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Photochemical and Microbial Degradation of 2,4,6-Trinitrotoluene (TNT) in a Freshwater Environment

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2,4,6-Trinitrotoluene (TNT) is a nitro-aromatic compound that has been widely used by the military in the production of shells, bombs, and grenades. The wastewater from munition plants was discarded on the ground or in lagoons, which lead to an extensive contamination of the soil, groundwater, rivers, and lakes (Boopathy et al. 1994). This brought about serious concerns as to the environmental fate of TNT, because of the possibility that it would find its way into the food chain. Concern about the chemical was compounded because of its toxicity, formation of potentially more toxic or recalcitrant products, and transformation into insoluble fractions that are undefined in terms of environmental fate and effects (Funk et al. 1996; Ederer et al. 1997; Lewis et al. 1997;.Kaplan 1998). The disposal of large quantities of TNT in an environmentally acceptable manner poses serious difficulties. Despite the diverse processes being investigated in natural and engineered systems, it has been demonstrated that TNT is usually not completely mineralized although it may undergo certain transformation through biotic or abiotic processes (Sheremata et al. 1999).

Photolysis and microbial degradation have been recognized as two important removal forces of many organic pollutants in natural surface waters (Hwang et al. 1986; Hwang et al. 1998; Mabey et al. 1983). In natural environment, photodegradation of many organic contaminants are strongly enhanced due to the presence of sensitizers (Tsao and Eto 1994). Nevertheless, the photoinduced toxicity to both eukaryotes and prokaryotes at low concentrations has been reported for a wide variety of organic contaminants (Khan et al. 1973; McConkey et al. 1997; Pelletier et al. 1997; Swartz et al. 1997). The extent of this problem suggests these compounds and their photoproducts may be jeopardizing environmental health. Microbial bioassays were widely applied to toxicity measurements based on the assumption that microorganisms can act as surrogates for higher organisms in the ecosystem and microbial tests are relatively simple, rapid and inexpensive. In this study, the relative contribution of photolysis and microbial assemblages to TNT degradation was measured with a simultaneous incubation system. The effects of TNT and its photoproducts on microbial assemblages were assessed with measurements of viable bacteria count and heterotrophic mineralization of glucose. In addition, the effect of riboflavin (i.e., an environmentally friendly and biodegradable photosensitizer) on TNT photolysis was also studied.

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MATERIALS AND METHODS

Surface freshwater samples were collected from the Mississippi River near Vicksburg, Mississippi. The pH of the water samples ranged from 7.0 to 7.2, and the temperature ranged from 25°C to 28°C. TNT (analytical grade; Chem Service Co., Pennsylvania) was dissolved in acetonitrile (HPLC grade; Fisher Scientific) and added to 50 mL of water sample in 150-mL quartz flasks (GM Associates, Inc., Oakland, California) and incubated in triplicate. Degradation of TNT was measured by quantifying the amount of $^{14}CO_2$ production (mineralization rate) and disappearance of the parent compound (transformation rate). The final concentration of TNT was either 14 µg/L (for mineralization study) or 10 mg/L (mainly for transformation study with HPLC analysis). The higher concentration (10 mg/L) was chosen based on TNT aqueous solubility and instrumental sensitivity for detecting the parent compound and degradation products. The quartz flasks allowed 85% and 100% transmission of light at wavelength of 285 and $\geq$ 300 nm, respectively. The flasks were suspended in an outdoor tub which contained continuous running water for maintaining the water temperature at 27±3°C. The water level in the flask was about 3 cm below the surface of the cooling water. A research radiometer (model IL 1700, International Light Inc., Newburyport, Massachusetts) was placed beside the tub to measure UV irradiance. The dark exposure group consisted of flasks being wrapped with aluminum foil. Killed control was accomplished with the water samples being poisoned with formaldehyde (0.4% final concentration; for assuring sterile conditions during mineralization study) or autoclaved (for transformation study) at 250°F at 15 psi for 20 minutes. All bottles were capped with silicon stoppers. At the termination of incubation, aliquots of water samples were filtered (0.45 µm; Schleicher and Schuell, Keene, New Hampshire) and the separation of TNT and its residues was performed by using a Waters 996 HPLC system. The system is equipped with a photodiode array detector and a reverse-phase Supelcosil LC-8 column (Supelco Co., Bellefonte, Pennsylvania), under isocratic condition with methanol-water (50:50, v/v) at a flow rate of 1 mL/min. Detection wavelength was set at 228 nm. Difference in experimental data between different treatment groups was determined with student-t-test ($p \leq 0.05$). Riboflavin (Analytical grade; Aldrich Chemical Co.) was dissolved in water (HPLC grade; Fisher Scientific) and added to the subsamples (final concentration 10 mg/L) to assess its effect on TNT (10 mg/L) photo-transformation rate.

