APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1996, p. 2651–2656 Vol. 62, No. 7
0099-2240/96/$04.00+0

Microbial Transformation of 2,4,6-Trinitrotoluene in Aerobic Soil Columns

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Received 2 January 1996/Accepted 6 May 1996

2,4,6-Trinitrotoluene (TNT)-contaminated soil material of a former TNT production plant was percolated aerobically in soil columns. Nineteen days of percolation with a potassium phosphate buffer supplemented with glucose or glucose plus ammonium sulfate caused an over 90% decline in the amount of extractable nitroaromatics in soils containing 70 to 2,100 mg of TNT per kg (dry weight). In the percolation solution, a complete elimination of TNT was achieved. Mutagenicity and soil toxicity were significantly reduced by the percolation process. 4-*N*-Acetylamino-2-amino-6-nitrotoluene was generated in soil and percolation fluid as a labile TNT metabolite.

Throughout the world, the soil and groundwater of former munition plants and their dumping sites have often been highly contaminated with the explosive 2,4,6-trinitrotoluene (TNT). In Germany, TNT was the main explosive produced during World War II; about 60 former production sites are known (27). Because of its toxicity and persistence, the grounds of former manufacturing facilities are an environmental hazard. TNT is toxic to numerous organisms (3, 11, 12, 19, 25, 29), induces frameshift mutations in *Salmonella typhimurium* (1, 30, 32, 35), and might cause cancer (14).

It is well-known that TNT is cometabolically transformed under aerobic as well as anaerobic conditions to aromatic amines by a variety of microorganisms (4, 6, 8–10, 16, 26). A complete reduction to triaminotoluene, however, occurs only under strictly anaerobic conditions (E_h, <-200 mV) (28). Furthermore, the generation of azoxy derivatives as dead-end products has been described for aerobic liquid culture experiments (36) and compost systems (21). Significant mineralization of TNT by microorganisms has been found by only a few authors. Fernando et al. (13) showed that the fungus *Phanerochaete chrysosporium* mineralized this explosive. However, this organism does not seem to be suitable for bioremediation (24, 31). Bradley et al. (6) reported mineralization of [^{14}C]TNT by up to 11% by soil microorganisms; this, however, was inhibited when cellobiose or syringate was added (5). Vanderberg et al. (33) postulated ring cleavage of TNT by *Mycobacterium vaccae* since they found 50% of [^{14}C]TNT in the cellular lipid fraction of this organism. They identified 2,4-diamino-6-nitrobenzyl methyl ether and 4-amino-2,6-dinitro-benzoic acid as new oxidative TNT metabolites. Recently, 4-*N*-acetylamino-2-amino-6-nitrotoluene (4-*N*-AcANT) was found as a dead-end TNT metabolite generated by *Pseudomonas fluorescens* under anoxic as well as aerobic conditions (15). Different composting methods (7, 18, 20, 21) and anaerobic slurry reactor systems (22) have been tested for the bioremediation of TNT-contaminated soil. Here, we report an efficient aerobic circulating percolation reactor system for the microbial detoxification of TNT-contaminated soil.

TNT-contaminated surface soil samples were collected from the grounds of the former TNT production plant, Tanne, near Clausthal-Zellerfeld, Lower Saxony, Germany. Soil samples were sieved immediately after collection (<8 mm) and stored in the dark at 4°C prior to experiments. The soil contained 32% clay and silt, 63% sand, and 5% gravel. The collected soil was contaminated with about 4,800 ± 2,100 mg of TNT, 49 ± 32 mg of 4-amino-2,6-dinitrotoluene (4-ADNT), and 40 ± 19 mg of 2-amino-4,6-dinitrotoluene (2-ADNT) per kg of dry soil (means ± standard deviations of six parallel analyses). The soil pH was about 7. In the first percolation experiments, soil contaminated with about 3,000 ± 800 mg of TNT per kg of dry soil (means ± standard deviations of five parallel analyses) was used. In these experiments, no reduction in the amount of nitroaromatics could be achieved within 57 days when buffer or tap water was used as the percolation solution. Therefore, further experiments were carried out with less contaminated soil. To obtain lower concentrations of TNT, contaminated soil samples were mixed with a similar type of soil with no history of nitroaromatic exposure.

