Bioremediation of Nitroaromatic and Haloaromatic Compounds

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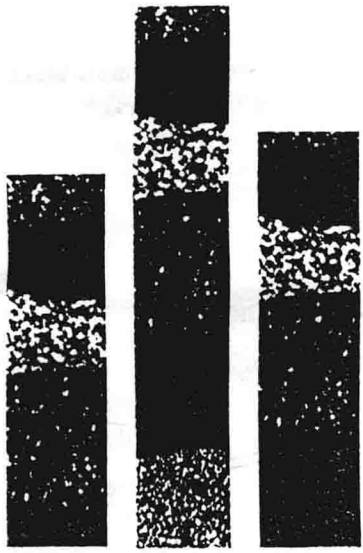

Editors

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and Andrea Leeson
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FOREWORD

The Fifth International In Situ and On-Site Bioremediation Symposium was held in San Diego, California, April 19–22, 1999. The program included approximately 600 platform and poster presentations, encompassing laboratory, bench-scale, and full-scale field studies being conducted worldwide on a variety of bioremediation and supporting technologies used for a wide range of contaminants.

The author of each presentation accepted for the program was invited to prepare a six-page paper, formatted according to specifications provided by the Symposium Organizing Committee. Approximately 400 such technical notes were received. The editors conducted a review of all papers. Ultimately, 389 papers were accepted for publication and assembled into the following eight volumes:

Natural Attenuation of Chlorinated Solvents, Petroleum Hydrocarbons, and Other Organic Compounds – Volume 5(1)
Engineered Approaches for In Situ Bioremediation of Chlorinated Solvent Contamination – Volume 5(2)
In Situ Bioremediation of Petroleum Hydrocarbon and Other Organic Compounds – Volume 5(3)
Bioremediation of Metals and Inorganic Compounds – Volume 5(4)
Bioreactor and Ex Situ Biological Treatment Technologies – Volume 5(5)
Phytoremediation and Innovative Strategies for Specialized Remedial Applications – Volume 5(6)
Bioremediation of Nitroaromatic and Haloaromatic Compounds – Volume 5(7)
Bioremediation Technologies for Polycyclic Aromatic Hydrocarbon Compounds – Volume 5(8)

Each volume contains comprehensive keyword and author indices to the entire set.

This volume deals with the application of bioremediation technologies at sites contaminated with explosives, pesticides, herbicides, PCBs, and other aromatic compounds. Such sites present formidable technical, regulatory, and financial challenges. Bioremediation offers the promise of cost-effective site remediation that can serve as a key component of a well-formulated strategy for achieving site closure. This volume presents the results of bench-, pilot-, and field-scale projects focused on the use of biological approaches to remediate many problem compounds, such as RDX, HMX, TNT, DDT, 2,4-D, nitro- and chlorobenzenes, nitroaniline, chloroaniline, hexachlorobenzene, PCPs, PCBs, and dichlorophenol in soils and groundwater.

We would like to thank the Battelle staff who assembled the eight volumes and prepared them for printing. Carol Young, Lori Helsel, Loretta Bahn, Gina Melaragno, Timothy Lundgren, Tom Wilk, and Lynn Copley-Graves spent many hours on production tasks—developing the detailed format specifications sent to each author; examining each technical note to ensure that it met basic page layout requirements and making adjustments when necessary; assembling the

volumes; applying headers and page numbers; compiling the tables of contents and author and keyword indices, and performing a final check of the pages before submitting them to the publisher. Joseph Sheldrick, manager of Battelle Press, provided valuable production-planning advice and coordinated with the printer; he and Gar Dingess designed the covers.

The Bioremediation Symposium is sponsored and organized by Battelle Memorial Institute, with the assistance of a number of environmental remediation organizations. In 1999, the following co-sponsors made financial contributions toward the Symposium:

Celtic Technologies
Gas Research Institute (GRI)
IT Group, Inc.
Parsons Engineering Science, Inc.
U.S. Microbics, Inc.
U.S. Naval Facilities Engineering Command
Waste Management, Inc.

