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RDX (Hexahydro-1,3,5-trinitro-1,3,5-triazine) Biodegradation in Aquifer Sediments under Manganese-Reducing Conditions

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ABSTRACT A shallow, RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine)–contaminated aquifer at Naval Submarine Base Bangor has been characterized as predominantly manganese-reducing, anoxic with local pockets of oxic conditions. The potential contribution of microbial RDX degradation to localized decreases observed in aquifer RDX concentrations was assessed in sediment microcosms amended with [U-^{14}C] RDX. Greater than 85% mineralization of ^{14}C-RDX to $^{14}CO_2$ was observed in aquifer sediment microcosms under native, manganese-reducing, anoxic conditions. Significant increases in the mineralization of ^{14}C-RDX to $^{14}CO_2$ were observed in anoxic microcosms under NO_3-amended or Mn(IV)-amended conditions. No evidence of ^{14}C-RDX biodegradation was observed under oxic conditions. These results indicate that microbial degradation of RDX may contribute to natural attenuation of RDX in manganese-reducing aquifer systems.

KEYWORDS anoxic, aquifer, biodegradation, metal, reduction, microbial degradation, RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), sediment microcosms

INTRODUCTION

A fundamental obstacle to the broad application of biodegradation-based strategies for RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) remediation in soil and groundwater systems is the lack of information on environmental factors affecting RDX biodegradation and metabolism despite the fact that the cyclic nitroamines RDX and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) are the most common military explosives in use today (Hawari, 2000). In situ redox conditions have long been identified as a significant factor effecting the efficiency of RDX biodegradation (Hawari, 2000).

RDX biodegradation has been demonstrated under aerobic and anaerobic conditions (Hawari, 2000; Kitts et al., 1994; McCormick et al., 1981; Price et al., 2001; Young et al., 1997). Possible microbial, metabolic functions of RDX include (1) electron donor for metabolic and respiratory redox reactions, (2) carbon substrate for growth and metabolism, or (3) nitrogen substrate for

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growth and metabolism (Hawari, 2000). Microorganisms are known to degrade RDX as a nitrogen source under nitrogen-limited, oxic conditions (Binks et al., 1995; Fournier et al., 2002; Jones et al., 1995; Osmon and Klausmeier, 1972; Seth-Smith et al., 2002; Sheremata and Hawari, 2000), but the relevance of aerobic RDX biodegradation under non-nitrogen-limiting conditions remains controversial (Hawari, 2000). The potential for anaerobic, microbial degradation of RDX has been demonstrated under nitrate-reducing (Freedman and Sutherland, 1998; AEM), sulfate-reducing (Boopathy et al., 1998a, 1998b), and methanogenic conditions (Boopathy et al., 1998b). To the authors' knowledge, RDX biodegradation under predominantly metals-(Mn(IV) or Fe(III)) reducing conditions has not been investigated previously. The potential for RDX biodegradation in aquifer sediments under metal-reducing conditions was assessed in this study.

METHODS

Chemicals

RDX biodegradation was investigated using uniformly labeled [U-^{14}C] RDX (Perkin Elmer Life Sciences, Boston, MA). The radiochemical composition of the [U-^{14}C] RDX stock (20.0 mCi/mmol specific activity) was evaluated in our laboratory by radiometric detection HPLC (high performance liquid chromatography) (HPLC/RD) and found to be 97.9 $\pm$ 0.3% as ^{14}C-RDX and 2.3% $\pm$ 0.4% as an unidentified polar peak. $H^{14}CO_3^-$ (Sigma Biochemicals, St. Louis, MO) and $^{14}CH_4$ (New England Nuclear, Boston, MA) were used as ^{14}C calibration standards (radiochemical purities >98%). Authentic standards for RDX and the nitroso degradation products (MNX, DNX, and TNX) were obtained from Ultra Scientific (North Kingstown, RI) and Dr. R. J. Spanggord (SRI International, Menlo Park, CA), respectively.

