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ORIGINAL PAPER

Isolation and characterization of RDX-degrading *Rhodococcus* species from a contaminated aquifer

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Abstract Groundwater contamination by the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a global problem. Israel's coastal aquifer was contaminated with RDX. This aquifer is mostly aerobic and we therefore sought aerobic bacteria that might be involved in natural attenuation of the compound in the aquifer. RDX-degrading bacteria were captured by passively sampling the indigenous bacteria onto sterile sediments placed within sampling boreholes. Aerobic RDX biodegradation potential was detected in the sediments sampled from different locations along the plume. RDX degradation with the native sampled consortium was accompanied by 4-nitro-2,4-diazabutanal formation. Two bacterial strains of the genus *Rhodococcus* were isolated from the sediments and identified as aerobic RDX degraders. The *xplA* gene encoding the cytochrome P450 enzyme was partially (~500 bp) sequenced from both isolates. The obtained DNA sequences had 99% identity with corresponding gene fragments of previously isolated RDX-degrading *Rhodococcus* strains. RDX degradation by both strains was prevented by 200 μM of the cytochrome P450 inhibitor metyrapone, suggesting that cytochrome P450 indeed mediates the initial step in RDX degradation. RDX biodegradation activity by the T7 isolate was inhibited in the presence of nitrate or ammonium concentrations above 1.6 and 5.5 mM, respectively (100 mg l^{-1}) while the T9N isolate's activity was retarded only by ammonium concentrations above 5.5 mM. This study shows that bacteria from the genus *Rhodococcus*, potentially degrade RDX in the saturated zone as well, following the same aerobic degradation pathway defined for other *Rhodococcus* species. RDX-degrading activity by the *Rhodococcus* species isolate T9N may have important implications for the bioremediation of nitrate-rich RDX-contaminated aquifers.

Keywords Groundwater · RDX · 4-nitro-2,4-diazabutanal · *Rhodococcus* · *xplA* · Cytochrome P450

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Introduction

The explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) pollutes soil and groundwater adjacent to its production plants worldwide. Due to its toxicity, the

US Environmental Protection Agency (EPA) has set a recommended drinking water limit for RDX at 2 μg l^{-1} (US EPA 2006). However, the potential for microbial biodegradation of RDX in the subsurface has been observed in different contaminated sites (e.g. Beller and Tiemeier 2002; Bradley and Dinicola 2005; Pennington et al. 2001) and by various bacterial species possessing different degradation pathways (Hawari et al. 2000). In recent years, considerable effort has been invested in disclosing the aerobic biochemical pathway of RDX biodegradation that is carried out by various *Rhodococcus* species. Denitration has been identified as the initial step in this pathway, with an indication of the involvement of the enzyme cytochrome P450 (Tekoah et al. 1999) encoded by the gene *xplA* (Indest et al. 2007; Jackson et al. 2007; Seth-Smith et al. 2008). In vitro, this aerobic enzymatic activity with NADPH as an electron donor results in the formation of 4-nitro-2,4-diazabutanal (NDAB) (Jackson et al. 2007). This activity has also been demonstrated in vivo for the *Rhodococcus* strains DN22 (Bhushan et al. 2003; Coleman et al. 2002; Fournier et al. 2002), 11Y (Seth-Smith et al. 2008) and YH1 (Nejidat et al. 2008; Tekoah et al. 1999) as well as under microaerophilic conditions by various other *Rhodococcus* strains (Fuller et al. 2010).

In all previous studies, RDX-degrading *Rhodococcus* strains were isolated from explosive-contaminated soils: strain DN22 was isolated from contaminated soils in Australia (Coleman et al. 1998), strain 11Y and strains HS1-HS19 from contaminated soils in England (Seth-Smith et al. 2008; Seth-Smith et al. 2002), and strains YH1 and GYY1 from contaminated soils in Israel (Brenner et al. 2000; Ronen et al. 2008; Tekoah et al. 1999). In addition, Roh et al. (Roh et al. 2009) demonstrated the incorporation of ^{15}N isotope from ^{15}N labeled RDX into *xplA* gene amplified from total DNA extracted from groundwater enrichment culture. However, to the best of our knowledge, no RDX-degrading *Rhodococcus* strains have been isolated from contaminated groundwater.

