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Enhanced bioremediation of RDX and Co-Contaminants perchlorate and nitrate using an anaerobic dehalogenating consortium in a fractured rock aquifer

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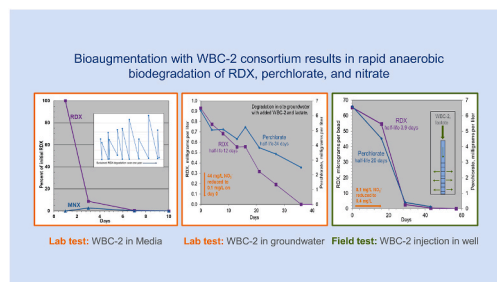
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HIGHLIGHTS

- High nitrate and perchlorate may impede anaerobic RDX biodegradation in aquifers.
- Organic donor did not stimulate biodegradation of RDX, perchlorate, and nitrate.
- Adding an anaerobic mixed culture with the donor resulted in rapid biodegradation.
- WBC-2 culture sustained RDX degradation for a year without metabolite accumulation.
- Field tests verify bioaugmentation as feasible remedy for RDX and co-contaminants.

GRAPHICAL ABSTRACT



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ABSTRACT

The potential neurotoxic and carcinogenic effects of the explosives compound RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) on human health requires groundwater remediation strategies to meet low cleanup goals. Bioremediation of RDX is feasible through biostimulation of native microbes with an organic carbon donor but may be less efficient, or not occur at all, in the presence of the common co-contaminants perchlorate and nitrate. Laboratory tests compared biostimulation with bioaugmentation to achieve anaerobic degradation of RDX, perchlorate, and nitrate; a field pilot test was then conducted in a fractured rock aquifer with the selected bioaugmentation approach. Insignificant reduction of RDX, perchlorate, or nitrate was observed by the native microbes in microcosms, with or without biostimulation by addition of lactate. Tests of the RDX-degrading ability of the microbial consortium WBC-2, originally developed for dehalogenation of chlorinated volatile

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organic compounds, showed first-order biodegradation rate constants ranging from 0.57 to 0.90 per day (half-lives 1.2 to 0.80 days). WBC-2 sustained degradation without daughter product accumulation when repeatedly amended with RDX and lactate for a year. In microcosms with groundwater containing perchlorate and nitrate, RDX degradation began without delay when bioaugmented with 10% WBC-2. Slower RDX degradation occurred with 3% or 5% WBC-2 amendment, indicating a direct relation with cell density. Transient RDX daughter compounds included methylene dinitramine, MNX, and DNX. With WBC-2 amendment, nitrate concentrations immediately decreased to near or below detection, and perchlorate degradation occurred with half-lives of 25–34 days. Single-well injection tests with WBC-2 and lactate showed that the onset of RDX degradation coincided with the onset of sulfide production, which was affected by the initial perchlorate concentration. Biodegradation rates in the pilot injection tests agreed well with those measured in the microcosms. These results support bioaugmentation with an anaerobic culture as a remedial strategy for sites contaminated with RDX, nitrate, and perchlorate.

1. Introduction

The nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common contaminant at sites where munitions have been used, manufactured, or disposed of, including 37 sites on EPA's National Priorities List (as of 2020) (U.S. Environmental Protection Agency, 2021). Because of low sorption and moderate solubility, RDX is highly mobile in soil and groundwater, potentially resulting in long and persistent contaminant plumes (Hatzinger et al., 2004; Lewis et al., 2004). Federal guidelines established for RDX, due to health concerns that include neurotoxic and carcinogenic potential effects, are a 2 micrograms per liter ($\mu\text{g/L}$) lifetime health advisory level for drinking water and a 0.97 $\mu\text{g/L}$ screening level for tap water (U.S. Environmental Protection Agency, 2021). Anaerobic degradation was recognized as a potential bioremediation approach for RDX after laboratory studies identified anaerobic degradation pathways for RDX (Hawari et al., 2000a, b; Hawari and Halasz, 2002; Halasz et al., 2012) (Fig. S1) and showed that native microbial populations in several contaminated aquifers could be stimulated to degrade RDX through the addition of a carbon substrate (Hatzinger and Lippincott, 2012). Pilot tests conducted in RDX-contaminated aquifers further demonstrated the feasibility of biostimulation to enhance degradation, using injections of a range of carbon substrates that included whey (Hatzinger and Lippincott, 2012), acetate (Wani et al., 2003; Livermore et al., 2013), emulsified vegetable oil (Hatzinger and Fuller, 2016), and fructose (Michalsen et al., 2016).

Microbes capable of RDX anaerobic degradation have been shown to utilize RDX as a terminal electron acceptor, a nitrogen source, or both (Zhao et al., 2003; Hatzinger and Fuller, 2014; Cho et al., 2015, 2016). Anaerobic degradation proceeds primarily by two pathways— one pathway that produces nitroso derivatives (MNX, DNX, TNX), and a second pathway that involves direct enzymatic ring cleavage of RDX to methylenedinitramine (MEDINA) (Fig. S1). The ring cleavage product 4-nitro-2,4-diazabutanal (NDAB) has been observed primarily under aerobic conditions or from abiotic reactions (Halasz et al., 2012; Hatzinger and Fuller, 2014). Although stimulation of aerobic degradation of RDX with carbon substrate addition also has been shown in laboratory and field studies, most studies have indicated that anaerobic biostimulation results in higher degradation rates for RDX than aerobic biostimulation (Michalsen et al., 2016).

Sulfate or methanogenic conditions have been indicated as necessary for effective *in situ* anaerobic bioremediation of RDX (Hatzinger and Fuller, 2014). Perchlorate and nitrate are common co-contaminants of explosives compounds that can also be degraded under anaerobic conditions and potentially hinder RDX degradation. Perchlorate is a common energetics booster and oxidant in a variety of rockets and munitions, and breakdown of nitrate-containing explosives in soil can result in elevated nitrate concentrations in groundwater at explosives-contaminated sites. These co-contaminants, which have similar reduction potentials (Guan et al., 2015), can act as more favorable electron acceptors for microbial metabolism than sulfate or RDX and delay the onset of sufficiently reducing conditions for RDX degradation. The presence of nitrate also provides a more readily available

nitrogen source for microbial metabolism than available in RDX. In June 2020, EPA withdrew a 2011 regulatory determination for perchlorate, stating that perchlorate no longer meets the criteria for regulation as a drinking water contaminant. However, co-located reduction of perchlorate and nitrate concentrations likely are needed for effective *in situ* RDX biodegradation under anaerobic conditions. Multiple field tests have evaluated anaerobic bioremediation of RDX and perchlorate, separately or together, but studies with comingled RDX, perchlorate, and nitrate are limited. In addition, previous laboratory and field bioremediation studies have been conducted in unconsolidated sand aquifers and with relatively low concentrations of RDX and comingled constituents (generally <1 mg/L of each) (e.g., Hatzinger and Fuller, 2016).

Although many RDX bioremediation studies have focused on biostimulation with an organic donor, recent studies have turned to bioaugmentation as an alternative bioremediation method to address the wide variety of site characteristics and the low cleanup goals for RDX. Initial bioaugmentation studies involved addition of aerobic RDX-degrading microbes (Crocker et al., 2015; Fuller et al., 2015, 2017; Michalsen et al., 2016). Achieving and maintaining aerobic conditions may be difficult in many contaminant plumes, however, especially when an organic donor source also needs to be added to support aerobic degradation. Recent laboratory tests (Fuller et al., 2017) and a field test (Michalsen et al., 2020) used bioaugmentation with a combined inoculation of the aerobic RDX-degrading microbial species, *Gordonia* sp. KTR9, and a facultative RDX-degrading strain, *Pseudomonas fluorescens* strain I-C cells ("Strain I-C") for degradation of RDX, without the presence of nitrate or perchlorate co-contaminants. These studies indicated that bioaugmentation is a viable technology for enhancing RDX cleanup and that use of the facultative Strain I-C promoted RDX degradation under anoxic conditions (Fuller et al., 2017; Michalsen et al., 2020).

For the study reported here, the feasibility of *in situ* anaerobic bioremediation utilizing bioaugmentation with a mixed culture is examined for a plume of RDX in a fractured rock aquifer that also contains relatively high nitrate and perchlorate (>5 mg/L of each). Preliminary tests at this site indicated that biostimulation with donor alone was not sufficient to achieve RDX degradation but that addition of a mixed anaerobic culture, WBC-2, in conjunction with added carbon donor could be effective (Lorah et al., 2008b). WBC-2 was originally developed for dehalogenation of chlorinated solvents (Jones et al., 2006; Lorah and Voytek, 2004; Lorah et al., 2008a, 2015). The objectives of the study reported in this paper were to (1) compare biodegradation of RDX, perchlorate, and nitrate under natural, biostimulated, and bioaugmented conditions in microcosms, (2) conduct laboratory microcosms to define the ability of the bioaugmentation culture WBC-2 to degrade RDX by quantifying RDX degradation rates, determining primary daughter products, and optimizing cell density, and (3) verify laboratory results by applying the bioremediation technology in a field pilot test in a fractured rock aquifer in New Mexico. Tests with the site groundwater and the native and bioaugmented microbial communities included a range of RDX concentrations, from site-specific

concentrations of less than about 0.150 mg/L to relatively high concentrations of RDX (>1 mg/L).

2. Materials and methods

2.1. Field site

The field site used to obtain materials for laboratory testing and for the pilot test is in southern New Mexico at the Hazardous Test Area (HTA), about 10 miles north of the main base at White Sands Missile Range (WSMR), where two Open Burn/Open Detonation (OB/OD) pits were constructed in 1953 (Langman et al., 2008) (Fig. S2). The site is at an altitude of approximately 5700 ft above NAVD88 (North American Vertical Datum of 1988) on the pediment slope of the San Augustin and San Andres mountains. The OB/OD pits were used until 2000 for the demolition of ordnance that contained explosives and propellants and resulted in release of RDX, perchlorate, and nitrate to the soil and underlying fractured-granite aquifer. The fractured-granite aquifer at HTA is in a Precambrian granite pluton that is bounded on the north by the Bear Peak Fold and Thrust Zone, where aquifer recharge likely occurs along the pediment-mountain interface (Langman et al., 2008). Groundwater flow is generally east-southeast across the site (Fig. S2) toward the valley floor of the Tularosa Basin, but groundwater flow-paths are along hydraulically conductive fractures that have variable orientation and dip (Langman et al., 2008).

Previous studies at the site showed that RDX, nitrate, and perchlorate concentrations in groundwater from wells downgradient from the OB/OD pits were relatively constant (Langman et al., 2008). Changes in RDX, nitrate, and carbon dioxide concentrations and variations in reduction-oxidation conditions indicated that contaminant degradation was occurring in the downgradient direction, in addition to dilution, but degradation processes were not elucidated.

Four wells were selected for use in the current study, one for groundwater for the laboratory tests (HTA-10A) and three wells for the field injection test (HTA-11, HTA-13, HTA-16) (Table S1; Fig. S2). The OB/OD pits were directly upgradient of wells HTA-10A and HTA-11. Between 1996 and 2006, groundwater from wells HTA-10A and HTA-11 had similar, but variable, concentrations of RDX (about 22–90 µg/L), perchlorate (about 4–14 mg/L), and nitrate plus nitrite (about 6–12 mg/L) (Langman et al., 2008). Dissolved-oxygen concentrations were variable in the groundwater, ranging from about 0.1 to 7.2 mg/L. Background nitrate concentrations in groundwater at the HTA ranged

from 1 to 4 mg/L (Langman et al., 2008).

2.2. Laboratory tests

Laboratory tests are briefly summarized here, and additional detail, and all data are available in Naglieri and Lorah (2021). All microcosms were prepared, incubated, and sampled in an anaerobic glove box at room temperature. Prepared microcosms were covered to inhibit contaminant losses due to photodegradation and inverted by hand to gently mix every workday. The first set of experiments for this study consisted of microcosms prepared in serum bottles with undiluted WBC-2 in its mineral medium, amended with lactate as an electron donor and with varying target RDX concentrations to verify RDX degradation capability and obtain degradation rates (Table 1). WBC-2 was obtained from SIREM Lab, where it is maintained and available as KB-1 Plus (SIREM Lab, Guelph, Ontario). WBC-2 is typically maintained by feeding with chlorinated volatile organic compounds (VOCs), but the culture was analyzed to ascertain that all VOCs had been degraded prior to use in the experiments. Triplicate bottles were prepared for sterile controls by repeated autoclaving over three consecutive days and amended with target RDX concentrations of 1.0 mg/L. Samples were removed at selected time intervals for duplicate analyses of RDX and potential daughter compounds.

