Journal of Hazardous Materials 297 (2015) 42–51

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Application of ^{13}C and ^{15}N stable isotope probing to characterize RDX degrading microbial communities under different electron-accepting conditions

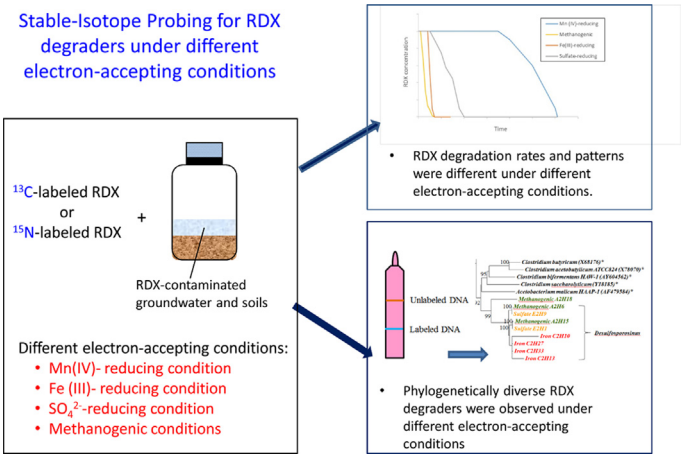
Kun-Ching Cho[a], Do Gyun Lee[a], Mark E. Fuller[b], Paul B. Hatzinger[b], Charles W. Condee[b], Kung-Hui Chu[a,*]

[a] Zachry Department of Civil Engineering, Texas A&M University, College Station, TX 77843-3136, USA
[b] CB&I Federal Services, Lawrenceville, NJ, USA

HIGHLIGHTS

- SIP characterized RDX-degrading communities under different e-accepting conditions.
- Dominant RDX degradation pathways differed under different e-accepting conditions.
- More complete detoxification of RDX occurred under methanogenic and sulfate-reducing conditions than under manganese(IV) and iron(III)-reducing conditions.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 October 2014
Received in revised form 26 February 2015
Accepted 20 April 2015
Available online 22 April 2015

Keywords:
SIP
RDX
Electron acceptors
Biodegradation
Explosives

ABSTRACT

This study identified microorganisms capable of using the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) or its metabolites as carbon and/or nitrogen sources under different electron-accepting conditions using ^{13}C and ^{15}N stable isotope probing (SIP). Mesocosms were constructed using groundwater and aquifer solids from an RDX-contaminated aquifer. The mesocosms received succinate as a carbon source and one of four electron acceptors (nitrate, manganese(IV), iron(III), or sulfate) or no additional electron acceptor (to stimulate methanogenesis). When RDX degradation was observed, subsamples from each mesocosm were removed and amended with $^{13}C_3$- or ring-$^{15}N_3$-, nitro-$^{15}N_3$-, or fully-labeled $^{15}N_6$-RDX, followed by additional incubation and isolation of labeled nucleic acids. A total of fifteen 16S rRNA sequences, clustering in α- and γ-Proteobacteria, Clostridia, and Actinobacteria, were detected in the ^{13}C-DNA fractions. A total of twenty seven sequences were derived from different ^{15}N-DNA fractions, with the sequences clustered in α- and γ-Proteobacteria, and Clostridia. Interestingly, sequences identified as *Desulfosporosinus* sp. (in the Clostridia) were not only observed to incorporate the labeled ^{13}C or ^{15}N from labeled RDX, but also were detected under each of the different electron-accepting conditions.

* Corresponding author. Tel.: +1 979 845 1403.
E-mail address: kchu@civil.tamu.edu (K.-H. Chu).

http://dx.doi.org/10.1016/j.jhazmat.2015.04.059

The data suggest that ^{13}C- and ^{15}N-SIP can be used to characterize microbial communities involved in RDX biodegradation, and that the dominant pathway of RDX biodegradation may differ under different electron-accepting conditions.

1. Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is an explosive that is frequently detected in soil and groundwater at military sites, particularly at Department of Defense (DoD) training and testing ranges [1–5]. RDX is moderately soluble and has a relatively low octanol–water partitioning coefficient, and thus, can migrate rapidly from soil to groundwater. Due to its potential toxicity, the U.S. Environmental Protection Agency (EPA) recently listed RDX on Contaminant Candidate List 3 (CCL 3: http://water.epa.gov/scitech/drinkingwater/dws/ccl/ccl3.cfmcfm). The EPA has established a lifetime health advisory level of 2 μg/L for RDX in drinking water [6].