In Israel, RDX was found contaminating the groundwater of the coastal aquifer in a ca. 1.35-km-long contamination plume (Bernstein et al. 2010). Biodegradation was examined as a potential remediation strategy to deal with the threat of this contamination to the future water supply. The use of compound-specific isotope analysis indicated that biodegradation is indeed an important process reducing the extent of RDX contamination in the aquifer (Bernstein et al. 2010). It was also suggested that RDX is degraded through the aerobic pathway due to the high dissolved oxygen concentrations detected in most boreholes along the contamination plume. RDX degradation through the anaerobic pathway is unlikely to occur because the high nitrate which can inhibit anaerobic metabolism of RDX, as nitrate may serve as a more favorable electron acceptor than RDX (Beller 2002; Ronen et al. 1998; Wani and Davis 2003). Although aerobic biodegradation of RDX was suggested to play an important role in reducing the extent of contamination within this aquifer, there was no microbial proof that aerobic degrading bacteria actually existed spatially along the plume. Aerobic RDX-degrading *Rhodococcus* strains (YH1 and GYY1) were previously isolated from the vadose zone overlying this aquifer. These isolates degraded RDX following the aerobic denitration pathway, which was shown to be catalyzed by *xplA*-encoded cytochrome P450 (Nejidat et al. 2008; Ronen et al. 2008).

RDX biodegradation can be beneficial to microorganisms in a nutrient-poor environment, as the high nitrogen content of RDX makes it a potentially good nitrogen source (Brenner et al. 2000; Ronen et al. 1998). Indeed, RDX degradation by *Rhodococcus* strains is inhibited in the presence of inorganic nitrogen compounds (Coleman et al. 1998; Nejidat et al. 2008). However, significant RDX degradation (Bernstein et al. 2010) was observed along the contaminated plume within the Israeli coastal aquifer despite the high nitrate concentrations (up to 3.2 mM, or 200 mg l^{-1}) adjacent to the contamination source; see Table S1 in the Supplemental Material). Therefore, the presence of RDX-degrading activity that is not inhibited by inorganic nitrogen species was hypothesized. The aim of this study was to isolate and characterize aerobic RDX-degrading bacteria from the contaminated plume and examine the effect of inorganic nitrogen (ammonium and nitrate) on their activity.

Materials and methods

Bacterial sampling

Passive sampling of the aquifer's bacteria was carried out in 17 different boreholes, spatially scattered

throughout the contaminated site (see Fig. S1 in Supplemental Material). In the past, RDX contamination had been detected in some, but not all of these boreholes. Polyester mesh (Sefar, PET 1000 48T-70W, Sefar Inc., Freibach, Switzerland) was used to prepare ca. 3 × 15 cm bags filled with sediment. Each bag contained local sediments originating from the material drilled from the borehole into which it was later placed. Bags of sediments were sterilized by autoclaving for two sequential days and then placed at the bottom of their respective boreholes, tied to a nylon line. Bags were left inside the boreholes for 6.5 months. They were then collected and stored in sterile polypropylene containers at 4°C until use. Subsamples (ca. 10 g) were aseptically transferred into 15-ml tubes and kept at −80°C.

RDX biodegradation potential

Laboratory slurry experiments were conducted using the sediments from the mesh bags. Aerobic incubation was carried out in 125-ml Erlenmeyer flasks. Each flask contained 50 ± 2.5 ml of liquid growth medium. The mineral medium contained a phosphate buffer (5.74 mM K_2HPO_4 and 3.67 mM KH_2PO_4, pH 7.1), 1.66 mM $MgSO_4$, and trace elements (16.5 μM $FeSO_4$, 45.1 μM $CaCl_2$, 8.28 μM $MnSO_4$, 1.56 μM $CuSO_4$, and 1.21 μM Na_2MoO_4). Glucose and citric acid served as the carbon source (5.55 and 5.21 mM, respectively) and the final pH was 7.2. RDX was added to the growth medium at an initial concentration of 85 μM, and was the only nitrogen source added. The medium was inoculated with 3.0 ± 0.2 g of soil from the mesh bags. Replicates with addition of 15.4 mM sodium azide were used as abiotic controls.