Additional microcosms were prepared with WBC-2 in mineral medium and amended with RDX repeatedly for one year. Samples were collected for analysis of RDX and daughter products before re-spiking with RDX.

A second set of laboratory experiments was aimed at evaluating RDX degradation in site groundwater, comparing treatments with and without added WBC-2 and in the presence of nitrate and perchlorate naturally occurring at the site. Each treatment was prepared in triplicate in 40 mL glass VOC vials with Teflon-lined septa and using groundwater collected from well HTA-10A (Table S1). Microcosms were inoculated with WBC-2 at three different cell densities, given as the percent WBC-2 added by volume groundwater in the microcosms, and amended with RDX to give target initial concentrations of 1.0 mg/L, or 4.5 µmol per liter (µM) (Table 1). Cell counts of WBC-2 were consistently around 10^{10} cells/mL (SIREM Lab). For one of the non-bioaugmented treatments and one of the WBC-2 bioaugmented treatments, 20 mg/L nitrate (as N) was also added to obtain higher initial nitrate concentrations than present in the site groundwater. Control treatments with unamended native groundwater (live controls) and biostimulated treatments with lactate

Table 1

Laboratory and field pilot test results for RDX degradation with first-order degradation rate constants (k), associated half-lives, and daughter products. [GW, groundwater; RDX_i, initial RDX concentration in units of micromoles per liter (µM) for culture and GW tests and in units of micromoles per bead for field tests. Note that field tests also had perchlorate and nitrate present. Dashes indicate RDX degradation or daughter product was not detected. TNX (not shown) was detected in one sample (RWBC-2-H, 0.07% on day 3). Correlation coefficients for rate constant regressions were between 0.822 and 0.985].

Description	Name	RDX _i (µM)	k (per day)	Half-life (days)	MXN %; days	DNX %; days	MEDINA %; days
Culture microcosms, 100% WBC-2							
100% WBC-2+lactate, low RDX	RWBC-2-L	3.111	0.574	1.2	3.9%; 3	–	–
100% WBC-2+lactate, medium RDX	RWBC-2-M	17.167	0.902	0.8	1.0%; 1	–	–
100% WBC-2+lactate, high RDX	RWBC-2-H	23.360	0.684	1.0	2.6%; 3	–	–
GW microcosms, no WBC-2							
Site GW	GW	0.707	–	–	–	–	–
Site GW + RDX, perchlorate	GW-RP	4.444	–	–	0.43%; 4	0.56%; 4	–
Site GW + RDX, perchlorate, lactate	GW-LRP	4.133	–	–	0.51%; 16	0.65%; 8	–
Site GW + RDX, perchlorate, lactate, nitrate	GW-LNRP	4.840	–	–	0.60%; 8	0.65%; 8	–
GW microcosms, WBC-2 amended							
GW+3% WBC-2, RDX, perchlorate, lactate	3% WBC-2	4.354	0.015	47	2.1%; 36	1.0%; 8	32%; 50
GW+5% WBC-2, RDX, perchlorate, lactate	5% WBC-2	3.160	0.017	42	4.2%; 27	0.65%; 4	30%; 50
GW+10% WBC-2, RDX, perchlorate, lactate	10% WBC-2	4.182	0.056	12	2.0%; 27	0.75%; 4	30%; 13
GW+10% WBC-2, RDX, perchlorate, lactate, nitrate	10% WBC-2-N	3.530	0.075	9.0	3.3%; 16	1.0%; 8	33%; 13
Field pilot tests, WBC-2 injected							
Well HTA-11, RDX on Bio-Sep beads	HTA-11	0.246	0.176	3.9	–	–	–
Well HTA-13, RDX on Bio-Sep beads	HTA-13	0.295	0.194	3.6	–	–	–
Well HTA-16, RDX on Bio-Sep beads	HTA-16	0.244	0.187	3.7	0.34%; 42	–	–

(0.1 mM) added as an organic donor were prepared to compare to the WBC-2 bioaugmented (plus donor) microcosms.

2.3. Field pilot test

Because of limited time to conduct the pilot test and the constraint to use the existing monitoring well network, the field pilot test consisted of single-well injection tests in three wells that ranged from 85 to 120 feet (25.9–36.6 m) in depth (Table S1). The single well injection tests were similar to single well “push-pull” tests that have been used often for cost-effective pilot tests (Pitterle et al., 2005; Harrison et al., 2011) but incorporated passive sampling. Injection volumes were calculated using the well characteristics and known porosity (Table S1) to achieve desired amendment concentrations in a 1.5-m radius of influence from the well. After collection of background samples, 300 gallons of water was pumped from each well into a tank and amended with 5 gallons of a 60 percent sodium lactate solution to provide an organic carbon source and induce reducing conditions in the groundwater. At each well, the amended water in the tank was sampled before being pumped back into the well. Immediately following the lactate injection, the WBC-2 culture was injected into each well from a tank under nitrogen pressure; a volume of culture required to achieve a 7% cell density in the radius of influence was injected in each well.

Groundwater in the three wells was sampled again immediately following WBC-2 injection to establish day 0 concentrations for the field test. Sets of four diffusion samplers (Rigid Porous Polyethylene Samplers, Columbia Analytical, Columbia, SC) that are suitable for deeper wells were then placed in each well. To obtain groundwater samples during the pilot test monitoring, one diffusion sampler was removed from each well on the specified sampling date, and the water was immediately transferred to 40-mL glass vials and sealed without headspace. Groundwater samples during the field test were analyzed for RDX and potential metabolites, perchlorate, nitrate, ammonia, sulfide, and ferrous and total dissolved iron. All field test data are available in Naglieri and Lorah (2021); groundwater concentrations measured from immediately following injection through the end of the test also are shown in Table S2.

Because of the relatively low RDX concentrations at the site, a known quantity of RDX sorbed on Bio-Sep beads (Microbial Insights, Knoxville, TN) was placed in a contained unit in each of the three wells along with the passive diffusion water samplers. Bio-Sep beads were pre-loaded with RDX, and test analyses were done by the University of Maryland Baltimore County (UMBC) prior to deployment in the wells to establish the mass of RDX on the beads and the ability to remove the RDX on the beads for analysis. Table 1 shows the initial measured RDX concentrations on the beads for the field tests. Bio-Sep beads were removed for analysis at the same time as the groundwater samples. The Bio-Sep bead and water samples were shipped overnight on ice to the USGS Maryland-Delaware-District of Columbia Water Science Center, Baltimore, MD (USGS MD-DE-DC) and UMBC for analysis.

2.4. Chemical analyses

Microcosms were prepared at USGS MD-DE-DC, and analyses were done at USGS MD-DE-DC and UMBC. RDX and its potential daughter products and perchlorate were analyzed at UMBC. The analytical method developed for RDX and intermediates, which allowed the use of small sample volumes, used high performance liquid chromatography with ultra-violet absorbance and photo-assisted electrochemical detection (Fedorowski et al., 2011, 2012). RDX metabolites analyzed by this method included MNX, DNX, TNX, and MEDINA; detection limits were <0.001 mg/L, except for a detection limit of <0.010 mg/L for MEDINA. Triplicate analyses of RDX and the metabolites MNX, DNX, and MEDINA were used to calculate the relative standard deviation (RSD) or relative percent difference (RPD) if the sample had three or two detectable concentrations, respectively, of the analyte (Naglieri and Lorah, 2021).

The combined average for the RSDs and RPDs was 10.4% for RDX, 12.0% for MNX, 21.1% for DNX, and 36.8% for MEDINA. Insufficient detections were available to calculate the RSD or RPD for TNX.

The RDX standard used for amendments was dissolved in acetonitrile; however, the acetonitrile was removed by sparging with nitrogen gas before use in amending all culture and site groundwater microcosms, except for one treatment prepared with acetonitrile for comparison purposes in the first culture tests. RDX degradation and daughter product formation was nearly identical in the microcosms prepared with or without acetonitrile in the RDX standard (Naglieri and Lorah, 2021). Acetonitrile was not removed from the RDX standard used to spike the year-long microcosms.

Perchlorate was analyzed at UMBC using ion chromatography and had a detection limit of 0.01 mg/L. Ammonia, nitrate, iron, and sulfide in groundwater samples were analyzed immediately using Hach (Loveland, CO) colorimetric methods and analyzed with a spectrophotometer in the USGS MD-DE-DC laboratory within 24 h of sample collection. For microcosms, ammonia, nitrate, and hydrazines were analyzed using Hach colorimetric methods and spectrophotometer in the USGS MD-DE-DC laboratory. Hydrazine concentrations, which had been proposed as unstable products of degradation of the three nitroso-derivatives in early research of RDX anaerobic biodegradation (McCormick et al., 1981), were less than the detection limit of 4 µg/L in all microcosm samples.

3. Results and discussion

3.1. RDX degradation in WBC-2 culture tests

RDX degradation occurred in WBC-2 culture in media (100% WBC-2) without lag and with apparent first-order kinetics (Fig. 1a; Fig. S3). RDX concentrations decreased to below detection (<0.001 mg/L, or <0.00045 µM) within 10 days in the three microcosm treatments with WBC-2, while concentrations in the killed controls had about a 20% removal of RDX over the 10 days (Fig. 1a). For initial RDX concentrations between 0.691 and 5.189 mg/L, first-order rate constants for RDX degradation by WBC-2 ranged from 0.57 to 0.90 per day, corresponding to half-lives between 0.8 and 1.2 days (Table 1).

MNX was the primary daughter product detected in the live WBC-2 microcosms, where it was detected at low concentrations that accounted only for 1–4% of the initial RDX concentrations (Fig. 1b; Table 1). Maximum MNX concentrations (4%) were observed in the microcosms with the lowest initial RDX concentration, which also showed the lowest RDX degradation rate (RWBC-2-L in Fig. 1; Table 1). It is likely that the higher MNX accumulation in treatment RWBC-2-L resulted from a slower MNX degradation in this treatment compared to the two treatments with higher initial RDX concentrations. Besides MNX, the only other daughter product detected in the WBC-2 in culture microcosms was one detection of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (0.07% of the initial RDX) at day 3 in the treatment with the highest initial RDX concentration (5.189 mg/L or 23.4 µM). MNX was not detected in killed controls. MEDINA was the only detected daughter product in the killed controls, occurring only at day 7 in two of the three killed control treatments (average of 0.233 mg/L, or 1.71 µM for detections) (Naglieri and Lorah, 2021).

