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Impact of Ferrihydrite and Anthraquinone-2,6-Disulfonate on the Reductive Transformation of 2,4,6-Trinitrotoluene by a Gram-Positive Fermenting Bacterium

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Batch studies were conducted to explore differences in the transformation pathways of 2,4,6-trinitrotoluene (TNT) reduction by a Gram-positive fermenting bacterium (*Cellulomonas* sp. strain ES6) in the presence and absence of ferrihydrite and the electron shuttle anthraquinone-2,6-disulfonate (AQDS). Strain ES6 was capable of TNT and ferrihydrite reduction with increased reduction rates in the presence of AQDS. Hydroxylaminodinitrotoluenes, 2,4-dihydroxylamino-6-nitrotoluene (2,4-DHANT), and tetranitroazoxytoluenes were the major metabolites observed in ferrihydrite- and AQDS-free systems in the presence of pure cell cultures. Ferrihydrite enhanced the production of amino derivatives because of reactions with microbially produced surface-associated Fe(II). The presence of AQDS in the absence of ferrihydrite promoted the fast initial formation of arylhydroxylamines such as 2,4-DHANT. However, unlike in pure cell systems, these arylhydroxylamines were transformed into several unidentified polar products. When both microbially reduced ferrihydrite and AQDS were present simultaneously, the reduction of TNT was more rapid and complete via pathways that would have been difficult to infer solely from single component studies. This study demonstrates the complexity of TNT degradation patterns in model systems where the interactions among bacteria, Fe minerals, and organic matter have a pronounced effect on the degradation pathway of TNT.

Introduction

Contamination of groundwater and soil with explosives, such as 2,4,6-trinitrotoluene (TNT), is a widespread environmental problem because of the mutagenic, carcinogenic, and toxic effects of TNT and its metabolites (*1*−*4*). TNT is extremely recalcitrant to microbial mineralization in aerobic environments (*5*), however, many studies have reported that TNT can be bioreduced to amino derivatives (*6*). Hydroxylaminodinitrotoluenes (HADNTs) are commonly observed intermediates during both chemical and biological transformation of TNT to aminodinitrotoluenes (ADNTs). The reduction of all three nitro groups to form 2,4,6-triaminotoluene (TAT) has been shown to occur under strictly anaerobic conditions (*6*). Understanding the TNT degradation pathway is important, since some metabolites (e.g., TAT) may bind more strongly to soils and others may be more toxic than TNT (e.g., 2,2′,6,6′-tetranitro-4,4′-azoxytoluene; 4,4′-Azoxy) (*2*, *7*−*11*). Given that the complete mineralization of TNT by microorganisms does not readily occur in natural environments, immobilization of reduced TNT metabolites is one mechanism for minimizing transport and enhancing the natural attenuation of TNT in soils (*9*). However, the mechanisms and pathways of TNT biotransformation in heterogeneous systems such as soils containing organic matter, different mineral phases, and a variety of microorganisms are not well studied.

The reduction of Fe(III) (hydr)oxides by dissimilatory metal-reducing bacteria (DMRB) has received considerable attention since the resulting ferrous iron has proven to be an effective reductant of highly toxic metals such as Cr(VI) or U(VI) and organic pollutants such as chlorinated solvents or nitroaromatic compounds (*12*−*16*). The reduction rate of Fe(III) (hydr)oxides by DMRB can increase in the presence of humic acids (HA) and anthraquinone-2,6-disulfonate (AQDS). Both HA and AQDS have been shown to function as electron shuttles, thus increasing the rate and extent of Fe(III) reduction by diminishing the need for direct contact between microorganisms and Fe(III) surfaces (*14*, *17*−*21*).

While the influence of electron-shuttling compounds on Fe(III) mineral reduction has been investigated intensively, very few studies have examined their direct influence on the reductive transformation of organic pollutants. The reduction of chlorinated compounds and azo dyes by reduced humic acids and the reduced form of AQDS (anthrahydroquinone-2,6-disulfonate, AHQDS) has been reported (*22*, *23*). Hofstetter et al. (*12*) demonstrated that TNT can be reduced to its amino derivatives by Fe(II) present at the surface of goethite (α-FeOOH) and, less efficiently, by 8-hydroxy-1,4-naphthoquinone (juglone), in the presence of H_2S. They also demonstrated that *Geobacter metallireducens* and a mixed Fe(III)-reducing consortium can indirectly contribute to the reduction of nitroaromatics via production of Fe(II) in ferrogenic columns (*12*).

The majority of research investigating the effect of electron shuttles and Fe(III) mineral reduction on the fate of environmental contaminants has been conducted with the two highly studied, metal-reducing genera *Shewanella* and *Geobacter (12*, *13*, *15*, *24)*. However, little is known regarding the reduction of Fe(III) minerals mediated by other common soil organisms. Benz et al. (*25*) demonstrated that several Gram-positive and Gram-negative fermenting bacteria can reduce Fe(III) minerals via electron-shuttling compounds and Kappler et al. (*26*) recently reported that such organisms can have a significant influence on electron transfer in freshwater sediments. Sani et al. (*27*) described many isolates, identified as *Cellulomonas* spp., that were the predominant organisms in Cr(VI)-reducing enrichment cultures from aquifer samples of the U.S. Department of Energy's Hanford site. *Cellulomonas* sp. strain ES6, a facultative anaerobic bacterium capable of fermentation of complex carbon sources, was recently shown to reduce Cr(VI), U(VI), Fe(III)-NTA, goethite, maghemite, magnetite, hematite, ferrihydrite, TNT, and AQDS (*3*, *27*−*29*).

Given the importance of Fe(II) and reduced electron shuttles on the fate of TNT, the primary goal of the present

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study was to utilize *Cellulomonas* sp. strain ES6 to investigate the contribution of (i) direct microbial, (ii) Fe(II)-mediated, and (iii) electron-shuttle-mediated nitroaromatic reduction on the fate of TNT in multicomponent systems containing bacteria, ferrihydrite, and AQDS.