The experimental setup is shown in Fig. 1. Soil columns consisted of glass tubes (55-mm inner diameter; 250-mm length). Columns were filled with a mixture of 80% TNT-contaminated soil and 20% gravel (<8 mm) (wt/wt). It was necessary to add gravel to the soil to ensure good aeration and flow. Four hundred fifty grams (wet weight) of the soil-gravel mixture was put into each glass column. The circulation of percolating solutions was carried out by a peristaltic pump with a flow rate of 11.5 ml/h. Air was supplied to the columns by a diaphragm pump at a flow rate of 6 to 8 liters/h. Two hundred fifty milliliters of percolating solution was added to 500-ml brown glass bottles which were used as the reservoir. A silicone tubing was fixed to the glass tubing at the bottom of the glass columns, which reached into the percolating fluid reservoir to aerate the fluid.

As percolating fluid, 50 mM potassium phosphate buffer (pH 7) was applied with the following modifications: additions

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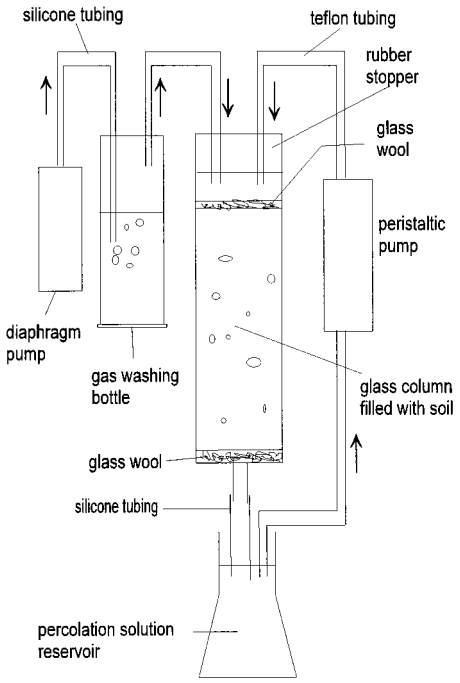

FIG. 1. Experimental setup of the aerobic percolation reactor.

of 7.5 mM ammonium sulfate, 20 mM glucose, and 20 mM glucose plus 7.5 or 15 mM ammonium sulfate.

In all percolation experiments, the pH was measured daily and, if necessary, adjusted to 7 by the addition of 10 N NaOH. Furthermore, in all percolating fluids containing ammonia, the concentration was determined daily by the Merkoquant ammonia test (Merck, Darmstadt, Germany). It was necessary to add 1 ml of a 0.75 M ammonia stock solution every fourth or fifth day to adjust the ammonium concentration. The glucose concentration in the percolation solution was determined daily with Uristix (Bayer Diagnostics, Munich, Germany). It was necessary to add 1 to 2 ml of a 2 M glucose stock solution about every second day to adjust the glucose concentration. When samples were withdrawn from the percolating solution reservoir, the sample volume (approximately 5 ml) was replaced with fresh percolating solution. Liquid loss because of evaporation was balanced by the addition of demineralized water. During percolation, the soil columns were kept in the dark at 22 ± 2°C. Nitroaromatics were determined in the column soil before and after percolation. Methanol extractions of soil were carried out as described elsewhere (7). Three to five parallel extractions per datum point were performed.

Soil extracts were analyzed by gas chromatography-electron capture detection (GC-ECD) and by high-pressure liquid chromatography–diode array detection (HPLC-DAD). The GC-ECD technique was the most rapid method (7 min per analysis) for the determination of TNT, 4-ADNT, and 2-ADNT and was suitable to analyze all contaminations present in soil samples. For the determination of additional microbially generated TNT transformation products, such as diaminonitrotoluenes (DANT) and azoxy derivatives, combined samples of the parallel extractions were analyzed by HPLC-DAD.