WBC-2 in culture media that was spiked with RDX and lactate repeatedly (monthly or bi-monthly) was able to maintain degradation of RDX for over one year (Fig. 2 and S4). Initially, RDX was added to give a target concentration of 5.0 µM but was increased over time with the repeated spiking (Fig. 2). In most cases, RDX concentrations generally decreased to below detection before the next sampling and amendment date. In the last month before completing the long-term test, sampling and re-amendment was done on a weekly basis. RDX was completely degraded within a week during the weekly amendment period in one microcosm bottle, although a duplicate bottle showed slightly slower degradation during the weekly amendment (Fig. 2 and S4). During the period of monthly-bimonthly amendment, only low concentrations of

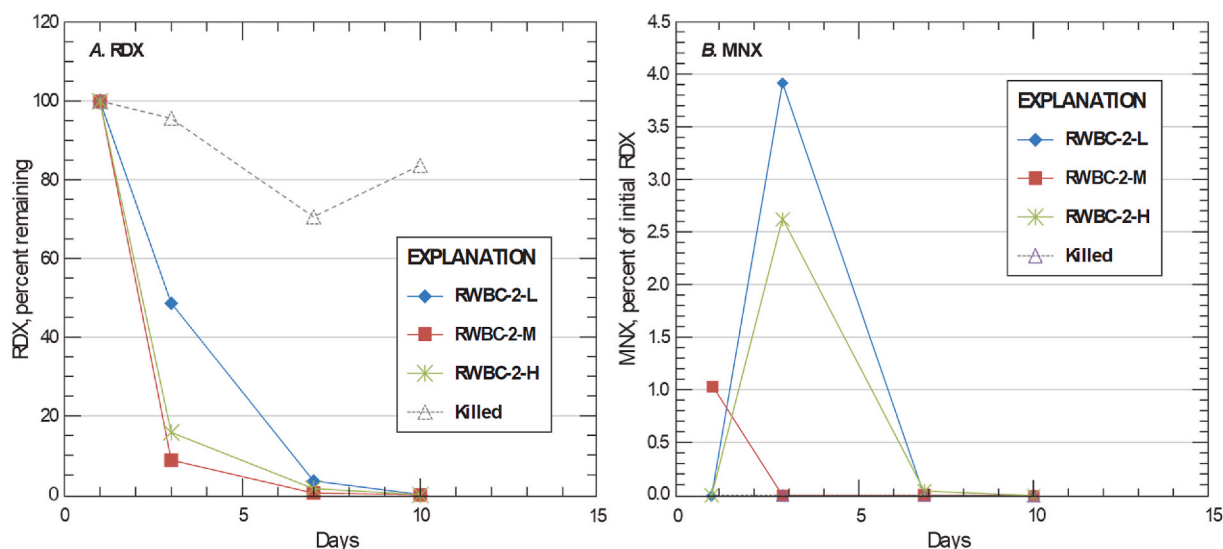


Fig. 1. (A) Percent removal of RDX and (B) percent production and removal of MNX in WBC-2 and culture media in microcosms with low (-L), medium (-M), and high (-H) initial concentrations of RDX. The killed control is the average of 3 autoclaved controls. See Table 1 for treatment descriptions and initial concentrations.

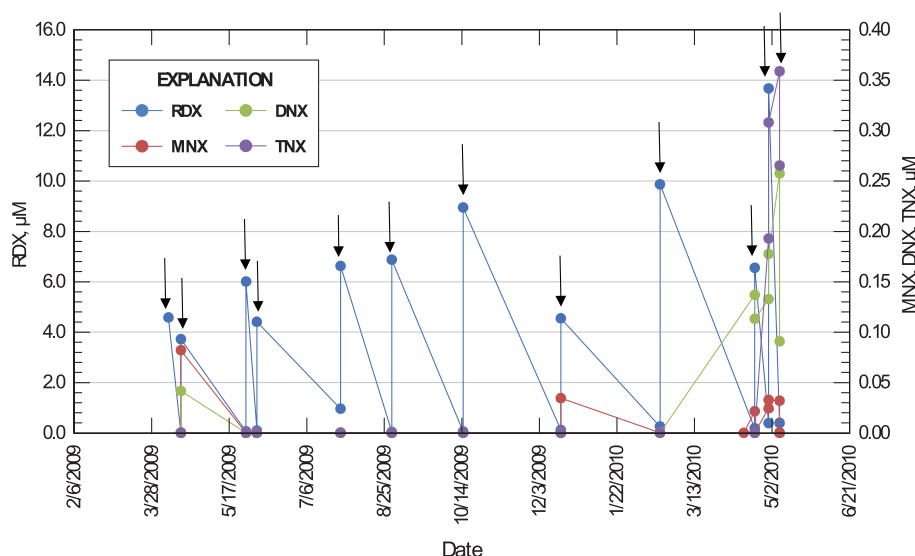


Fig. 2. Concentrations of RDX and daughter products MNX, DNX, and TNX in a microcosm bottle that contained WBC-2 in anaerobic culture media and was repeatedly amended with RDX and lactate. Arrows show dates of amendment.

daughter products were detected sporadically in the test bottles, with total concentrations of MNX, DNX, and TNX less than $0.4 \mu\text{M}$ on any sampling date. When amendment was increased to weekly intervals, higher concentrations of daughter products were observed but with decreasing concentrations showing their degradation (Fig. 2; Fig. S4). MEDINA was not detected throughout this long-term test. Although the test was not designed in a manner to optimize RDX degradation, it does show the feasibility of sustaining the WBC-2 culture on RDX, rather than chlorinated VOCs.

3.2. RDX degradation in laboratory experiments with site groundwater

Anaerobic microcosms with the unamended site groundwater (treatment GW) had less than $1.0 \mu\text{M}$ RDX and did not show any removal of RDX or production of daughter compounds over a 50-day test (Fig. 3a and Table 1). In addition, none of the RDX-amended (about 1.0 mg/L , or $4.5 \mu\text{M}$) groundwater microcosms that were not bioaugmented with WBC-2 showed removal of RDX over a 50-day test (Fig. 3a).

Biostimulation alone was ineffective in promoting RDX degradation, as shown by the treatment with addition of lactate and RDX (GW-LRP in Fig. 3a). A slow increase in RDX concentrations was instead observed in the non-bioaugmented groundwater microcosms, most likely because of slow dissolution of the amended RDX and desorption from the microcosm containers and septum. The lack of biostimulation of RDX degradation with carbon donor alone contrasts with multiple previous studies that have shown biostimulation with various organic donors (e.g., Hatzinger and Lippincott, 2012; Livermore et al., 2013; Hatzinger and Fuller, 2016), including lactate (Schaefer et al., 2007). Except for the study reported by Schaefer et al. (2007), however, nitrate and perchlorate either were absent or in low concentrations. The groundwater microcosms in our study contained nitrate concentrations (as N) (see next section) that were about a factor of 10 higher than the Schaefer et al. (2007) study and thus would be more likely to negatively affect biodegradation of RDX.

In contrast to the unamended and lactate only groundwater treatments, RDX concentrations decreased to below detection by day 50 in

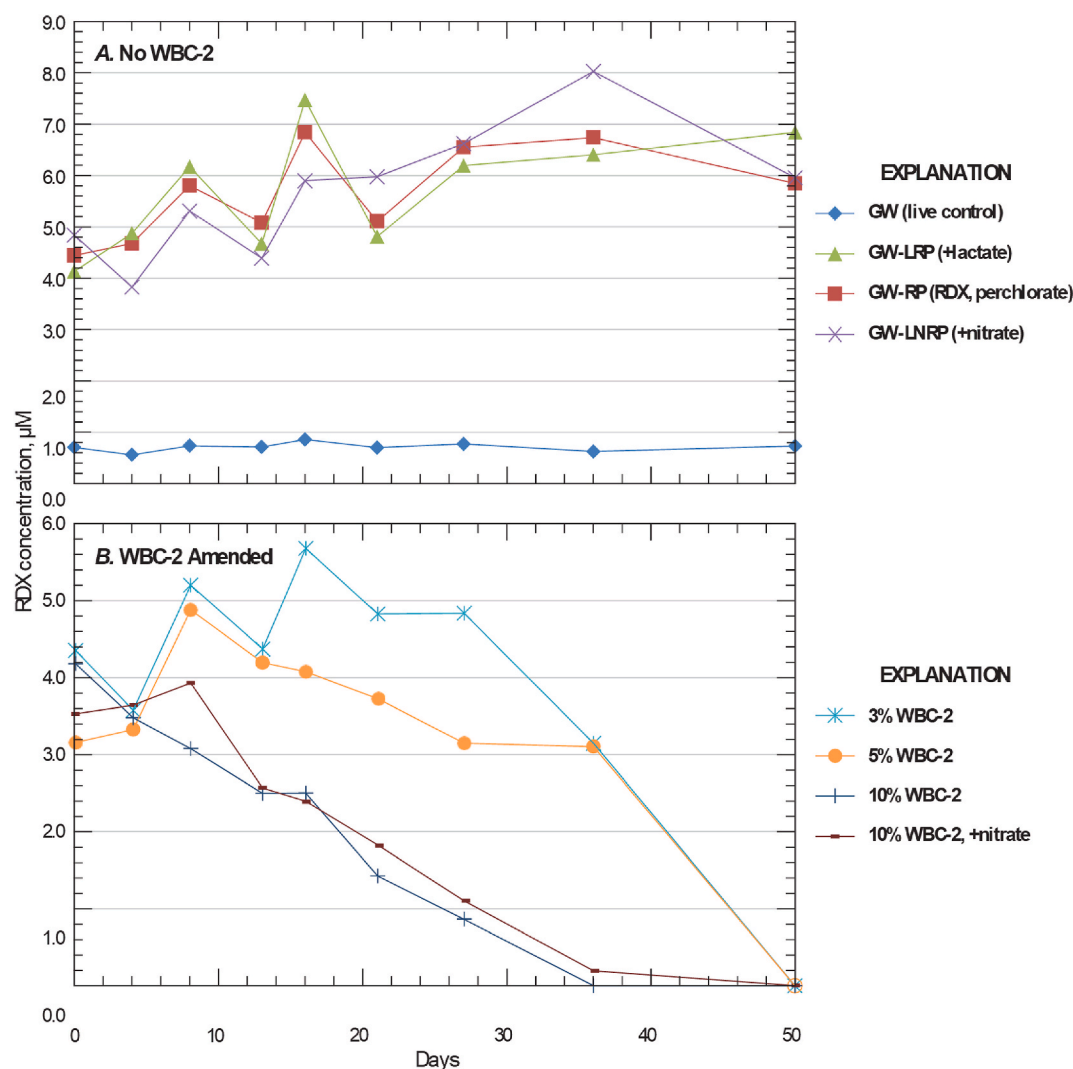


Fig. 3. RDX concentrations in microcosms with site groundwater (GW) that (A) was not amended with WBC-2 and (B) was amended with WBC-2 at 3, 5 or 10% by volume. Except for the live control, all microcosms were amended with RDX and contained perchlorate; all except the live control and treatment GW-RP were also amended with lactate (Table 1).

the WBC-2 bioaugmented microcosms (Fig. 3b). RDX degradation was delayed until about days 16 and 8, respectively, in the treatments amended with 3% and 5% WBC-2 by volume but occurred rapidly and without delay in the treatment with 10% WBC-2 and no added nitrate. When 20 mg/L nitrate (as N) was added to the 44 mg/L nitrate (as N) already present in the site groundwater in a treatment, the onset of RDX removal appeared to be delayed until day 13 (Fig. 3b, treatment 10% WBC-2, + nitrate). The overall RDX degradation rate, however, was unaffected by the additional nitrate in the WBC-2-amended microcosm (Table 1). First-order rate constants for RDX degradation in the groundwater microcosms were 0.056 and 0.075 per day with 10% WBC-2, with and without added nitrate, corresponding to half-lives between 9 and 12 days (Table 1). The degradation rate constants for RDX in groundwater amended with 10% WBC-2 were about a factor of 10 lower than those measured in microcosms with 100% WBC-2 (in mineral medium) (Table 1), indicating a positive relation between WBC-2 population density and RDX degradation. In the Schaefer et al. (2007) study with comingled RDX, perchlorate, and nitrate, the first order degradation rate constant determined for RDX in microcosms with emulsified vegetable oil, which they found to be a more effective carbon donor than lactate for RDX biodegradation, was more than a factor of 10 lower (0.0048 per day) than the rate constants determined here with 10% WBC-2 and lactate. The RDX biodegradation rate constant of 0.0048 per

day corresponds to a half-life of 144 days, compared to the 9–12 days determined with 10% WBC-2 and lactate. Another explosives compound, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), was also present in the Schaefer et al. (2007) study but had substantially lower degradation rates than the RDX.

RDX degradation products were below detection in the unamended live control with site groundwater (treatment GW) (Table 1), consistent with the lack of RDX removal observed. In groundwater microcosms amended with RDX but not WBC-2, production of MNX and DNX was observed within the first 16 days of the experiment with total concentrations of about 1% of the initial RDX concentrations (Table 1). In groundwater microcosms bioaugmented with WBC-2, the maximum molar percentages of MNX, DNX, and MEDINA were similar regardless of the amount of WBC-2 added (Table 1). The molar percent DNX in the bioaugmented groundwater microcosms (0.65–1.0%) was consistent with the production observed in those microcosms without added WBC-2, whereas higher molar percentages of MNX (2.0–4.2%) and MEDINA (30–33%) were observed in the bioaugmented groundwater (Table 1). Figs. S5–S7 illustrate the production and degradation of RDX daughter products in the groundwater microcosms.