Study Site

The study site is located on the Naval Submarine Base (NSB) Bangor, Washington. The site is characterized by unconsolidated glacial and interglacial deposits over 450 m in thickness and underlain by bedrock (Easterbrook and Anderson, 1968; Jones, 1998). The RDX-contaminated, shallow groundwater-system consists of a 6 m thick seasonal, high-permeability aquifer perched atop a low-permeability glacial till, and an underlying, 60 m thick moderate- to low-permeability unit of silty, sandy, glaciolacustrine ice-contact deposits. The water table in the deeper unit, referred to locally as the shallow aquifer, is approximately 25 m below ground surface (Kahle, 1998). Dissolved RDX concentrations up to 1000 μg/L and 660 μg/L have been observed in the perched and shallow aquifers, respectively. In situ redox conditions, assessed at the time of sediment collection on the basis of geochemical redox indicators (Chapelle et al., 1995), indicated that the shallow aquifer at NSB Bangor was characterized by predominantly anoxic (dissolved [O_2] <0.5 mg/L), Mn(IV)-reducing conditions with local pockets of oxic (dissolved [O_2] $\geq$ 0.5 mg/L) conditions occurring near active pump-and-treat extraction wells (Dinicola, unpublished results). Aquifer sediment was collected with a flame-sterilized, split-spoon sampler from 30 m below ground surface in a location characterized by anoxic, manganese-reducing conditions and RDX concentrations between 20 and 110 μg/L.

Microcosm Studies

Bed sediment microcosms were prepared as described previously (Bradley et al., 1998) in 20-mL serum vials with 10 mL (15 g wet weight) of saturated sediment, 3 mL of sterile, anoxic water, and an atmosphere of air (oxic treatment) or helium (anoxic treatments). Anoxic KNO_3 solution was added to NO_3-amended, anoxic treatments to yield initial dissolved NO_3^- concentrations of 7.4 $\pm$ 0.1 mM. Mn(IV)- and Fe(III)-amended, anoxic treatments were prepared by adding 0.3 mL (approximately 70 mg) of an anoxic, sterile slurry of poorly crystalline MnO_2 (Lovley and Phillips, 1988a, 1988b) or $Fe(OH)_3$ (Lovley and Phillips, 1988b), respectively, as described (Bradley et al., 1998). Additional NO_3-amended, anoxic and unamended, anoxic treatments were prepared as above and amended with sterile sodium acetate (4 mM initial concentration). Triplicate experimental microcosms were prepared for each sediment treatment. Duplicate killed control microcosms and a single sediment-free control microcosm were prepared for each sediment treatment and autoclaved twice for 1 h at 15 PSI and 121°C. All microcosms were amended with 0.16 μCi of [U-^{14}C] RDX to yield an approximate, initial dissolved RDX concentration of

1.5 ± 0.2 μM (330 μg/L). Microcosms were incubated in the dark at 20°C for 296 days.

Chemical Analyses

Headspace concentrations of N_2, N_2O, CH_4, $^{14}CH_4$, CO_2, and $^{14}CO_2$ were monitored by radiometric/thermal conductivity detection gas chromatography as described in detail (Bradley et al., 2001). Dissolved phase concentrations of $^{14}CO_2$ and $^{14}CH_4$ were estimated based on experimentally determined Henry's partition coefficients, as described (Bradley et al., 2001). The radioactivity associated with ^{14}C-RDX and its potential nitroso degradation products (^{14}C-MNX, ^{14}C-DNX, and ^{14}C-TNX) and short-chain organic acid products were assessed using HPLC/RD as described by Oh et al. (2001) and Bradley and Chapelle (2000), respectively. The radioactivities associated with adsorbed and gaseous phases were estimated based on adsorption and Henry's partition coefficients that were determined experimentally as described (Bradley et al., 2001). The short-term (72-h) adsorption coefficient (K_{ads} in mL/g) for ^{14}C-RDX under these conditions was 0.13 ± 0.01. The dimensionless Henry's partition coefficient for ^{14}C-RDX was insignificant. Initial and final concentrations of NO_3^- and SO_4^{2-} were determined by ion chromatography. Initial and final concentrations of dissolved Mn(II) (Table 1) and Fe(II) were determined colorimetrically according manufacturers instructions (Hach Chemical Co.).

