

## RDX degradation using an integrated Fe(0)-microbial treatment approach

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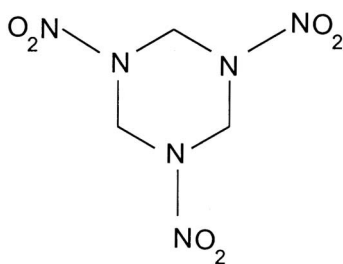
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**Abstract** RDX is a persistent and highly mobile groundwater contaminant that represents a major remediation challenge at numerous munitions manufacturing and load-assembly-package facilities. This work presents proof of concept that permeable reactive iron barriers might be a viable approach to intercept and degrade RDX plumes. Specifically, RDX was rapidly reduced in aquifer microcosms amended with Fe(0) powder, and in flow-through columns packed with steel wool. The rate and extent of RDX degradation in microcosms was enhanced by anaerobic bacteria that feed on cathodic hydrogen (i.e., H<sub>2</sub> produced during anaerobic Fe(0) corrosion by water). Apparently, the hydrogenotrophic consortium that exploits Fe(0) corrosion as a metabolic niche participated in the further degradation of heterocyclic intermediates produced by the reaction of RDX with Fe(0). Reductive treatment of RDX with Fe(0) also reduced its toxicity to microorganisms and enhanced its subsequent biodegradability under either anaerobic or anaerobic conditions. Therefore, a combined or sequential Fe(0)-biological treatment approach might improve treatment efficiency.

**Keywords** Aquifer; bioaugmentation; biodegradation; explosives; iron; Microtox; permeable reactive barriers; RDX

### Introduction

Careless disposal of munitions waste has created serious soil and groundwater contamination problems at numerous military sites. In the past, water used to wash production equipment was discarded into unlined trenches or lagoons where contaminants accumulated at very high levels (Garg *et al.*, 1991). RDX, which is one of the most persistent toxic compounds in the subsurface, was often discarded in this manner (Testud *et al.*, 1996). RDX (Figure 1) is classified as a Class C (possible human) carcinogen, and can cause unconsciousness and epileptiform seizures (Harvey *et al.*, 1991). RDX is also used as a rodenticide, and the Surgeon General recommends a 24-h maximum RDX concentration of 0.3 mg l<sup>-1</sup> to protect aquatic life (McLellan *et al.*, 1988). In the U.S., the Office of Drinking Water has set a limit for lifetime exposure to RDX at 0.1 mg l<sup>-1</sup>.



**Figure 1** Structure of RDX (Hexahydro-1,3,5-trinitro-1,3,5-triazine). RDX, which is the most important high explosive used in the U.S. today, is the British code name for Research Department Explosive (Testud *et al.*, 1996)

Removing RDX from contaminated sites is a very difficult task because of its recalcitrance to microbial degradation, low volatility (dimensionless Henry's constant,  $H' = 2 \times 10^{-11}$ ), moderate solubility ( $42 \text{ mg l}^{-1}$ ), and high mobility in aquifers ( $\log K_{ow} = 0.8$ ) (Goronzy *et al.*, 1994). Current practices to treat RDX-contaminated soil include incineration, composting, alkaline hydrolysis/oxidation, and aqueous thermal decomposition (Garg *et al.*, 1991). These *ex situ* approaches, however, are not cost-effective to treat large volumes of RDX-contaminated groundwater. In addition, complete destruction of RDX is not always achieved, giving rise to the possibility that products of equal or greater toxicity may accumulate (e.g., dimethylhydrazine, dimethylnitrosamine, and azoxymethane) (McCormick *et al.*, 1981). Thus, there is a need for a remediation strategy that is easy, cost-effective, less prone to accumulate toxic by-products, and addresses both chemical and microbiological advantages and constraints.

In the last five years, there has been an explosion of activity directed at the use of zero-valent iron (Fe(0)) as a reactive material to remove redox-sensitive contaminants from groundwater. Semipermeable Fe(0) barriers are particularly attractive for *in situ* remediation in that they conserve energy and water, and through long-term low operating and maintenance costs, have the potential to be considerably less costly than conventional cleanup methods. Fe(0) can be placed in the path of a contaminant plume, either in a trench (O'Hannesin and Gillham, 1998), buried as a broad continuous curtain (Blowes *et al.*, 1995), or injected as colloids (Kaplan *et al.*, 1996), to name a few options. Although Fe(0) barriers are mainly used to treat waste chlorinated solvents and hexavalent chromium plumes (Vidic *et al.*, 1996), it has been recently reported that Fe(0) can also chemically reduce RDX in contaminated soil (Singh *et al.*, 1998). Thus, Fe(0) barriers could also be applied to intercept and degrade RDX plumes.