RDX biodegradation has been observed under various electron-accepting conditions, including manganese-reducing [7], iron-reducing [8,9], sulfate-reducing [10], acetogenic [11], and methanogenic [12] conditions. RDX degradation also has been reported in denitrifying enrichments [13], but only after all nitrate had been degraded, suggesting that nitrate can be inhibitory to RDX degradation in some circumstances. Because of its complex structure, RDX can potentially be utilized by microorganisms as an electron donor, a source of nitrogen or carbon, and/or as a terminal electron acceptor. Thus, the processes contributing to RDX biodegradation in the environment can be difficult to interpret. For example, Bradley and Dinicola [7] reported that RDX served as a microbial nitrogen source under manganese-reducing conditions in aquifer microcosms, whereas Beller [11] hypothesized that the RDX was used as a terminal electron acceptor by microorganisms in aquifer enrichments fed hydrogen as a sole electron donor.

Biodegradation of RDX under aerobic and anoxic conditions can be described via three known degradation pathways (Fig. S1) [14]. Under aerobic conditions, one nitro group of the RDX is first removed before the ring destabilizes and spontaneously cleaves, leading to the production of formaldehyde, carbon dioxide, and 4-nitro-2,4-diazabutanal (NDAB) [15]. Under anoxic conditions, RDX can be degraded through sequential nitro-reduction to produce hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) before ring cleavage, or through direct ring cleavage to produce methylenedinitramine (MEDINA) and bis(hydroxymethyl) nitramine, followed by production of terminal end products, including nitrous oxide and formaldehyde [16].

Numerous RDX-degrading microorganisms have been isolated from soil or sludge under anoxic conditions; however, only a few strains are known to use RDX as a nitrogen source [17–21]. *Desulfovibrio desulfuricans* EFX-DES is the only known anaerobic strain capable of using RDX as a carbon and nitrogen source under anoxic conditions [22]. Studies of these isolates in the laboratory have provided insight into RDX microbiology; however, it remains unclear if these microbial species are responsible for RDX degradation observed in the field. Since only a small fraction of environmental organisms can be isolated and cultivated in the laboratory [23], it is possible that many active but uncultivable RDX-degrading microorganisms are present in the field awaiting discovery.

Stable isotope probing (SIP) is a powerful tool to identify active degradative microorganisms in complex microbial communities without cultivation [24–26]. Recent applications of SIP with labeled RDX have provided interesting insights into anoxic RDX-degrading bacteria and microbial communities in soil and groundwater [27–29]. However, these studies did not systematically examine the metabolically active RDX utilizers under different electron-accepting conditions. The goal of this study was to identify RDX-degrading microorganisms capable of using RDX or RDX-metabolites as carbon and/or nitrogen sources under different dominant electron-accepting conditions using both ^{13}C- and ^{15}N-SIP. This is the first study to employ both ^{13}C- and ^{15}N-SIP to characterize active RDX-degrading microbial communities in aquifer samples under different electron-accepting conditions.

2. Materials and methods

2.1. Chemicals

Fully-labeled $^{13}C_3$-RDX, ring- and nitro-$^{15}N_3$-RDX, and fully-labeled $^{15}N_6$-RDX (all 99% chemically pure via HPLC analysis) were synthesized by Dr. Steve Fallis, US Naval Air Weapons Station, China Lake, CA, starting with relevant precursors of 99 atm% purity. The RDX breakdown products MNX, DNX, and TNX wereobtained from SRI international (Menlo Park, CA).

2.2. Sample collection and experimental set-up

Groundwater and saturated aquifer materials were collected from shallow wells (screen ~3–6 mbgs) at a US Department of Defense explosives testing range in Virginia with a history of RDX contamination. The groundwater in the silty sand aquifer contained 0.05 mg/L RDX, 0.6 mg/L perchlorate, 150 mg/L sulfate, and about 1 mg/L nitrate-N. Shallow aquifer solids (~3–6 mbgs) were collected in acetate sleeves using a direct push rig. The acetate sleeves were opened in the field, and the aquifer solids were transferred to plastic bags, which were sealed in a second bag and then placed on ice. Core samples and groundwater were stored at 4 °C until use.

Mesocosms were prepared by adding homogenized saturated aquifer solids (mixture of sand, silts and clay; 0.5 kg wet wt), and groundwater (8 L) to each of five 10 L mini-kegs (bleached and autoclaved). The slurry in each keg was purged with nitrogen for 3 h to remove most of the oxygen. Five mesocosms were amended with sodium succinate and RDX to achieve initial concentrations of 40 mg/L (~0.3 mM) and 0.15 mg/L (~0.7 μM), respectively. Four of the kegs were amended with one of four different electron acceptors at 1 mM each; KNO_3, MnO_2, $FeCl_3{\cdot}6H_2O$ or K_2SO_4. The fifth keg was not amended with any additional electron acceptor and was designed to stimulate methanogenic conditions. Degradation of RDX was not observed in microcosms under aerobic conditions in preliminary studies with site samples, so an aerobic mesocosm was not set-up. All kegs were pressurized to 30 psi with sterile nitrogen gas and incubated at 15 °C, to simulate groundwater temperature at site. Kegs were mixed at least twice a week by manual shaking. Aqueous samples were collected from the mesocosms over time and analyzed for RDX, RDX degradation products, succinate,