To determine the amount of nitroaromatics in percolating fluids, samples were withdrawn regularly from the percolation solution reservoir and analyzed by HPLC-DAD. Samples of 1 ml were centrifuged (18,000 × *g*, 20 min) and transferred into 2-ml autosampler vials. NaN_3 (50 μg) was added to prevent bacterial growth in vials. Previous analyses had shown that NaN_3 has no effect on nitroaromatics.

GC-ECD analyses were performed as described elsewhere (7). Methanolic soil extracts were diluted 10- to 100-fold with toluene prior to analyses. The following substances were used as external standards: TNT, 4-ADNT, 2-ADNT, 2-nitrotoluene, 3-nitrotoluene, 4-nitrotoluene, 2,6-dinitrotoluene (2,6-DNT), 2,4-DNT, and 3,4-DNT.

For HPLC analyses, we used a system obtained from Gynkotek (Munich, Germany) which consisted of an M 480 pump, a Gina 50 automatic sampler, a 340 S diode array detector, and a column oven (set to 25°C). A Nucleosil 120-3 C_{18} column (3 mm by 25 cm; CS-Chromatographie Service, Langerwehe, Germany) was used for separation. Aliquots of 10 μl were injected. A water-methanol gradient starting with 70% water–30% methanol (vol/vol) was run. This eluent was held for 10 min. A linear gradient was then run to 50% water–50% methanol (vol/vol) over 5 min. This solvent composition was held for 20 min. Then a linear gradient to 100% methanol was run over 35 min, which was maintained for 10 min. Thereafter, the column was equilibrated for 15 min with the initial eluent of 70% water–30% methanol (vol/vol). The flow rate was set at 0.3 ml/min. Peak detection was performed at 230 nm, and the three-dimensional field bandwidth was set from 200 to 400 nm. Nitroaromatics were determined via comparison of the retention times and UV spectra with those of an external standard. Integration was performed with Gynkosoft 5.32 software (Gynkotek). The following substances were used as external standards: TNT, 4-ADNT, 2-ADNT, 2,4-diamino-6-nitrotoluene (2,4-DANT), 2,6-diamino-4-nitrotoluene (2,6-DANT), 4-hydroxylamino-2,6-dinitrotoluene, 2-*N*-acetylamino-4,6-dinitrotoluene, 4-*N*-acetylamino-2,6-dinitrotoluene, 2,2′,6,6′-tetranitro-4,4′-azoxytoluene, 4,4′,6,6′-tetranitro-2,2′-azoxytoluene, 2,4-DNT, and 2,6-DNT.

TNT was purchased from Fluka (Buchs, Switzerland) and recrystallized five times in ethanol. 2,4-DNT, 2,6-DNT, and 3,4-DNT were obtained from Merck. The DNT isomers were recrystallized three times in ethanol before use. 4-ADNT, 2-ADNT, 2,4-DANT, 2,6-DANT, 4-hydroxylamino-2,6-dinitrotoluene, 2,2′,6,6′-tetranitro-4,4′-azoxytoluene, 4,4′,6,6′-tetranitro-2,2′-azoxytoluene, 2-*N*-acetylamino-4,6-dinitrotoluene, 4-*N*-acetylamino-2,6-dinitrotoluene, and a UV spectrum of 4-*N*-AcANT were provided by the Department of Chemistry, Philipps University Marburg, Marburg, Germany. The purity of each chemical used as a standard was 99% or more.