MEDINA was the dominant daughter product in the groundwater microcosms bioaugmented with WBC-2 with a maximum of about 1.2 µM, which corresponded to about 30% of the initial RDX concentration

(Table 1). MEDINA production was about a factor of 10 higher than the percent MNX or DNX (Table 1). Compared to MNX and MEDINA, DNX was present only early in the microcosm experiment, with concentrations peaking by day 4–8 and then rapidly declining and not appearing again in the 50-day test. TNX was not detected in any of the groundwater microcosm treatments, with or without WBC-2 bioaugmentation. The patterns in daughter product concentrations indicate that RDX and MNX primarily degraded to MEDINA, and other ring cleavage and terminal products that were not detected, rather than through the pathway that produces DNX and TNX (Fig. S1). The dominance of MEDINA in the groundwater microcosms bioaugmented with 3%, 5%, and 10% WBC-2 contrasts with the lack of MEDINA in the 100% culture microcosms (Table 1).

It is likely that MEDINA, a relatively unstable compound (Hatzinger and Fuller, 2014), was produced but degraded too rapidly to be observed in the 100% culture microcosms, while the more stable MNX had consistent molar percentages in the culture and groundwater microcosms. The dominance of MEDINA as a transient metabolite in the groundwater microcosms is consistent with other studies that have shown denitration or ring cleavage as the predominant RDX degradation pathway under strongly reducing conditions (Adrian and Arnett, 2004; Hatzinger and Fuller, 2014). Concentrations of nitroso-RDX derivatives

observed in RDX contaminant plumes commonly are low and identifying other possible metabolites to determine if biodegradation is occurring has been a problem, partly because of the difficulty in measuring unstable ring cleavage products (Hatzinger and Fuller, 2014).

3.3. Perchlorate and nitrate in laboratory experiments with site groundwater

The co-contaminants perchlorate and nitrate were present in site groundwater used for the microcosms, and two treatments (with and without added WBC-2) were amended with additional nitrate. Perchlorate concentrations in the anaerobic microcosm treatment with unamended site groundwater (treatment GW) ranged from 7.3 to 8.9 mg/L over 36 days (Fig. 4a). Addition of lactate or RDX to the site groundwater (treatments GW-LRP and GW-RP) did not affect perchlorate removal in the microcosms (Fig. 4a). The treatments that were amended with WBC-2 showed substantially higher perchlorate removal than the treatments without WBC-2 (Fig. 4). The apparent absence of microorganisms capable of reducing perchlorate in the site groundwater, despite organic donor addition, contrasts with their recognition as being generally ubiquitous in the subsurface (Stroo et al., 2008). The only groundwater microcosm treatment that was not amended with

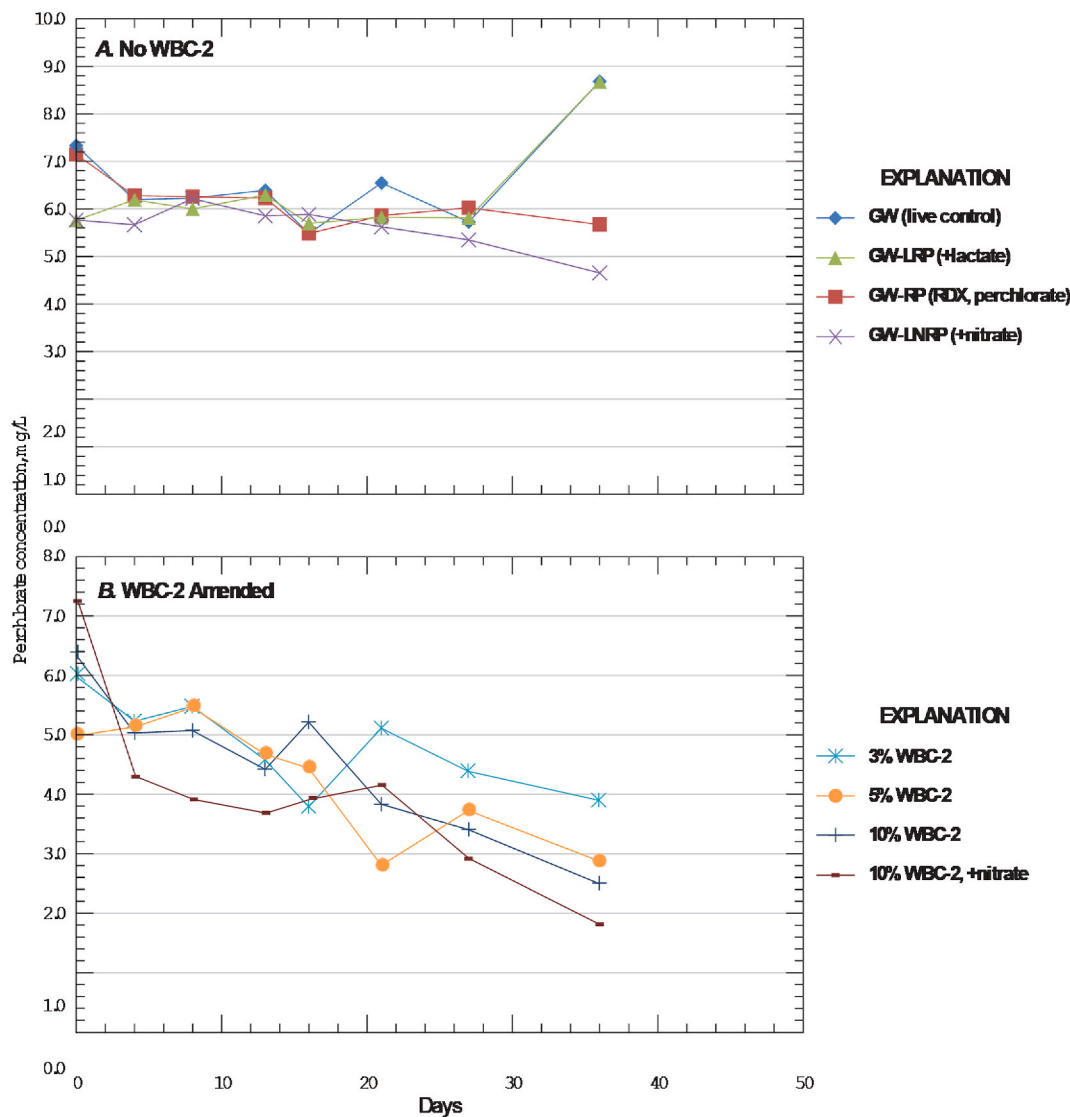


Fig. 4. Perchlorate concentrations in microcosms with site groundwater (GW) that (A) was not amended with WBC-2 and (B) was amended with WBC-2 at 3, 5 or 10% by volume.

WBC-2 but showed a decline in perchlorate concentrations was the one that received additional nitrate (GW-LNRP, Fig. 4a). Although nitrate can be a competing electron acceptor for perchlorate reduction, high nitrate concentrations also have been shown to stimulate growth of perchlorate-reducing bacteria when nitrate is also reduced (Guan et al., 2015). The treatment GW-LNRP showed a gradual decline in perchlorate concentrations after 20 days of incubation (Fig. 4a); however, nitrate concentrations only decreased slightly during this period (Fig. S8). A microcosm study of perchlorate degradation rates with soil or sediment from multiple sites, encompassing the effect of a range of nitrate concentrations (1–22.8 mg/L), showed that perchlorate degradation

kinetics are highly site-specific (Tan et al., 2004).

Each of the bioaugmented treatments showed similar removal of perchlorate and did not appear to be dependent on the amount of WBC-2 added, as was observed for RDX degradation (Figs. 3b and 5b). Perchlorate degradation occurred without a lag in the bioaugmented microcosms (Fig. 4b). In contrast, another microcosm study that evaluated biostimulation with several different organic donors, including lactate, to enhance degradation of comingled RDX, perchlorate, and nitrate showed a substantial lag period before perchlorate degradation occurred, although nitrate reduction was rapid (Schaefer et al., 2007). Perchlorate degradation was best estimated as zero order kinetics

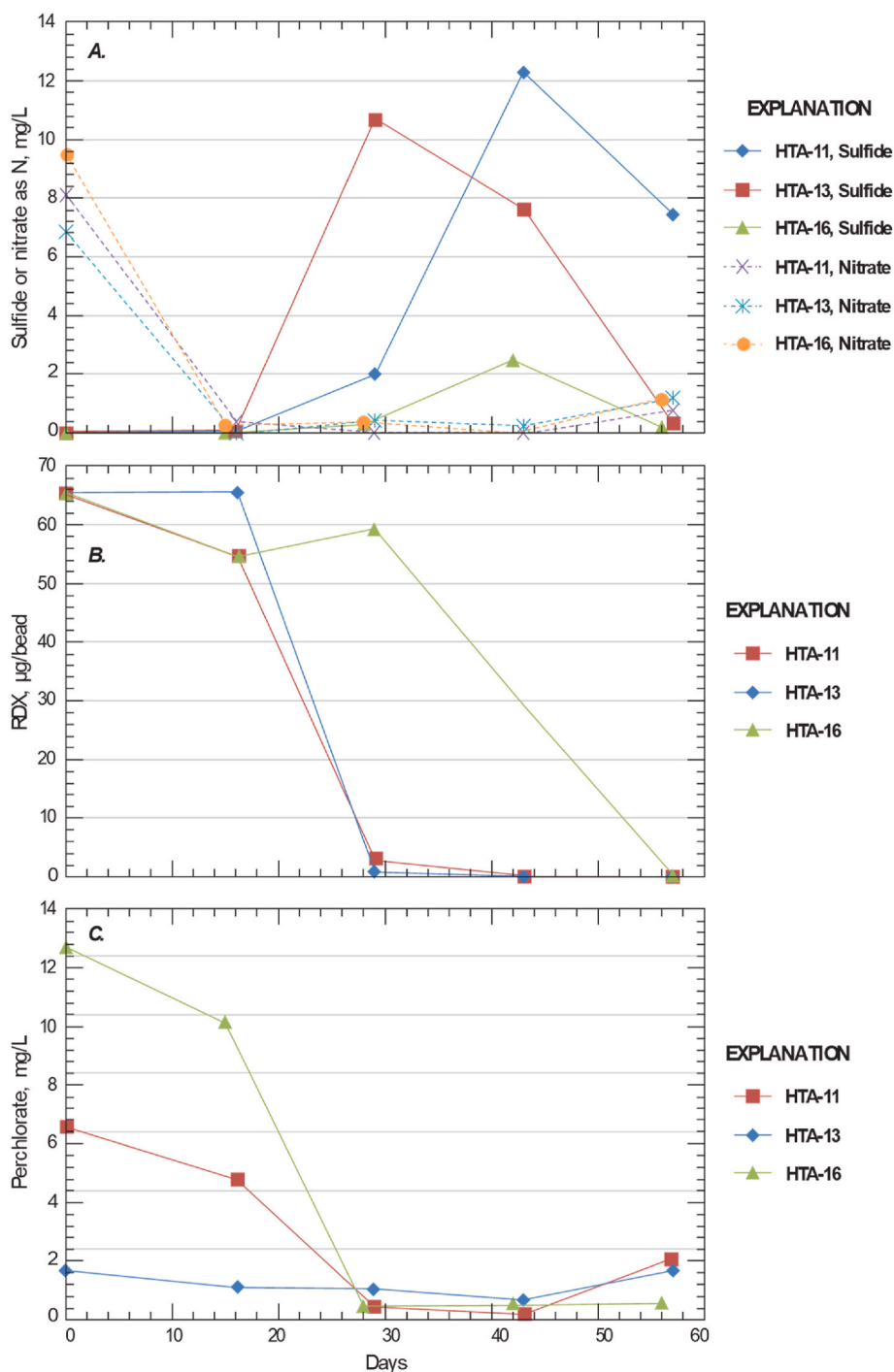


Fig. 5. Concentrations of (A) sulfide and nitrate in groundwater samples, (B) RDX in Bio-Sep bead samples, and (C) perchlorate in groundwater samples collected during the pilot injection tests in wells HTA-11, HTA-13, and HTA-16, White Sands Missile Range, New Mexico.