TABLE 1 **Production of Dissolved Mn(II) (nmoles) in Experimental (EXP) and Autoclaved Control (AC) Microcosms Containing Bangor Sediment and in Sediment-Free Control (SFC) Microcosms After 296 Days**[a]

Amendment	Carbon addition	Dissolved Mn(II) Production (nmoles)		
		EXP	AC	SFC
O_2	—	4 ± 4	2 ± 2	NS[b]
NO_3	—	20 ± 16	NS	NS
	Acetate	11 ± 9	NS	NS
Mn(IV)	—	560 ± 47	34 ± 25	NS
Fe(III)	—	69 ± 5	10 ± 1	NS
Unamended	—	45 ± 7	13 ± 4	NS
	Acetate	207 ± 6	49 ± 17	NS

[a]Calculated as the difference in dissolved Mn(II) content measured in microcosms sacrificed at the beginning and end of the study. Experimental data for each treatment are means $\pm$ *SD* for triplicate microcosms. Control data are from duplicate autoclaved microcosms (means $\pm$ *SD*) and a single-sediment-free microcosm.

[b]NS: Differences between initial and final dissolved Mn(II) contents not significant ($p < .05$; Kruskal-Wallis one-way analysis of variance on ranks).

Quantitative PCR Analyses

DNA was extracted from 1-g (fresh weight) samples of fresh Bangor sediment (triplicate samples) prior to microcosm preparation and from 1-g samples of unamended, anoxic microcosm sediment after the 296-day incubation period using the UltraClean Soil DNA Kit (MO BIO Laboratories, Inc., Solana Beach, CA). Quantitative polymerase chain reaction (Q-PCR) targeted at *Geobacteraceae* 16S rDNA was carried out using the *Geobacteraceae*-specific primer pair 5′-AGG AAG CAC CGG CTA ACT CC-3′ (GEO494F) and 5′-TAC CCG CRA CAC CTA GT-3′ (GEO825R), which yields an approximate 330-bp amplicon (Holmes et al., 2002). SYBR Green Q-PCR was conducted as described previously (Chapelle et al., 2002) on a Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with *Geobacter sulfureducens* DNA as calibrant. Thermocycler conditions were 10 min activation at 95°C; followed by 50 cycles of 30 s at 95°C, 60 s at 55°C, 60 s at 72°C, and a final extension step of 10 min at 72°C.

RESULTS AND DISCUSSION

Oxic Conditions

No significant ^{14}C-RDX biodegradation was observed in aquifer microcosms under oxic conditions (Table 2, Figure 1). Biological O_2 consumption was apparent, but no indication of NO_3 reduction, Mn(IV)-reduction, Fe(III)-reduction, SO_4 reduction, or methanogenesis was observed. Only ^{14}C-RDX was detected in experimental and control microcosms after 296 days of incubation (Table 2), but the recovery of ^{14}C-RDX was approximately 70% in experimental and autoclaved sediment control microcosms, compared with complete recovery in sediment-free controls. This discrepancy between sediment microcosms and sediment-free controls was attributed to significant, long-term adsorption of ^{14}C-RDX not accounted for by the relatively short-term (3-day) adsorption coefficient estimated in this study. The presence of 30 ± 6 μM of dissolved NO_3^- in oxic, aquifer sediment microcosms and the accompanying lack of RDX biodegradation are consistent with previous