In a separate experiment, the effect of TNT and its photoproducts on bacterial assemblages was measured with spread plate counting (nutrient agar) and heterotrophic mineralization of ^{14}C-UL–D-glucose (S.A.: 265mCi/mmol; Moravek Biochemicals, Brea, California). TNT was added to 25 mL of autoclaved river water in quartz flasks to make the final concentration 20 mg/L. The water samples containing TNT were exposed to midday sunlight outdoors for up to 1.5 hr. Equal amount of fresh unamended river water was then added to the photo-exposed water to make the final concentration 10 mg/L. Water samples were incubated in darkness for 1 day at 25°C in the laboratory. Treatments included: darkness control (no TNT), darkness exposure (exposure to TNT in darkness) and exposure to sunlight (with and without TNT) for 0.5 hr, 1 hr, and 1.5 hr respectively. Radiolabeled glucose (final concentration 1 µg/L) dissolved in ethanol was added to the water samples after the preexposure and incubated at 25°C in darkness for 1 hr. At the termination of the incubation, 0.5 mL of 2N H_2SO_4 was added to the sample, and the $^{14}CO_2$ produced was trapped with 2-phenylethylamine-soaked filter papers (Hwang and Maloney 1996). The

radioactivity of the filter paper was measured with liquid scintillation spectrometry (Packard Instrument; model TR 1600). To measure the relative contribution of bacterial assemblages and photolysis to TNT mineralization, ring-^{14}C-UL-TNT (purity 98%; S.A. 11.85 mCi/mmol; NEN Life Science Products, Boston, Massachusetts) was added to the water sample and incubated outdoors for up to 3 days in the flask system described as above. The $^{14}CO_2$ generated was collected with a two-trap system involving NaOH and 2-phenylethylamine sequentially (Hwang et al. 1986). The first-order rate constants are calculated from the equation: $\ln (C_0/C_t) = k_p t$, where C_0 and C_t refer to the concentrations of TNT at t_0 and t , respectively, and k_p is the first-order rate constant of photolysis, in unit of hr^{-1}.

RESULTS AND DISCUSSION

In April, Mississippi River water samples were incubated with radiolabeled TNT (14 µg/L) outdoors for up to 3 days. Negligible amount (< 1%) of TNT was mineralized to CO_2 in poisoned dark exposure and dark exposure groups, indicating the lack of abiotic and biotic degradations in the dark (Table 1). However, after 3 days TNT was mineralized by 40.7% and 73% in poisoned light exposure group and light exposure group respectively (Table 1). Mathematically microbial degradation accounted for 44% of the total TNT mineralization. Its contribution was more significant (i.e., 79%) among all degradative processes in day 2. Nevertheless, bacterial assemblages (live dark exposure group) failed to significantly mineralize TNT without the initital transformation by photolysis (Table 1). To contrast with the transformation study with HPLC analysis, mineralization study was also conducted at the same concentration (10 mg/L). A similar pattern in synergistic interaction between the two processes was also observed (Table 1). This is important for realistic persistence modeling and remediation designing for surface waters contaminated by TNT and related compounds. Considering the cost factor, speed of degradation and extent of elimination, the concept of combining solar photolysis and microbial technology for TNT remediation is worth promoting. The advantage of this technology becomes even more apparent since no acclimated or UV-resistant microbes are required for the remediation system (Katayama and Matsumura 1991). The amount of TNT mineralization in the photolysis treatment only (poisoned light) group was very significant, indicating that sunlight-induced reactive species (e.g., singlet oxygen, hydroxyl radicals, superoxide, hydrogen peroxide) were actively involved in TNT elimination process in natural surface water (Zepp and Baughman 1977; Cooper et al. 1989).

