

Thermophilic Biotransformations of 2,4,6-Trinitrotoluene Under Simulated Composting Conditions

D. L. KAPLAN* AND A. M. KAPLAN

U.S. Army Natick Research and Development Command, Science and Advanced Technology Laboratory, Environmental Protection Group, Natick, Massachusetts 01760

Received 4 March 1982/Accepted 25 May 1982

The biotransformations of ^{14}C -labeled 2,4,6-trinitrotoluene by thermophilic microorganisms in a compost system were determined. The reduction products identified in solvent extracts were similar to those identified in mesophilic systems. A significant percentage of the ^{14}C -labeled products were bound to humus fractions.

Soil (12) and groundwater (21) contamination by 2,4,6-trinitrotoluene (TNT) has resulted from munitions manufacturing, loading, assembling, and packing operations. TNT represents an environmental hazard because it has toxicological effects on a number of organisms (14, 18, 20, 22, 26), and it is mutagenic (6, 9).

Previous biological treatment systems for TNT-contaminated wastes have demonstrated the resiliency of this compound to microbial attack. The aromatic nucleus is not cleaved, and the nitro groups are sequentially reduced (15-17). Insoluble macromolecules have also been characterized as microbial reduction products (4). TNT reduction products also pose environmental hazards, since they have been reported to be toxic and mutagenic (6, 7). Initial research on the composting of TNT (19) was terminated before a definitive determination of the fate of TNT could be made. Accordingly, it was necessary to further examine the transformations of TNT in the composting process. The purpose of our studies was to define the pathway for TNT degradation in compost. These findings would be instrumental in evaluating the composting process as an alternative treatment for TNT-contaminated wastes.

TNT was purchased from Eastman Kodak (Rochester, N.Y.) and recrystallized. J. C. Hoff-sommer, U.S. Naval Surface Weapons Center, Silver Spring, Md., supplied samples of 2-amino-4,6-dinitrotoluene (2A), 4-amino-2,6-dinitrotoluene (4A), 2,4-diamino-6-nitrotoluene (2,4DA), 2,6-diamino-4-nitrotoluene (2,6DA), 4-hydroxylamino-2,6-dinitrotoluene (4OHA), 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'Az), and 4,4',6,6'-tetranitro-2,2'-azoxytoluene (2,2'Az). 2',4,6,6-Tetranitro-2,4'-azoxytoluene (2,4'Az) was synthesized according to Sitzmann (23). [$U\text{-}^{14}\text{C}$]TNT 11.3 μCi per mg, was synthe-

sized from [^{14}C]toluene (New England Nuclear Corp., Boston, Mass.)

The composting mixture consisted of horse manure, alfalfa hay, grass clippings, dead hardwood leaves, and garden soil. All components were air dried, mixed, and shredded, and 112 g (dry weight) was added to 1-liter Erlenmeyer flasks. Trinitrotoluene, 1.7 g (1.5% TNT by dry weight of compost), was added to the test flasks. The ^{14}C -labeled TNT (1.69 μCi) diluted with unlabeled TNT was dissolved in acetone. The acetone was evaporated under a stream of nitrogen. Sufficient distilled water was added to the control and test systems to bring the moisture level up to 60%. A seed of active compost was added to all flasks.

The composting systems were set in an oven at 55°C and agitated twice weekly. The flasks were continuously flushed with a stream of CO_2 -free air, which entered the composting system via a perforated aeration loop in the bottom of the flask. The stream then exited successively through an empty flask, 100 ml of 1 N HCl, an empty flask, 100 ml of 1 N NaOH, a drying tube, and an activated carbon trap. The acid and base traps were changed twice weekly during the first 3 weeks and weekly thereafter. In a separate experiment, temperature readings were recorded to the nearest 0.5°C for 31 days with a YSI model 425C tele-thermometer (Yellow Springs, Ohio). Equal portions of the solutions from the traps were dissolved in 15 ml of Aquasol 2 scintillation cocktail (New England Nuclear Corp.), and the radioactivity was assayed in a Packard model 3255 Tri-Carb liquid scintillation counter. Each vial was corrected for quench, with an external standard.