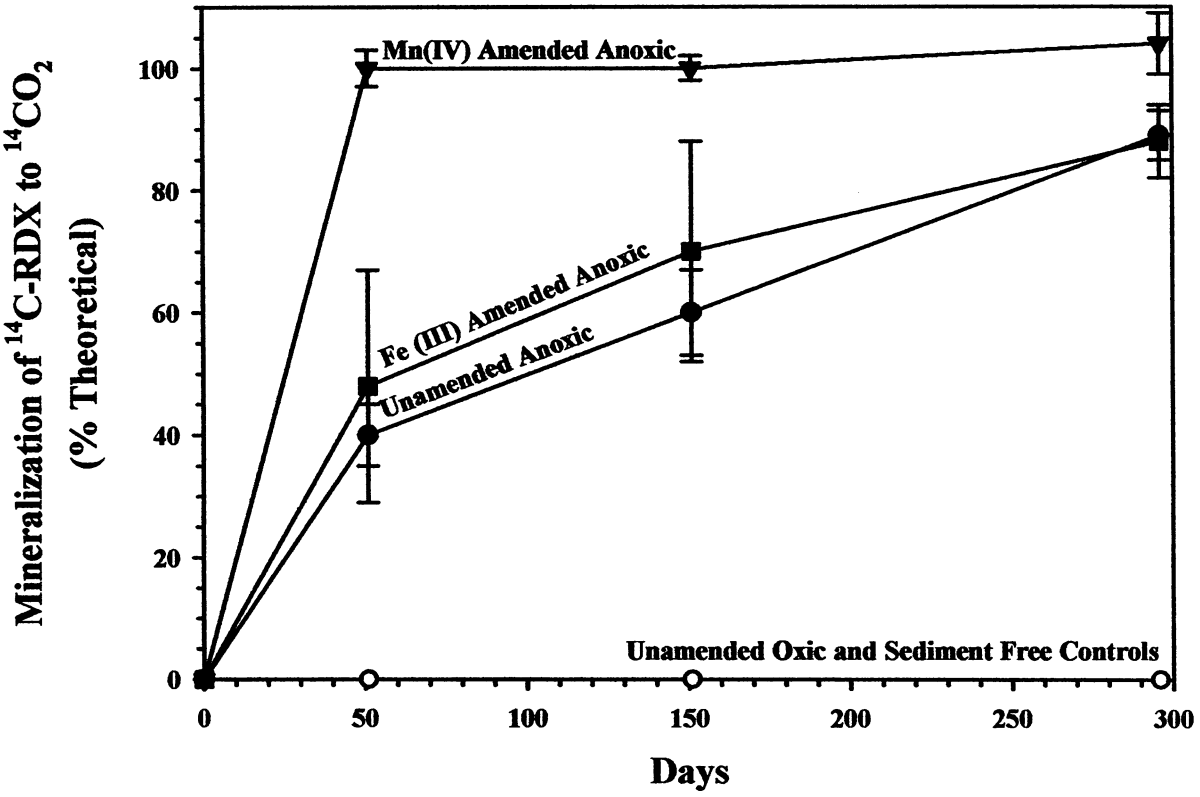

FIGURE 1 **Effect of oxygen, Mn(IV), and Fe(III) amendment on mineralization of ^{14}C-RDX to $^{14}CO_2$ in NSB Bangor sediment microcosms and sediment-free control microcosms. Unamended anoxic microcosms were Mn(IV)-reducing.**

reports suggesting that RDX biodegradation under oxic conditions is restricted to meeting nitrogen requirements in nitrogen-limited environments (Binks et al., 1995; Fournier et al., 2002; Hawari, 2000; Jones et al., 1995; Osmon and Klausmeier, 1972; Seth-Smith et al., 2002; Sheremata and Hawari, 2000). Based on these results, biodegradation is not expected to contribute significantly to the natural attenuation of

TABLE 2 **Final Percentage Distribution of ^{14}C Radioactivity in Experimental (EXP) and Autoclaved Control (AC) Microcosms Containing Bangor Sediment and in Sediment-Free Control (SFC) Microcosms After 296 Days[a]**

