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Microbial Transformation of Nitroaromatics in Surface Soils and Aquifer Materials

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Microorganisms indigenous to surface soils and aquifer materials collected at a munitions-contaminated site transformed 2,4,6-trinitrotoluene (TNT), 2,4-dinitrotoluene (2,4-DNT), and 2,6-dinitrotoluene (2,6-DNT) to amino-nitro intermediates within 20 to 70 days. Carbon mineralization studies with both unlabeled (TNT, 2,4-DNT, and 2,6-DNT) and radiolabeled ([^{14}C]TNT) substrates indicated that a significant fraction of these source compounds was degraded to CO_2.

The groundwater, soils, and sediments at many military sites are contaminated with nitroaromatic wastes as a result of the manufacture, loading, assembling, packing, and unloading of 2,4,6-trinitrotoluene (TNT)-based munitions (10). TNT is an environmental hazard because of its toxicity to fish (17, 23), algal species (23, 28), microorganisms (13, 28), and other organisms (28). The principal impurities created during TNT manufacture, 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT), are also listed as U.S. Environmental Protection Agency priority pollutants on account of environmental toxicity (12).

Numerous investigators have examined microbial transformation of nitroaromatic compounds (1–5, 8, 9, 11, 14–18, 22, 24, 25, 27, 29). However, these studies utilized artificially selected microbial communities, such as sewage and activated sludge systems (4, 9, 14), bioslurry and composting mixtures, (8, 11), and isolated cultures of bacteria (1, 2, 13, 15–17, 24, 29) and fungi (5, 18, 25, 27). Surprisingly little is known about the capability of native microbial communities to degrade nitroaromatic compounds in situ. In particular, transformation of nitroaromatics by microorganisms indigenous to aquifer systems has not been demonstrated conclusively. Here, we report the potential for in situ biodegradation of TNT, 2,4-DNT, and 2,6-DNT by microbial communities indigenous to the surface soils and aquifer materials at an inactive munitions plant near Weldon Spring, Mo.

Even though munitions manufacturing and handling at Weldon Spring ceased circa 1945, recent surveys at the site revealed widespread contamination of the surface soils and underlying aquifer by nitroaromatic compounds. Of the 6,200 surface soil samples collected at the site, 15.9% contained concentrations of TNT that were greater than 30 mg/kg of dry soil (6). The U.S. Army has detected dissolved concentrations of as high as 19 μg/liter for TNT and 8.5 μg/liter for DNT in the underlying aquifer (22). This groundwater contamination may be a relic of past waste disposal practices when the contaminants are stable in situ and not subject to significant microbial degradation. Alternatively, when microorganisms capable of nitroaromatic degradation are present, a continuous supply of nitroaromatics from leaching of contaminated surface soils is indicated. The presence of significant amounts of 4-amino-2,6-DNT and 2-amino-4,6-DNT in the shallow soils and groundwater collected beneath areas of high surface TNT contamination is suggestive of in situ degradation of TNT, because these compounds are commonly reported products of microbial TNT transformation (1, 11, 14, 16, 28). However, the presence of these compounds does not provide conclusive evidence of biodegradation, because trace amounts of these compounds may be produced as impurities during munitions manufacture. Thus, an evaluation of the potential for degradation of nitroaromatic compounds by indigenous microorganisms is essential to resolving the source of groundwater contamination at Weldon Spring.

TNT, 2,4-DNT, 2,6-DNT, 4-amino-2,6-DNT, and 2-amino-4,6-DNT were obtained from SRI International (Menlo Park, Calif.). 2-Amino-4-nitrotoluene, 2-amino-6-nitrotoluene, and 4-amino-2-nitrotoluene were obtained from Aldrich Chemical Company (Milwaukee, Wis.). The purity of all standards was 99% or greater. Uniformly labeled [^{14}C]TNT (26.3 mCi mmol^{-1}) was obtained from Du Pont (NEN Research Products, Boston, Mass.). The purity of the radiolabeled TNT was determined by thin-layer chromatography and high-performance liquid chromatography (HPLC) and was found to be greater than 99%.