Experimental Section

Preparation of ES6 Cultures. *Cellulomonas* sp. strain ES6 was maintained in frozen stock cultures containing tryptic soy broth (TSB) amended with 20% glycerol and stored at −70 °C (*27*). Cells were precultured in TSB (30 g/L, Difco Laboratories) at 21 ± 2 °C and 150 rpm on a horizontal shaker for 24 h, transferred into fresh TSB, and grown again for 18 h under aerobic conditions. Cultures were harvested during the late log/early stationary phase by centrifugation at 5860*g* for 20 min at 4 °C, washed once in O_2-free synthetic groundwater (SGW), and resuspended in SGW to obtain the desired cell concentration. Cell culture densities were determined using colony forming units (CFU), protein assays (Coomassie Plus, Pierce, Rockford, IL), or optical density (OD) measurements at 540 nm. CFU were determined after dilution in phosphate-buffered saline solution (pH 7) and spread plating of 100-μL aliquots onto tryptic soy agar plates (Difco Laboratories) in triplicate. Plates showing between 30 and 300 colonies after 48 h of incubation at 30 °C were counted, counts were averaged, and cell numbers were calculated with corresponding standard deviations.

Media Composition. Synthetic groundwater (SGW) of the following composition was used in all experiments (*30*) (all concentrations in mg/L): $Na_2SiO_3-9H_2O$ (455), Na_2CO_3 (160), Na_2SO_4 (6), yeast extract (50), casamino acids (100), KCl (26.5), $CaCl_2-2H_2O$ (8), $Mg(OH)_2$ (33.5), $NaHCO_3$ (2519.7), LiCl (0.021), $CuSO_4-5H_2O$ (0.08), $ZnSO_4-7H_2O$ (0.106), H_3BO_4 (0.6), $Al_2(SO_4)_3-18H_2O$ (0.123), $NiCl_2-6H_2O$ (0.11), $CoSO_4-7H_2O$ (0.109), Na_2SeO_4 (0.05), $MnCl_2-4H_2O$ (0.629), KBr (0.03), KI (0.03), Na_2MoO_4 (0.01), and $FeSO_4-7H_2O$ (0.3). The $Na_2SiO_3-9H_2O$, Na_2CO_3, Na_2SO_4, yeast extract, and casamino acids were dissolved in deionized (DI) water and were autoclaved. The autoclaved solution was boiled for 10 min and was cooled to room temperature under an O_2-free atmosphere of N_2/CO_2 (80:20). Sterile O_2-free stock solutions of KCl, $NaHCO_3$, $CaCl_2-2H_2O$, $Mg(OH)_2$, and the trace minerals were added using purged syringes and needles. The medium was dispensed under the N_2/CO_2 atmosphere to maintain O_2-free conditions.

Iron Synthesis and Analysis. A ferrihydrite suspension was synthesized by dissolving 10.8 g $FeCl_3$ in 100 mL of DI water and adjusting the pH to 7.0 using dropwise addition of 6 N NaOH over a period of 1 h (*31*). This ferrihydrite suspension was diluted 1:40 in SGW, aged for 1 month, and 10 mL of the resulting suspension was added to ferrihydrite-containing treatments. XRD analysis indicated that 2-line ferrihydrite was the major phase after aging, but incipient 6-line diffraction features were also present (data not shown; (*32*)). The N_2-BET surface area (*33*) of the aged ferrihydrite was 38.6 ± 5.8 m^2/g. This relatively low surface area compared to other studies may be caused by aging, which can lead to aggregation and formation of 6-line ferrihydrite (*32*, *34*). Total iron (extraction with 2.5 N HCl + 0.25 N NH_2OH for 24 h), mild acid extractable $Fe(II)_{0.5N\ HCl}$ (extraction with 0.5 N HCl for 2 h), and aqueous $Fe(II)_{aq}$ (filtered through a 0.2-μm filter) were determined using the Ferrozine method (*35*).

AHQDS Production and Analysis. AHQDS was produced from anthraquinone-2,6-disulfonate (AQDS, Sigma-Aldrich, MO) by reducing it in the presence of palladium-coated silica particles and hydrogen gas at 90 °C. After filtration (0.2 μm) in an oxygen-free glovebag, the extent of AQDS reduction was determined by adding a known amount of Fe(III)−citrate, measuring the amount of Fe(II) produced, and calculating the amount of electrons transferred from the reduced AQDS to Fe(III). No differentiation was made between the two possible reduced forms of AQDS, anthradihydroquinone-2,6-disulfonate and anthrahydroquinone-2,6-disulfonate, and the sum of both was referred to as AHQDS throughout this research.

TNT Transformation Experiments. Reaction solutions (25 mL) containing O_2-free SGW, sucrose (10 mM), and unlabeled TNT (40−65 μM) were prepared anaerobically. SGW and sucrose were dispensed under the N_2/CO_2 atmosphere, and TNT was added in a glovebag (90% N_2; 5% CO_2; 5% H_2) to 30-mL test tubes (Bellco Glass, NJ) in the presence and absence of 6 mM ferrihydrite, as determined by the Ferrozine assay, and 100 μM AQDS. The test tubes were crimp sealed with polytetrafluoroethylene-faced butyl rubber septa (West Pharmaceutical Services, PA) and were inoculated with strain ES6 at initial population densities of 1.8×10^9 (fresh cell systems) or 3.8×10^8 (preincubated systems) colony forming units (CFU) per mL. The tubes were pressurized with 10 psi of N_2/CO_2 (80/20) to achieve a media pH of 7. The vials were incubated in the dark on a rotator at 21 ± 2 °C, and triplicate sets of vials were sampled periodically in the O_2-free glovebag and were analyzed for dissolved TNT and metabolites. Several experiments were conducted using U-ring-^{14}C-TNT (9×10^5 dpm/mL; radiochemical purity >99%; ChemSyn Laboratories, KS) at an initial cell density of 1.5×10^7 CFU/mL to track the fate of TNT in the presence of AQDS where we previously observed that several TNT reaction products were not identifiable using an exhaustive analytical protocol for determination of TNT metabolites (*3*).