The potential for microorganisms to enhance the efficiency of Fe(0) barriers was recently demonstrated, and one biogeochemical interaction that enhances contaminant removal seems to be cathodic depolarization (Till *et al.*, 1998; Weathers *et al.*, 1997). Specifically, anaerobic Fe(0) corrosion by water produces cathodic hydrogen:



Cathodic hydrogen can serve as electron donor for the biotransformation of a wide variety of reducible contaminants. This suggests that a hydrogen-utilizing microbial consortium could be established around Fe(0) barriers to exploit cathodic depolarization and RDX degradation as metabolic niches. In addition, the nitro groups attached to the RDX ring (Figure 1) attract electrons released during iron corrosion. Such electron transfer might remove the nitro groups from the ring releasing  $\text{NO}_2^-$  (which can be used as a N source by bacteria) and create potentially more biodegradable compounds (Singh *et al.*, 1998). Therefore, RDX reduction by Fe(0) might enhance the participation of microorganisms in the cleanup process.

This paper addresses the potential benefits of an integrated microbial-Fe(0) system to intercept and treat RDX-contaminated groundwater. Emphasis was placed on evaluating if bioaugmentation of Fe(0) barriers could enhance RDX degradation kinetics and reduce the toxicity of degradation products. Experiments were also conducted to determine if pretreatment of RDX with Fe(0) alone would enhance its subsequent biodegradability under either aerobic or anaerobic conditions. In doing so, information was obtained to evaluate the potential for natural attenuation of any RDX byproducts that could break through an Fe(0) barrier, and to assess the feasibility of a sequential Fe(0)-biological treatment approach.

## Methods

### Batch degradation assays

Reactors were prepared using 250-ml amber serum bottles capped with Mininert valves. Bottles were filled with 100 ml of an acetate-enriched anaerobic culture (250 mg l<sup>-1</sup> VSS) and fed RDX at 12 mg l<sup>-1</sup>. Five reactor sets were prepared in triplicate: sterilized cultures (poisoned with 350 mg l<sup>-1</sup> HgCl<sub>2</sub>), sterilized culture plus Fisher Fe(0) powder (10 g, 2.0127 m<sup>2</sup> g<sup>-1</sup>, 325 mesh), viable culture alone, viable anaerobic culture plus H<sub>2</sub> gas (5 ml at 1 atm), and viable culture plus Fe(0) powder. Reactors fed H<sub>2</sub> gas were used as positive controls to determine if the cathodic H<sub>2</sub> (produced during anaerobic corrosion of Fe(0) by water) could serve as an electron donor for microbial reduction of RDX.

### Effect of pre-treatment with Fe(0) on RDX biodegradability

Experiments were also conducted to determine if pre-treatment of RDX with Fe(0) enhances its subsequent biodegradability under either aerobic or anaerobic conditions. This information is relevant to assess the potential for natural attenuation of any RDX metabolites that could break through an Fe(0) barrier, and to assess the feasibility of a sequential chemical-biological treatment approach. To this end, we first determined how the ratio of the biochemical oxygen demand (BOD) to the chemical oxygen demand (COD) changed after treatment with Fe(0). The BOD/COD ratio is commonly an indication of biodegradability, with higher ratios corresponding to more biodegradable compounds (Miller *et al.*, 1996).

The ultimate BOD of Fe(0)-treated and untreated RDX was determined using a HACH BODTrak(tm) instrument. This apparatus is equipped with six bottles. Two of these bottles were fed 420 ml of an RDX solution (30 mg l<sup>-1</sup>), two contained Fe(0)-treated RDX (i.e., a 30 mg l<sup>-1</sup> RDX solution that was filtered after reacting with 100 mg l<sup>-1</sup> Fe(0) powder for 4 days), one contained Fe<sup>0</sup>-treated DI water to control for any oxygen demand exerted by ferrous iron, and the last one was a blank to control for the oxygen demand exerted by the seed. No RDX was present in the samples pre-treated with Fe(0). All bottles were amended with BOD nutrient pillows, and were seeded with 20 ml of primary effluent from the Iowa City North Wastewater Treatment Facility. The bottles were then sealed and incubated on the BODTrak(tm) instrument, which automatically monitored the BOD continually over 11 days. The samples were continually stirred at 20 °C using magnetic stir bars. The initial COD of these samples was also measured using a HACH kit.