The abilities of microorganisms indigenous to Weldon Spring to transform TNT, 2,4-DNT, and 2,6-DNT were investigated by using contaminated surface soil (red tank soil), uncontaminated surface soil (top soil), fractured carbonate bedrock material (carbonate), and material from a weathered, semiconsolidated, water-bearing zone (residuum) that occurs at the top of the carbonate bedrock. The contaminated red tank soil was collected within an inactive red-water holding lagoon. The top soil was collected at an adjacent site with no detectable nitroaromatic contamination and no history of nitroaromatic exposure. Both surface samples were collected with flame-sterilized hand tools to excavate a core of soil. Cores of the residuum and carbonate aquifer materials (approximately 15-cm diameter by 40-cm length) were collected by rotary drilling with a steam-sterilized bit. All samples were stored at 4°C prior to beginning the microcosm studies.

The sample cores were broken to expose undisturbed material, and the radial centers were subsampled with sterilized instruments. About 20 g (wet weight) of core material was placed in sterile 40-ml serum vials, and 20 ml of sterile test solution was added. Test solutions consisted of TNT, 2,4-DNT, or 2,6-DNT dissolved in 10% methanol and diluted 100-fold with distilled water to yield a concentration of 100 μM. Experimental microcosms were prepared in triplicate for each combination of sample and nitroaromatic test substrate. In

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addition, triplicate live controls were prepared in the same manner without the addition of TNT, 2,4-DNT, or 2,6-DNT. Duplicate abiological controls were prepared for each soil and sediment sample by sterilizing the microcosms (5 mM $HgCl_2$; autoclaved at 121°C for 1 h) prior to addition of the test substrate. The microcosms were incubated statically in the dark and at room temperature. The microcosms were regularly sampled for dissolved nitroaromatics and headspace CO_2 over a period of approximately 70 days.

TNT, 2,4-DNT, and 2,6-DNT were analyzed by HPLC. A few hours before sampling, the treatment vials were shaken by hand to thoroughly mix the interstitial and standing water. Previous studies demonstrated that the dissolved and adsorbed phases of these compounds reached equilibrium within 1 to 2 h of disturbance (6, 19). For sampling, 0.5 ml of standing water was removed, filtered (0.2-μm-pore size filter), and analyzed. The sample volume was replaced with 99.9% O_2 gas. The various nitroaromatic compounds were analyzed on a Beckman Ultrasphere C_{18} column at 40°C with an acetonitrile-distilled water-methanol mobile phase. 2,4-DNT and 2,6-DNT and their amino-nitro intermediates (2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, and 2-amino-6-nitrotoluene) were analyzed isocratically with a 15:45:40 acetonitrile-distilled water-methanol mobile phase. TNT and its amino-nitro intermediates (2-amino-4,6-DNT and 4-amino-2,6-DNT) were analyzed by gradient elution with a 15:80:5 acetonitrile-distilled water-methanol mobile phase ramping up to 47:48:5 over 25 min and holding for 5 min. The amino-nitro intermediates were identified by fortification and coelution with known standards. All compounds were detected by UV absorption at 250 nm. To estimate overall microbial metabolism, the rate of microcosm CO_2 production was measured by thermal conductivity detection gas chromatography. Dissolved CO_2 concentrations were estimated from Henry's law coefficients (26). Rates of CO_2 production were estimated by linear regression of the amount of CO_2 produced per gram of dry soil or sediment against time (20).

The potential for microbial mineralization of nitroaromatic compounds was investigated by using uniformly labeled [^{14}C]TNT. Red tank soil, top soil, and residuum samples were collected and subsampled as described for the disappearance studies. About 3 g (wet weight) of material was placed in sterile 40-ml serum vials. Stopper and base trap assemblies were prepared by attaching 500-μl plastic cups to Teflon-coated butyl rubber stoppers and autoclaving (121°C for 1 h). Microcosms were then sealed with the empty base trap suspended inside. Abiological controls were prepared by adding $HgCl_2$ (5 mM) and autoclaving the microcosms (121°C for 1 h).

