ASSESSING THE EFFECT OF PHOTOPRODUCTS OF 2,4,6-TRINITROTOLUENE (TNT) ON MICROBIAL ASSEMBLAGES IN NATURAL AQUATIC ENVIRONMENT

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INTRODUCTION

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. Wastewater from munitions 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 and its metabolites because of worry that it would find its way into the food chain (Harvey 1990). Concern about the chemical was compounded because of its toxicity and recalcitrance to biodegradation (Funk, Crawford, and Crawford 1996). The disposal of large quantities of TNT in an environmentally acceptable manner poses serious difficulties. One past manner of disposal included the dumping of obsolete munitions in the sea. Open burning and detonation were also used, but were deemed ineffective because they added particulates, NO_2, and other products to the already polluted air (Yinon 1990).

Researchers began to look at other methods for the remediation of the chemical including photolysis and microbial degradation. Both of these processes have been shown to be effective degrading processes affecting selected nitro-aromatic pollutants in aquatic environments (Hwang et al. 1986). The use of microbial bioassays has been widely used, based on the assumption that microorganisms can act as surrogates for higher organisms in the environment. In addition, microbial bioassays are desirable because they are relatively simple, inexpensive, and swift. Furthermore, the use of sensitizers has been shown, in some cases, to enhance the photolysis rate of organic compounds (Halmann 1996).

In this study, the effects of TNT and its photoproducts on microbial activity were analyzed with measurements of viable bacterial numbers and bacterial mineraliza- tion of glucose. In addition, the effects of the sensitizer riboflavin on TNT were also studied. The photolysis of TNT in direct sunlight was studied using the HPLC to measure the final concentration of the chemical after exposure.

MATERIALS AND METHODS

Surface water samples were obtained from the Mississippi River near Vicksburg, Mississippi. At the time of collection, the temperature and pH of the samples were measured. The temperature of the samples ranged from 23°C to 29°C, and the pH ranged from 7.0 to 7.5. 2,4,6-Trinitrotoluene was obtained from Chem Service Company. Acetone, acetonitrile (HPLC grade and water (HPLC grade) were obtained from Fisher Scientific Company, Atlanta, Georgia. ^{14}C-UL-D-glucose (S.A.:265 mCi / mmol) was obtained from Moravek Biochemicals, California.

2,4,6-Trinitrotoluene was dissolved in acetone and added to fifty milliliters of sample water in 150 ml quartz flasks (GM Associates Incorporation, Oakland, California), with the final concentration of TNT being 10 mg/L. In experiments to determine if riboflavin acts as a photosensitizer for TNT, 10 mg/L of riboflavin (Aldrich Chemical Company, Milwaukee, Wisconsin) was added to the samples. Dark controls were also used with 50 ml of sample water being placed in 160 ml pyrex bottles (Corning Company). The pyrex bottles were covered with aluminum foil to ensure that no light would reach the sample. All of the samples were then placed outside in a tub which contained continuously flowing water to maintain an approximate temperature of 28°C. The water level in the tub was two to four cm above the level of water in the flasks. A research radiometer (model IL 1700, International Light Incorporation) was placed outside near the tub to measure the amount of ultraviolet radiation being received by the samples. After incubation, recovery of TNT and its photoproducts was obtained by filtering 5ml of the sample through a membrane filter with pore size 0.45 μm filter (Schleicher and Schuell, Keene, New Hampshire). Viable bacterial numbers were obtained by using the spread plate method while total bacterial numbers were measured with Acridine Orange Direct Counting (AODC) of epifluorescence microscopy technique (Hobbie et al. 1977; Hwang and Maloney 1996).

Trinitrotoluene concentration was measured with high performance liquid chromatography (HPLC). The column used was the reverse phase Supelcosil LC-8 column (Supelco Company, Bellefonte, Pennsylvania).

The system used consisted of a Waters 510 HPLC Pump and a Waters U6K Universal Liquid Chromatography Injection fitted with a 2 ml injection loop. Detection was attained at 254 nm using a Waters 996 photodiode array detector. The mobile phase was 0.50 ml/min of HPLC grade acetonitrile and 0.50 ml/min of HPLC grade water. Difference in experimental data between sample groups was determined with student t-test ($p \leq 0.05$).

In a similar experiment, the effect of TNT and its photoproducts on bacterial assemblages was assessed with heterotrophic mineralization of ^{14}C-D-glucose and total bacterial numbers. TNT was added to 25 mL of autoclaved river water in quartz flasks to make the final concentration 20 mg/L. The TNT water samples were placed outside and exposed to midday sunlight for up to 1 hr and 30 min. An equal amount of fresh unamended river water was added to the photo-exposed water which was then incubated in darkness for 1 day at 25°C in the laboratory. Treatments included: darkness control, darkness exposure, light control, and light exposure. The light samples were exposed to sunlight for 30 min, 1 hr, and 1 hr 30 min, respectively. Radiolabeled glucose (final concentration 1 µg/L) dissolved in ethanol was added to the water samples after pre-exposure and incubated at 25°C in darkness for 1 hr. At the termination of the incubation, 0.5 mL of 2 N H_2SO_4 was added to the sample, and the $^{14}CO_2$ produced was trapped by 2-phenylethylamine-soaked filter papers (Hwang and Maloney 1996). The radioactivity of the filter paper was measured with liquid scintillation spectrometry (Packard model TR 1600).

RESULTS AND DISCUSSION

Freshwater samples were collected from the Mississippi River near Vicksburg, Mississippi, and incubated with 2,4,6-trinitrotoluene at 28°C for 1.5 hours in midday sunlight. After incubation, the effects of TNT on bacterial assemblages were measured with spread plate technique, which measures the viable bacterial colonies in a water sample. Also, the photolysis rate of TNT was measured by assessing the concentration of TNT remaining in the samples using High Performance Liquid Chromatography (HPLC).