TNT Metabolite Analysis. Aliquots of 500 μL were withdrawn from the vials over time and were filtered (0.2 μm, Spartan 13/A, regenerated cellulose, Schleicher & Schuell MicroScience, Inc., FL). Losses of nitroaromatics because of filtration with these filters are reported to be less than 2% (*12*), and all HPLC samples were analyzed immediately as described previously (*3*). Sample withdrawal and filtration was performed inside the oxygen-free glovebag to avoid sample oxidation. Calibration standards were obtained from authentic compounds, and multipoint standard calibration curves were established for quantification of the compounds as described by us previously (*3*). Pure compounds (i.e., standards) of HADNTs and DHANTs are very unstable and minor degradation of the standard compounds occurring during shipment and handling could have caused slightly skewed calibration equations for these compounds (*3*, *36*). A Spectra/Chrom CF-1 Fraction Collector (Spectrum Chromatography, TX) was used to collect radiolabeled fractions of the HPLC eluent. ^{14}C in HPLC fractions and stock solutions was quantified with a Packard scintillation analyzer (Tri-Carb 1900CA) after addition of samples to Ultima Gold scintillation cocktail (Packard Instrument, CT).

Results and Discussion

Influence of Ferrihydrite and AQDS on the Transformation of TNT by ES6. TNT was completely reduced within 24 h in the presence of strain ES6 with 10 mM sucrose as the electron donor resulting in the concurrent production of mostly 4-HADNT (step 1.1, Figure 1). Smaller amounts of 2-HADNT (step 1.2, Figure 1) and 4,4′-Azoxy (step 4, Figure 1; Figure 2a) were also observed. The production of the dimer 4,4′-Azoxy was likely a result of spontaneous hydroxylamino-nitroso condensation reactions (*3*, *6*, *36*). Further reduction of 4-HADNT lead primarily to 2,4-DHANT (step 2), 4-ADNT (step 6), and one unidentified compound. On the basis of abiotic experiments described previously (*3*), in which a standard mixture containing 2- and 4-HADNT was allowed to react in the presence of oxygen, the unidentified compound is believed to be either the 2′,4,6,6′-tetranitro-2,4′-azoxy-toluene (2,4′-Azoxy) or the 2,4′,6,6′-tetranitro-2′,4-azoxy-toluene (2′,4-Azoxy; Figure 1). 2-HADNT, 4-HADNT, and their

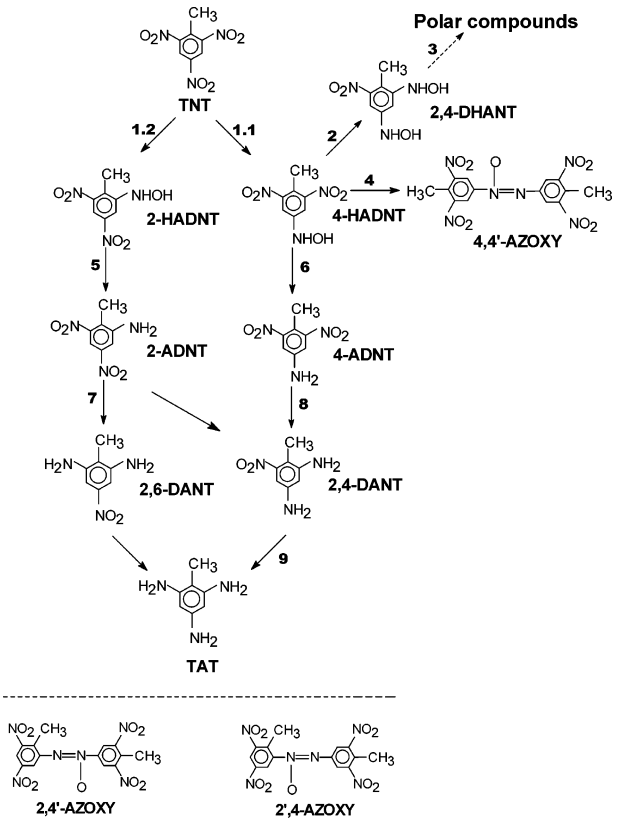

FIGURE 1. TNT degradation pathways and metabolites observed in this study under biotic or abiotic conditions.