The microcosms were amended with approximately 4×10^5 dpm of uniformly labeled [^{14}C]TNT and were shaken by hand to thoroughly disperse the label. The microcosms were incubated statically at room temperature and in the dark. At each time point, triplicate experimental vials and a single control vial were acidified with 1,000 μl of 2 N H_3PO_4. Evolved $^{14}CO_2$ was collected by placing 200 μl of 1 N KOH in the suspended base traps and shaking the acidified microcosms overnight. The ^{14}C recovered in the base solution was quantified by liquid scintillation counting. The recovery efficiency of $^{14}CO_2$ in the sample material was determined with $H^{14}CO_3$. Reported values were corrected for recovery efficiencies and the activity that was detected in sterilized control vials.

Nitroaromatic transformations. The results of the present study indicate that the microbial communities associated with surface soils and aquifer materials at Weldon Spring are capable of transforming TNT, 2,4-DNT, and 2,6-DNT. In most cases, complete disappearance of the source compound from the dissolved phase was achieved in 20 to 70 days (Fig. 1 to 4). Under the conditions used in this study, the detection limit for TNT, 2,4-DNT, and 2,6-DNT was 0.05 μM. In the carbonate and residuum treatments, in which the test substrate was not completely transformed, the initial concentrations of 2,6-DNT were reduced by 90 and 60%, respectively (Fig. 1 and 2). In all cases, disappearance of the test substrate could be attributed to biological activity, because no significant decrease in concentration occurred when the microcosms were sterilized before the addition of substrate (Fig. 1 to 4).

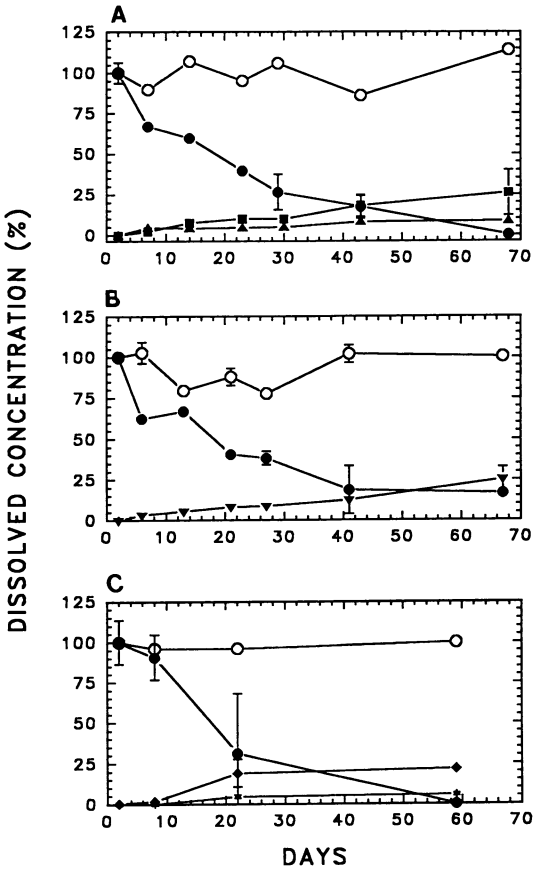

FIG. 1. Microbially mediated disappearance of nitrotoluene compounds from carbonate microcosms. (A) Dissolved concentrations of 2,4-DNT in experimental microcosms (●) and killed controls (○) and accumulation of 4-amino-2-nitrotoluene (■) and 2-amino-4-nitrotoluene (▲) in the dissolved phase; (B) dissolved concentrations of 2,6-DNT in experimental microcosms (●) and killed controls (○) and accumulation of 2-amino-6-nitrotoluene (▼) in the dissolved phase; (C) dissolved concentrations of 2,4,6-TNT in experimental microcosms (●) and killed controls (○) and accumulation of 4-amino-2,6-DNT (◆) and 2-amino-4,6-DNT (*) in the dissolved phase. Datum points are means of triplicates ± standard deviations (SD).