### Effect of pre-treatment with Fe(0) on RDX toxicity

The toxicity of untreated versus Fe(0)-treated RDX was compared using a Microtox™ assay. This assay measures the toxicity to the bioluminescent bacterium, *Photobacterium phosphoreum*, and reports the “effective concentration” of a sample that results in a light output reduction of 50% (EC<sub>50</sub>). Higher EC<sub>50</sub> values relate to higher toxicity (Microtox™ Manual, 1992).

Biochemical methane potential of Fe(0)-treated and untreated RDX was also measured to evaluate differences in anaerobic toxicity and the feasibility of anaerobic post-treatment. Methanogenic batch reactors were prepared in 120-ml serum bottles with 25 ml of the acetate-enriched culture. Reactors were fed acetate (2100 mg l<sup>-1</sup>) and either Fe(0)-treated or untreated RDX at 0, 1, 5, 10, or 15 mg l<sup>-1</sup>. Reactors were incubated in a Coy anaerobic box at 25°C for 50 days. Methane production was monitored regularly over this time.

### Analytical methods

RDX and its degradation products were measured using high performance liquid chromatography (HPLC). The equipment used included a Gilson 307 isocratic pump, a Spectra

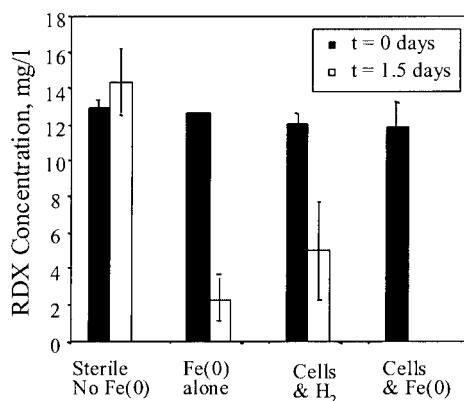
100 UV-Vis Detector (240 nm), an Alltech 570 Autosampler, and an Alltech Econosphere C18 5U column. The signal was processed with Chemstation Software. RDX standards were obtained from Chem Service in West Chester, PA. Standards for RDX metabolites (i.e., 1,3,5-trinitroso-1,3,5-triazacyclohexane (TNX), 1,3-dinitroso-5-nitro-1,3,5-triazacyclohexane (DNX), and 1-nitroso-3,5-triazacyclohexane (MNX)) were obtained from Ronald Spanggord, at SRI International, Menlo Park, CA). Samples were collected from the reactors using gas-tight syringes, and transferred to 1.8-ml amber autosampler vials (Kimble) where they were mixed 50:50 with methanol before being analyzed. The mobile phase for the HPLC analysis was a 50:50 mixture of DI water and methanol. The flow rate used was  $1.0 \text{ ml min}^{-1}$ . Absorbance readings were taken at 240 nm and samples were run for 8 minutes with the RDX peak eluting at 4.6 minutes. Other retention times were 3.6 minutes for TNX, 4.0 minutes for DNX, and 4.4 minutes for MNX. The limit of detection was approximately  $0.1 \text{ mg l}^{-1}$  for each of these compounds.

Methane and hydrogen gas concentrations were also monitored in the microcosms. Headspace samples were collected using a  $100\text{-}\mu\text{L}$  gas tight syringe (VICI Precision Sampling, Inc.) and injected immediately into a gas chromatograph. Methane analysis was conducted with a HP 5890 Series II GC equipped with a flame ionization detector and a DB-WAX capillary column (J&W Scientific).  $\text{H}_2$  was analyzed with a HP 5890 series II GC equipped with a thermal conductivity detector and a Hayesep Q packed column (Alltech Associates).

## Results and discussion

These laboratory experiments suggest that permeable reactive Fe(0) barriers might be a viable approach to intercept and degrade RDX plumes, and that this process can be enhanced by the participation of anaerobic (hydrogen consuming) microorganisms.