Amendment	Carbon addition	EXP			AC	SFC
		$^{14}CO_2$	^{14}C-RDX	^{14}C-Total[b]	^{14}C-RDX[c]	^{14}C-RDX[d]
O_2	—	ND[e]	72 ± 11	72 ± 11	69 ± 3	108
NO_3	—	100 ± 4	ND	100 ± 4	97 ± 4	100
	Acetate	90 ± 1	16 ± 22	106 ± 23	—	98
Mn(IV)	—	104 ± 5	ND	104 ± 5	ND	107
Fe(III)	—	88 ± 6	ND	88 ± 6	98 ± 4	101
Unamended[f]	—	89 ± 4	ND	89 ± 4	98 ± 7	110
	Acetate	95 ± 2	ND	95 ± 2	—	96

[a]Recoveries are given as the percentage of radioactivity initially added to the sediment microcosms as ^{14}C-RDX. Experimental data for each treatment are means ± *SD* for triplicate microcosms. Control data are from duplicate autoclaved microcosms (means ± *SD*) and a single sediment-free microcosm. Radiolabeled C1–C4 organic acids were analyzed at the completion of this study but not detected (MDL was 2%). Radiolabeled MNX, DNX, and TNX were only detected in trace amounts (<3%) in experimental microcosms sacrificed at the beginning of the study.

[b]Total ^{14}C recovery as $^{14}CO_2$ and ^{14}C-RDX in microcosms.

[c]Mn(IV)-amended, autoclaved control microcosms exhibited complete mineralization to $^{14}CO_2$. Mineralization was not detected (MDL of 2%) until after 151 days incubation. No autoclaved control data for acetate amended microcosms.

[d]Only ^{14}C-RDX was detected in sediment-free control microcosms.

[e]Not detected. The MDLs were 2% and 4% for $^{14}CO_2$ and ^{14}C-RDX, respectively.

[f]Unamended treatment. The unamended treatment was Mn-reducing.

RDX in oxic portions of the shallow aquifer at NSB Bangor.

Anoxic Conditions

The predominant terminal electron–accepting process in unamended, anoxic microcosms appeared to be metals reduction (Mn(IV)-reduction and potentially Fe(III)-reduction). The lack of significant dissolved O_2 ($[O_2] < 2\ \mu M$), NO_3 ($[NO_3^-] < 1.0\ \mu M$), and SO_4 ($[SO_4^{2-}] < 20\ \mu M$); the lack of significant production of CH_4 (not detected, $[CH_4] < 1\ \mu mol/L$ headspace), dissolved sulfide (not detected, $[HS^-] < 0.2\ \mu M$), and dissolved Fe(II) (1 ± 2 nmoles Fe(II) produced); and the significant accumulation of dissolved Mn(II) (45 ± 7 nmoles; Table 1) indicated that Mn(IV)-reduction was the predominant terminal electron–accepting process under unamended, anoxic conditions. Because others (Lovley and Phillips, 1988a) have shown that Fe(III)-reduction can proceed under Mn(IV)-reducing conditions without significant accumulation of dissolved Fe(II), the possibility of coincident Fe(III)-reduction could not be ruled out. Consistent with the apparent importance of metal, reduction in these sediments under anoxic conditions, *Geobacteraceae*-targeted 16S rDNA Q-PCR analyses indicated that this population of well-known Mn(IV)/Fe(III) reducers increased from $0.24\% \pm 0.03\%$ to $3.00\% \pm 0.91\%$ of the total microcosm bacterial population during the study.