Additional participating organizations assisted with distribution of information about the Symposium:

Ajou University, College of Engineering
American Petroleum Institute
Asian Institute of Technology
Conor Pacific Environmental Technologies, Inc.
Mitsubishi Corporation
National Center for Integrated Bioremediation Research & Development (University of Michigan)
U.S. Air Force Center for Environmental Excellence
U.S. Air Force Research Laboratory Air Base and Environmental Technology Division
U.S. Environmental Protection Agency
Western Region Hazardous Substance Research Center (Stanford University and Oregon State University)

The materials in these volumes represent the authors' results and interpretations. The support of the Symposium provided by Battelle, the co-sponsors, and the participating organizations should not be construed as their endorsement of the content of these volumes.

Bruce Alleman and Andrea Leeson, Battelle
1999 Bioremediation Symposium Co-Chairs

CONTENTS

BIODEGRADATION OF RDX AND HMX BY A METHANOGENIC ENRICHMENT CULTURE

Neal R. Adrian and *Anna Lowder* (US Army CERL, Champaign, IL)

ABSTRACT: The biotransformation of RDX and HMX by a methanogenic enrichment culture was studied. The enrichment culture only degraded RDX when ethanol was included as an electron donor. Methane production, however, was only observed after RDX had been depleted. The addition of BESA inhibited methane production, but not ethanol fermentation nor RDX degradation. The addition of H_2 gas supported RDX degradation. HMX was also degraded, but the degradation rate (0.12 μM day^{-1}) was nearly 10-fold less than observed for RDX (1.6 μM day^{-1}). HMX degradation slowed when ethanol was depleted, but resumed after reamending the bottles with ethanol. The total number of anaerobes in the enrichment culture ranged from 1.5 x 10^5 to 1.6 x 10^6 ml^{-1}. Ethanol-fermenting syntrophs ranged from 1.5 x 10^4 to 1.6 x 10^5 ml-1; H_2-utilizing methanogens ranged from 7.0 x 10^1 to 7.6 x 10^2 ml^{-1}; acetoclastic methanogens were $< 1.0 \times 10^1$ ml^{-1}; and H_2-utilizing acetogens ranged from 3.8 x 10^3 to 4.2 x 10^4 ml^{-1}. These findings indicate ethanol may serve as a source of H_2 for the RDX and HMX degrading bacteria. The presence of acetogens and a lack of CH_4 production during ethanol degradation may indicate their involvement in the biodegradation of nitramine explosives.

INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetrazocine (HMX) are nitramine explosives widely used by the military (Gorontzy et al., 1994). Improper disposal of RDX and HMX contaminated wastewater in the past has led to environmental contamination near ordnance sites and Army ammunition manufacturing plants (Funk et al., 1993). Despite the Army's apparent need for information on the biodegradation of nitramine explosives, relatively little is known (Gorontzy et al., 1994). RDX is reported to be more easily biodegraded under anaerobic, rather than aerobic conditions (Funk et al., 1993; Kitts et al., 1994; McCormick et al., 1981). The few exceptions include RDX biodegradation by a white rot fungus (Fernando and Aust, 1991), by *Stenotrophomonas maltophilia* PB1 when using RDX as the sole source of nitrogen (Binks et al., 1995), and during composting of explosives contaminated soil (Williams et al., 1992). Studies demonstrating RDX biodegradation under anaerobic conditions, however, were carried out where the electron donors and electron acceptors were not firmly established. The lack of adequate information makes it difficult to develop a biological approach for wastewater treatment or cleanup of contaminated groundwater. Our primary objectives for this study were to study the microbiology and biodegradation of RDX and HMX in a

methanogenic enrichment culture. In this presentation, we report on the biodegradation of RDX and HMX and the potential involvement of acetogens in their degradation.

MATERIALS AND METHODS

A five-tube most-probable-number procedure was used to enumerate total anaerobes, H_2-utilizing and acetoclastic methanogens, ethanol-metabolizing syntrophs, and H_2-utilizing acetogens. The MPN tubes were incubated at ambient room temperature and scored after 21 days incubation. Biodegradation studies were carried out in serum bottles (160 ml) containing 80 ml of a basal salts medium and 20 mls of the RDX-degrading enrichment culture. Filter-sterilized resazurin (0.0002%) was added as a redox indicator. The basal salts medium and enrichment culture were dispensed to the serum bottles using strict anoxic techniques, sealed with butyl rubber stoppers and then crimped with aluminum seals. The headspace of the bottles was evacuated and filled three times with N_2:CO_2 (80:20), and then pressurized to 1.3 ATM. The study was conducted in triplicate and employed sterile and RDX unamended controls.