Table 2

Laboratory and field pilot test results for perchlorate degradation with zero-order degradation rate constants (k) and associated half-lives. [GW, groundwater; Perchlorate_i, initial perchlorate concentration in units of milligrams per liter (mg/L). Dashes indicate perchlorate degradation was not detected].

Description	Name	Perchlorate _i (mg/L)	k (mg/ L-day)	Half- life (days)	Correlation coefficient
GW tests, no WBC-2					
Site GW	GW	7.33	–	–	–
Site GW + RDX, perchlorate	GW- RP	7.15	–	–	–
Site GW + RDX, perchlorate, lactate	GW- LRP	5.76	–	–	–
Site GW + RDX, perchlorate, lactate, nitrate	GW- LNRP	5.76	0.03	100	0.611
GW tests, WBC-2 amended					
GW+3% WBC-2, RDX, perchlorate, lactate	3% WBC-2	5.98	0.12	25	0.869
GW+5% WBC-2, RDX, perchlorate, lactate	5% WBC-2	4.99	0.10	26	0.633
GW+10% WBC-2, RDX, perchlorate, lactate	10% WBC-2	6.34	0.09	34	0.885
GW+10% WBC-2, RDX, perchlorate, lactate, nitrate	10% WBC- 2-N	7.27	0.11	34	0.694
Field pilot tests, WBC-2 injected					
Well HTA-11	HTA- 11	6.8	0.17	20	0.927
Well HTA-13	HTA- 13	1.7	0.02	43	0.910
Well HTA-16	HTA- 16	14.5	0.35	21	0.905

(Table 2), unlike the first-order kinetics observed for RDX degradation (Table 1). A comparison of perchlorate degradation rates measured in biological reactors across a wide range of influent concentrations indicated a transition from first order to zero order kinetics above perchlorate concentrations of 10 mg/L (Logan and LaPoint, 2002). Initial perchlorate concentrations were less than 10 mg/L in the current microcosms, ranging from 5 to 8 mg/L (Table 2). However, it should be noted that perchlorate data were not available for the end of the groundwater microcosms (day 50) in this study, and perchlorate was not fully degraded within the 36 days measured to obtain a complete degradation profile. Degradation rate constants for perchlorate ranged from 0.09 to 0.12 mg/L-day in the WBC-2 bioaugmented groundwater, corresponding to half-lives of 25–34 days, and were unaffected by additional nitrate amendment (Table 2). These half-lives for perchlorate are lower than the RDX half-lives in groundwater treatments amended with 10% WBC-2, which were the only ones that did not show a delay in the onset of degradation. In the groundwater treatment without WBC-2, the perchlorate half-life was 100 days, substantially higher than in the bioaugmented treatments (Table 2).

Perchlorate is degraded to chlorate (ClO_3^-) and chlorite (ClO_2^-), which can further degrade to chloride and oxygen (Logan and LaPoint, 2002; Coates and Achenbach, 2004). It is possible that oxygen released during concurrent perchlorate and nitrate reduction could result in re-oxidation of ammonium and other reduced compounds, although it has been reported that consumption by perchlorate respiring bacteria prevents oxygen accumulation in solution (Logan and LaPoint, 2002).

Nitrate and ammonium are the only nitrogen species that were

analyzed in the microcosm samples. In the treatments without WBC-2, nitrate concentrations remained high throughout the test, about 35–50 mg/L as N, indicating that native bacteria capable of denitrification were absent or had a low population density (Fig. S8). Note that nitrate concentrations in the site groundwater used in the microcosms were higher than those previously measured at the site (Langman et al., 2008) and exceeded the U.S. EPA Maximum Contaminant Level and Maximum Contaminant Level Goal for drinking water of 10 mg/L for nitrate as N. Ammonium concentrations also were low or undetected (<0.10 mg/L) in the treatments without WBC-2 (Naglieri and Lorah, 2021), supporting the lack of nitrate reduction.

In contrast, the microcosms constructed with the same site groundwater and amended with WBC-2, had low (maximum of 0.163 mg/L) or undetected (<0.001 mg/L) nitrate concentrations throughout the test, indicating that nitrate reduction was rapid and complete (Naglieri and Lorah, 2021). Thus, nitrate reduction occurred more rapidly than perchlorate reduction in these bioaugmented microcosms. Even in the WBC-2 treatment amended with an additional 20 mg/L nitrate, nitrate was undetected. Ammonium was detected in the WBC-2-amended treatments, with maximum concentrations of about 1 mg/L in the microcosms amended with 3% or 5% WBC-2 and about 23–30 mg/L in the treatments with 10% WBC-2 (Fig. S8). Production of other reduced forms of nitrogen besides ammonium, including nitrite and nitrogen gas, likely account for the remaining nitrogen that was initially present in the microcosms as nitrate, along with microbial uptake of nitrogen.

RDX degradation under both anaerobic (McCormick et al., 1981; Hawari et al., 2000b) and aerobic (Fournier et al., 2002; Thompson et al., 2005) conditions can result in production of nitrate and ammonia. However, the low RDX concentrations in the microcosms would make this nitrogen contribution insignificant compared to the groundwater nitrate concentrations. In addition, a possible reason for microbial breakdown of RDX and other nitrogen containing explosives may be to obtain the nitrogen in the explosives for metabolic uptake (Zhao et al., 2003; Hatzinger and Fuller, 2014). The molar concentration of nitrogen available from ammonium in the WBC-2-amended microcosms (maximum of 2120 μM) was orders of magnitude higher than the nitrogen available from the initial RDX concentrations of about 3.2–4.4 μM (Table 1).

3.4. Pilot injection tests

Initial concentrations of the redox-sensitive constituents nitrate, ammonia, and sulfide were similar in samples from the three wells prior to injection of lactate and WBC-2 (day 0, Table S2). The relatively high nitrate concentrations (6.9–9.5 mg/L as N), low ammonium (0.12–0.15 mg/L as N), and undetectable sulfide (<0.10 mg/L) indicated oxic to mildly reducing conditions prior to injections. pH was near neutral (7.31–7.43) in the three wells. Reduction of the initial nitrate concentrations occurred within 16 days after injection, indicating effective distribution of the lactate and culture around the injection wells (Fig. 5). Similar to the results of the WBC-2 amended microcosms, ammonium concentrations remained low (<0.010–0.67 mg/L as N) throughout the field test, indicating that other reduced forms of nitrogen and microbial uptake accounted for the decreased nitrogen concentrations (Table S2). Production of sulfide was delayed in well HTA-16 compared to wells HTA-11 and HTA-13, and the maximum sulfide concentration in well HTA-16 (2.46 mg/L) was about five times lower than the concentrations in the other wells (10.7–12.3 mg/L) (Table S2).

In all the test wells, the onset of RDX degradation (measured by analysis of the Bio-Sep beads) coincided with the onset of sulfide production. The delay before the onset of RDX degradation was greatest in well HTA-16 (Fig. 5). Perchlorate concentrations also were highest in well HTA-16, with an initial concentration greater than 12 mg/L compared to 1.69 and 6.59 mg/L in wells HTA-13 and HTA-11 (Fig. 5). Rapid RDX removal occurred in well HTA-16 beginning at day 29, after perchlorate concentrations had decreased from 10 mg/L to 0.5 mg/L

and sulfide concentrations began to increase. The high perchlorate concentrations in HTA-16 most likely delayed the onset of highly reducing conditions favorable for RDX degradation by WBC-2. In contrast, rapid RDX removal was observed after day 16 in test wells (HTA-11 and HTA-13), which had lower initial perchlorate concentrations (Fig. 5).

Degradation rates of RDX and perchlorate in the pilot injection tests agreed well with those measured in the WBC-2 bioaugmented laboratory microcosm results (Tables 1 and 2). Note that degradation rates were calculated from the point of decrease in RDX or perchlorate concentrations. RDX half-lives measured in the three wells in the field test were nearly equal, ranging from 3.6 to 3.9 days, and were in between the range measured in laboratory tests with the WBC-2 in its culture media (0.8–1.2 days) and those measured in laboratory tests with WBC-2 added to site groundwater (9.0–47 days). Perchlorate half-lives in the field injection tests (20–43 days) were also very consistent with the microcosms bioaugmented with WBC-2 (25–34 days). The agreement in laboratory- and field-measured degradation rates, coupled with the other geochemical data collected, indicates that no inhibiting factors for WBC-2 degradation of RDX were present in the site groundwater after sulfate-reducing conditions were reached. Except for one detection of MNX, no RDX daughter compounds (MNX, DNX, TNX, or MEDINA) were detected on the Bio-Sep beads or in the groundwater during the field test (Table 1).

4. Implications for bioremediation

Prior data for the groundwater plume in fractured rock at WSMR indicated that RDX degradation was not occurring naturally at the site and that intervention would be needed to achieve biodegradation. Our study identified the factors inhibiting natural degradation and verified amendments that could be used to overcome these inhibitions. The results of the combined laboratory and field tests reported here demonstrate that bioaugmentation with the mixed anaerobic culture WBC-2, along with an added carbon donor, is a viable remediation approach for RDX and the co-contaminants perchlorate and nitrate. Microcosm experiments with WBC-2 in its culture media showed that anaerobic RDX biodegradation is rapid and complete. In laboratory tests with WSMR site groundwater and in field pilot tests, rapid RDX biodegradation was achieved along with reduction of relatively high concentrations of nitrate and perchlorate. Use of this anaerobic culture could be extended to other RDX-contaminated sites, with or without perchlorate and nitrate co-contaminants.

Other tests of *in situ* anaerobic bioremediation of RDX have indicated that biostimulation alone was sufficient to achieve anaerobic biodegradation; however, comingled perchlorate and nitrate were present in low concentrations or were absent in these studies (e.g., Hatzinger and Fuller, 2016), except for the study reported by Schaefer et al. (2007). Because bioaugmentation injections were made simultaneously with lactate as a carbon donor in the field pilot tests at WSMR, it was not possible to determine from the field data how effective biostimulation alone would have been. The lack of nitrate, perchlorate, or RDX removal by the native microbial community in groundwater microcosms with added lactate indicated the need for bioaugmentation at this site. In the microcosm experiments reported by Schaefer et al. (2007) with comingled contaminants, lactate addition resulted in about 50% reduction of the added RDX concentration and showed performance equal to emulsified vegetable oil for nitrate and perchlorate reduction. It is possible that longer incubation times with lactate in the microcosms with the WSMR groundwater may have allowed sufficient growth of nitrate and perchlorate reducing bacteria. The bioaugmentation pilot tests at WSMR showed that perchlorate had a greater lag time for reduction to begin than nitrate reduction, and high perchlorate concentrations (>10 mg/L) caused an increased lag time before RDX biodegradation occurred (Fig. 5). Based on the microcosm experiments, lag time for RDX degradation would have been substantially higher, if it occurred at all, if only

biostimulation was used.

Michalsen et al. (2020) showed effective bioaugmentation with a combination of a single anaerobic RDX-degrading strain and an aerobic-degrading species for a plume of high concentrations of RDX, without co-contaminants, in a sand aquifer. Presence of the co-contaminants nitrate and/or perchlorate may make the use of a mixed consortia more desirable than a single strain, providing a wider diversity of microbes for the diversity of degradation reactions needed for multiple contaminants. Although microbial community analyses could not be conducted in this study to determine specific microbes involved in the degradation reactions, WBC-2 characterization has shown a wide variety of microorganisms (Jones et al., 2006; Lorah et al., 2008a; Molenda et al., 2016). Microbial groups in WBC-2; span many of the groups identified as containing RDX-degraders, including *Pseudomonas*, *Clostridia*, and *Geobacteraceae* (Kwon and Finneran, 2006; Cho et al., 2013, 2015, 2016; Wang et al., 2017; Niedzwiecka et al., 2020). Another study showed that anaerobic cultures developed to degrade chlorinated ethenes (predominantly trichloroethene), similar to WBC-2, could also degrade RDX (Young et al., 2006). A chlorate-reducing isolate, *Pseudomonas chloritidismutans*, also has been identified in WBC-2 (Jones et al., 2006; Lorah et al., 2008a).