Residual RDX concentrations in batch reactors amended with Fe(0), acetate-enriched anaerobic cultures (initially fed also  $15 \text{ ml H}_2$ ), or both, are shown in Figure 2. Following the 1.5-day incubation period, RDX was significantly removed in all reactors prepared with either the mixed culture or Fe(0), but not in Fe(0)-free sterile controls. Residual RDX concentrations in reactors prepared with the mixed culture or Fe(0) alone were between 2 and 8 mg/l. Yet, no RDX was detected in reactors amended with both Fe(0) plus the mixed culture. Interestingly, HPLC analysis (UV detector @254 nm) found heterocyclic metabolites such as TNX, DNX, and MNX (up to about  $2 \text{ mg l}^{-1}$ ) in reactors prepared with viable bacteria or Fe(0) alone, even after 9 days of incubation. Yet, no metabolites were detected in reactors amended with both bacteria plus Fe(0) at 5 = 15 days (limit of detection  $0.1 \text{ mg l}^{-1}$ ),



**Figure 2** RDX removal in reactors with Fe(0) ( $100 \text{ mg l}^{-1}$ ), anaerobic cells ( $250 \text{ mg l}^{-1}$  VSS), or both. Bars depict  $\pm$  one standard deviation from the mean of triplicate reactors

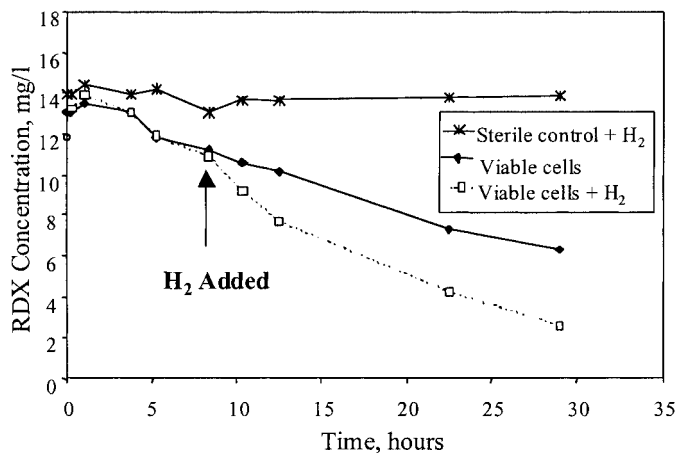
suggesting that a more complete degradation of RDX (beyond ring fission) occurred. This suggests that the combination of Fe(0) and anaerobic bacteria might enhance the treatment efficiency. Specifically, bioaugmenting Fe(0) barriers or reactive Fe(0) filters with acclimated anaerobic cultures might enhance both the rate and extent of RDX transformation.

Batch experiments were also conducted to determine if H<sub>2</sub> production by Fe(0) corrosion (equation 1) could enhance anaerobic RDX biodegradation. Three incubations were prepared with anaerobic cultures as described previously. No Fe(0) was added to preclude confounding effects associated with abiotic RDX reduction. RDX was biodegraded at similar rates in two reactors with live cells during the first 8 h, but not in the sterile control (Figure 3). At this time, H<sub>2</sub> (5 ml) was added to one of the two variable reactors. This stimulated RDX degradation. H<sub>2</sub>, however, did not reduce RDX in sterile controls, indicating that live cells served as electron shuttles to transfer H<sub>2</sub>-derived electrons to RDX. This shows that cathodic H<sub>2</sub> could be used as electron donor to support microbial reduction of RDX.

The ability of Fe(0) treatment to reduce RDX toxicity and increase its subsequent biodegradability was evaluated by comparing BOD to COD ratios and Microtox™ EC<sub>50</sub> values. Pretreatment of RDXs with Fe(0) nearly doubled the COD, indicating that RDX was chemically reduced (Table 1). Untreated RDX did not exert any BOD, reflecting its recalcitrance to (aerobic) microbial degradation. In fact, only a few microorganisms that degrade RDX have been isolated, most of which are fermenters or strict anaerobes (Boopathy *et al.*, 1998; Kitts *et al.*, 1994; Young *et al.*, 1997). No aerobic RDX biodegradation had been reported until recently, a couple of studies found that *Stenotrophomonas maltophilia* PB1 (Binks *et al.*, 1995) and *Rhodococcus* sp. strain DN22 (Coleman *et al.* 1998) degraded RDX using it as the sole N source.

Treatment of RDX with Fe(0) increased the BOD to COD ratio from 0 to 0.31 (Table 1). The resulting ratio is relatively low. This indicates that while the reaction products are potential substrates for microorganisms, they are not readily biodegradable. Yet they are more biodegradable than the parent compound, RDX.

Pre-treatment with Fe(0) also decreased RDX toxicity, as reflected by lower EC<sub>50</sub> values (Table 2). No toxicity was detected in Fe(0)-treated RDX that was subject to subsequent aerobic biodegradation during the BOD assay, which illustrates a potential advantage of sequential Fe(0)-biological treatment approaches.