Decreases in the dissolved concentrations of the TNT or DNT test substrates were accompanied by accumulation of amino-DNT or amino-mononitrotoluene compounds, respectively (Fig. 1 to 4). The initial and quantitatively most significant intermediate products of TNT transformation were 4-amino-2,6-DNT and 2-amino-4,6-DNT. The fact that the dissolved concentration of 4-amino-2,6-DNT was at least twice that of 2-amino-4,6-DNT indicates that the *para*-nitro group was reduced most readily. This pattern is consistent with the findings of previous investigations of microbial TNT transfor-

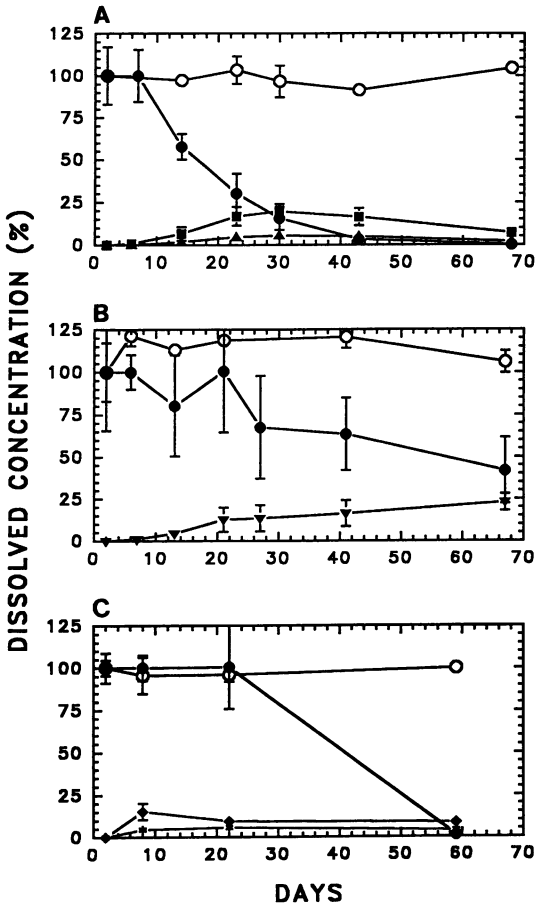

FIG. 2. Microbially mediated disappearance of nitrotoluene compounds from residuum microcosms. (A) Dissolved concentrations of 2,4-DNT in experimental microcosms (●) and killed controls (○) and accumulation and subsequent disappearance of 4-amino-2-nitrotoluene (■) and 2-amino-4-nitrotoluene (▲) in the dissolved phase; (B) dissolved concentrations of 2,6-DNT in experimental microcosms (●) and killed controls (○) and accumulation of 2-amino-6-nitrotoluene (▼) in the dissolved phase; (C) dissolved concentrations of 2,4,6-TNT in experimental microcosms (●) and killed controls (○) and accumulation and subsequent disappearance of 4-amino-2,6-DNT (◆) and 2-amino-4,6-DNT (*) in the dissolved phase. Datum points are means of triplicates ± SD.