Transformation study was conducted with incubation for up to 1.5 hr. After incubation the effect of TNT and intermediate compounds on bacterial assemblages was measured with spread plate counting and heterotrophic mineralization of D-glucose. Glucose mineralization reflects bacterial heterotrophic activities, while spread plate counting measures viability of heterotrophic bacterial populations. The concentration of TNT remaining in the samples was measured with HPLC. Sample solutions turned pink after exposing to sunlight and HPLC analysis indicated that TNT was rapidly degraded photochemically. After photolysis proceeded for 0.5 hr, 1 hr and 1.5 hr in August, there was 95%, 81%, and 49% of TNT remaining respectively. Surprisingly more TNT was degraded in the winter than in the summer, with the first-order rate constants being 1.0 hr^{-1} and 0.3 hr^{-1}, respectively for the winter and summer photolysis process (Table 2). The UV irradiance received (integrated between

325 and 375 nm) by radiometer was 1. 3 x 10^{-3} and 2.4 x 10^{-6} watts/cm^2 for the August experiment and December experiment, respectively. The water sample collected in August was very muddy. Since photolysis is relatively inert to temperature effect, we speculate that lower photolysis rate in the summer was due to higher light attenuation by the suspended particulates present in the water. TNT has been reported to be biodegraded to reduced products such as 2,4-diamino-6-nitrotoluene (2,4-DANT), 2,6-diamino-4-nitrotoluene (2,6-DANT), 4-amino-2,6-dinitrotoluene (4-ADNT) and 2-amino-4,6-dinitrotoluene (2-ADNT) (Kaplan 1998; Sheremata et al. 1999). These compound were not detected in any sample during our transformation study. Instead, an oxidized product, 1,3,5-trinitrobenzene, was found in poisoned and live light samples. Therefore, photo-oxidation process appears to predominate in the sun-lit degradation pathways during this study and microbes were "directed" to mineralize these intermediates into final inorganic products such as carbon dioxide. Several minor peaks were observed in the chromatogram of the samples following the combined solar photolysis and microbial degradation. Trinitrobenzaldehyde and trinitrobenzoic acid were reported as the major reaction intermediates for the photocatalytic degradation of TNT in pure aqueous solutions (Lang et al. 1998). Formation of conjugates between TNT and TNT degradation interemediates with other organic or inorganic fractions in river water was likely to occur (Kaplan 1998). Identification of these TNT residues is underway by using HPLC/photodiode array detector, GC/MS and LC/MS/MS systems.

Table 1. Mineralization (%) of TNT under different exposure treatments.*

Treatment	14 µg/L (% mineralized)	10 mg/L (% mineralized)
Poisoned dark (1 day)	0.230 ± 0.006	0.16 ± 0.02
Poisoned dark (3 days)	0.36 ± 0.07	0.30 ± 0.02
Live dark (1 day)	0.10 ± 0.01	0.24 ± 0.04
Live dark (3 days)	0.6 ± 0.4	0.40 ± 0.06
Poisoned light (1 day)	0.100 ± 0.006	0.50 ± 0.07
Poisoned light (2 days)	13.5 ± 9.4	0.6 ± 0.2
Poisoned light (3 days)	40.7 ± 4.2	1.8 ± 0.5
Live light (1 day)	0.20 ± 0.04	1.5 ± 0.3
Live light (2 days)	63.0 ± 18.4	1.7 ± 0.4
Live light (3 days)	73 ± 14	3.7 ± 0.9

*TNT was added at a final concentration of 14 µg/L (all radiolabeled form) or 10 mg/L (mixture of radiolabeled and unlabeled forms). Numbers are expressed as mean ± 1 standard deviation (n = 3).