**Figure 3** Enhanced anaerobic RDX degradation by H<sub>2</sub>

**Table 1** Ultimate BOD and COD of various samples

Sample	Ultimate BOD, mg l <sup>-1</sup> (corrected for seed)	COD, mg l <sup>-1</sup>	BOD/COD ratio
Fe(0)-treated water (control)	0	-0.56 ± 1.8	0
Untreated RDX (30 mg/l)	0	11.1 ± 2.7	0
Fe(0)-treated RDX (30 mg/l)	6.6	21.3 ± 2.9	0.31

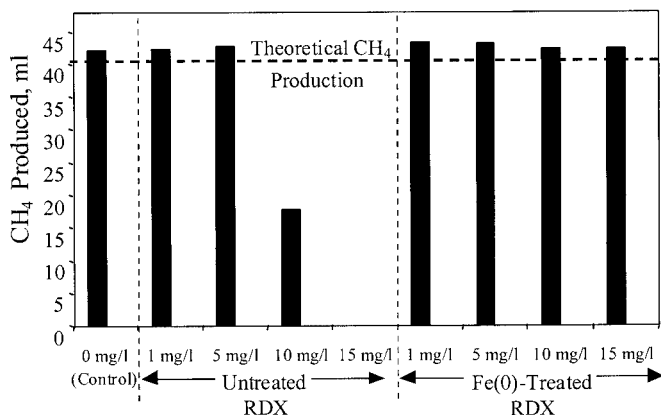
The higher BOD/COD ratio reflects enhanced aerobic biodegradability of RDX after Fe(0) treatment

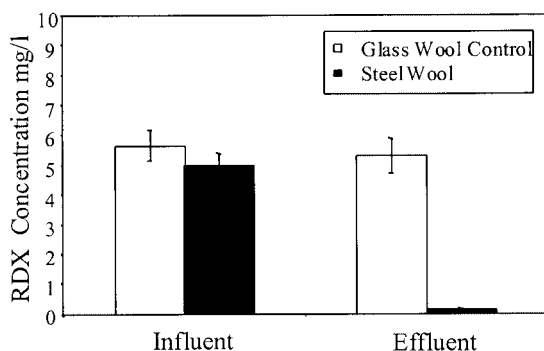
**Table 2** Reduction of toxicity after RDX treatment with Fe(0)

Sample	Microtox(tm) EC <sub>50</sub> (mg l <sup>-1</sup> )
RDX	45.8 ± 5.2
Fe(0)-treated RDX	15.2 ± 4.3
Fe(0)-treated RDX after aerobic biodegradation	No toxicity

Batch anaerobic toxicity assays were also conducted. These assays compared methane production in reactors amended with Fe(0)-treated versus untreated RDX. All reactors were seeded with a methanogenic mixed culture, and were fed acetate (2100 mg l<sup>-1</sup>) plus either Fe(0)-treated or untreated RDX, as described previously. Untreated RDX inhibited methanogenesis when fed at 10 mg l<sup>-1</sup>, and no methane was produced when fed at 15 mg l<sup>-1</sup> (Figure 4). In contrast, no inhibition was observed with 15 mg l<sup>-1</sup> RDX that had been pre-treated with Fe(0). Thus, contacting RDX with Fe(0) increased the feasibility of anaerobic post-treatment. Interestingly, RDX was degraded in the reactors that did not produce methane. For example, the residual RDX concentration was 3 mg l<sup>-1</sup> in the reactor fed 15 mg l<sup>-1</sup>. Apparently, microbial reduction of RDX resulted in a buildup of metabolites that inhibited methanogenesis. This corroborates the finding of other researchers, who reported the inhibitory potential of RDX metabolites (McCormick *et al.*, 1981).

Continuous flow columns (2.5-cm diameter × 26.5-cm long) were also used to evaluate the potential for Fe(0) to remove RDX in a flow-through system mimicking a permeable reactive barrier or filter. One column was packed with a 8 g of steel wool, as described elsewhere (Till *et al.*, 1998). The second (control) column was packed with glass wool. Both columns were fed in an upflow mode at a Darcy velocity of 1 ft day<sup>-1</sup>, using Harvard syringe pumps. The steel wool column (hydraulic retention time = 0.75 d) removed more

**Figure 4** Anaerobic toxicity assays. Pre-treatment with Fe(0) decreased RDX toxicity to negligible compared to that from the added 2100 mg l<sup>-1</sup> acetate