To investigate the effects of different percolation solutions, soil samples containing about 70 mg of TNT per kg (dry weight) were used. GC-ECD analyses of methanolic soil extracts revealed that for all percolating solutions, 19 days of percolation strongly reduced the amount of extractable nitroaromatics (Fig. 2). The infiltration of potassium phosphate buffer supplemented with glucose or glucose plus ammonium sulfate was the most effective treatment. With these additives, the amount of extractable TNT in soil could be reduced by more than 90% and the quantity of ADNT was also diminished (Fig. 2D and E). The number of aerobic and facultative anaerobic bacteria minus fastidious soil bacteria (called viable cells hereafter) was determined on Merck standard II media by the most probable number method (2) before and after percolation. The number of viable cells was increased by about 1 order of magnitude by the percolation process using a glucose-containing solution. Furthermore, water extracts of soil were prepared as described elsewhere (7) and used in a bioluminescence test with *Vibrio fischeri* (Dr. Lange, Berlin, Germany). The inhibition of bioluminescence was reduced from an initial value of about 40% to around 15% when the percolation

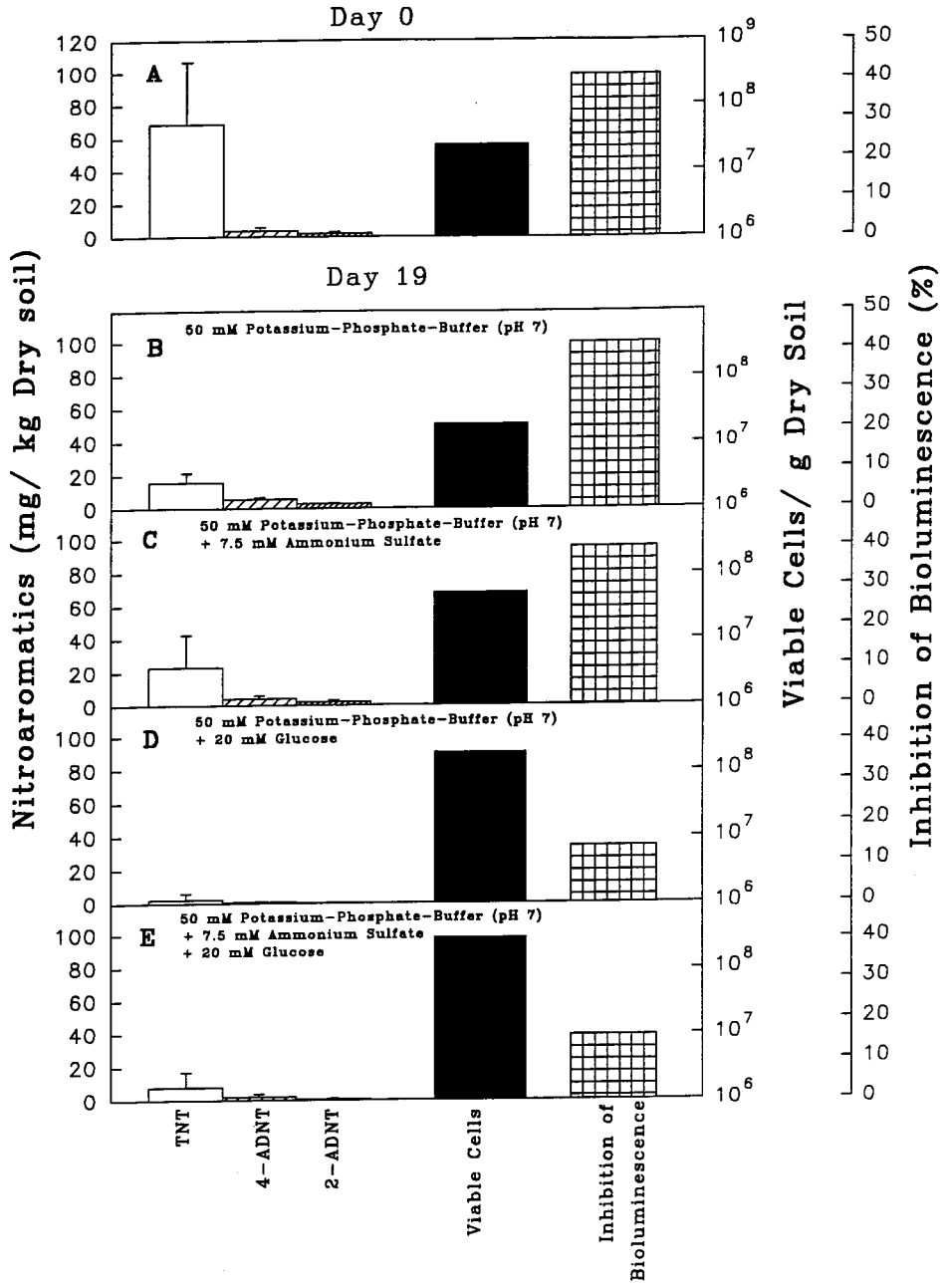

FIG. 2. Nitroaromatic compound contents, numbers of viable cells, and inhibition of the bioluminescence of *V. fischeri* by aqueous soil extracts of TNT-contaminated soil before and after 19 days of aerobic percolation with different percolation solutions as indicated. (A) Starting conditions on day 0; (B through E) conditions after 19 days. Nitroaromatic data are the means ± standard deviations of five parallel extractions.

solution contained a C source. According to German standard DIN 38412 L34, aqueous samples inhibiting bioluminescence by 20% or less are declared to be nontoxic.

For further analyses, the soil extracts of each column obtained after 19 days of percolation were combined and analyzed by HPLC-DAD. These analyses confirmed the results of the GC-ECD analyses except that the extracts of soil column D also contained 0.5 mg of 2,4-DANT per kg of dry soil and the extracts of column E contained 1.1 mg of 2,4-DANT per kg of dry soil. Azoxy derivatives were not detectable. Furthermore, an initially unknown peak occurring at about 20 min was found by comparison through UV spectrum and HPLC-mass spectrometry analyses to have 100% identity with 4-*N*-AcANT (data not shown). Unfortunately, this TNT transformation product was not available in an amount sufficient for use as an external standard to allow quantification.

Percolation with a potassium phosphate buffer or a potassium phosphate buffer supplemented with ammonium sulfate caused a TNT elimination of about 75 and 60%, respectively (Fig. 2B and C), but the concentration of ADNT remained constant. 2,4-DANT and 4-*N*-AcANT were not generated in these soil samples. This treatment had no positive effect on the number of viable cells, and the toxic effects on *V. fischeri* before and after percolation were the same.