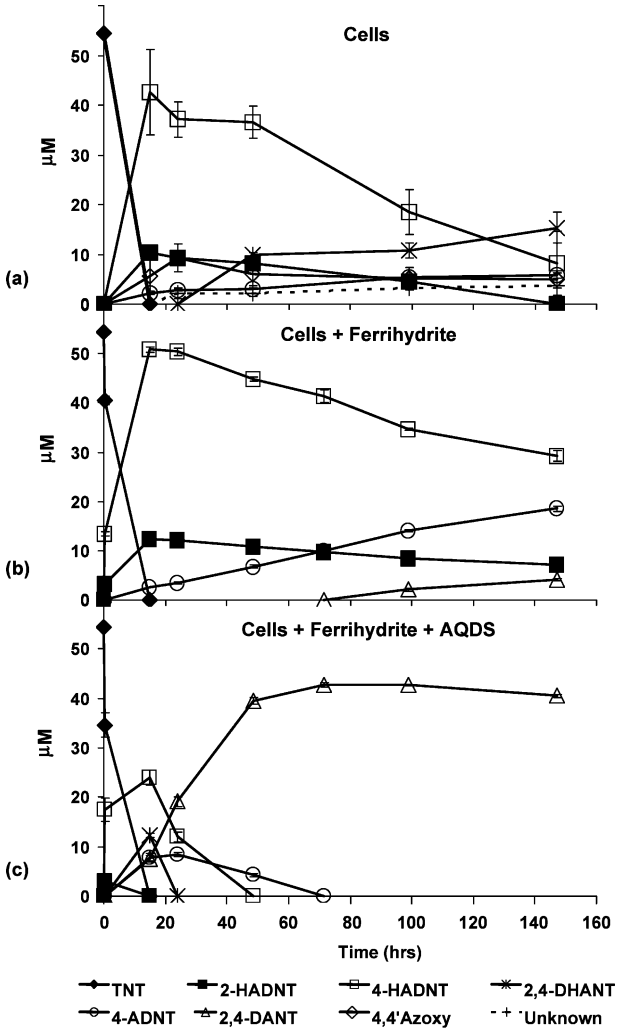

FIGURE 2. Transformation of 2,4,6-trinitrotoluene (TNT) with (a) strain ES6, (b) ES6 + ferrihydrite, and (c) ES6 + ferrihydrite + 100 μM anthraquinone-2,6-disulfonate (AQDS). The unknown compound observed in Figure 2a may be 2′,4-Azoxy or 2,4′-Azoxy (see discussion in text and Figure 1). Inoculation and TNT addition occurred simultaneously at 0 h. Sucrose (10 mM) was used as the electron donor. Experiments were conducted in triplicate and error bars represent one standard deviation. Where not visible, error bars are smaller than the symbol size.

nitroso analogues are known precursors of azoxy compounds (*36*, *37*).

The observed TNT degradation pattern in the presence of ES6 is similar to that obtained with other fermenting bacteria although we detected azoxy derivatives, which had not been reported by others. Lewis et al. (*38*) demonstrated that *Clostridium bifermentans* was capable of completely reducing TNT to TAT with a transient appearance of HADNTs, 4-ADNT, 2,4-DHANT, 2-hydroxylamino-4-amino-6-nitrotoluene (2-HA4ANT), and 2,4-DANT. *Clostridium thermoaceticum* and *C. acetobutylicum* were both reported to degrade TNT via 2-HADNT, 4-HADNT, and 2,4-DHANT to a Bamberger rearrangement product (*39*−*42*).

The addition of ferrihydrite to ES6 cultures caused a significant shift in the TNT degradation pathway (Figure 2b). No 2,4-DHANT or azoxy compounds were observed; however, degradation was accelerated via the para-substituted intermediates including 4-HADNT, 4-ADNT, and eventually 2,4-DANT (steps 1.1, 6, and 8; Figure 1). Importantly, 2-HADNT is also formed in the presence of ferrihydrite, but as will be discussed below, ortho-substituted metabolites (such as 2-HADNT, 2-ADNT, and 2,6-DANT) become more important in systems where higher concentrations of surface-associated Fe(II) are present. Only 79 ± 16 μM of surface-associated Fe(II) was formed within 24 h in these systems containing cells and ferrihydrite.

Results in the presence of ES6, ferrihydrite, and 100 μM AQDS (Figure 2c) showed further alterations in the TNT transformation pattern. Increased transformation rates of 4-HADNT and 4-ADNT to 2,4-DANT were observed in the presence of AQDS (steps 1.1, 6, and 8, Figure 1). Only traces of 2-HADNT and 2,4-DHANT were observed and by 70 h, 2,4-DANT was the dominant product accounting for 79% of the mass balance. The accelerated and more complete reduction of TNT metabolites in the presence of cells, ferrihydrite, and AQDS represents a significant shift in the degradation pattern compared to single component systems. The observed decrease in mass balance of 25% indicated that AQDS also caused the production of metabolites not identifiable with the robust HPLC-DAD method utilized in this study and that was designed to maximize metabolite identification (*3*). Because of the loss in mass balance and the potential for direct reduction of TNT by AHQDS, ^{14}C-labeled TNT degradation studies were conducted to further investigate the fate of TNT in the presence of AQDS (described in a later section).

No significant difference in bacterial growth (CFU, protein, and OD) was observed among treatments with or without AQDS or ferrihydrite; consequently, the transformation patterns shown in Figure 2 (a−c) indicate that strain ES6, ferrihydrite, and AQDS influence the transformation of TNT via several possible reaction mechanisms: (1) TNT and its metabolites can be directly reduced by strain ES6 to form 4-HADNT, 2,4-DHANT, and azoxy compounds, (2) TNT degradation can be enhanced primarily via formation of 4-HADNT and 4-ADNT in the presence of ferrihydrite and strain ES6, (3) AQDS can influence the degradation of TNT by ES6 directly via enhanced electron transfer to TNT, and

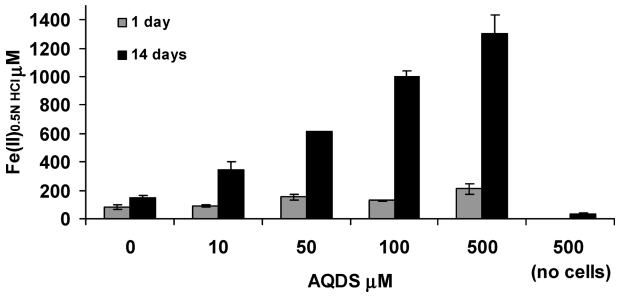

FIGURE 3. Reduction of ferrihydrite (6 mM) by ES6 as a function of AQDS after 1 and 14 days of incubation. Sucrose (10 mM) was used as the electron donor. Experiments were conducted in triplicate and error bars correspond to one standard deviation.

(4) AQDS can contribute to TNT degradation indirectly via enhanced electron transfer from ES6 to ferrihydrite resulting in a higher concentration of surface-associated redox-active Fe(II) and increased importance of Fe(II)-mediated TNT reduction (*12*, *43*). An evaluation of the relative contribution of these four different mechanisms to the fate of TNT will be the focus of the remainder of this manuscript.