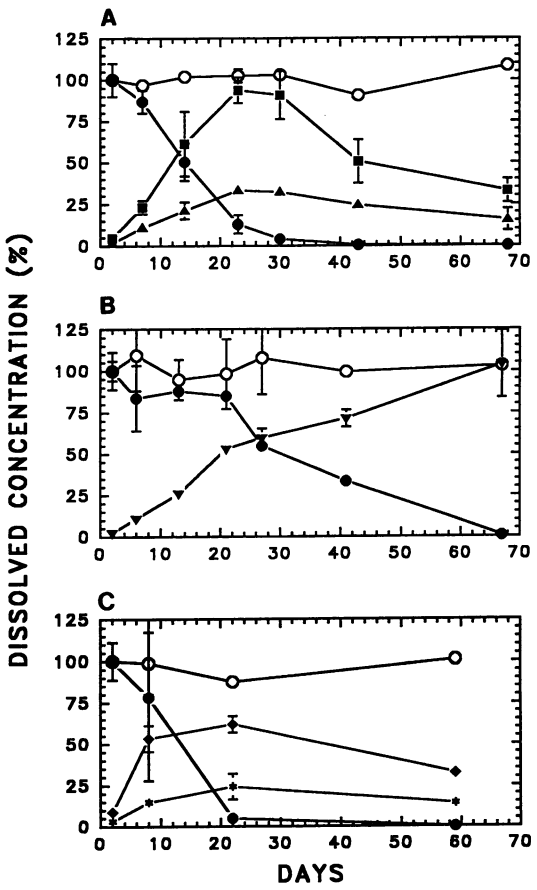

FIG. 3. Microbially mediated disappearance of nitrotoluene compounds from red tank soil microcosms. (A) Dissolved concentrations of 2,4-DNT in experimental microcosms (●) and killed controls (○) and accumulation and subsequent disappearance of 4-amino-2-nitrotoluene (■) and 2-amino-4-nitrotoluene (▲) in the dissolved phase; (B) dissolved concentrations of 2,6-DNT in experimental microcosms (●) and killed controls (○) and accumulation of 2-amino-6-nitrotoluene (▼) in the dissolved phase; (C) dissolved concentrations of 2,4,6-TNT in experimental microcosms (●) and killed controls (○) and accumulation and subsequent disappearance of 4-amino-2,6-DNT (◆) and 2-amino-4,6-DNT (*) in the dissolved phase. Datum points are the means of triplicates ± SD.

mation (8, 10, 16). Likewise, 2,4-DNT was transformed immediately to 4-amino-2-nitrotoluene and, to a lesser extent, 2-amino-4-nitrotoluene as described previously (14, 15). In contrast, the major intermediate product of 2,4-DNT transformation by the white rot fungus *Phanaerochaete chrysosporium* was 2-amino-4-nitrotoluene (27). In the present study, 2,6-DNT was transformed to 2-amino-6-nitrotoluene.

The transformation of nitrotoluene-based compounds to amino-nitrotoluenes under aerobic conditions has been reported elsewhere (11, 15, 16, 28). Others have concluded that transformation of 2,4-DNT and 2,6-DNT to amino-nitrotoluenes occurs only under anaerobic conditions (9, 14). Recently, it has been suggested that reduction of the nitro group to form an amino-nitrotoluene is the initial process in TNT degradation under both aerobic and anaerobic conditions but that under aerobic conditions the hydroxylamino and nitroso precursors tend to dimerize to azoxy compounds which are resistant to further degradation (8). Microbial production of azoxy dimers has been widely reported under aerobic conditions (8, 11, 15, 18, 29).

The groundwater at the Weldon Spring site contains measurable quantities of oxygen. However, because of microbial activity and the low diffusion coefficient of oxygen in water, soils and sediments that contain less than 5 to 10% aerated pore spaces tend to be anaerobic (7). To simulate the aerobic-anaerobic heterogeneity which dominates the subsurface environment at Weldon Spring, microcosms were created with a layer of standing water and an aerobic headspace. Given the microbial activity of these samples, the aerobic zone associated with microcosm sediments was probably less than 10 μm thick (7). Thus, in the red tank and top soil microcosms (Fig. 3 and 4), the approximately stoichiometric conversion of 2,4-DNT and 2,6-DNT to their respective amino-nitro intermediates is consistent with previous observations of nitroaromatic transformation to amino-nitrotoluenes under anaerobic conditions (9, 14).