The compost incubated with ^{14}C -labeled TNT and the corresponding control were cured for 91 days. Samples, taken at 24 and 91 days, were

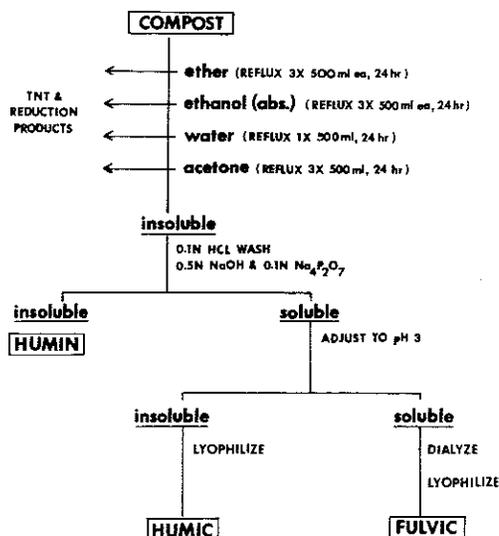


FIG. 1. Extraction scheme for test and control compost.

oven dried at 50°C and then exhaustively extracted (Fig. 1). These solvent extracts were counted for radioactivity and analyzed by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and Fourier Transform Infrared Spectroscopy (FTIR). The solvent systems for TLC (11) and the HPLC conditions (10) were reported previously. FTIR analyses were conducted on a Nicolet model 7000 FTIR, using potassium bromide pellets. After solvent extraction, the remaining insoluble materials were fractionated (Fig. 1 [13, 24]). Samples of the organic matter fractions were counted for radioactivity and analyzed by infrared spectroscopy (Perkin-Elmer model 283) and mass spectroscopy (Finnegan model 4000) by probe analysis.

No significant ^{14}C -labeled compounds, $^{14}\text{CO}_2$, or volatile amines were recovered in the effluent alkali, acid, or activated carbon traps. Temperature probes revealed little variation (55.0 to 56.5°C) in temperatures throughout the experiment once equilibration had occurred. The distribution of radioactivity recovered in the four extracts is presented in Table 1. Most of the counts were in the ether fraction, and the total counts recovered in these fractions decreased from 86.6 to 61.5% from 24 to 91 days of curing. TNT, 2A, and 4A were identified in the ether extract from the test compost after 24 days of curing. The presence of these compounds was confirmed by TLC, HPLC, scintillation counting, and FTIR analysis. The FTIR analysis indicated that 4A accounted for the majority of the two amines. After 91 days, TNT, 2A, 2,4DA,

TABLE 1. Solvent fractions from the test compost

Solvent	% of total ^a radioactivity recovered	
	24-day compost	91-day compost
Ether ^b	67.4	48.3
Ethanol (absolute) ^c	2.1	1.5
Water	13.5	9.7
Acetone ^c	3.6	2.0

^a 3.75×10^6 dpm (initial).

^b Compounds identified included 4,4'Az, 2,4'Az, TNT, 2A, and 4A.

^c Compounds identified included 2,4DA, 2,6DA, TNT, 2A, and 4A.

2,6DA, 4,4'Az, and 2,4'Az were identified in the solvent extracts by TLC, HPLC, and scintillation counting. The ether fractions contained the more nonpolar metabolites (4,4'Az and 2,4'Az); the ethanol and acetone extracts contained the polar metabolites (2,4DA, 2,6DA), and TNT, 2A, and 4A were present in all three solvents.

The insoluble material which remained after solvent extraction was separated into three organic matter fractions, humic acid, fulvic acid, and humin, to determine whether the remaining ^{14}C -labeled material was associated with any one particular fraction. The radioactivity recovered in these three fractions accounted for 5.7 and 22.1% of the total recovered after 25 and 91 days of composting, respectively (Table 2).

Thermophilic bacteria, actinomycetes, and fungi were isolated from the control and test composts. The bacteria isolated at 55°C included *Bacillus stearothermophilus* Donk, *B. subtilis* Cohn, and *B. coagulans* Hammer. These were identified in both systems. A number of other *Bacillus* isolates were obtained as well as a number of different actinomycetes which were not identified. The hyphomycete *Thermomyces lanuginosa* Tsiklinskaya was identified in both the test and control composts. Bacterial identifications were according to Breed et al. (3) and Gordon et al. (8), and fungal identifications were according to Cooney and Emerson (5).

TABLE 2. Organic matter fractions from the test compost

Fraction	% of total ^a radioactivity recovered	
	24-day compost	91-day compost
Humic acid	4.0	7.8
Fulvic acid	0.4	0.4
Humin	1.3	13.9

^a 3.75×10^6 dpm (initial).