In addition, fractured rock aquifers can be a challenging environment for bioremediation because of frequently oxic or fluctuating redox conditions from recharge through fracture flow and lower microbial population densities than typically observed in unconsolidated (granular) aquifers (Kinner and Eighmy, 2004). The higher electron acceptor demand from the presence of the mobile co-contaminants nitrate and perchlorate could exacerbate the difficulty of achieving and maintaining suitable reducing conditions for degradation of RDX in fractured rock and affect the survival of RDX-degrading microorganisms. Previous tests with WBC-2 have shown an unusual tolerance to oxygen exposure, demonstrating a continued ability to degrade chlorinated solvents through reductive dechlorination even after saturation with oxygen (Majcher et al., 2009). Perchlorate concentrations began to rise near the end of the field test, most likely because fresh groundwater entering the injection radius increased contaminant concentrations and diluted the injected donor below effective concentrations. However, RDX concentrations remained below detection in the groundwater and on the Bio-Sep beads. The injected culture likely colonized the Bio-Sep beads and the aquifer rock surfaces during the field test and was able to maintain degradation of RDX. Thus, the field tests, as well as the year-long, repeated amendment of the culture with RDX in a laboratory test, indicated that bioaugmentation with WBC-2 could be a sustainable bioremediation approach for RDX, as well as comingled perchlorate and nitrate.

It is noted that a final remedy design would need to incorporate knowledge of the hydrogeology, including fracture locations, orientations, and connectivity, that were not addressed in these field tests. Ultimate success would be linked to the ability to distribute the amendments within the contaminated groundwater, as in any *in situ* bioremediation. The single well injection tests, however, provided validation under field environmental conditions for many critical parameters, including the donor and culture amounts needed to achieve reducing conditions for RDX and co-contaminant degradation and the biodegradation rates. Importantly, the selection of three wells with different geochemical characteristics for the pilot injection tests provided representation of the geochemical heterogeneity at the site, which is largely related to groundwater flow and fracture heterogeneity.

Credit author statement

Michelle M. Lorah: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Project administration, Funding acquisition. **Eric Vogler:** Project administration, Investigation, Funding acquisition. **Fredrick E. Gebhardt:** Investigation. Duane Graves: Methodology, Investigation, Writing – review & editing. **Jennifer F.**

Grabowski: Formal analysis, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2022.133674>.

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Enhanced Bioremediation of RDX and Co-Contaminants Perchlorate and Nitrate Using an Anaerobic Dehalogenating Consortium in a Fractured Rock Aquifer

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Supplementary Data

Table S1. Wells used for study at Open Burn/Open Detonation Site, Hazardous Test Area (HTA), White Sands Missile Range, New Mexico (from Langman et al, 2008). [USGS, U.S. Geological Survey; NAD 83, North American Datum of 1983. Porosity was previously determined to be 0.12 by a single-well injection and withdrawal tracer test in well HTA-11 (Gebhardt, F.E., 2010, unpublished data). [ft bls, feet below land surface; NA, not available. All wells 4-inch diameter with polyvinyl casing.]

Well	USGS site identification number	Latitude (NAD 83)	Longitude (NAD 83)	Screen (ft bls)	Well Depth (ft bls)
HTA-10A	322941106311502	32 29 39.82N	106 31 15.08W	NA	80
HTA-11	322941106311301	32 29 40.74N	106 31 15.04W	60-80	85
HTA-13	322938106311601	32 29 37.13N	106 31 15.84W	95-115	120
HTA-16	322937106310901	32 29 37.77N	106 31 08.50W	78-98	103

Table S2. Concentrations measured in groundwater samples collected during the field pilot test, White Sands Missile Range HTA site, New Mexico.

[Day 0 samples were collected immediately after injection of amendments. All concentrations in milligrams per liter (mg/L). Nitrate and ammonium reported as mg/L as nitrogen. Ferric iron was calculated by subtracting ferrous iron from total dissolved iron (total iron). RDX and perchlorate concentrations in duplicate groundwater samples were averaged, with one half the detection limit used for RDX non-detects. <, less than; >, greater than; --, no data.]

Days after injection	pH	Nitrate	Ammonium	Sulfide	Total iron	Ferrous iron	Ferric Iron	RDX	Perchlorate
Well HTA-11									
0	7.43	8.1	0.15	<0.010	--	--	--	0.185	6.59
16	--	0.45	0.08	0.075	2.9	1.1	1.8	0.086	4.80
29	--	<0.02	0.13	>2.0	1.1	1.0	0.1	<.001	0.48
43	--	<0.02	0.18	12.3	0.90	--	--	<.001	0.18
57	--	0.78	0.67	7.46	4.5	4.0	0.5	<.001	2.11
Well HTA-13									
0	7.31	6.9	0.13	<.010	--	--	--	0.009	1.69
16	--	<0.02	0.03	0.030	0.21	0.80	<0.01	0.004	1.10
29	--	0.42	<0.01	10.7	2.2	1.9	0.3	<.001	1.05
43	--	0.23	0.14	7.64	2.3	--	--	<.001	0.70
57	--	1.2	0.43	0.346	20.3	16.0	4.3	<.001	1.68
Well HTA-16									
0	7.38	9.5	0.12	<.010	--	--	--	0.041	12.7
15	--	0.27	0.16	<.010	0.32	0.90	<0.01	0.021	10.1
28	--	0.36	0.16	0.284	1.0	1.0	<0.1	<.001	0.50
42	--	<0.02	0.15	2.46	0.70	--	--	<.001	0.50
56	--	1.2	0.18	0.224	1.3	0.9	0.4	<.001	0.57

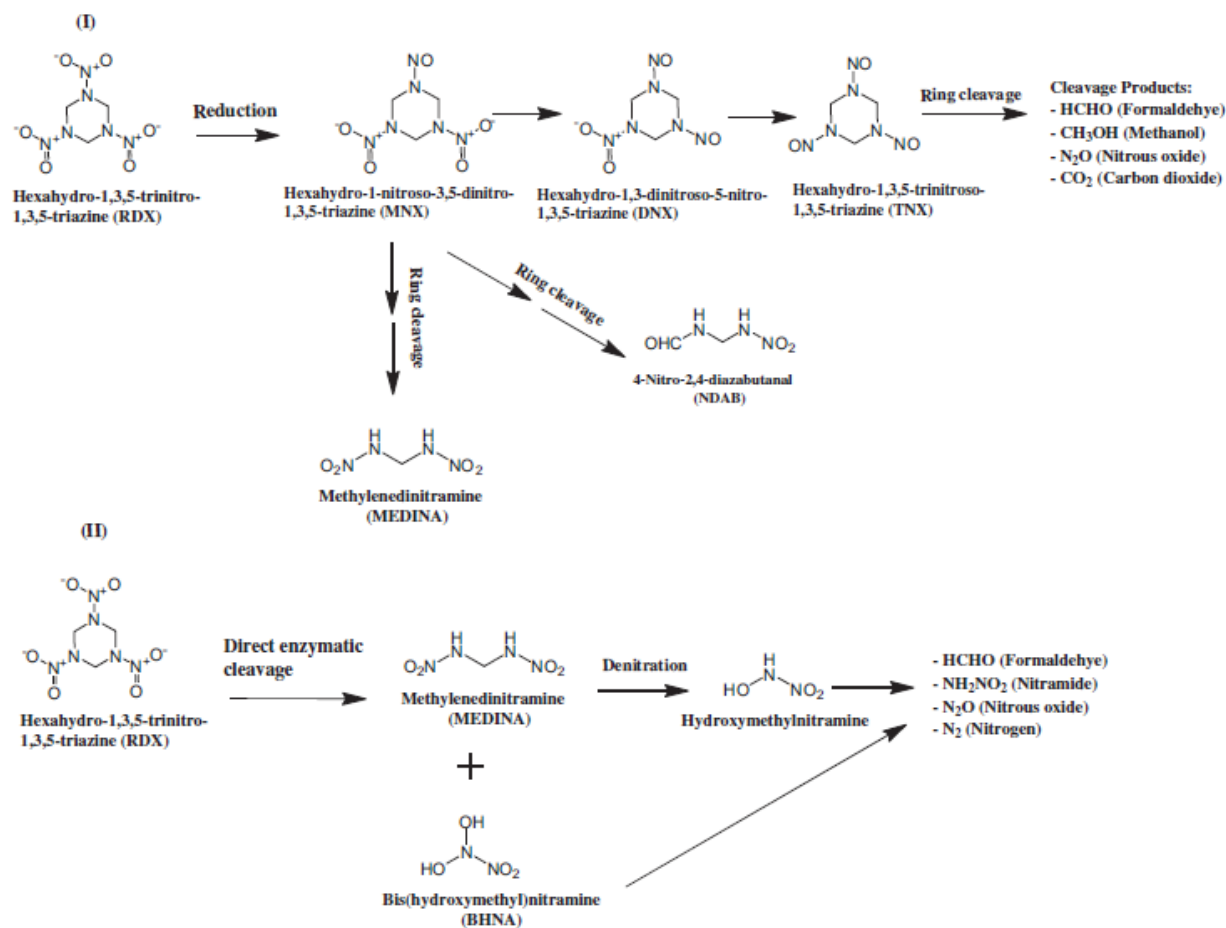


Figure S1. RDX biodegradation pathways under anaerobic conditions. Reprinted from Environmental Pollution, Vol. 178, Cho, K.-C., Lee, D.G., Roh, H., Fuller, M.E., Hatzinger, P.B., Chu, K.-H., Application of ¹³C-stable isotope probing to identify RDX-degrading microorganisms in groundwater, Pages 350-360, Copyright 2013, with permission from Elsevier, 2013.