Addition of riboflavin at 10 mg/L significantly increased photolysis rates of TNT in the water samples collected from both seasons, with the first-order rate constants increasing from 0.3 to 0.6 hr^{-1} for the summer sample and from 1.0 to 1.5 hr^{-1} for the winter sample respectively (Table 2). The enhancement effect began to decrease when riboflavin was added at higher concentrations, possibly due to the increased light quenching by darker solutions. Riboflavin is highly unstable when irradiated with visible light and the riboflavin sensitized photolysis was also observed for chloroanilines in our laboratory (unpublished data). The enhancement was possibly mediated through a "Type I" sensitized reaction mechanism or the production of singlet oxygen (Halmann 1996; Dunlap and Susic 1986). Since riboflavin is photo-labile, biodegradable and environmentally friendly, it has the potential to be used as an efficient and safe

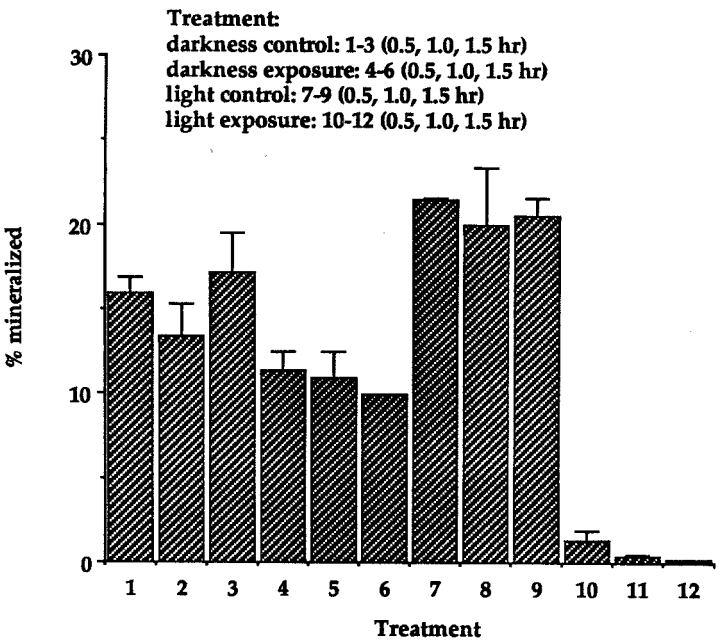

Figure 1. Comparison of glucose mineralization rates of bacterial assemblages in different exposure groups.

Table 2. Time course photolysis of TNT in Mississippi River water in different seasons.#

Sample/ time point	August/ amount left (mg/L)	December/ amount left (mg/L)	August/ 1st-order rate constant (hr^{-1})	December/ 1st-order rate constant (hr^{-1})
0.5 hr	9.5 ± 0.2	7.8 ± 2.1	0.10 ± 0.04	0.5 ± 0.5
0.5 hr/ +riboflavin	7.6 ± 0.6^	4.6 ± 0^*	0.5 ± 0.2^	1.5 ± 0^*
1 hr	8.07 ± 0.08	3.3 ± 0.2*	0.21 ± 0.01	1.11 ± 0.07*
1 hr/ +riboflavin	5.78 ± 0.07^	2.6 ± 0.2^*	0.55 ± 0.01^	1.34 ± 0.09^*
1.5 hr	4.9 ± 0.8	1.2 ± 0.2*	0.5 ± 0.1	1.4 ± 0.1*
1.5 hr/ +riboflavin	2.9 ± 0.4^	0.80 ± 0.09^*	0.82 ± 0.09^	1.68 ± 0.07^*
average of 1st-order rate constant			0.3 ± 0..2	1.0 ± 0.5
average of 1st-order rate constant (+riboflavin)			0.6 ± 0.2	1.5 ± 0.2

*Significantly different from the August sample ($p < 0.05$).
^ Significantly different from the sample without riboflavin ($p < 0.05$).
#TNT and riboflavin were added at an initial concentration of 10 mg/L respectively. Numbers are expressed as mean ± 1 standard deviation (n= 3).