Liquid samples were taken periodically and analyzed by reverse phase high-pressure liquid chromatography (HPLC) and gas chromatography (GC) for RDX and acetate, and ethanol, respectively. The headspace of the serum bottles was monitored for the formation of CH_4 by GC. Methane produced from unamended controls was subtracted from that produced in substrate-amended bottles. This amount was compared to the theoretically expected amount of CH_4 (Gottschalk, 1986).

RESULTS

The number of total anaerobes and those comprising four major metabolic groups of bacteria were enumerated in an RDX-degrading culture (Table 1). Total anaerobes ranged from 7.0×10^5 to 7.6×10^6 ml^{-1}. Interestingly, we did not observe any colony forming units on the plate count agar plates incubated aerobically, indicating there were no aerobic bacteria or facultative anaerobes capable of growing under the test conditions. Acetoclastic methanogens were present at relatively low numbers (<10 ml^{-1}) compared to the H_2-utilizing methanogens (3.3×10^2 ml^{-1}). H_2-utilizing acetogens were present in numbers similar to the hydrogenotrophic methanogens, ranging from 3.9×10^2 to 4.2×10^3 ml^{-1}. The ethanol fermenting syntrophs ranged from 3.9×10^2 to 4.2×10^3 ml^{-1}.

A major band was observed in denaturing gradient gel electrophoresis (DGGE) of the 16S rDNA gene in a sample taken from the culture. This band was sequenced and closely resembled *Acetobacterium malicum*. A second major band was observed and sequenced, closely resembling the sequence for *Geobacter akaganeitreducens*.

The enrichment culture only degraded RDX when ethanol was included as an electron donor (Figure 1). Approximately 32 μM RDX was biodegraded in 17 days. Methane production was only observed after RDX had been depleted, while RDX unamended controls experienced no lag in methane production.

Table 1. The number of total anaerobes and those bacteria comprising four major metabolic groups in the RDX-degrading culture. Numbers reported are bacteria ml^{-1}. ND = not detected.

Metabolic Group	Culture A
Aerobes	ND
Total anaerobes	2.3×10^6
Proton-reducing syntrophs	1.3×10^3
H_2-using methanogens	3.3×10^2
Acetoclastic methanogens	$< 1 \times 10^1$
Acetogens	*2.3×10^2*

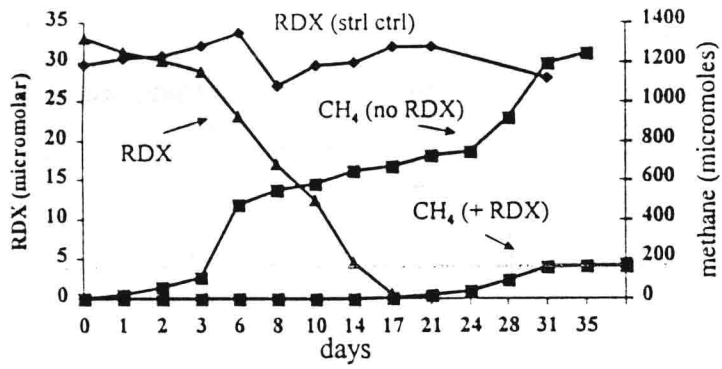

Figure 1. RDX degradation and methane formation by culture. Initial ethanol concentration was 10 mM.

Acetate formation was only observed in bottles amended with ethanol (Figure 2). In bottles amended with ethanol and RDX, on day 21 106% of the expected amount of acetate was produced from the transformation of ethanol to acetate and H_2 (Figure 2). Acetate continued to accumulate to approximately 11.5 mM, accounting for 114% of the expected amount of acetate (Figure 2). After day 35, methane production started to increase and acetate concentrations decreased, indicating acetoclastic methanogens were responsible for the observed activity.

Approximately 110% of the expected amount of methane was observed from ethanol in RDX unamended bottles, while only 14% in bottles containing RDX (Table 2).

The addition of BESA inhibited methane production, but not ethanol fermentation nor RDX degradation. RDX degradation was more rapid when H_2 gas was the electron donor (4.4 μM day^{-1}) compared to ethanol (1.9 μM day^{-1}). There was no loss of RDX in sterile or electron donor unamended controls.