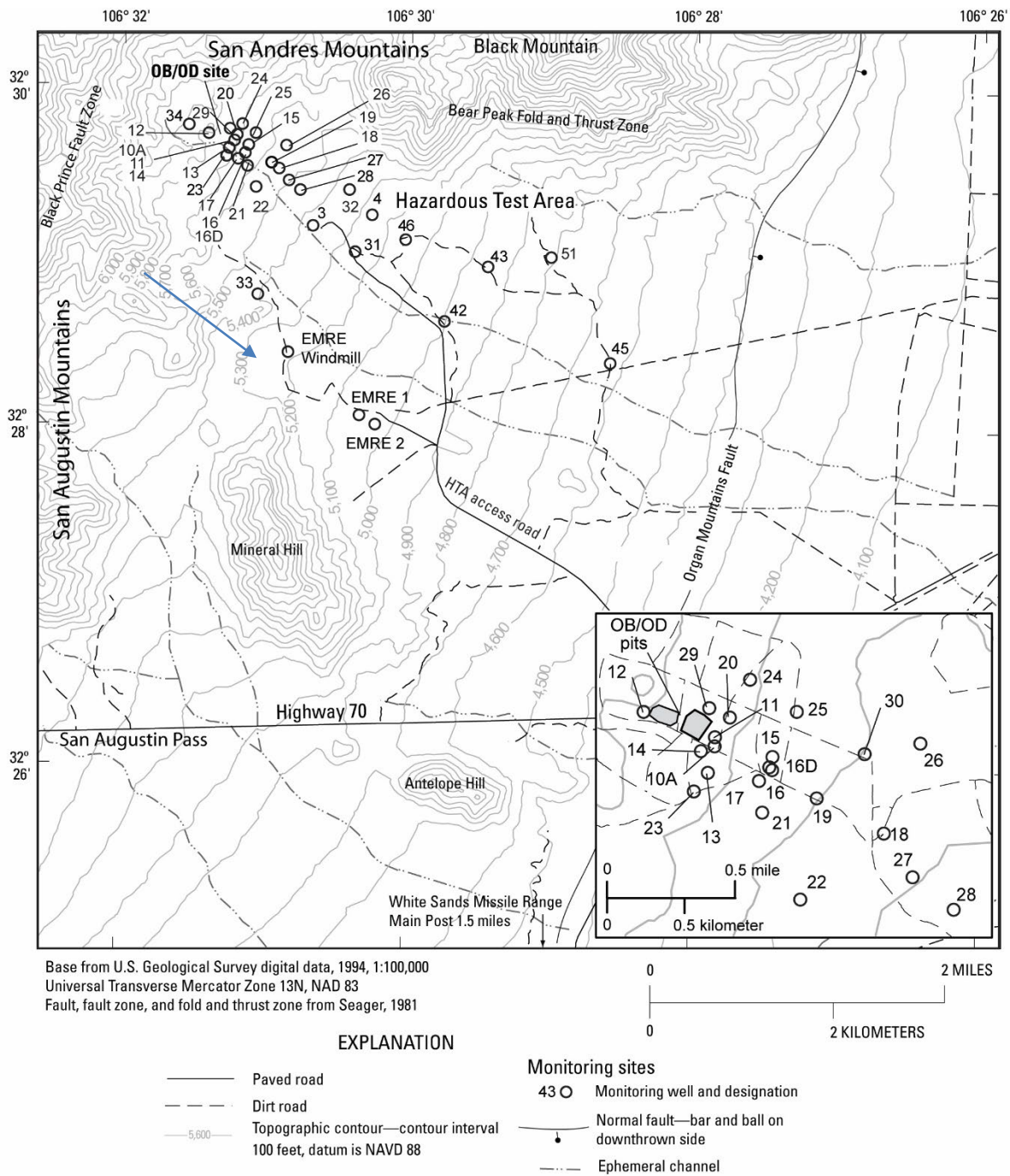


Figure S2. Location of Hazardous Test Area (HTA) and the Open Burn, Open Detonation (OB/OD) pits, White Sands Missile Range, New Mexico. All well names are preceded by “HTA-” in name. Groundwater for microcosms was collected from well HTA-10A, and pilot injection tests were conducted at HTA-11, -13, and -16. Blue arrow shows overall flow direction, but flow is contained in fracture systems. Modified from figures 2 and 26 in Langman et al. (2008).

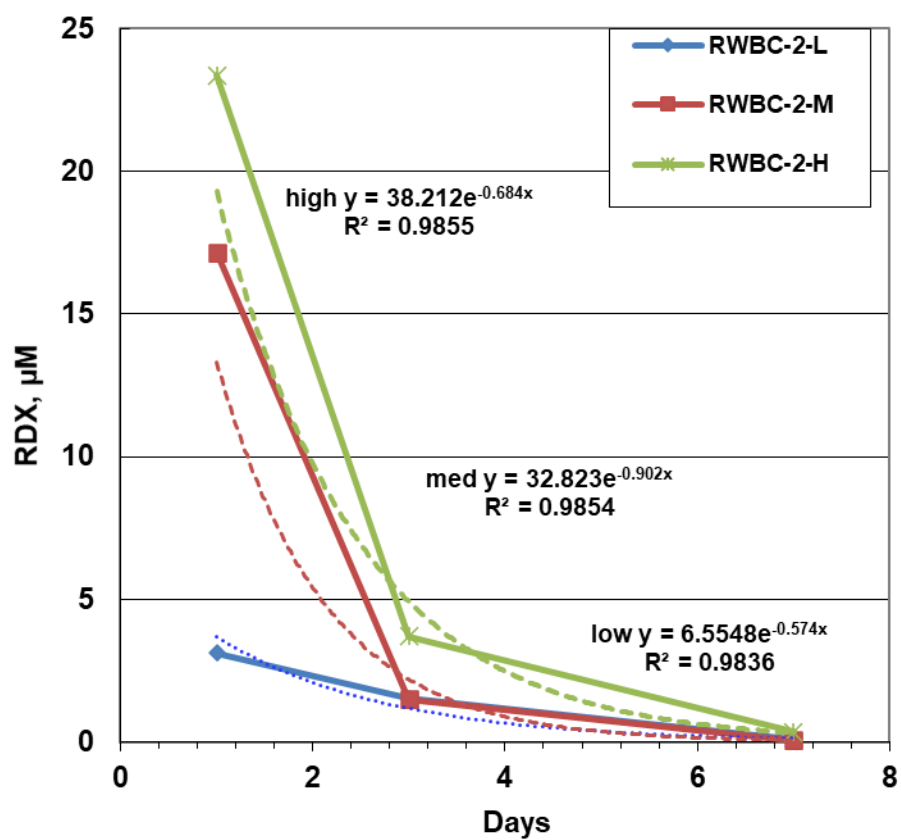


Figure S3. First-order degradation kinetics for RDX removal in WBC-2 and culture media in microcosms with low (-L), medium (-M), and high (-H) initial concentrations of RDX. Dashed lines show the exponential fit to the data shown in the respective solid color to obtain the first-order degradation rates.

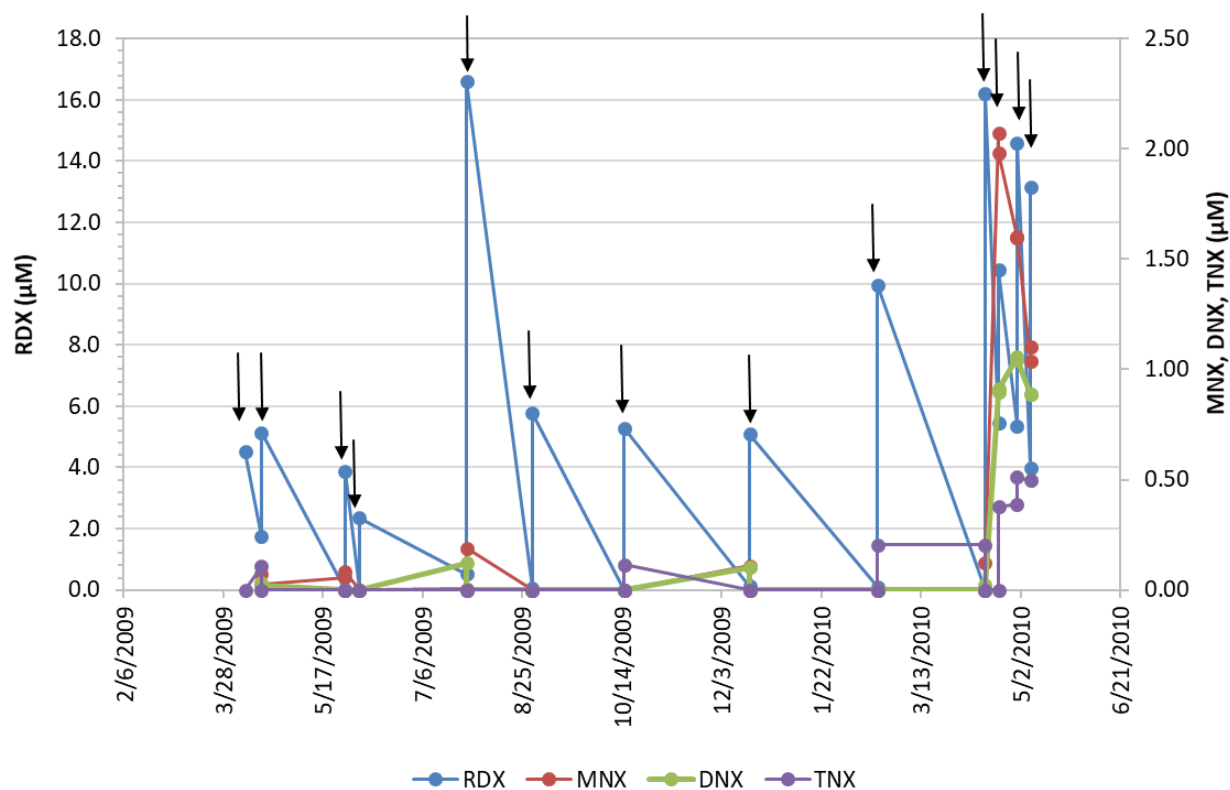


Figure S4. Concentrations of RDX and daughter products MNX, DNX, and TNX in the duplicate microcosm bottle that contained WBC-2 in anaerobic culture media and was repeatedly amended with RDX and lactate. Arrows show dates of amendment.

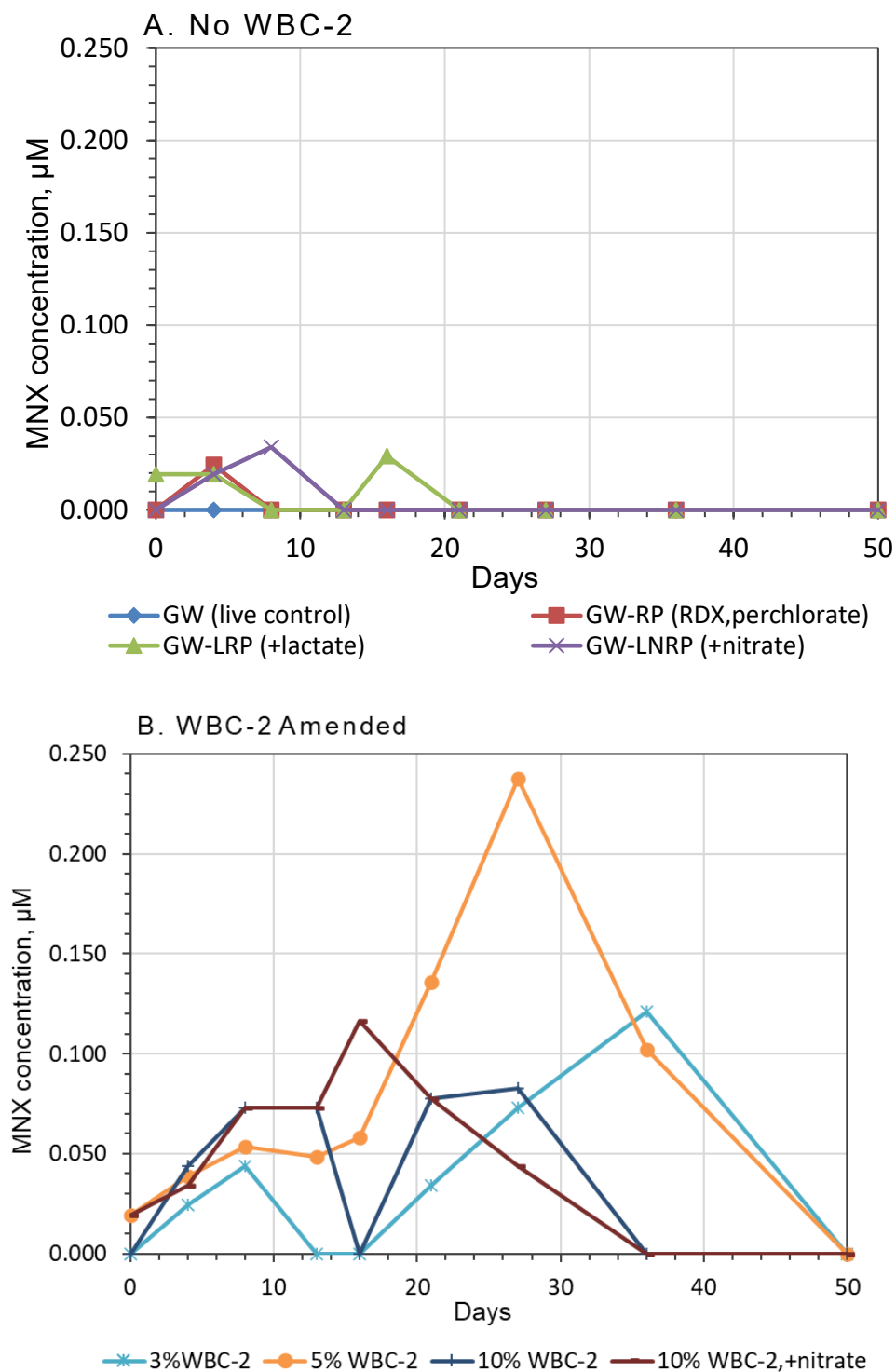


Figure S5. MNX concentrations in microcosms with site groundwater (GW) that (A) was not amended with WBC-2 and (B) was amended with WBC-2 at 3, 5 or 10% by volume. Except for the GW live control, all microcosms were amended with RDX and contained perchlorate; except for the GW live control and treatment GW-RP, all microcosms were also amended with lactate.