Aerobic slurries were kept in the dark at 25°C and were continuously shaken on a rotary shaker at 150 rpm. Samples were taken once a day and analyzed for RDX and NDAB concentrations by high-performance liquid chromatography (HPLC).

Bacterial isolation and characterization

Sediment slurries from boreholes T7 and T9N were transferred (10% v/v) to fresh medium containing glucose and citric acid (5.55 and 5.21 mM, respectively) as the carbon source and 85 μM RDX as sole nitrogen source. After two successive transfers, the cultures were streaked on the above medium solidified with 1.5% (w/v) agar (Acumedia Manufacturers Inc., Lansing, MI). Single colonies were isolated by repeated streaking on the agar plates. Phylogenetic identification of the isolates was carried out at Hy Labs (Rehovot, Israel) based on 16S rRNA gene sequences.

The 16S rRNA gene sequences (600 bp) of the current isolates as well as those of other known RDX degraders were uploaded onto the RDP 10 website (Cole et al. 2009). After alignment, closest relatives were selected using RDP 10's SeqMatch tool. A phylogenetic tree was constructed using the Mega 4.1 program based on the results of the neighbor-joining analysis method (Tamura et al. 2007). The 16S rRNA gene sequences obtained in this study were submitted to GenBank and assigned accession nos. FJ790674 for isolate T9N and FJ790675 for isolate T7.

Amplification of *xplA* gene fragments

Whole genomic DNA was extracted from cell cultures using GeneElute bacteria genomic DNA kit (Sigma Rehovot Israel) according the manufacture instructions. The *xplA* gene fragments (500 bp) were amplified using xplAF and xplAR primers (Roh et al. 2009). The PCR product was gel purified using HiYield Gel/PCR DNA Fragments Extraction Kit (RBC BioScience Taipei, Taiwan) and then cloned into pTZR/T vector (Fermentase. Burlington Canada). Clones with the correct size insert were then sent for sequencing (Macrogen, Seoul Korea). The DNA sequences were deposited in the Gene Bank under the accession numbers HQ588902 and HQ588903 for strain T7 and T9N isolates respectively. Search for similarity in the data base was performed using the blastn algorithm (Altschul et al. 1990).

Effect of cytochrome P450 inhibitor on RDX degradation

To test whether cytochrome P450 was involved in the initial step of RDX degradation, the effect of metyrapone, a cytochrome P450 inhibitor, was studied (Nejidat et al. 2008). Briefly, cultures (100 ml) were pre-grown with RDX (100 μM) until its complete degradation. Then the cultures were harvested by centrifugation (10,000×*g* for 15 min at 4°C), washed once and resuspended in 10 ml mineral medium. From these cultures, 200 μl were used to inoculate

mineral medium (50 ml) containing RDX (100 μM) only, RDX and metyrapone (200 μM), ammonium (0.56 mM) or ammonium (0.56 mM) and metyrapone (200 μM). After 6 days of incubation, samples were taken for RDX and NDAB analysis, as well as optical density (OD) measurements. All treatments were run in triplicate.

Effect of nitrate and ammonium on RDX biodegradation

Bacteria used for these experiments originated from the T7 and T9N isolates. Bacteria were initially recovered from frozen glycerol stocks by cultivation in LB medium (Sigma) for 3 days, and placed on a rotary shaker, 125 rpm at 25°C in the dark. Then 200 μl of the turbid culture was transferred to a selective medium (100 ml) in which RDX (100 μM) served as sole nitrogen source. This medium was similar to that described above for the slurry experiments, except for the carbon source which consisted of 5.21 mM glucose. A second transfer of 2 ml was made to fresh medium with similar properties. When the cultures of the second transfer actively degraded RDX (as determined by HPLC), the cultures (100 ml for each strain) were harvested by centrifugation (10,000×g for 15 min at 4°C), washed once with mineral medium without carbon and then resuspended in the mineral medium (50 ml for each strain). From this suspension, 2 ml was used to initiate the degradation experiments that were carried out in 250-ml Erlenmeyer flasks with 100 ml growth medium as described above for the slurry experiments. Treatments were amended with 130 μM RDX with or without 1.61 mM (100 mg l^{-1}) nitrate as an external nitrogen source. Each treatment was carried out in triplicate. The same experimental protocol was repeated with ammonium at concentrations of 0.56, 5.56 and 55.6 mM (10, 100 and 1000 mg l^{-1}).