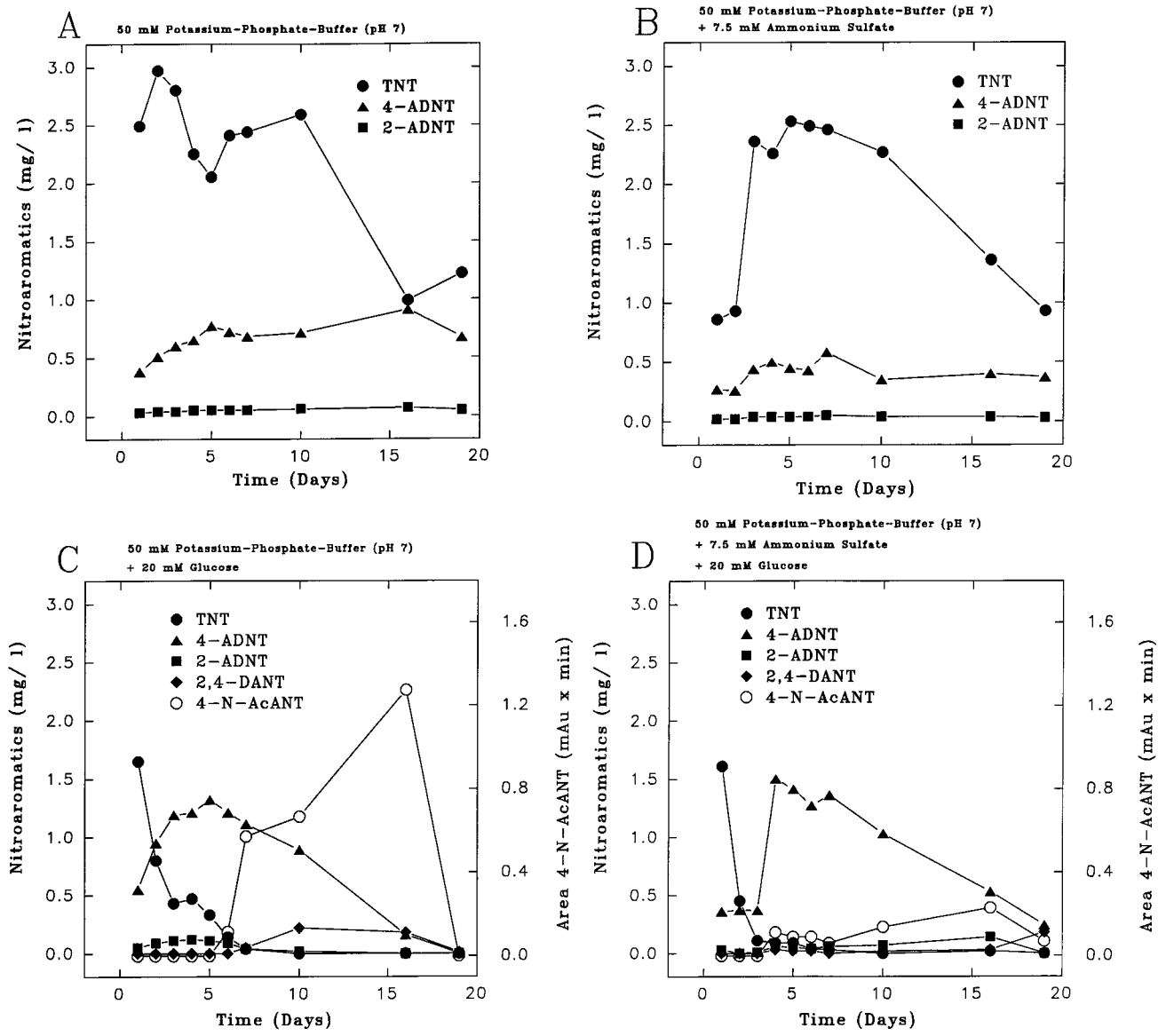

FIG. 3. Time course of the appearance of TNT and its metabolites in the different solutions used for the percolation of the soils shown in Fig. 2B through E.

The time course of nitroaromatics appearing in percolating fluids is shown in Fig. 3. TNT represented the main contaminant in glucose-free solutions, with concentrations of up to 2.5 to 3 mg/liter (Fig. 3A and B) which declined to about 1 mg/liter within 19 days. In addition, minor amounts of ADNT were generated. In glucose-containing percolating fluids, the initial TNT concentration of about 1.5 mg/liter declined rapidly within 6 or 7 days to almost zero (Fig. 3C and D); the addition of ammonium sulfate appeared to accelerate this process. Along with the disappearance of TNT, major amounts of ADNT were formed; however, they almost completely vanished as percolation went on. Furthermore, minor amounts of DANT were detectable in percolating fluids (Fig. 3C and D). It was particularly notable that after 4 to 6 days of percolation, 4-*N*-AcANT became detectable in percolating fluids that contained glucose (Fig. 3C and D). When additional ammonium sulfate was added, 4-*N*-AcANT was present in much smaller quantities (Fig. 3D). In both percolation systems, 4-*N*-AcANT was almost completely degraded by the last day of percolation. This stands in contrast to the results of Gilcrease and Murphy (15), who found 4-*N*-AcANT as a dead-end product in liquid culture experiments. As did Gilcrease and Murphy, we could identify only 4-*N*-AcANT, not acetylated ADNT. The probable formation of 4-*N*-AcANT is shown in Fig. 4. The percolation solution reservoir represented a C- and N-containing aerobic liquid culture into which bacteria could migrate from the soil. However, bacterial growth in the reservoir was not quantitatively determined. We assume that the degradation of 4-*N*-

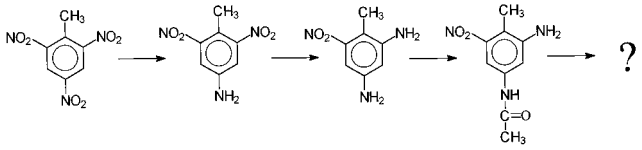

FIG. 4. Probable formation of 4-*N*-AcANT in aerobic soil percolation columns and percolation fluid.