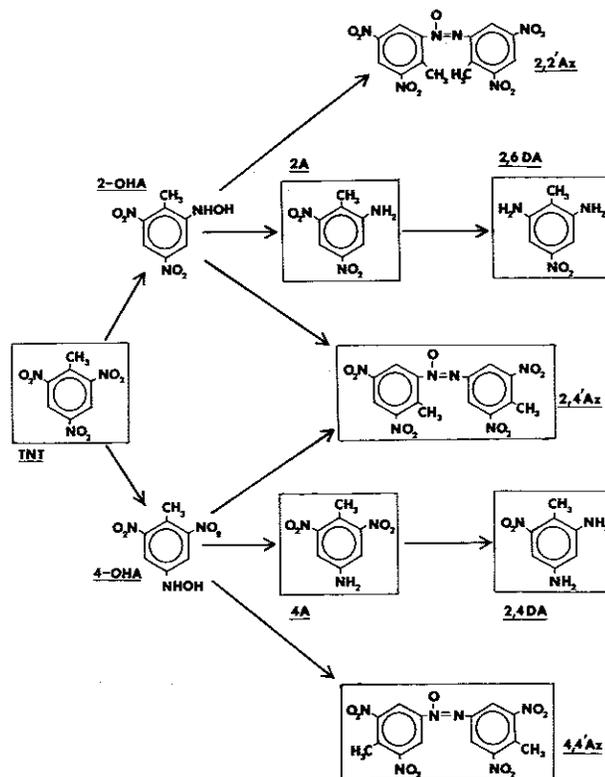


FIG. 2. Biotransformation scheme for TNT in compost. Compounds boxed were identified in solvent extracts.

A significant percentage of ^{14}C -labeled material was found in the organic matter fractions after solvent extraction. The radioactivity associated with the fulvic acid fraction may be a low figure because some of the lower-molecular-weight fulvic acids were probably lost during the dialysis. The humin fraction may also contain higher-molecular-weight insoluble TNT conjugates (4), which would have been insoluble in our extraction scheme. There is a progressively greater percentage of TNT transformation products bound up or conjugated into the organic matter fractions as the compost curing or stabilization process proceeds. Similar processes may occur with TNT in soil as well, as has been reported for the fate of various pesticides (1, 2, 25).

The metabolic products identified in the solvent extracts from the 91-day-old compost can be assembled into the biotransformation scheme presented in Fig. 2. This pathway is similar to that previously reported for mesophilic systems (16). The aromatic ring is not cleaved, and the nitro groups are reduced to amino groups. The hydroxylamino intermediates can also couple to form azoxy compounds. The reduction in the

para or 4 position of TNT is preferred to the *ortho* or 2 position.

We thank S. Cowburn and P. Riley for their technical assistance, B. Wiley for the identification of the thermophilic fungus, J. H. Cornell for the synthesis of 2,4'Az, C. DiPietro for mass spectral analysis, and J. Walsh and R. Bagalawis for the FTIR analyses.

LITERATURE CITED

- Bartha, R., and T. Hsu. 1974. Interaction of pesticide-derived chloroaniline residues with soil organic matter. *Soil Sci.* 115:444-452.
- Bollag, J. M., P. Blattmann, and T. Laanto. 1978. Adsorption and transformation of four substituted anilines in soil. *J. Agric. Food Chem.* 26:1302-1306.
- Breed, R. S., E. G. D. Murray, and N. R. Smith (ed.). 1957. *Bergey's manual of determinative bacteriology*, 7th ed. The Williams & Wilkins Co., Baltimore.
- Carpenter, D. F., N. G. McCormick, J. H. Cornell, and A. M. Kaplan. 1978. Microbial transformation of ^{14}C -labeled 2,4,6-trinitrotoluene in an activated-sludge system. *Appl. Environ. Microbiol.* 35:949-954.
- Cooney, D. G., and R. Emerson. 1964. *Thermophilic fungi*. W. H. Freeman and Co., San Francisco.
- Dilley, J. V., C. A. Tyson, and G. W. Newell. 1979. Mammalian toxicological evaluation of TNT wastewaters. VIII. Acute and subacute mammalian toxicity of condensate water. SRI International, Menlo Park, Calif.
- Ellis, H. V. III, J. R. Hodgson, S. W. Hwang, L. M. Halfpapp, D. O. Helton, B. S. Anderson, D. L. Van-