Analytical methods

RDX concentrations were monitored by HPLC (Agilent 1100 series, Palo Alto, CA) according to EPA method 8330 (US EPA 1997). The aerobic ring-cleavage product NDAB was determined by HPLC using a Synergi 4μ Hydro-RP column (250 × 4.6 mm, Phenomenex, Torrance, CA), a mobile phase that consisted of 20 mM KH_2PO_4, pH 3.4, and a flow rate of 0.8 ml min^{-1}. NDAB was detected at 210 nm and quantified by a calibration curve that ranged from 1 to 100 μM. Verification of the NDAB peak was based on retention time and UV adsorption spectra in comparison to authentic standards. The analysis error was estimated as ±2% for RDX and ±10% for NDAB. RDX standards were prepared from solid powder (Ronen et al. 2008). 4-nitro-2,4-diazabutanal was purchased from SRI International (Menlo Park, CA).

Bacterial growth was measured as OD at 600 nm (BioMate 5, Thermo Scientific, England). Nitrate concentrations in the treatments which were amended with 1.61 mM (100 mg l^{-1}) nitrate were determined by the second-derivative method (Ferree and Shannon 2001).

Results and discussion

Potential for aerobic degradation of RDX

The potential for aerobic biodegradation was detected by slurry experiments in sediments from 5 of the 17 boreholes tested (Fig. S1 and S2 in Supplemental Material). In all five of the positive slurries, RDX was completely degraded within 5–8 days (Fig. 1; Fig. S2 in Supplemental Material). All slurries showed a lag time of 4–6 days followed by rapid RDX degradation. The degradation of RDX was accompanied by delayed NDAB formation. In four out of the five

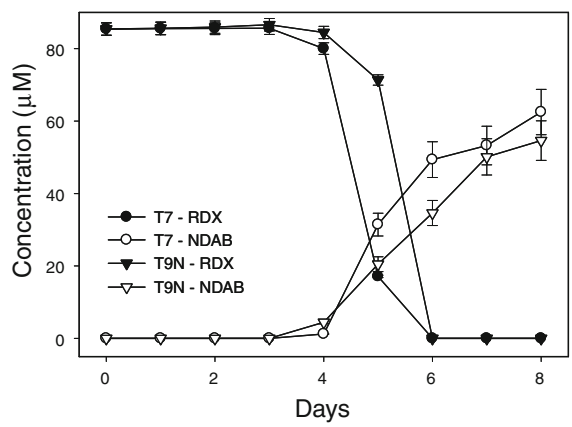

Fig. 1 RDX degradation and NDAB accumulation in slurries originating from borehole T7 and T9N. Similar trends for slurries originating from other boreholes are provided in the Supplemental Material (Fig. S2). For the spatial location of these boreholes see Fig. S1 in the Supplemental Material

slurries, 64 ± 10% to 78 ± 10% of the RDX was recovered as NDAB after 8 days. No significant loss of RDX or accumulation of NDAB was observed in any of the controls (data not shown).

No correlation was found between the potential aerobic RDX degradation by the slurries and the dissolved oxygen concentrations measured in the wells from which they were sampled. For instance, potential for aerobic biodegradation was detected using sediments recovered from borehole T101, even though measurements of dissolved oxygen concentrations in this well were lower than 1 mg l^{-1} (Table S1 in Supplemental Material). Furthermore, the potential for aerobic biodegradation activity was also not correlated with the origin of the sediments along the contamination plume: potential for aerobic biodegradation was detected using sediments from borehole T4, a borehole in which RDX was never detected during a monitoring project carried out from 2003 to 2008 in the contaminated site (detection limit = 0.045 μM). The detection of RDX degradation potential in boreholes in which RDX was not detected during sampling may be due to previous exposure to RDX contamination. As suggested by Bernstein et al. (2010), the groundwater flow directions in the aquifer change due to alterations in pumping, and this may be why RDX was not detected in borehole T4 in the past, but the potential for its degradation was detected in this study. The detection of aerobic degradation potential in microaerophilic sections of the aquifer (borehole T101) can be explained by the fact that under these conditions, degradation of RDX by microorganisms carrying the *xplA* gene can occur via the methylenedinitramine (MEDINA) pathway (Jackson et al. 2007; Fuller et al. 2010).