TABLE 1. Nitroaromatic contents, numbers of viable cells, inhibition of the bioluminescence of *V. fischeri* by aqueous soil extracts, and mutagenic potencies of soils contaminated with different amounts of TNT before and after 19 days of aerobic percolation[a]

Sample	Time (days)	Amt (mg/kg of dry soil)				Viable cells (10^6/g of soil)[e]	Inhibition of bioluminescence (%)[f]	Mutagenic potency (revertants/plate/μg of soil)[g]
		TNT	ADNT[b]	DANT[c]	Az[d]			
Soil	0	151	4.8	0	0	1	53	0.93
	19	6.7	1.5	2.2	0	110	17	0.33
Percolation solution	1	6.1	0.4	0	0	ND[h]	ND	ND
	19	0	0	0.5	0	ND	ND	ND
Soil	0	338	4.3	0	0	2.5	83	ND
	19	125	29	7.1	8.2	510	62	ND
Percolation solution	1	26	1.1	0	0	ND	ND	ND
	19	0	2.1	1.2	0	ND	ND	ND
Soil	0	2,139	18	0	0	10	85	2.56
	19	212	54	10	23	190	84	1.66
Percolation solution	1	27	1.0	0	0	ND	ND	ND
	19	0	10	3.6	0	ND	ND	ND

[a] Three parallel soil extractions per datum point were performed. Extracts from day 0 were separately analyzed by GC-ECD. The means from these analyses are presented. Extracts from day 19 were combined and analyzed by HPLC-DAD.
[b] Sum of 2-ADNT and 4-ADNT.
[c] Sum of 2,6-DANT and 2,4-DANT.
[d] Sum of 4,4′,6,6′-tetranitro-2,2′-azoxytoluene and 2,2′,6,6′-tetranitro-4,4′-azoxytoluene.
[e] Determined by the most probable number method.
[f] Inhibition of the bioluminescence of *V. fischeri* NRRL-B-11177 by aqueous soil extracts.
[g] Determined by the Ames test with *S. typhimurium* TA98 without S9 metabolic activation.
[h] ND, not determined.

AcANT is due to the activity of endogenous soil microflora in the soil column as well as in the percolation solution reservoir. It has been shown previously that these organisms are able to degrade TNT (8–10).

To assess the bioremediation potential of higher contamination levels, another set of percolation experiments was performed. A 50 mM potassium phosphate buffer (pH 7) containing 20 mM glucose plus 15 mM ammonium sulfate was used as the percolation solution. As shown in Table 1, 19 days of percolation caused a reduction of about 90% in the amount of extractable TNT in soil originally contaminated with 2,139 mg of TNT per kg (dry weight). Significant amounts of ADNT, DANT, and azoxy derivatives were also formed in this soil. About 63% of the extractable TNT in soil contaminated with about 340 mg of TNT per kg (dry weight) vanished. In this system, significant amounts of ADNT, DANT, and azoxy derivatives were also detectable. Over 95% of TNT in soil initially containing about 150 mg of TNT per kg (dry weight) was eliminated. In this soil, the amount of ADNT was reduced from 4.8 (initially) to 1.5 mg/kg (dry weight) and 2.2 mg of DANT per kg of dry soil was generated. Furthermore, no azoxy derivatives were detectable. As in the first set of experiments, 4-*N*-AcANT was formed in all soils.

In soil originally containing 2,139 mg of TNT per kg (dry weight), percolation caused an increase in the number of viable cells by 1 order of magnitude, whereas in less contaminated soils an increase of about 2 orders of magnitude was detectable. Reductions in the toxicity of aqueous extracts of soil samples, as measured by bioluminescence inhibition, were observed only for soils originally containing about 340 mg or less of TNT per kg of dry soil. Ames tests were carried out with methanol extracts of two soils according to the instructions of Maron and Ames (23). Methanol extracts (100, 50, 20, and 10 μl) were used in these tests. For soils containing 2,139 and 150 mg of TNT per kg (dry weight), percolation caused a significant reduction, 2.8- and 1.5-fold, respectively, in the mutagenic potential.