Complete biological removal of ^{14}C-RDX was observed in aquifer microcosms under unamended anoxic conditions (Table 2, Figure 1). Mineralization of ^{14}C-RDX to $^{14}CO_2$ was apparent within 51 days (Figure 1) and $^{14}CO_2$ was the sole ^{14}C-compound detected in experimental microcosms after 296 days (Table 2). The lack of ^{14}C-RDX depletion in autoclaved and sediment-free control microcosms indicated that the ^{14}C-RDX degradation observed under experimental conditions was biologically mediated. To our knowledge, these results constitute the first demonstration of RDX biodegradation in predominantly metals-reducing aquifer sediments. These results suggest that in situ biodegradation of RDX may contribute substantially to natural attenuation of RDX within anoxic, Mn(IV)-reducing portions of the shallow aquifer at NSB Bangor. Moreover, the apparent lack of accumulation of the characteristic RDX reduction products (MNX, DNX, TNX) and the near complete mineralization of

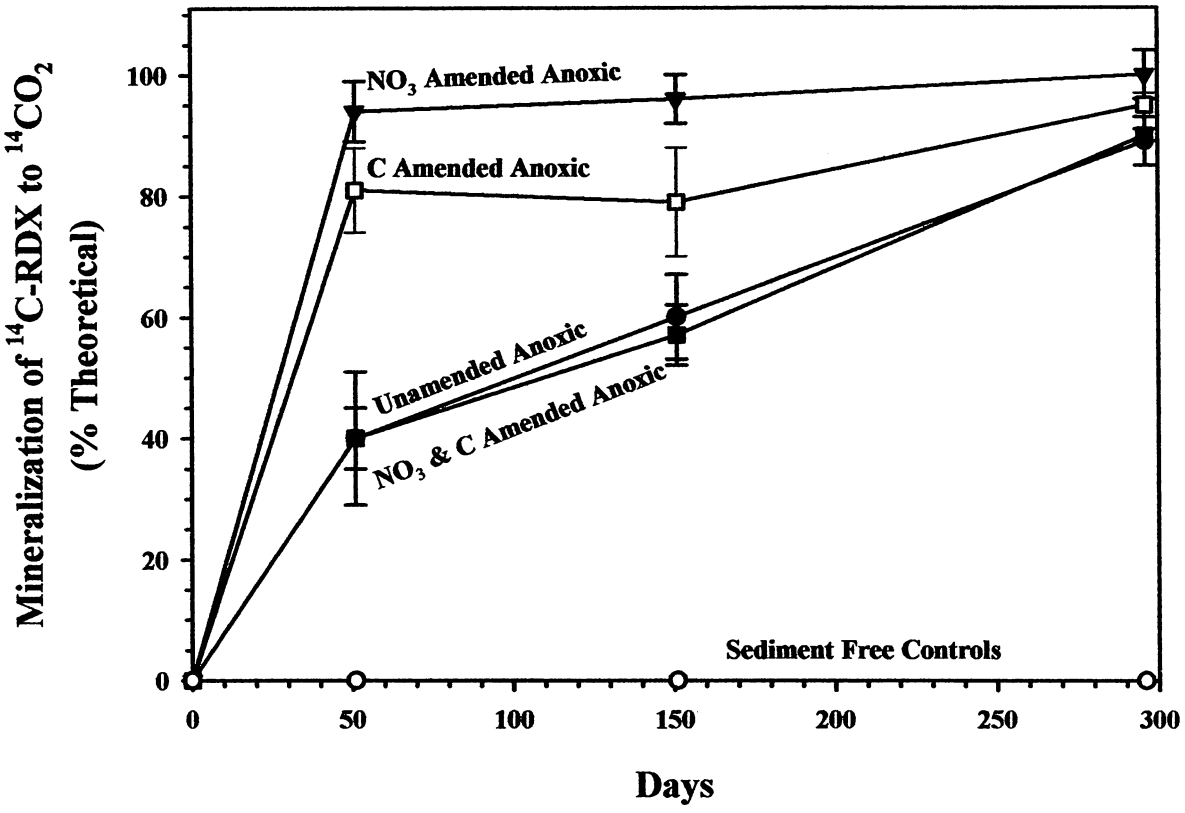

FIGURE 2 Effect of NO_3^- and carbon (C) amendment on mineralization of ^{14}C-RDX to $^{14}CO_2$ in NSB Bangor sediment microcosms and sediment-free control microcosms. Unamended anoxic microcosms were Mn(IV)-reducing.