The use of sterile sediment as a substratum for colonization by RDX-degrading bacteria thus proved to be an effective means of retrieving the relevant bacteria. Nevertheless, although this passive sampling method is likely to be preferable to using groundwater samples for obtaining native groundwater microflora because it mimics the aquifer environment (Alfreider et al. 1997), the further incubation conditions may be selective to the isolated strains only, and discriminate other potential strains that were not retrieved in this study. Thus, it is possible that other RDX-degrading strains exist along the studied plume.

Bacterial identification

Two isolates (T7 and T9N) that degrade RDX under aerobic conditions were successfully isolated and further identified based on their 16S rRNA gene sequences, as rhodococci. Strain T7 (accession number FJ790675) showed 100% identity with *Rhodococcus* sp. NJUST16 (EF635425), which is capable of mineralizing 2,4,6-trinitrophenol (Shen et al. 2009), and strain T9N (accession number FJ790674) showed 100% identity with *Rhodococcus* sp. 7/1, a cold-tolerant alkane-degrading *Rhodococcus* species from Antarctica (Bej et al. 2000). Pair-wise alignment of 16S rRNA gene sequences from strains T7 and T9N revealed 94.5% identity. Phylogenetic analysis revealed that strain T9N clusters with the known RDX-degrading *Rhodococcus* strain DN22, whereas strain T7 did not cluster with any of the known RDX-degrading rhodococci (Fig. 2). Thus, although *Rhodococcus* strains are ubiquitous in the environment and were found in contaminated groundwater (Bell et al. 1998; Martínková et al. 2009), this is the first study that reports the isolation of aerobic RDX-degrading strains (T7 and T9N) of this genus in the saturated zone of a contaminated aquifer.

The *Rhodococcus* strain YH1 was previously isolated from the surface soil overlying this contaminated aquifer (Brenner et al. 2000; Nejidat et al. 2008). However, the 16S rRNA gene sequences of the newly isolated strains T7 and T9N have only 94 and 92% identity with that of strain YH1 respectively, suggesting that different RDX-degrading *Rhodococcus* species were evolved in the contaminated aquifer than those in the contaminated vadose zone. Priestley et al. (2006) have shown that the transport of RDX-degrading *Rhodococcus* sp. in a sand column (similar to the unsaturated zone at the studied site) is limited, which may explain the discontinuity between the surface RDX-degrading *Rhodococcus* population and that in the groundwater.

Involvement of cytochrome P450 in RDX degradation

DNA fragments related to the gene (*xplA*) encoding the cytochrome P450 enzyme that catalyze the first step of aerobic RDX degradation (Jackson et al. 2007; Nejidat et al. 2008; Seth-Smith et al. 2008; Indest et al. 2010) were PCR-amplified from whole