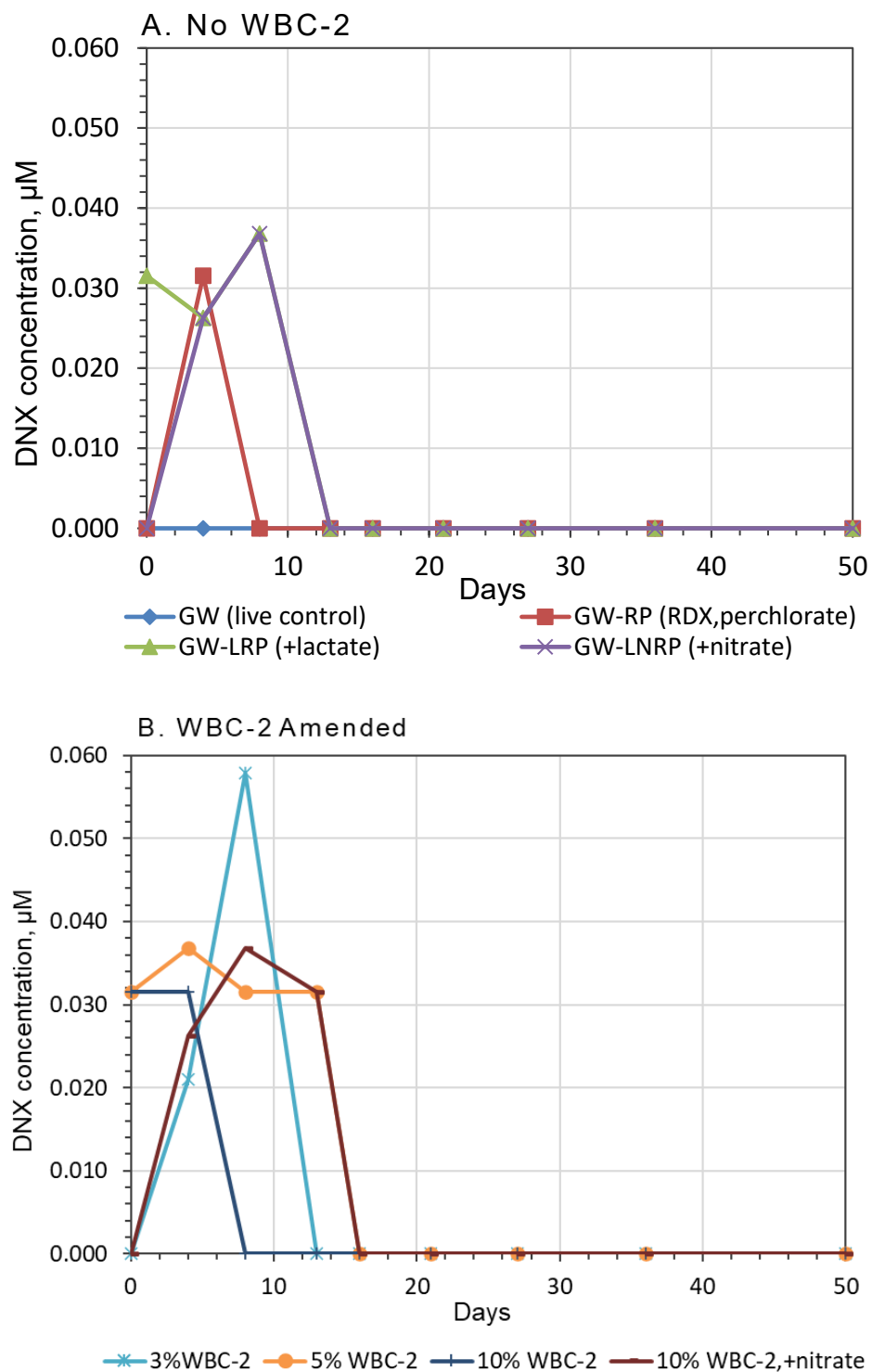


Figure S6. DNX concentrations in microcosms with site groundwater (GW) that (A) was not amended with WBC-2 and (B) was amended with WBC-2 at 3, 5 or 10% by volume. Except for the GW live control, all microcosms were amended with RDX and contained perchlorate; except for the GW live control and treatment GW-RP, all microcosms were also amended with lactate.

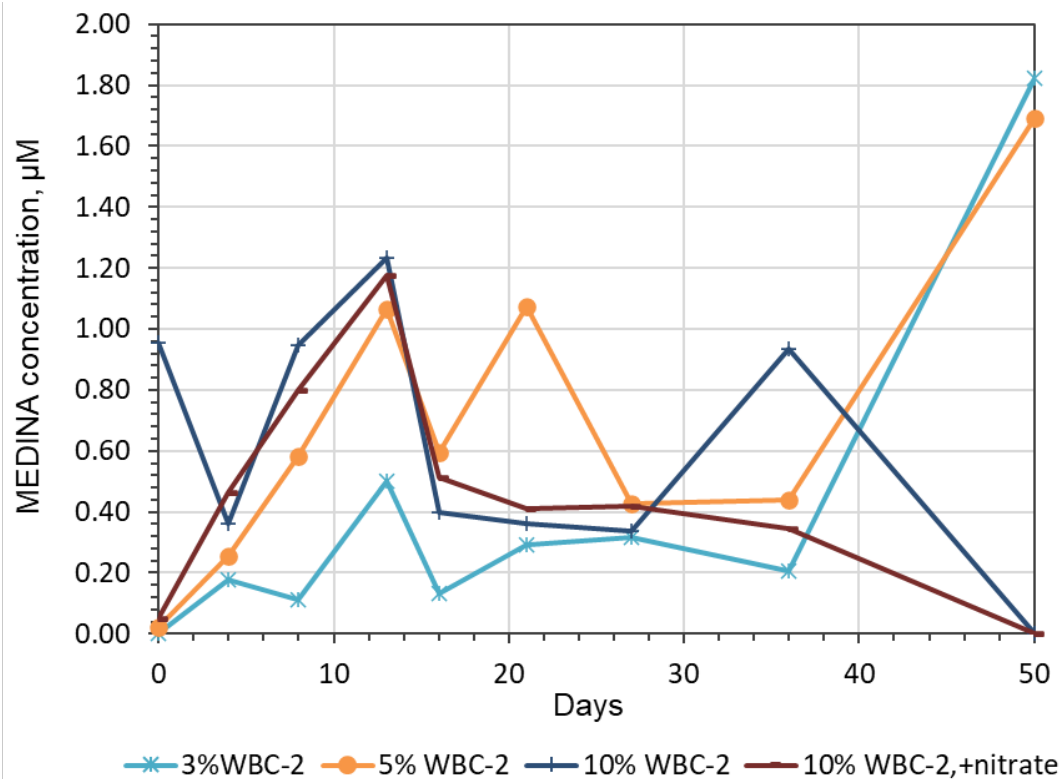


Figure S7. MEDINA concentrations in microcosms with site groundwater that was amended with WBC-2 at 3, 5 or 10% by volume. MEDINA concentrations were below detection in all microcosms that were not amended with WBC-2 and are not shown (Naglieri and Lorah, 2021). All microcosms were amended with RDX and lactate; all microcosms also contained perchlorate that was present in the site groundwater used to construct microcosms.

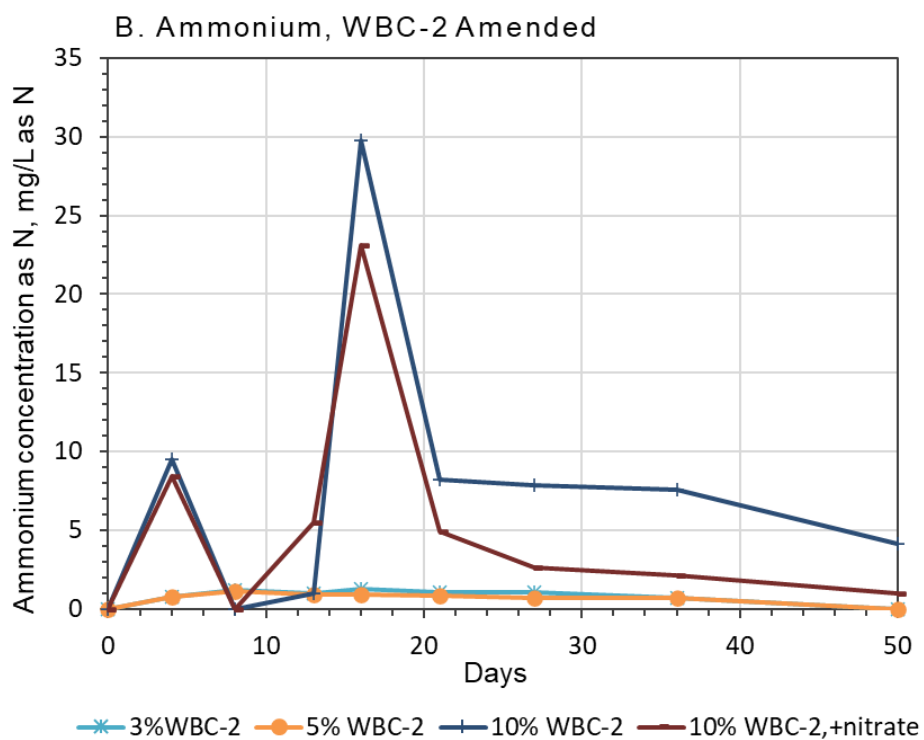
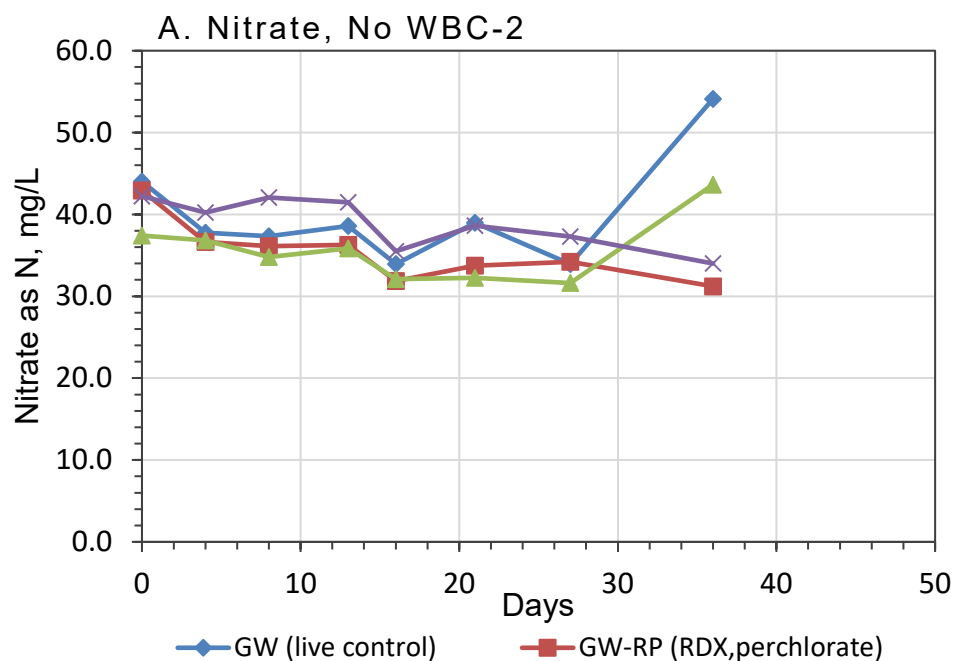


Figure S8. Concentrations of (A) nitrate in microcosms with site groundwater (GW) that was not amended with WBC-2 and (B) ammonium in microcosms with site groundwater that was amended with WBC-2 at 3, 5 or 10% by volume. Nitrate concentrations were below detection (<0.001 mg/L) in the WBC-2 amended microcosms (Naglieri and Lorah, 2021) and are not shown. Note that nitrate was not measured at the last sampling point (day 50).