^{14}C-RDX to a nondiagnostic product, $^{14}CO_2$, indicate that a reliance on the accumulation of diagnostic intermediates as an indicator of in situ RDX biodegradation may be problematic.

Insight into the potential contribution of Fe(III)-reduction to the biodegradation of ^{14}C-RDX under metal reducing conditions was provided in Fe(III)-amended treatments. The lack of significant dissolved O_2, NO_3^-, or SO_4^{2-}, the lack of accumulation of methane, dissolved sulfide, or dissolved Fe(II) and the clear accumulation of dissolved Mn(II) (Table 1) again indicated the predominance of metals-reducing conditions. A 53% increase in the accumulation of dissolved Mn(II) was observed in Fe(III)-amended anoxic microcosms compared with unamended, anoxic microcosms (Table 1). The fact that Fe(III) amendment had no apparent effect on dissolved Mn(II) concentrations in autoclaved control microcosms indicated that the stimulation of Mn(IV)-reduction in Fe(III)-amended, experimental microcosms was attributable to biological activity. This result is consistent with the previous conclusion (Lovley and Phillips, 1988a) that Fe(III)-reduction can stimulate net Mn(IV)-reduction as the result of reoxidation of the product Fe(II) coupled to Mn(IV)-reduction. However, Fe(III) amendment had no significant effect on the final products (Table 2) or the final extent (Table 2, Figure 1) of ^{14}C-RDX biodegradation. These results indicate that RDX biodegradation in anoxic NSB Bangor sediments was not limited by Fe(III) availability and suggest that the mineralization of ^{14}C-RDX observed under anoxic conditions was associated with Mn(IV)-reduction rather than Fe(III)-reduction.

Consistent with this hypothesis, addition of MnO_2 substantially increased the efficiency of ^{14}C-RDX biodegradation under anoxic conditions. The fact that the accumulation of dissolved Mn(II) in Mn(IV)-amended, anoxic microcosms was more than 10× greater than that observed in unamended or Fe(III)-amended, anoxic microcosms (Table 1) indicated that Mn(IV)-reduction in anoxic, NSB Bangor sediments was limited by the bioavailability of reducible Mn. Addition of MnO_2 more than doubled the initial mineralization of ^{14}C-RDX $^{14}CO_2$ (Figure 1). ^{14}C-RDX mineralization was essentially complete within 51 days under Mn(IV)-amended anoxic conditions (Figure 1). No significant decrease in ^{14}C-RDX recovery was observed in sediment-free control microcosms over 296 days (Table 2) and no production of $^{14}CO_2$ was observed in autoclaved control microcosms during the first 151 days of incubation (footnote c, Table 2). However, ^{14}C-RDX mineralization was apparent in the autoclaved control microcosms at 296 days (Table 2) and was attributable to a recovery of biological activity between 151 and 296 days of incubation. This response was unique to Mn(IV)-amended, autoclaved controls. These results demonstrate that effective microbial degradation and mineralization of RDX can occur under Mn(IV)-reducing, anoxic conditions. The fact that ^{14}C-RDX biodegradation was limited by the bioavailability of Mn(IV) but not Fe(III) is consistent with the hypothesis that RDX biodegradation under metals-reducing conditions was coupled to Mn(IV)-reduction.