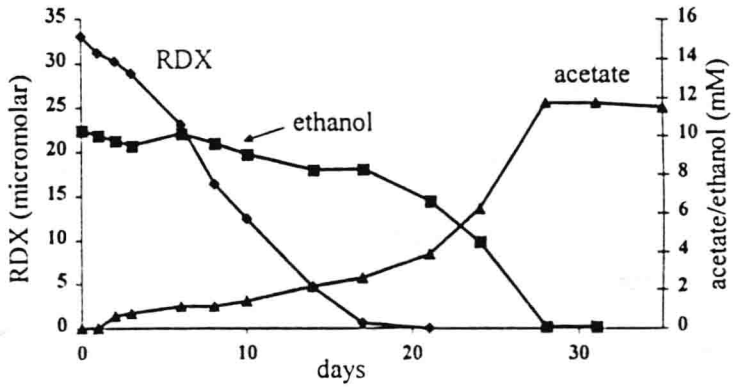

Figure 2. RDX degradation and acetate formation in methanogenic enrichment culture fed ethanol.

Table 2. Methane formation by the enrichment culture fed ethanol only or ethanol and RDX.

	Initial μmoles	Final μmoles[a]	μmoles depleted	CH_4 Exp.[b]	CH_4 Observed	% CH_4 Recovered[c]
Ethanol						
Ethanol	1030	177	853	426		
Acetate	0	619	233	233		
RDX	0	-	-	-		
Total				660	729	110
Ethanol + RDX						
Ethanol	1030	775	255	127		
Acetate	0	272	0	0		
RDX	3	0	3			
Total				127	18	14

(a) μmoles substrate remaining after 21 days incubation

(b) CH_4 expected based on the following equations: 2 ethanol + HCO_3^- ↔ 2 acetate$^-$ + CH_4 + H_2O + H^+; acetate ↔ CH_4 + CO_2

(c) % CH_4 recovered = (expected / observed)*100

HMX was more resistant to degradation (Figure 3). Approximately 8 μM HMX was degraded in 55 days (0.15 μM day^{-1}). HMX degradation slowed when ethanol was depleted, but resumed after reamending the bottles with ethanol. There was no loss of HMX in sterile or ethanol unamended controls.

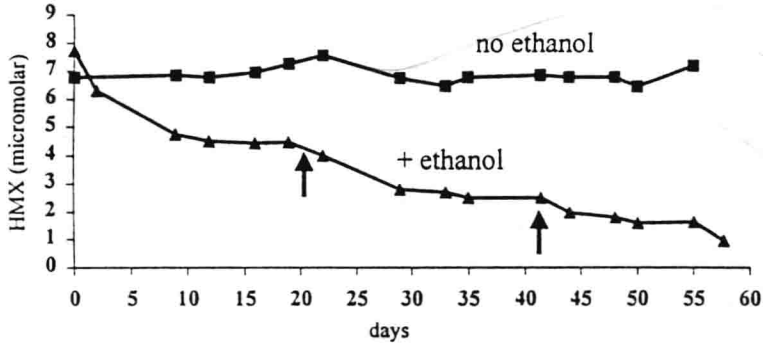

Figure 3. HMX degradation by methanogenic enrichment culture. The initial ethanol concentration was 7 mM. Arrows indicate the reamendment of ethanol to the culture.

DISCUSSION

Our results demonstrate ethanol is required by the enrichment culture for RDX and HMX degradation. In a methanogenic culture, such as the RDX-degrading enrichment one, H_2 must be removed for ethanol degradation to occur, and when CO_2 is the only available electron acceptor, CH_4 production will be observed. However, there was no methane production by the culture from ethanol in the presence of RDX. Ethanol was degraded (Figure 2) and acetate concentrations increased (Figure 3), therefore H_2 must have been removed for ethanol to be degraded. We believe the acetogens may be responsible for this, as well as degrading the explosives. In support of this, a major band in DGGE gels closely matched the sequence from *Acetobacterium malicum*, an acetogen common to fresh water sediments (Tanaka and Pfennig, 1988). Interestingly, RDX degradation rates for culture A are almost 2-fold greater than culture B, a culture similar to A in many respects, but lacks a major band in DGGE gels corresponding to an acetogen. Future studies will investigate in more detail the role of acetogens in degrading nitramine explosives.