Influence of Surface-Associated Fe(II) on the Transformation of TNT. Hofstetter et al. (*12*) investigated the fate of TNT in Fe(III)-reducing aquifer columns and found that surface-bound Fe(II) was responsible for TNT reduction. Thus, we postulated that surface-associated Fe(II) also plays an important role in TNT reduction in the presence of ferrihydrite. AQDS is known to enhance the biogenic production of Fe(II) by a wide range of bacteria (*14*, *17*−*21*), which can in turn increase the abiotic reduction of TNT by surface-associated Fe(II). Hence, a brief study was conducted in which the production of Fe(II) was analyzed as a function of AQDS concentration to verify that AQDS promoted Fe(III) reduction by ES6. Figure 3 clearly indicates that the production of Fe(II) from ferrihydrite increases with increasing AQDS concentrations after 2 weeks of incubation with ES6. A control containing 500 μM AQDS without cells did not show significant Fe(II) production. No differences in bacterial growth (turbidity and CFU) were observed between systems with and without AQDS (data not shown).

The abiotic contributions of ferrihydrite−Fe(II)-mediated reduction of TNT were elucidated in additional studies where Fe(II) was added to cell-free batch systems in the presence and absence of ferrihydrite. Three abiotic TNT treatments were established under anaerobic conditions at pH 7: (i) 6 mM ferrihydrite in SGW, (ii) 6 mM ferrihydrite + 0.89 mM Fe(II) in SGW, and (iii) 0.89 mM Fe(II) in DI water (to rule out reactions between SGW and dissolved Fe(II) in the absence of ferrihydrite). In treatment ii, 92% of the Fe(II) added was sorbed to the ferrihydrite surface (after 24 h of preequilibration) at the time of TNT injection. While no TNT transformation was observed in systems containing only ferrihydrite or only dissolved Fe(II), TNT was degraded rapidly (within 5 h) in the combined Fe(II)−ferrihydrite system (Figure 4). These observations are in agreement with Hofstetter et al. (*12*) who reported that TNT was only reduced by Fe(II) if it was associated with goethite surfaces. Further, these results show that abiotic TNT degradation by Fe(II)−ferrihydrite proceeds preferentially via the ortho-substituted isomer 2-HADNT relative to the para-substituted isomer 4-HADNT, followed by the formation of 2-ADNT (5.2 μM), 4-ADNT (5.9 μM), 2,6-DANT (2.7 μM), and 2,4-DANT (3.7 μM) after 24 h (Figure 4). The persistence of HADNTs during the 24-h reaction time suggests that a significant fraction of the Fe(II) added was not reactive with TNT or its reduced metabolites. The production of the observed TNT metabolites required 316 μM of electrons which corresponds to an oxidation of 39% of the added Fe(II). It has been noted that

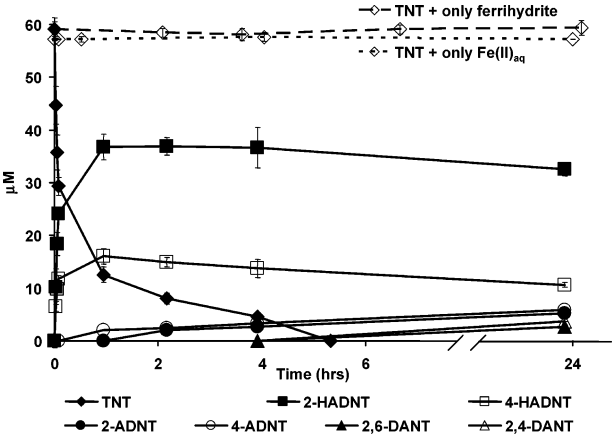

FIGURE 4. Abiotic transformation of TNT in the presence 6 mM ferrihydrite and 0.89 mM $Fe(II)_{tot}$ at pH 7. No TNT metabolites were observed for treatments containing only 6 mM ferrihydrite or only 0.89 mM $Fe(II)_{aq}$. Experiments were conducted in triplicate and error bars correspond to one standard deviation. Where not visible, error bars are smaller than the symbol size.

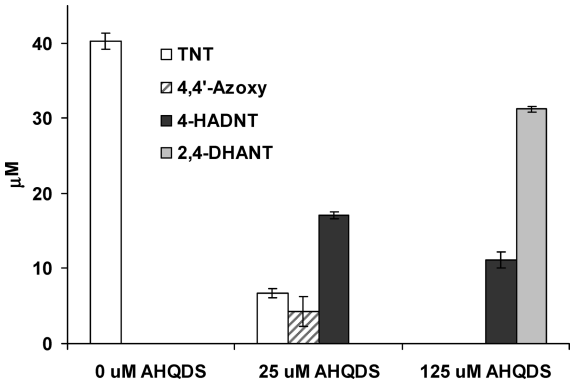

FIGURE 5. Reduction of TNT by abiotically produced AHQDS showing the product distribution after 16 h reaction time. Experiments were conducted in triplicate and error bars correspond to one standard deviation.

in mixed Fe(III)/Fe(II)-mineral systems not all sorbed Fe(II) is always available for redox reactions because of possible electron delocalization within the bulk phase and that aqueous Fe(II) is necessary to drive the reduction reactions (*44*−*46*). It is also possible that optimum $Fe(II)_{aqueous}$:$Fe(II)_{surface}$ concentrations were not achieved for maximum reduction rates in our study (*46*). However, the shift in degradation pathway to the ortho-substituted isomer (2-HADNT) appeared to be an important metabolite signature for TNT reduction via Fe(II)−ferrihydrite.