The effect of NO_3 amendment on ^{14}C-RDX biodegradation was examined because dissolved NO_3^- was detected in a few locations in the shallow aquifer at NSB Bangor. Thus, the possibility that in situ RDX biodegradation was driven by microbial nitrogen demands (Hawari, 2000) raised concerns that RDX biodegradation might be inhibited in areas of detectable NO_3^- concentrations. The lack of significant dissolved O_2 and SO_4, the insignificant production of CH_4, dissolved sulfide, and dissolved Fe(II); the decreased accumulation of dissolved Mn(II) (56% less than that in unamended, anoxic microcosms, Table 1), combined with a 51% decrease in dissolved NO_3^- (3.6 ± 0.3 mM final concentration), significant accumulation of dissolved NO_2^- (250 ± 105 μM), transient production of N_2O, and stoichiometric accumulation of N_2 within experimental microcosms indicated a fundamental shift from metals reduction to predominantly denitrification under NO_3-amended, anoxic conditions. NO_3 amendment resulted in a substantial increase in the efficiency of ^{14}C-RDX mineralization compared with that observed under unamended, anoxic conditions (Table 2, Figure 2). The initial production of $^{14}CO_2$ under NO_3-amended conditions was comparable to that observed in MnO_2-amended, anoxic microcosms and ^{14}C-RDX mineralization was essentially complete by day 51 (Figure 2). This result is consistent with previous reports of efficient RDX biodegradation under NO_3-reducing conditions (Freedman and Sutherland, 1998, Hawari, 2000). The fact that NO_3^- was present in millimolar concentrations in this treatment suggests that ^{14}C-RDX was not biodegraded as a nitrogen substrate but was utilized as a carbon substrate or electron

donor under NO_3-amended conditions. From a field perspective, these results indicate that a some potential for denitrification-associated RDX biodegradation may exist in NO_3-containing portions of the shallow aquifer.

The results of the acetate-amended treatment indicate that RDX serves different microbiological functions under denitrifying and Mn(IV)-reducing conditions at NSB Bangor (Table 2, Figure 2). Under NO_3-amended conditions, acetate appeared to inhibit Mn(II) accumulation (Table 1) while stimulating denitrification, as indicated by a 98% decrease in dissolved NO_3^- concentrations, transient N_2O production and stoichiometric accumulation of N_2. The increased rate of denitrification observed in acetate-amended microcosms suggests that denitrification was carbon/electron donor limited under these conditions. The fact that acetate amendment inhibited the production of $^{14}CO_2$ (Table 2), and the final percentage loss of ^{14}C-RDX (Table 2) in NSB Bangor sediments under NO_3-amended, anoxic conditions is consistent with the hypotheses that RDX functioned primarily as carbon substrate or electron donor under denitrifying conditions. In contrast, addition of acetate to unamended (no NO_3^-, MnO_2 or $Fe(OH)_3$ added), anoxic microcosms apparently stimulated Mn(IV)-reduction (Table 1) and the initial rate of ^{14}C-RDX mineralization (Figure 2). The approximately 5-fold increase in dissolved Mn(II) accumulation observed in acetate-amended, anoxic microcosms indicates that Mn(IV)-reduction was limited by the availability of carbon substrate and/or electron donor (Table 1). Acetate amendment doubled the initial production of $^{14}CO_2$, resulting in approximately 80% mineralization of ^{14}C-RDX to $^{14}CO_2$ within the first 51 days (Figure 2). The stimulation of ^{14}C-RDX biodegradation by carbon amendment indicates that ^{14}C-RDX biodegradation was carbon/electron donor limited under the in situ, metals-reducing conditions. This observation is consistent with the hypothetical role for RDX as a nitrogen substrate during biodegradation under nitrogen-limiting conditions (Hawari, 2000).

This study demonstrates the potential for effective RDX biodegradation in predominantly anoxic, metals-reducing aquifer sediments. This observation and the apparent predominance of metals-reducing conditions at NSB Bangor suggest that the potential for in situ biodegradation of RDX is significant within the shallow aquifer at NSB Bangor. The lack of detectable accumulation of MNX, DNX, or TNX in the laboratory and in the field (Dinicola, unpublished results), combined with the stoichiometric mineralization of ^{14}C-RDX to the nondiagnostic product $^{14}CO_2$, indicate that a reliance on the accumulation of diagnostic intermediates as an indicator of in situ RDX biodegradation may be problematic under metals-reducing conditions.

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