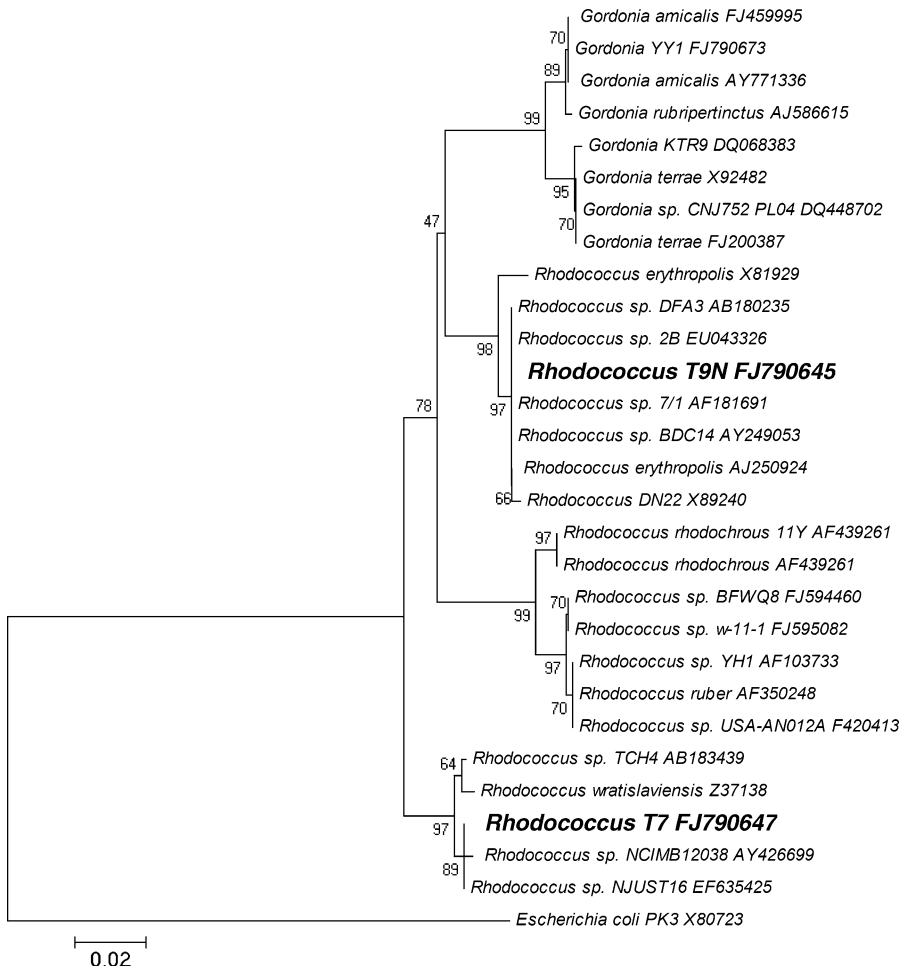

Fig. 2 Phylogenetic relationships between the isolated strains (*bold*) and other RDX-degrading microorganisms from the genera *Rhodococcus* and *Gordonia*. The tree was constructed using the neighbor-joining method and bootstrap values based on 1,000 replications are indicated at the nodes. The tree was rooted with 16 rRNA gene sequences of *E. coli*

genomic DNA of strain T7 and strain T9N cells and sequenced. The obtained DNA sequences had 99% identity with their corresponding *xplA* gene fragments from *Gordonia* sp. KTR9, harboring the pGKT2 plasmid (Indest et al. 2010) and *Rhodococcus* sp. YH1 that was previously isolated from the surface soil above the aquifer (Nejidat et al. 2008). The *xplA* gene has now been identified in 24 isolates belonging to the *Corynebacterineae* across the globe with near sequence identity (Rylott et al. 2011). The 99% identity of the *xplA* gene sequences from T7 and T9N isolates to *xplA* genes from other RDX-degrading rhodococci is a further support for the conservation of this gene and its horizontal spread (Seth-Smith et al. 2008; Indest et al. 2010). The sequence identity of *xplA* gene from the two isolates to that sequence from *Rhodococcus* strain YH1, isolated from the surface soil above the aquifer, is in line with this observation nevertheless, based on 16S rRNA gene sequences they are not identical strains (Fig. 2).

Metyrapone, a cytochrome P450 inhibitor (Nejidat et al. 2008; Seth-Smith et al. 2008), inhibited RDX degradation and growth of the T7 and T9N isolates almost completely (93 and 90% respectively) when RDX was used as sole nitrogen source (Fig. S3 in the Supplemental Material). The facts that both isolated strains posses the *xplA* gene and their RDX degradation activity is inhibited by metyrapone support the involvement of cytochrome P450 in this aerobic activity.

Influence of nitrate and ammonium on aerobic RDX degradation

RDX degradation experiments were conducted using the bacterial isolates T7 and T9N with and without

nitrate as an additional nitrogen source (Fig. 3a). RDX degradation by the T7 isolate was strongly inhibited by nitrate while its degradation by T9N was fully achieved after 6 days of incubation (Fig. 3a). 4-nitro-2,4-diazabutanal measurement on day 6 revealed that RDX had been fully converted to NDAB by strain T9N while only 20 ± 23% had been converted by strain T7. However, both stains have consumed the added nitrate (Fig. 3b). Concomitant to RDX degradation and nitrate consumption significant bacterial growth was measured (Fig. 3c), that was more evident in the presence of nitrate. The decline in OD with this treatment was accompanied by aggregation of the cultures (Fig. 3c). Furthermore, RDX degradation by T7 isolate (Fig. S4 in the Supplemental Material) and T9 N isolate (data not shown) was inhibited by increasing ammonium concentrations. The addition of 55.6 mM of ammonium caused 90 and 92% inhibition of RDX degradation by strains T7 and T9N respectively.

