesting traits that include survival in both low and high pH environments [43-45] and heavy metal environments [35]. While the impact of these microorganisms upon ^{14}C removal is unclear, their presence suggests they occupy a niche only a handful of known bacteria can survive.

It was unclear what roles *Shewanella algae* BrY and

Geobacter metallireducens GS-15 played in these columns over time. The importance of general bacterial activity in these columns was more evident. The loss of soluble ^{14}C from solution measured on day 65 compared to the sterile control column suggests that some bacteria (although not necessarily the added DIRB) contributed to the degradation of RDX during the early days of operation. It is possible that both biotransformation and chemical reaction with biogenic iron surfaces could account for some loss of soluble ^{14}C. However, it is unknown to what extent, if any, these bacteria influenced the chemistry or reactivity iron surfaces at that time. Others have shown that DIRB may enhance the reactivity of rusted ZVI through bacterial reduction of Fe^{3+} surfaces [33].

Mineral Characterization

Siderite ($FeCO_3$) was the dominant iron mineral phase, and was found in similar abundance, in solids samples analyzed from the soil zones just "upstream" of the ZVI zones for each column. Mössbauer spectra from samples collected at three points from the column bioaugmented with *G. metallireducens* GS-15—the soil zone (2.5 cm), the ZVI-zone (14 cm), and the sand zone (22.5 cm)—all show siderite ($FeCO_3$) as the dominant iron species (Figure 5). The absorption
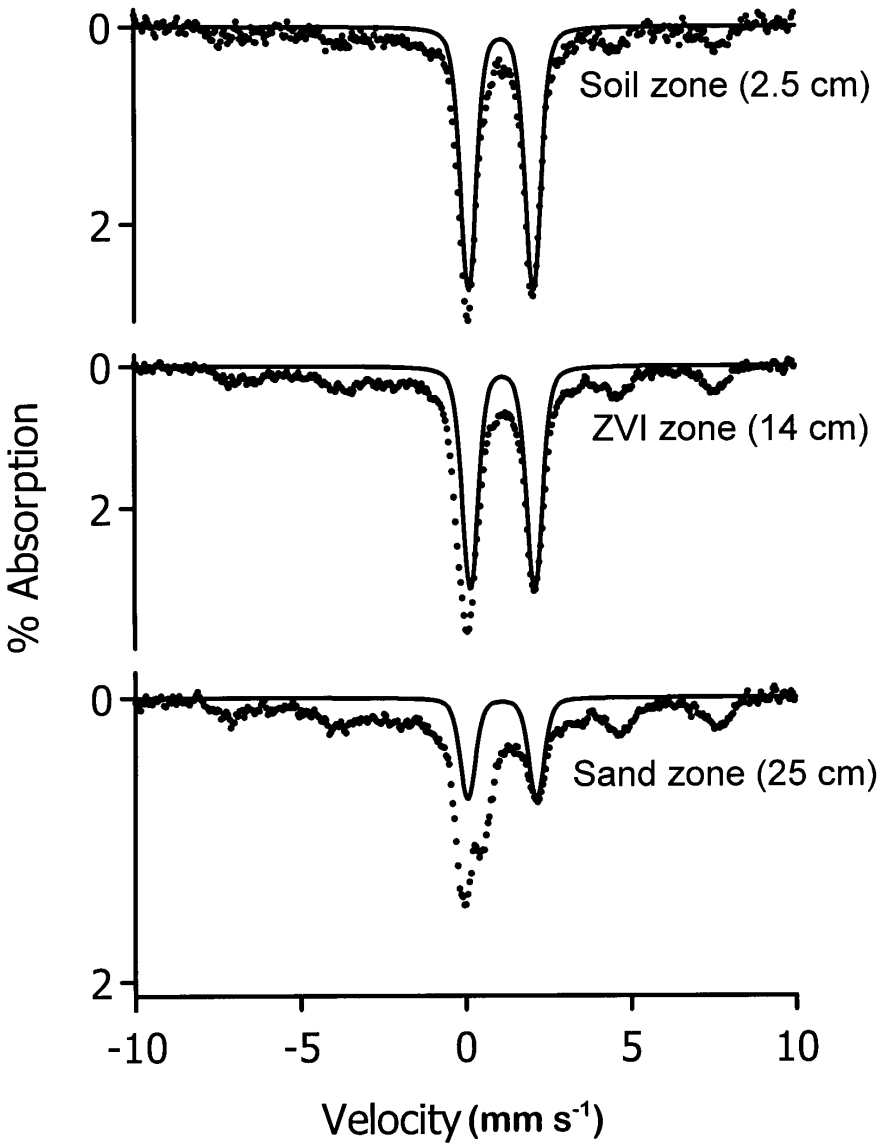