Influence of AQDS on the Fate of TNT. The impact of AQDS on TNT reduction in the presence and absence of ES6 was investigated in a series of experiments with both ^{14}C-labeled and unlabeled TNT. Instantaneous and direct chemical reduction of TNT by abiotically produced AHQDS was observed (Figure 5). AHQDS concentrations equal to or greater than 250 μM resulted in the production of yet unidentified metabolites eluting in the polar region of the HPLC chromatogram (retention time (t_r) = 2.2−2.9 min; data not shown). Mass balances of 80−105% were obtained at lower AHQDS concentrations (25 and 125 μM) after 16 h of reaction time. The primary transformation products observed were the hydroxylamino compounds, 4-HADNT and 2,4-DHANT, but a small amount of 4,4′-Azoxy was produced in the system amended with 25 μM AHQDS (Figure 5). An electron balance revealed that 116−136% of the electrons added as AHQDS was accounted for by the TNT metabolites observed, consistent with the good TNT mass balance. The

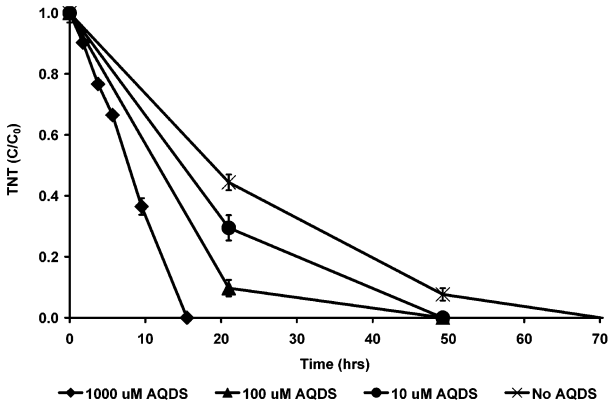

FIGURE 6. Reduction of 60 μM TNT by ES6 as a function of AQDS concentration. Sucrose (10 mM) was used as the electron donor. Experiments were conducted in triplicate and error bars correspond to one standard deviation. Where not visible, error bars are smaller than the symbol size.

higher than theoretically possible reduction of TNT may have been due to a slight underestimation of the amount of reduced AQDS present as determined via the Fe(III)−citrate assay.

In the presence of ES6, increasing AQDS concentrations resulted in increasing rates of TNT reduction (Figure 6). The initial cell concentration in these studies was approximately 2 orders of magnitude lower (1.5×10^7 CFU mL^{-1}) than in the previous studies (1.8×10^9 CFU mL^{-1}, Figure 2) to allow for clear separation of the impact of AQDS on the TNT degradation rate in the presence of ES6. The TNT degradation pathway in the presence of 1000 μM AQDS and ES6 was clearly dominated by the early production of 4-HADNT (peaking at 36 μM by 18 h), followed by formation of 4,4′-Azoxy and 2,4-DHANT (Figure 7a). Importantly, the TNT reduction patterns in the presence of strain ES6 and AQDS agree with TNT metabolites observed during TNT reduction with abiotically produced AHQDS (Figure 5). At 30 h, 2,4-DHANT accounted for approximately 90% of the added ^{14}C-TNT; however, the disappearance of 2,4-DHANT between 30 and 120 h (Figure 7a) resulted in no measurable TNT metabolites using HPLC-DAD. No significant mineralization of ^{14}C-TNT to $^{14}CO_2$ was observed and the total recovery of ^{14}C-TNT was 91 ± 4%. The possible identity of nondetectable metabolites was assessed by analyzing the solutions obtained at 240 h using HPLC-^{14}C detection (Figure 7b).

The majority of ^{14}C eluted between approximately 2.7 and 8 min and was not associated with TNT metabolites that are detectable using the HPLC-DAD method (Figure 7b). These retention times correspond to compounds with a polarity similar to or higher than 2,4-DANT (t_r of 6.7 min). Approximately 15% of the products appeared to have polarities similar to or higher than TAT (t_r of 4.1 min). Approximately 20% of the ^{14}C recovered was associated with HPLC peaks eluting near AQDS ($t_r \sim$ 4.6 min; Figure 7b). However, significant binding of metabolites to AQDS appeared unlikely in the present study because the area of the AQDS peak remained constant throughout all experiments. This is in agreement with previous studies showing no significant covalent binding of nucleophilic compounds, such as 2,4-DANT, to natural organic matter (NOM) in anaerobic sediments (*47*).

A significant fraction of the original ^{14}C-TNT (∼22%) was recovered during filtration prior to HPLC analysis indicating binding to or uptake by biomass or precipitation of TNT metabolites (losses of dissolved nitroaromatic compounds because of filtration is less than 2% (*12*)). The potential for binding of TNT metabolites to bacterial cells is consistent

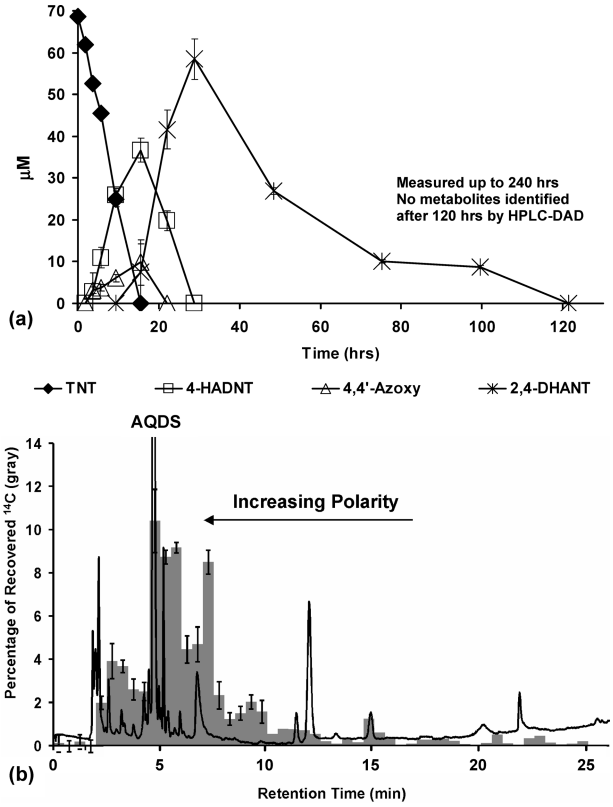

FIGURE 7. Transformation of ^{14}C-radiolabeled TNT in the presence of ES6 and 1000 μM AQDS (a) metabolite identification from 0 to 120 h on the basis of UV diode array detection and (b) superimposed HPLC chromatogram and ^{14}C radiogram (gray bars) obtained after 240 h of reaction time. Where not visible, error bars are smaller than the symbol size.

with previous studies showing that a fraction of the polar TNT metabolites clearly became associated with cells of *Pseudomonas pseudoalcaligenes* strain JS52 (*48*).