In light of the relatively rapid transformation of TNT and

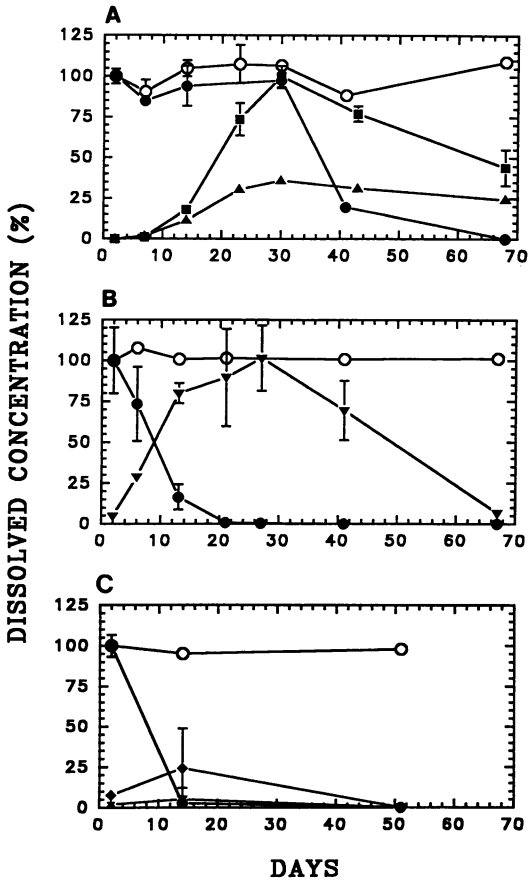

FIG. 4. Microbially mediated disappearance of nitrotoluene compounds from top soil microcosms. (A) Dissolved concentrations of 2,4-DNT in experimental microcosms (●) and killed controls (○) and accumulation and subsequent disappearance of 4-amino-2-nitrotoluene (■) and 2-amino-4-nitrotoluene (▲) in the dissolved phase; (B) dissolved concentrations of 2,6-DNT in experimental microcosms (●) and killed controls (○) and accumulation and subsequent disappearance of 2-amino-6-nitrotoluene (▼) in the dissolved phase; (C) dissolved concentrations of 2,4,6-TNT in experimental microcosms (●) and killed controls (○) and accumulation and subsequent disappearance of 4-amino-2,6-DNT (◆) and 2-amino-4,6-DNT (*) in the dissolved phase. Datum points are the means of triplicates ± SD.

DNT observed in this study, it is unlikely that the current groundwater contamination is a relic of contamination deposited circa 1945 during munitions manufacture. A close correlation between the dissolved concentration of nitroaromatic compounds and tritium levels in the groundwater at Weldon Spring also indicates that the groundwater contamination is more recent than circa 1945 (21). This observation in combination with the demonstrated capability of the indigenous microorganisms to degrade nitroaromatics indicates that the groundwater contamination at Weldon Spring is the result of leaching and subsequent in situ biotransformation of TNT, 2,4-DNT, and 2,6-DNT originating from the surface soils.

Influence of nitroaromatics on microbial metabolism. The overall metabolic activity (CO_2 production) of surface soils and aquifer materials from Weldon Spring responded quite differently to the addition of nitroaromatic test substrates (Table 1). The rate of CO_2 production by surface soil microcosms in the absence of a test substrate was either greater than (red tank soil) or within the range of (top soil) the rates observed with

TABLE 1. Rates of CO_2 production in Weldon Spring experimental microcosms[a]

Substrate added	Rate with the following sample materials[b]			
	Carbonate	Residuum	Red tank soil	Top soil
TNT	70 ± 16*	100 ± 6*	100 ± 10†	190 ± 14†
2,4-DNT	55 ± 8*	110 ± 11*	100 ± 10†	190 ± 9†
2,6-DNT	56 ± 6*	75 ± 5†	75 ± 5‡	230 ± 21*
None	0.3 ± 0.2†	4 ± 2‡	140 ± 23*	210 ± 13†

[a] Data are means ± SD of triplicate rates in nanomoles of CO_2 per gram of dry sample material per day.
[b] For a given sample material, means with same superscript are not significantly different according to the Student-Newman-Keuls test ($P \leq 0.05$).

test substrates. In contrast, very little CO_2 was produced by carbonate and residuum microcosms in the absence of added substrate.

The disparate responses of Weldon Spring soils and aquifer materials to the addition of nitroaromatics are probably attributable to differences in total organic carbon (TOC) content. The TOC content of the aquifer materials was 0.01% (percentage of dry weight) or less, while the surface soils contained 0.35 to 2.15% TOC content (Table 2 and this study) (6, 22). These observations suggest that the TOC content of the surface soils is sufficient to support microbial metabolism; however, the aquifer materials lack sufficient organic substrate to support significant microbial activity. The lack of CO_2 production by aquifer sediments in the absence of added substrate (i) suggests that the nitroaromatic test compounds were the sources of the CO_2 produced in the aquifer microcosms and (ii) demonstrates the dependence of aquifer microbial metabolism at this site on the transport of oxidizable carbon. These conclusions further support the hypothesis that groundwater contamination at the site is due to continuous leaching of surface contamination.