The availability of inorganic nitrogen sources such as ammonium and nitrate has been shown to inhibit RDX degradation by strain YH1 and to suppress accumulation of cytochrome P450 protein (Nejidat et al. 2008). Similarly the RDX degradation activity of *Rhodococcus* DN22 was inhibited by ammonium concentrations above 2 mM (Coleman et al. 1998). However, strains T7 and T9N exhibited differential sensitivities to ammonium and nitrate. Whereas the activity of T7 was inhibited by both ammonium and nitrate, T9N activity was inhibited by ammonium only, suggesting that T7 strain will preferentially utilize nitrate or ammonium over RDX as a nitrogen source in the aquifer. Furthermore, this differential response of the isolates to inorganic nitrogen supports the previously suggested hypothesis of multiple mechanisms controlling and regulating the expression of the aerobic RDX degradation pathway in different RDX-degrading *Rhodococcus* strains (Nejidat et al. 2008).

The fact that the isolates exhibited different sensitivities to nitrate (the major nitrogen in the aquifer) is important in explaining the distribution of residual RDX in the aquifer. As can be seen from Table S1 in the Supplemental Material, borehole T7 contained the highest concentrations of both nitrate and RDX. This suggests that as long as a high level of nitrate is present in this part of the aquifer (where strain T7 is the potential RDX degrader), aerobic degradation of RDX by this strain will be inhibited. In contrast, in the T9N borehole, RDX degradation by strain T9N is expected to continue despite the presence of nitrate. In previous work, a range of biodegradation rates were calculated for RDX, based on compound-specific isotope analysis (Bernstein et al. 2008; Bernstein et al. 2010). In that work, RDX was sampled from different boreholes along the plume and isotopically analyzed. Although the RDX and nitrate concentrations varied considerably between boreholes along the plume, a relatively narrow range of degradation rates was calculated for all shallow boreholes at the site, regardless of whether they were located in the center of the plume or at its fringes. Thus, we suggest that the differences in nitrate's inhibitory effect on RDX biodegradation observed in this study in the laboratory were masked in the field study by other limiting factors and left no significant mark on the biodegradation extents within this long and mature heterogeneous contamination plume. Additionally, it is possible that other RDX-degrading strains which are not inhibited by nitrate are

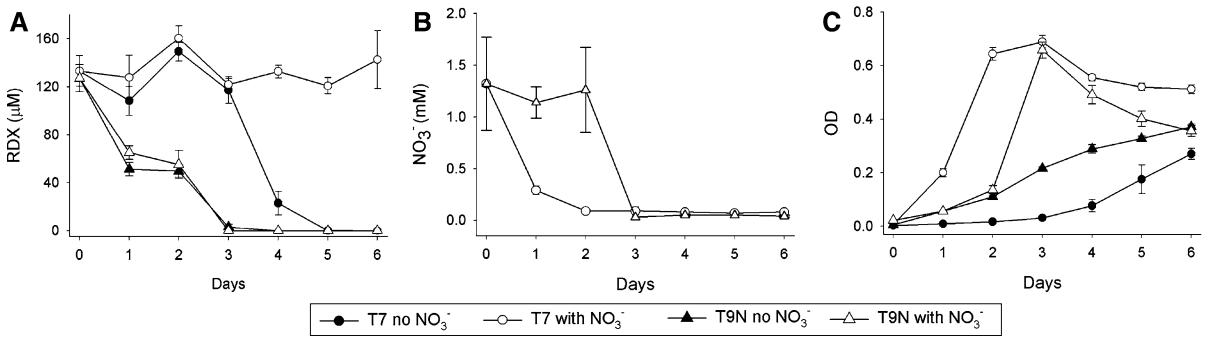

Fig. 3 Concentrations of RDX (**a**), nitrate (NO_3^-, presented only for treatments to which it was added) (**b**) and OD (**c**) during 6 days of incubation of strains T7 and T9N with and without added nitrate. Results are the average of triplicates ± SD

active along the plume, and were simply not retrieved in this study and were selected against by the medium used to establish the enrichment cultures.

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