Fiorella and Spain (*48*) also reported that TNT was converted to unidentified polar metabolites via the hydroxylamino intermediates 2,4-DHANT and 2-HA4ANT by *Pseudomonas pseudoalcaligenes* strain JS52. Later, Hughes et al. (*41*) reported that *Clostridium acetobutylicum* was able to reduce TNT to 2,4-DHANT and then to hydrophilic phenolic products (e.g., 2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene) via Bamberger rearrangement. The UV spectrum of the Bamberger rearrangement product described by Hughes et al. (*41*) was similar to one of the metabolites (t_r = 2.5 min) observed in this study. In addition, one of the unidentified chromatographic peaks (UV λ_{max} [nm], 221 and 329) with a retention time of 6.7 min (Figure 7b) was produced concomitantly with the disappearance of 2,4-DHANT after 16 h (data not shown). Consequently, the production of unidentified polar metabolites consistently correlates with the formation and subsequent disappearance of 2,4-DHANT.

Long-Term TNT Transformation in Preincubated Systems. In natural systems, it is expected that bacteria and Fe minerals will have been present for an extended period of time prior to contaminant introduction. Thus, it is likely that surface-associated Fe(II) is already present and available for reaction with contaminants such as heavy metals, radionuclides, or TNT (*49*). To simulate such a situation, strain ES6 was preincubated in the presence of ferrihydrite for 14 days to allow for the production of surface-associated Fe(II) prior to TNT injection. Similar to the experiments without preincubation, three treatments were established (a) cells only, (b) cells + ferrihydrite, and (c) cells + ferrihydrite + AQDS. The addition of AQDS was intended to increase Fe(II)

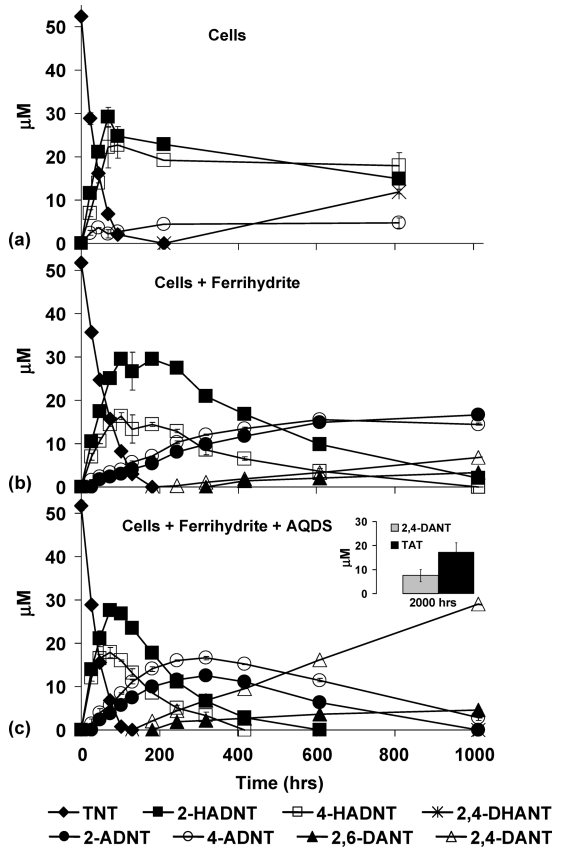

FIGURE 8. Transformation of TNT with (a) strain ES6, (b) ES6 + ferrihydrite, and (c) ES6 + ferrihydrite + 100 μM AQDS (inset shows metabolites (2,4-DANT and TAT) present after 2000 h reaction time). Sucrose (10 mM) was used as the electron donor. The systems were preincubated with strain ES6 for 2 weeks prior to injection of TNT. The treatments (a–c) contained (at time of TNT injection) 0, 176, and 704 μM of Fe(II)$_{0.5N\ HCl}$, respectively. Experiments were conducted in triplicate and error bars correspond to one standard deviation. Where not visible, error bars are smaller than the symbol size.

production by strain ES6 during the 14-day preincubation before addition of TNT. Preincubation with strain ES6 resulted in the production of 176 μM and 704 μM Fe(II)$_{0.5N\ HCl}$ in the presence of ferrihydrite and ferrihydrite + AQDS, respectively (equivalent to a reduction of 3% and 12% of the total Fe(III) available in these systems).

In the absence of ferrihydrite (Figure 8a), the TNT transformation pattern was quite similar to the transformation pattern with freshly grown ES6 cells (Figure 2a) with two notable exceptions: (i) the rates of TNT and metabolite reduction were lower and (ii) lower concentrations of 4-HADNT but higher concentrations of 2-HADNT were observed after 14 days of preincubation. The lower reduction rates may be explained by an approximately 7 times lower cell count (CFU) at the time of TNT addition after 14 days of preincubation. The higher relative concentration of 2-HADNT may be due to the presence of additional reductants in the cell suspension after 14 days of preincubation, which may be capable of nitroaromatic reduction. For example, it has been reported that the accumulation of microbially produced reductants such as ferredoxins, hydrogenases, and carbon monoxide dehydrogenase can rapidly reduce TNT to 2-HADNT, 4-HADNT, and 2,4-DHANT (*38*, *39*). The presence of such reductants after 14 days of microbial growth might have led preferentially to the reduction of the ortho-substituted nitro group (Figure 8a) and might also explain the decreased formation of azoxy compounds when compared to the fresh cell suspensions (Figure 2a).