In contrast to the response of the aquifer materials, the rate of CO_2 production in the red tank soil microcosms was apparently inhibited by the addition of nitroaromatics (Table 1). This inhibition of microbial activity is consistent with previous reports of the toxicity of nitroaromatic compounds to microorganisms. The transformation of TNT by *P. chrysosporium* was completely inhibited at dissolved concentrations of greater than 88 μM (25). About 10% of the label from [^{14}C]TNT was recovered as $^{14}CO_2$ in treatments containing 22 μM TNT; however, no $^{14}CO_2$ was recovered at TNT concentrations of greater than 66 μM (25). TNT caused frameshift mutations in and completely inhibited the growth of *Salmonella typhimurium* at concentrations of greater than 44 μM (28). On the basis of soil plate counts and pure-culture methods, concentrations of TNT that were 220 μM reduced the growth of gram-positive bacteria, actinomycetes, yeasts, and fungi (13).

TABLE 2. Carbon contents of soils and aquifer materials collected at Weldon Spring[a]

Sample material	Organic carbon (%)	Carbonate carbon (%)
Carbonate	<0.01	6.38
Residuum	<0.01	<0.01
Red tank soil	0.35	0.09
Top soil	1.52	<0.01

[a] Data are given as percentages of dry weights of samples.

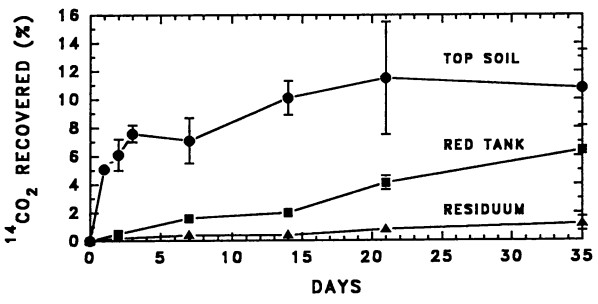

FIG. 5. Mineralization of [^{14}C]TNT to $^{14}CO_2$ in microcosms containing top soil, red tank soil, and residuum material. Datum points are means of triplicates ± SD.

Microbial mineralization of [^{14}C]TNT. Significant degradation of [^{14}C]TNT to $^{14}CO_2$ occurred within 35 days of incubation (Fig. 5). About 11% of the [^{14}C]TNT was mineralized in microcosms containing top soil. Approximately 1 and 6.5% of the [^{14}C]TNT in the residuum and red tank soil microcosms, respectively, were mineralized. These results are consistent with the disappearance studies in which relatively rapid transformation of TNT was observed in the top soil and red tank soil microcosms (Fig. 3 and 4). The lower rate of [^{14}C]TNT mineralization in the residuum microcosms also is consistent with the relatively long lag time (greater than 20 days) preceding a detectable decrease in the dissolved TNT concentration. However, the lack of CO_2 production by the residuum microcosms in the absence of added nitroaromatic compounds indicates that the aquifer microorganisms are also capable of mineralization of nitroaromatic compounds.

Several investigators have used radiolabeled substrates to examine nitroaromatic degradation by a variety of microorganisms (4, 11, 14, 18); however, only the white rot fungus *P. chrysosporium* produced significant amounts of $^{14}CO_2$ during degradation of nitrotoluene-based compounds (5, 25, 27). Approximately 34% of added [^{14}C]2,4-DNT was degraded to $^{14}CO_2$ by *P. chrysosporium* (27). Under simulated composting conditions and at a TNT concentration of about 6 μM, *P. chrysosporium* degraded 35% of the [^{14}C]TNT to $^{14}CO_2$ in 18 days (5). At concentrations typical of the field (about 44 μM), only 20% of the added label was recovered as $^{14}CO_2$ after 90 days (5). Spiker et al. (25) reported that *P. chrysosporium* degraded about 10% of added [^{14}C]TNT to $^{14}CO_2$ at a TNT concentration of about 22 μM. However, TNT mineralization was completely inhibited by TNT concentrations of greater than 66 μM, which are typical of munitions-contaminated soils (25). Consequently, the utility of *P. chrysosporium* as an agent for bioremediation of munitions contamination was questioned. The present results indicate that microorganisms indigenous to the Weldon Spring site are capable of partially mineralizing TNT and DNT at concentrations which completely inhibited nitrotoluene transformation and mineralization by *P. chrysosporium* (25).

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