Figure 5. Mössbauer spectra of iron solids sampled along the length of the ZVI column seeded with *Geobacter metallireducens* GS-15. Solid circles represent the extent of 14.4 keV g-ray absorption along the energy spectrum of +/- 10 mm s^{-1} relative to Fe(0) foil. Solid lines represent the portion of the total area within absorption data points that can be accounted as siderite.

pattern for siderite assumes the shape of a two-peaked doublet with a peak spacing of 1.95 mm s^{-1} (the quadrupole splitting) and a midpoint of 1.10 mm s^{-1} (the center shift). A decrease in relative abundance of siderite was observed along the length of the column. In the 2.5 cm sample, the area under the modeled siderite doublet (solid line) represents 67% of the total absorption area under the absorption data points (solid circles); therefore, siderite composes about 67% of the iron minerals within this sample. The samples at 14 cm and 22.5 cm contain 51% and 26% siderite, respectively. The remaining absorption area in each sample indicates the presence of a magnetite (Fe_3O_4)/ maghemite (Fe_2O_3) phase (as evidenced by six small peaks between +/- 8 mm s^{-1}; the two minerals are difficult to distinguish within Mössbauer spectra) and other minor mineral phases, but the relative abundance of each minor phase cannot accurately be determined due to the low total absorption.

Siderite was also detected as the dominant iron mineral in solid samples from the inlet ZVI zone within the naturally colonized column (59% siderite) and the column augmented with *S. algae* BrY (56% siderite) (data not shown). The abundance of siderite among all three biologically-active columns appears similar. Other minerals detected in minor amounts in all tested samples included vivianite ($Fe_3(PO_4)_2$) and a magnetite and/or maghemite phase.

It is likely that the chemical composition of the column feed solution partially governed the identity of precipitated iron minerals. The high concentrations of bicarbonate combined with dissolved Fe^{2+} provided favorable conditions for siderite formation, which diminished downgradient presumably as more bicarbonate becomes sequestered within precipitates. Bacteria may also have influenced mineral precipitation, for the biological reduction of Fe^{3+} by both *Geobacter* and *Shewanella* species has been shown to result in siderite, magnetite, or maghemite formation [46,47].

It is unclear whether iron corrosion products enhanced or hindered the reaction rates of RDX or its degradation products. Magnetite with surface-bound Fe^{2+} as well as green rusts can transform RDX [17,18,48-50]. However, Fe^{2+} bound to the magnetite/maghemite phase probably contributed little to RDX degradation because of its low abundance and its slower rate of reaction with RDX compared to those of ZVI [12,18,51]. No green rust compounds were detected within mineral samples. Siderite has been identified in ZVI-PRBs and in column studies with granular ZVI [19,21,52] but was shown to inhibit the reduction of nitrobenzene in batch reactors with granular ZVI [53]. Whether siderite alone can reduce RDX is unknown; however, a recent report [54] suggested that siderite may have only low reductive transformation activity. The effect of siderite upon the rate and extent of degradation of RDX and its metabolites is poorly understood at present. While several researchers have investigated the influence of medium composition upon iron speciation [47,55-57], it is not known if non-reactive iron species are recycled *in situ* to form more reactive species (over time or downstream). For example, it is possible that

biological respiration of Fe^{3+} produced reactive aqueous, surface bound, or solid Fe^{2+} species, but it is not possible to ascertain the contribution of such reactive phases on the rate of RDX degradation within these columns.