- Goethem, and C. C. Lee. 1978. Mammalian toxicity of munitions compounds phase I: acute irritation, dermal sensitization, disposition and metabolism, and ames tests of additional compounds. Project no. 3900-B, Midwest Research Institute, Kansas City, Mo.
8. Gordon, R. E., W. C. Haynes, and C. H. Pang. 1973. The genus *Bacillus*. U.S. Department of Agriculture agricultural handbook no. 427. U.S. Department of Agriculture, Washington, D.C.
9. Kaplan, D. L., and A. M. Kaplan. 1982. Mutagenicity of TNT-surfactant complexes. *Bull. Environ. Contam. Toxicol.* 28:33-38.
10. Kaplan, D. L., and A. M. Kaplan. 1982. Separation of mixtures of 2,4,6-trinitrotoluene reduction products with liquid chromatography. *Anal. Chim. Acta* 136:425-428.
11. Kaplan, D. L., and A. M. Kaplan. 1982. 2,4,6-trinitrotoluene-surfactant complexes: decomposition, mutagenicity and soil leaching studies, publication no. NATICK/TR-82/006. U.S. Army Natick Research and Development Laboratories, Natick, Mass.
12. Klausmeier, R. E., J. L. Osmon, and D. R. Walls. 1973. The effect of trinitrotoluene on microorganisms. *Dev. Ind. Microbiol.* 15:309-317.
13. Kononova, M. M. and N. P. Bel'chikova. 1961. Quick methods of determining the humus composition of mineral soils. *Trans. Soviet Soil Sci.* 1112-1121. (1960 *Pochvovedeniye* 11:1149).
14. Lee, C. C., J. V. Dilley, J. R. Hodgson, D. O. Helton, W. J. Wiegand, D. N. Roberts, B. S. Anderson, L. M. Halfpap, L. D. Kurtz, and N. West. 1975. Mammalian toxicity of munition compounds: acute oral toxicity, primary skin irritation, dermal sensitization and disposition and metabolism. Midwest Research Institute, Kansas City, Mo.
15. Lemberg, R., and J. P. Callahan. 1944. Metabolism of symmetrical trinitrotoluene. *Nature (London)* 154:768-769.
16. McCormick, N. G., F. E. Feeherry, and H. S. Levinson. 1976. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. *Appl. Environ. Microbiol.* 31:949-958.
17. Naumova, R. P., N. N. Amerkhanova, and V. A. Shayk-hutdinov. 1979. Study of the first stage of trinitrotoluene transformation by *Pseudomonas denitrificans*. *Prikl. Biokhim. Mikrobiol.* 15:45-50.
18. Nay, M. W. Jr., C. W. Randall, and P. H. King. 1974. Biological treatability of trinitrotoluene manufacturing wastewater. *J. Water Pollut. Control Fed.* 46:485-497.
19. Osmon, J. L. and C. C. Andrews. 1978. The biodegradation of TNT in enhanced soil and compost systems, U.S. Army Armament Research and Development Command, ARLCD-TR-77032, Dover, N.J. National Technical Information Service publication no. ADE400073. National Technical Information Service, Springfield, Va.
20. Osmon, J. L., and R. E. Klausmeier. 1972. The microbial degradation of explosives. *Dev. Ind. Microbiol.* 14:247-252.
21. Pereira, W. E., D. L. Short, D. B. Manigold, and P. K. Roscio. 1979. Isolation and characterization of TNT and its metabolites in groundwater by gas chromatograph-mass spectrometer-computer techniques. *Bull. Environ. Contam. Toxicol.* 21:554-562.
22. Sax, N. I. 1963. Dangerous properties of industrial materials, 2nd ed. Reinhold Publishing Corp., New York, N.Y.
23. Sitzmann, M. E. 1973. Chemical reduction of 2,4,6-trinitrotoluene (TNT) initial products, p. 1-9. National Technical Information Service publication no. AD-764060. National Technical Information Service, Springfield, Va.
24. Stevenson, F. J. 1965. Gross chemical fractionation of organic matter. p. 1409-1421. *In* C. A. Black (ed.), *Methods of soil analysis, part 2*. American Society of Agronomy, Inc., Madison, Wis.
25. Stevenson, F. J. 1976. Organic matter reactions involving pesticides in soil. p. 180-207. *In* D. D. Kaufman, G. G. Still, G. D. Paulson, and S. K. Bondal (ed.), *Bound and conjugated pesticide residues*. American Chemical Society Symposium Series 29, American Chemical Society, Washington, D.C.
26. Won, W. D., L. H. Disalvo, and J. Ng. 1976. Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Appl. Environ. Microbiol.* 31:576-580.