Acknowledgements

We are thankful to Greg Davis from Microbial Insights for identifying bacteria by DGGE analysis of polymerase chain reaction-amplified genes coding for 16S rDNA.

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FIELD DEMONSTRATION OF FBR FOR TREATMENT OF NITROTOLUENES IN GROUNDWATER

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ABSTRACT: A pilot-scale field demonstration was conducted to collect reliable cost and performance data for an aerobic, biological fluidized bed reactor (FBR) system that treats groundwater contaminated with nitrotoluenes. This project is an extension of a previous bench-scale FBR study on the simultaneous degradation of 2,4- and 2,6-dinitrotoluene. The FBR was inoculated with a mixed culture of bacteria that had been acclimated to a mixture of mono- and dinitrotoluenes. The contaminated groundwater was drawn from wells at the Volunteer Army Ammunition Plant (VAAP) in Chattanooga, TN. The nitrotoluene (NT) contaminants identified in the groundwater and their typical concentrations were: 2,4,6-trinitrotoluene (2,4,6-TNT) at 1.1 mg/L, 2,4-dinitrotoluene (2,4-DNT) at 9.2 mg/L, 2,6-dinitrotoluene (2,6-DNT) at 8.6 mg/L, 2-nitrotoluene (2-NT) at 2.9 mg/L, 3-nitrotoluene (3-NT) at 0.4 mg/L, and 4-nitrotoluene (4-NT) at 3.0 mg/L.

A range of loading rates and a variety of operating conditions were used to evaluate the FBR performance. The COD and NT loading rates, based on the volume of the fluidized bed, ranged from 0.6-11 kg COD/m^3/d and 0.45-4.5 kg NT/m^3/d. The best FBR performance was measured at the lower loading rates; a 96% reduction in NT and 93% reduction in COD were found at a loading rate of 1.0 kg NT/m^3/d (2.2 kg COD/m^3/d). The effluent at this loading rate contained an average of 0.5 mg 2,4,6-TNT and 0.8 mg 2,6-DNT per liter. The other NT compounds were below detection levels (0.05 mg/L). At the highest steady loading rate, 3.5 kg NT/m^3/d (7.6 kg COD/m^3/d), a 62% reduction in NT and 49% reduction in COD were measured. The effluent at this loading contained an average of 0.8 mg/L 2,4,6-TNT, 7.0 mg/L 2,6-DNT and 1.3 mg/L 2,4-DNT. The mononitrotoluenes were still below detection levels at the highest loading rate. Therefore, this FBR process can effectively remove mono- and dinitrotoluenes from contaminated groundwater, and at appropriate loading rates the FBR process is cost-effective compared to alternative technologies.

INTRODUCTION

The Volunteer Army Ammunition Plant in Chattanooga, TN (VAAP), produced and stored 2,4,6-trinitrotoluene (TNT) until 1977. As a result the

groundwater is contaminated with TNT and other nitrotoluenes. At VAAP and other DoD sites nitroaromatic contaminants in the groundwater are the target for remediation. The current treatment strategy involves carbon adsorption. Because of the high cost of carbon regeneration the DoD has identified a need to develop alternative treatment technologies for extracted groundwater at such sites.

This paper summarizes the findings of a demonstration of an aerobic dinitrotoluene (DNT) degradation process in a fluidized bed reactor (FBR) system. The demonstration, conducted over an 11-month period, evaluated the ability of the system to simultaneously degrade 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT), as well as the three mononitrotoluenes (MNTs: 2-NT, 3-NT, 4-NT), in groundwater extracted during remediation activities. Field demonstrations of FBR technology for the degradation of chlorinated solvents (e.g., TCE, chlorobenzene) in contaminated groundwater have been successfully conducted (Guarini and Folsom, 1996; Klecka, et al., 1986). This work was the first field demonstration of the FBR technology for treatment of recalcitrant nitroaromatic compounds, such as 2,4-DNT and 2,6-DNT.