**Figure 5** RDX removal in flow through columns packed with steel wool. Bars represent  $\pm$  one standard deviation of samples taken over a 15-day period

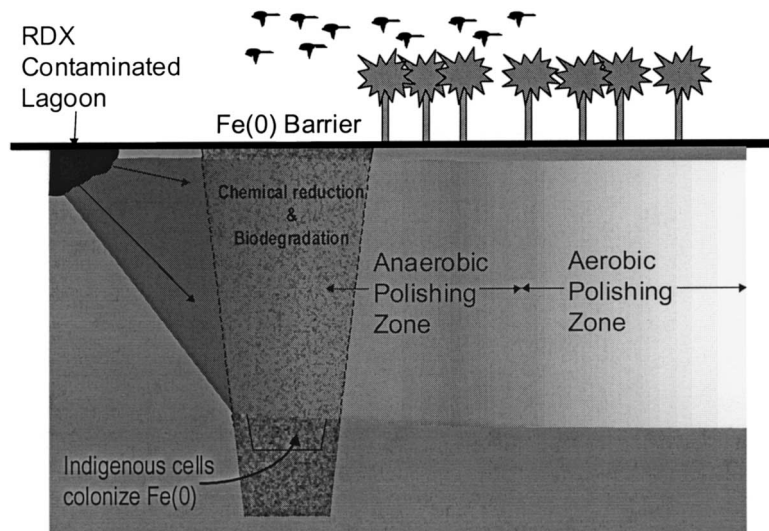
than 98% of the influent RDX (Figure 5). In contrast, little RDX removal was observed in the control column.

In summary, various forms of Fe(0) can rapidly reduce RDX. Furthermore, an Fe(0)-based bioremediation treatment system may offer significant advantages over approaches where either process is used alone. Specifically, bioaugmentation of Fe(0) barriers with pre-acclimated anaerobic bacteria may enhance the treatment of RDX plumes by increasing both the rate and extent of transformation and by yielding a more favorable product distribution when compared to treatment with Fe(0) alone or anaerobic bacteria alone. It is also possible that an indigenous hydrogenotrophic consortium may colonize Fe(0) barriers to exploit cathodic depolarization and RDX degradation as metabolic niches.

The benefits of combining Fe(0) with microorganisms is hypothesized to be due to several factors. Fe(0) corrosion rapidly induces anoxic conditions that favor RDX biotransformations. The production of cathodic (water-derived) hydrogen by Fe(0) corrosion increases the availability of an excellent primary substrate to support microbial reduction of RDX and the further degradation of some dead-end products that accumulate during abiotic reduction by Fe(0). Hydrogenotrophs could also remove the passivating cathodic H<sub>2</sub> layer from the Fe(0) surface, which could enhance the reactivity of Fe(0). Microbial consumption of H<sub>2</sub> gas bubbles could also enhance the permeability of the barrier to offset any decrease in permeability resulting from microbial growth. In addition, iron-reducing bacteria could enhance treatment efficiency by depassivating the iron surface through reductive dissolution of Fe(III) oxides. Alternatively, the reduction of RDX by Fe(0) was shown to reduce its toxicity to microorganisms and to enhance its subsequent biodegradability. Thus, a sequential scheme where a pre-treatment Fe(0) barrier is followed by down-gradient bioremediation of any products that break through might also be a practical approach to control RDX plumes (Figure 6).

## Conclusions

Permeable reactive Fe(0) barriers might be a viable alternative to intercept and degrade RDX plumes. This treatment approach can be enhanced by the concurrent participation of anaerobic microorganisms that feed on cathodic hydrogen and increase the rate and extent of RDX biotransformation. Such microorganisms could enhance both the rate and extent of RDX degradation. In addition, reductive treatment of RDX with Fe(0) reduces its toxicity and enhances its subsequent biodegradability under either aerobic or anaerobic conditions. Consequently, a sequential scheme where a (thinner) pre-treatment Fe(0) barrier is followed by down-gradient natural attenuation and passive bioremediation of any



**Figure 6** Integrated Fe(0)-bioremediation system to intercept and degrade RDX plumes

products that break through might also be a viable alternative to treat RDX-contaminated groundwater.

### Acknowledgements

We thank the Center for Health Effects of Environmental Contamination at the University of Iowa and SEDP for their financial support. Special thanks to Gene Parkin, Michelle Scherer, and Jerry Schnoor for useful discussions, and to Craig Just and Andrew Hawkins for their laboratory assistance.

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