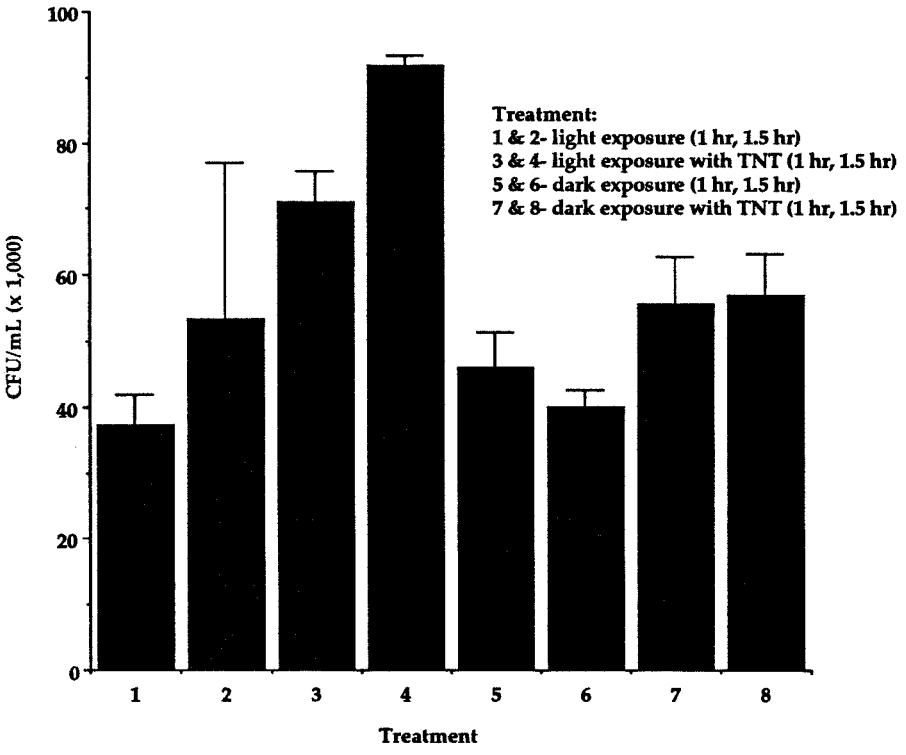

Figure 2. Comparison of viable counts of bacterial assemblages in different exposure groups.

sensitizer for removal of TNT and many other aromatic pollutants in contaminated surface waters in the presence of sunlight (Larson et al. 1989).

After exposure to TNT in darkness, bacterial mineralization rate of glucose was inhibited by up to 29% relative to darkness control (Figure 1). Exposure to sunlight only, however, enhanced bacterial heterotrophic activity by 19% to 49% with comparison to the darkness control group. The bacterial assemblages in the light exposure group, therefore, appeared to be in a more healthy state, possibly by utilizing photoproducts of natural dissolved organic matter. In comparison with the light control group the mineralization rate of glucose was inhibited by up to 99% after exposure to TNT in sunlight (Figure 1). However, the concurrent measurement of spread plate counting indicated that viability number increased by up to 129% after exposure to TNT and its photoproducts (Figure 2). Therefore, the light inhibition on glucose mineralization by TNT is probably due to the competitive utilization/uptake between glucose and mixtures of photoproducts present in the water samples (Hwang et al. 1998). In conclusion, both bacterial assemblages and photolysis were found to be important for TNT removal from contaminated surface waters. The relative importance of their contributions varied with time. The photoproducts of TNT could become the growth substrates for bacterial assemblages in natural waters. Addition of riboflavin at 10 mg/L significantly enhanced TNT photo-transformation rates.

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