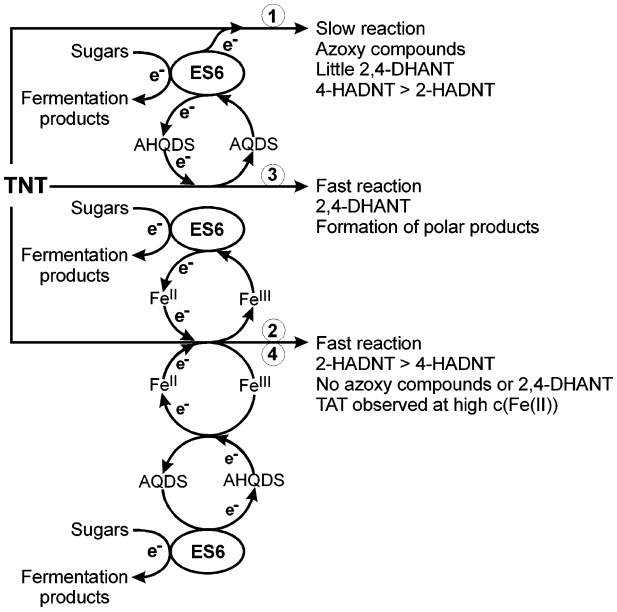

FIGURE 9. Conceptual model indicating four different processes influencing the transformation of TNT and its metabolites in model systems containing fermenting bacteria, iron minerals, and electron-shuttling compounds.

Degradation patterns of TNT in systems preincubated for 14 days in the presence of ferrihydrite (Figure 8b) also revealed a shift from the para-substituted metabolites (4-HADNT/4-ADNT) toward the ortho-substituted metabolites (2-HADNT/2-ADNT) (steps 1.2 and 5, Figure 1). Preincubated systems containing 176 μM microbially produced surface-associated Fe(II) showed higher concentrations of 2-HADNT and 2-ADNT than observed when TNT was added to fresh ferrihydrite-cell suspensions (Figure 2b).

TNT degradation patterns were further accelerated toward reduced metabolites in preincubated treatments containing AQDS and Fe(II)–ferrihydrite (704 μM Fe(II)$_{0.5N\ HCl}$) ultimately resulting in the formation of TAT (Figure 8c). In the presence of significant Fe(II)–ferrihydrite, a consistent shift in TNT degradation pathway was observed resulting in greater 2-HADNT production as well as the formation of 2-ADNT and 2,6-DANT. Abiotic controls focused on the reduction of TNT by ferrihydrite-associated Fe(II) (Figure 4) demonstrated a preferential reduction of the nitro group in the ortho position; consequently, the increase in 2-HADNT in Figure 8b and 8c is attributed primarily to the abiotic reduction of TNT by biologically produced Fe(II).

Environmental Significance. The microbial degradation of TNT was influenced by both electron-shuttling compounds and ferrihydrite. Since electron-shuttling compounds can influence the transformation pattern via several different reactions, depending on the presence of iron minerals, it is important to know which potential reductants are predominant in a given system. Microbial metabolism may be the ultimate source of electron flux, but the presence of ferrihydrite or electron-shuttling compounds has differential effects on TNT degradation patterns and rates via subsequent electron-transfer reactions. In the current study, at least four different mechanisms contribute to TNT degradation depending on what components are present in the system (Figure 9): (1) the direct reduction of TNT and its metabolites by strain ES6, (2) the microbial reduction of ferrihydrite resulting in surface-associated Fe(II), which then reductively transforms TNT, (3) the enhanced electron transfer from strain ES6 to TNT in the presence of AQDS, and (4) the enhanced electron transfer from strain ES6 to ferrihydrite via AQDS resulting in a higher concentration of surface-

associated redox-active Fe(II), which then increases the rate of Fe(II)-mediated TNT reduction.

Results of the current study indicate that the transformation patterns of TNT by fermenting bacteria such as strain ES6 can change remarkably in the presence of surface-associated Fe(II) and electron shuttles. In systems containing significant amounts of biogenically produced surface-associated Fe(II), an increased formation of ortho-substituted transformation products, such as 2-HADNT, and the formation of more reduced TNT metabolites were observed. The addition of AQDS lead to an accelerated and more complete reduction of TNT including the formation of TAT because of (i) higher concentrations of surface-associated Fe(II) and (ii) increased AQDS-mediated electron transfer from the bacterial cell to the nitroaromatics. The presence of AQDS in ferrihydrite-free cell suspensions resulted in an increase in the initial TNT reduction rate and a significant change in metabolite trajectory consisting of more polar metabolites, which are likely phenolic amines or protocatechuates (*50*). The combined presence of ferrihydrite and AQDS did not result in an additive metabolite pattern; the enhanced production of surface-associated Fe(II) in the presence of AQDS was more important in defining the metabolite trajectory than effects caused by AQDS alone.

The production of more reduced TNT metabolites in the presence of surface-associated Fe(II) is an important observation since these compounds (e.g., DANTs and TAT) have been reported to bind strongly to natural organic matter in the presence of oxygen (*2*, *7*−*11*). Such reactions could lead to the irreversible immobilization of TNT and thus represent a viable remediation strategy. The formation of more polar metabolites in the presence of electron shuttles is particularly interesting since ring cleavage and complete degradation of such compounds have been reported (*41*, *48*, *50*, *51*). While the use of AQDS as a bioremediation amendment has yet to be implemented, natural organic matter rich in quinone moieties could have a similar influence on the fate of TNT. Still, more studies are needed to characterize the nature of the formed polar metabolites in this study and the likelihood of ring cleavage.

Ultimately, the fate of TNT is controlled by the relative contribution of multiple individual mechanisms in mixed systems, and it is important to recognize that results from single component experiments may not be reliable predictors of the behavior of contaminants in complex environments. Certainly, further experiments that address the combined influence of abiotic and biotic processes are necessary for improving our understanding of the fate of TNT in soil−water systems.

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