The loss of ^{14}C in the three biologically active columns might be explained by $^{14}CO_2$ sequestration in iron carbonates such as siderite. RDX mineralization to CO_2 (up to 60%) has been observed in other ZVI systems containing non-sterile soil [11,51]. Minor amounts of CO_2 (<10%) were observed when RDX was reacted with Fe^{2+} bound to magnetite [18], but to date CO_2 formation has not been shown in reactors with RDX and ZVI alone. Based on prior reports RDX mineralization to CO_2 appears to be enhanced in biologically active soil and ZVI systems compared to sterile ZVI systems, which is consistent with our enhanced loss of ^{14}C in biologically active ZVI columns compared to complete ^{14}C recovery in the sterile ZVI column. We expect $^{14}CO_2$ formation and subsequent precipitation as iron carbonates to account for some of the ^{14}C loss. Lastly, direct mineralization of RDX to CO_2 by mineral surfaces seems unlikely within these columns. The most abundant carbon-containing RDX metabolite observed in magnetite/Fe^{2+} and green rust systems was formaldehyde (HCHO) [17,18]; while formaldehyde is generally soluble, it is subject to metabolism by bacteria via production of formic acid [3].

CONCLUSIONS

We tested the ability of bioaugmentation to enhance RDX degradation in simulated ZVI permeable reactive barriers using laboratory scale columns containing "aged" iron (previously reacted with oxidized pollutants other than RDX (Cr^{6+}, TCE, and nitrate). Two columns were bioaugmented with DIRB *Shewanella algae* BrY or *Geobacter metallireducens* GS-15 to investigate the potential for enhancing the reactivity of aged iron by DIRB through reductive dissolution of passivating iron oxides or though production of biogenic reactive minerals. A third column was allowed to be colonized by indigenous organisms present in bulk soil. All three columns provided sustained removal of RDX and a general increase over time to ≥50% removal of total soluble ^{14}C metabolites over one year. This loss of soluble ^{14}C suggested that mineralization of [^{14}C]-RDX and its metabolites occurred in these columns (with possible volatilization of $^{14}CO_2$, precipitation of [^{14}C]-carbonates, and formation of radiolabel bound residue). The benefits of bioaugmentation with DIRB appeared transient as RDX degradation and total soluble ^{14}C removal (from [^{14}C]-RDX) was similar in DIRB-bioaugmented and the indigenously-colonized column at the end of one year. Additionally, the presence of added *Shewanella algae* BrY and *Geobacter metallireducens* GS-15 could not be confirmed at the end of the experiment. However, removal of soluble [^{14}C]-RDX metabolites was only observed in biologically active columns as compared to a fourth sterile column that degraded RDX but did not reduce soluble ^{14}C. These results suggest the

benefits of bioaugmentation with specific bacterial strains may be most prevalent early in the design life of ZVI barriers to prevent breakthrough of contaminants by quickly establishing a degradation zone for removal of oxidized pollutants.

While RDX was predominantly removed within the soil layer, a significant fraction of soluble ^{14}C was removed in all columns within the ZVI layer. This demonstrated the efficacy of the combined biological-ZVI permeable barrier treatment strategy we investigated. The ^{14}C removal observed in the bioaugmented columns at 65 days and in all columns after one year suggested the growth and acclimation of bacteria capable of contributing to RDX and RDX-byproduct transformation and mineralization. Enhanced performance associated with the presence of microorganisms may also be due to reductive dissolution of passivating oxides and their biotransformation into minerals that are more reactive towards RDX and remove it below inhibitory levels to byproducts that facilitate microbial participation in the clean-up process. Such abiotic removal processes are important because RDX is toxic to some bacteria at less than 10 mg l^{-1} [7,58]. However, after one year, no highly-reactive oxides such as green rust were identified using Mössbauer analysis.

Microbial analysis using DGGE showed development of a diverse microbial population in these columns. Members of the Actinobacteria class contain known RDX-degrading organisms. The presence of Actinobacteria and other 16S rDNA bands obtained from the ZVI zones of these columns suggests that bacteria are capable of surviving in this harsh, high-pH, high Fe^{2+} environment.

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