In these experiments with soils containing high TNT concentrations, the time course of nitroaromatics in percolation fluids was similar to the one shown in Fig. 3D. The amount of TNT declined to zero within the first days of percolation, except for the highest TNT contamination, for which it took 17 days to eliminate TNT completely. After 2 to 3 days, ADNT appeared and were then degraded on the following days; finally, minor amounts of DANT were also detectable. The concentrations of nitroaromatics on days 1 and 19 are listed in Table 1. Furthermore, the 4-*N*-AcANT present in the percolating solutions of all three soil columns also disappeared during further incubation.

Our data demonstrate that aerobic percolation of TNT-contaminated soil with a phosphate buffer that contains glucose or glucose plus ammonium sulfate leads to a rapid decline in the amount of extractable nitroaromatics. Most importantly, this treatment detoxified the soil, as demonstrated by the inhibition of the bioluminescence of *V. fischeri* and by the Ames test. The percolation procedure was effective over a range of about 70 to 2,100 mg of TNT per kg of dry soil, except for the bioluminescence test, which indicated detoxification only for soils contaminated with about 340 mg or less of TNT per kg (dry weight). This is probably due to the fact that the inhibition of bioluminescence is not a linear process. Additionally, degradation of nitroaromatics in percolating solutions could be observed.

Obviously, stimulation of the biological activity in the soil was necessary to achieve detoxification of the soil. We assume that the transformation and elimination of this explosive was catalyzed by unspecific reactions of different soil bacteria and fungi, as has been previously postulated for soil-composting processes (7). In these systems, TNT is apparently transformed by microorganisms to ADNT or DANT, which in turn bind covalently to humic soil compounds. The binding of TNT metabolites to the biomass generated during percolation is also a possibility which has to be tested in further experiments. If detoxification via immobilization of the contaminant applies to

the percolation system, the binding structures must be of a very strong nature and resistant against hydrolysis, since even a drastic extraction method such as treatment with 8 N HCl (7) did not lead to the release of nitroaromatics from percolated soils. Nevertheless, mineralization of TNT cannot be completely excluded, although previous works about the aerobic microbiological degradation of TNT make ring cleavage and therefore complete breakdown of the molecule rather unlikely (4, 9, 26, 34). Further experiments with [^{14}C]TNT are therefore required to obtain more information about the fate of TNT under aerobic percolation conditions.

Anaerobic soil percolation may lead to different results since the washing out of TNT as amino derivatives has been found by Gorontzy (17). This observation lends support to our notion that TNT transformation and immobilization were catalyzed by oxygen-requiring cometabolic reactions.

In the future, scale-up experiments may prove that an aerobic percolation reactor could be useful for the cleanup of TNT-contaminated soil. Such a technique would present an alternative to existing biological sanitation methods, such as composting and slurry reactors. The major disadvantages of these more established techniques are the large amounts of additives (up to 90% of volume) that have to be added for composting and the costly drying of soil for slurry reactors. Neither of these inconveniences applies to percolation reactors. Furthermore, our present data indicate that microbial in situ sanitation of soil with low-level contamination might be possible by aerobic infiltration of nutrients into the soil. In that case, however, careful monitoring of the groundwater for possibly eluted nitroaromatics will be required.

This work was supported by the Bundesministerium für Forschung, Wissenschaft, Bildung und Technik (BMBF); by the state of Lower Saxony; and by the Industrieverwaltungsgesellschaft AG (IVG), Bonn, Germany. Project management was done by M. Fischer, Industrieanlagen-Betriebsgesellschaft mbH (IABG), Berlin, Germany.

We thank L. Kaminsky, M. Müller, and P. Asbach for technical assistance and J. Michels for his editorial advice on the manuscript.

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