Aerobic DNT Biodegradation Process. The aerobic biodegradation strategy involved a mixed culture of microorganisms that has been shown to simultaneously degrade 2,4-DNT and 2,6-DNT in a continuous flow bioreactor (Lendenmann, et al., 1998). Isolated strains of bacteria are capable of utilizing the individual isomers of DNT as growth substrates and the degradation pathways have been determined (Spanggord et al., 1991; Suen and Spain, 1993; Haigler, et al., 1994; Nishino and Spain, 1996). Burkholderia sp. DNT degraded 2,4-DNT at a hydraulic residence time (HRT) of 1 hour in a laboratory-scale fixed bed bioreactor (Heinze et al., 1995).

Although isolated strains were able to use 2,6-DNT as the sole growth substrate, low concentrations of 2,6-DNT inhibited growth of both 2,4-DNT and 2,6-DNT degrading strains (Nishino and Spain, 1996). Degradation of isomeric DNT mixtures was possible in continuous bioreactor systems if the concentration of 2,6-DNT could be maintained at a low steady-state level. The efficacy of the approach was proven in a study which demonstrated simultaneous degradation of 2,4-DNT and 2,6-DNT in a laboratory bench-scale FBR using a mixed culture of bacteria and sand as the support medium (Lendenmann, et al., 1998). Destruction efficiencies of greater than 98 percent for 2,4-DNT and 94 percent for 2,6-DNT were achieved at all loading rates.

FBR Process Description. The FBR system used in the present study coupled the aerobic biodegradation process with a high rate reactor design, using GAC as the support. The basic components of the FBR system include the bioreactor column, the GAC, a suitable biocatalyst, a fluid distribution system in the bottom of the reactor, an oxygenator, a high purity oxygen supply source, feed and influent pumps, a nutrient addition system, and a pH control mechanism. The pilot FBR system (EFB-02, 2" diameter) is designed to operate continuously, 24 hours a day, 7 days a week. The feed pump supplies contaminated groundwater to

the system on the suction side of the influent pump. The influent pump circulates the feed water plus recycle water through the oxygenator, where gaseous O_2 is dissolved in the liquid, then into the reactor column in an upward direction fluidizing the bed of GAC media. Microbes attach to the GAC media within the bioreactor and metabolize passing contaminants. A constant liquid level is maintained in the FBR and effluent leaves by gravity flow from the top of the reactor column. Nutrients and pH control chemicals are added in the recycle line. The influent flowrate is automatically controlled in order to achieve stable bed expansion.

EXPERIMENTAL DESIGN AND OPERATION

The field pilot demonstration was designed to meet the following objectives:

1. to determine the feasibility of implementing aerobic, microbial GAC FBR technology in the field to remediate groundwater contaminated with 2,4-DNT and 2,6-DNT, and 2-NT, 3-NT and 4.NT;
2. to obtain removal efficiencies and removal rates for (a) total organics, and (b) nitroaromatics as a function of inlet loading and expanded bed hydraulic retention times;
3. to obtain real-world operating characteristics for groundwater pump-and-treat applications to assess the extent of operator attention required;
4. to obtain data useful for the design of suitable full-scale FBR systems; and
5. to compare the capital and operating costs of the aerobic, microbial FBR technology to those of conventional treatment technologies.

The demonstration consisted of seven evaluation periods (referred to as phases) during the FBR operation and testing. For each evaluation period the conditions were varied to determine the effect on overall nitrotoluene (2,4-DNT, 2,6-DNT, 2,4,6,-TNT, 2-NT, 3-NT and 4-NT) removal. Each operating condition was maintained for a sufficient time to achieve stable effluent quality. The seven phases included five different feed flowrates and two different feed water compositions. The feed water composition was changed by varying the ratio of extracted groundwater from two wells which had different NT concentrations. A comparative analysis of the contaminant removal efficiency measured during each evaluation period was used to determine the optimal loading condition.

Phases 1 through 4 produced the data to evaluate the FBR loading capacity and treatment performance with different feed flowrates. Phase 5 produced the data to evaluate the impact of other FBR operating parameters (for example, the set point for dissolved oxygen concentration) on performance. Phases 6 and 7 produced the data to re-evaluate the performance after activity towards 2,6-DNT greatly improved. Under each operating condition overall DNT, TNT and MNT removal results were determined. Bench-top flask assays were performed at regular intervals to confirm that removal of the nitroaromatic compounds was due to biological activity and not adsorption to the GAC media or by stripping.