The effects of TNT on bacterial viability are shown in Figure 1. Results show that there was more colony growth in the light than in the dark with greater than 5000 cfu/ml in the light and less than 300 cfu/ml in the dark. This phenomenon could be due to the fact that the bacteria used the photoproducts of TNT as a growth substrate.

HPLC analysis of water samples showed that TNT degrades at a very fast rate in direct sunlight. In a study conducted by Mabey et al. (1983), it was shown that photolysis of TNT occurs rapidly in pure and natural waters irradiated with sunlight. In preliminary studies, we found that after three hours of exposure to sunlight, there was no measurable TNT in the sample waters; therefore, we cut sampling time to a total of 1.5 hours. After 1.5 hours of exposure to sunlight, river water samples contained only 12% TNT. We speculate that 78% of the TNT was degraded through a combination of photolysis and microbial degradation (Figure 2). When comparing studies from different seasons (summer and winter), we find that there is more degradation occurring in the winter (Table 1, Figure 3); the rate constants for the winter and summer photolysis were 1.53 hr^{-1} and .670 hr^{-1}, respectively without riboflavin and 1.53 hr^{-1} and .958 hr^{-1}, respectively with riboflavin (not shown). We speculate that this is due to more organics acting as natural sensitizers in the sample water in December or more light attenuation in August. In other studies comparing the photodegradation of TNT using river water with and without riboflavin added, we found that riboflavin at 10 mg/L significantly increased degradation at 1 hour in August, and at 1 hour and 1.5 hours in December (Figure 4). There was no significant difference in the degradation rates in either month after .5 hour. Larson et al. (1989) notes that riboflavin is an efficient sensitizer in the removal of several aromatic compounds found in contaminated waters in the presence of light.

The effects of TNT (final concentration 10 mg/L) and/or its photoproducts on natural bacterial assemblages in Mississippi River water were measured with glucose mineralization and acridine orange direct counting (AODC) technique. Glucose mineralization assesses the bacterial heterotrophic activities. AODC technique measures the total bacterial numbers (including dormant individuals) within a water sample. After exposure to TNT in darkness for one day, bacterial mineralization rate of glucose was inhibited by up to 29% in relative to darkness control (Figure 5). Exposure to sunlight only, however, enhanced bacterial heterotrophic activity by 19% to 49% relative to the darkness control group. We speculate bacterial assemblages were in a more healthy state by utilizing photoproducts of natural organic matters. Interestingly, by comparison to the light control group, the mineralization rate of glucose was inhibited by up to 98% by exposure to TNT in sunlight (Figure 5). Our preliminary data indicated that viable counts of heterotrophic bacteria increase over the control group by exposure to TNT light (Figure 1). Therefore, the light inhibition of glucose mineralization by TNT is probably due to the competitive utilization/uptake between glucose and a variety of TNT photoproducts

present in the water samples (Hwang et al. 1998). Identification of TNT photoproducts and measurement of the effects of specific photoproducts on bacterial assemblages will be conducted in future experiments. AODC results are not included at this time due to difficulties with the water samples.

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Table 1. Results of T-test showing significant difference between the LN Concentrations of TNT remaining in river water with and without riboflavin in December and August.

	AUGUST	DECEMBER	T-TEST*
T1+	2.25 +/- .014	1.67 +/- .631	
T1-R	2.02 +/- .078	1.54 +/- .063	*
T2	2.08 +/- .007	1.16 +/- .036	*
T2-R	1.75 +/- .014	.958 +/- .092	*
T3	1.59 +/- .170	.142 +/- .203	*
T3-R	1.05 +/- .139	-.227 +/- .110	*

* $p<0.05$; indicates significant difference
+ T1=.5hr, T2=1hr, T3=1.5hrs
Legend: T - River water
T-R - River water with riboflavin

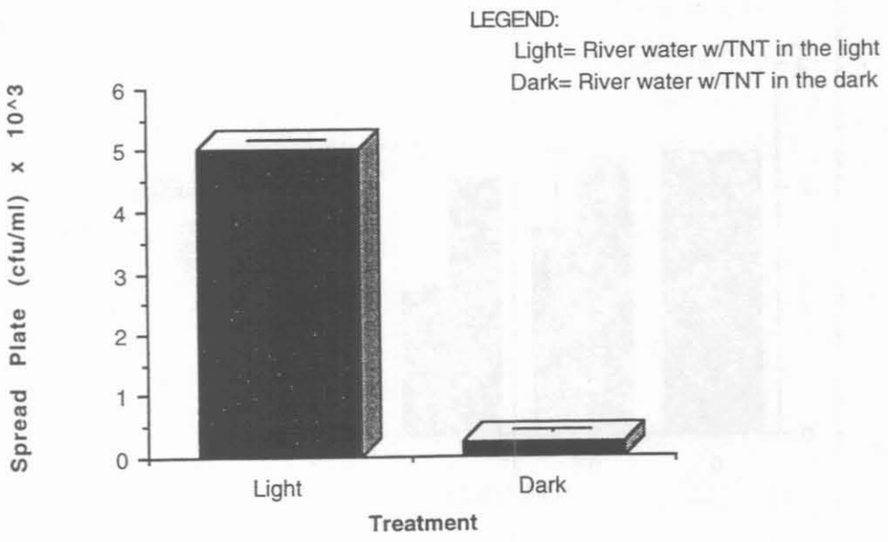

Figure 1. Effect of 2,4,6-Trinitrotoluene on bacteria viability.

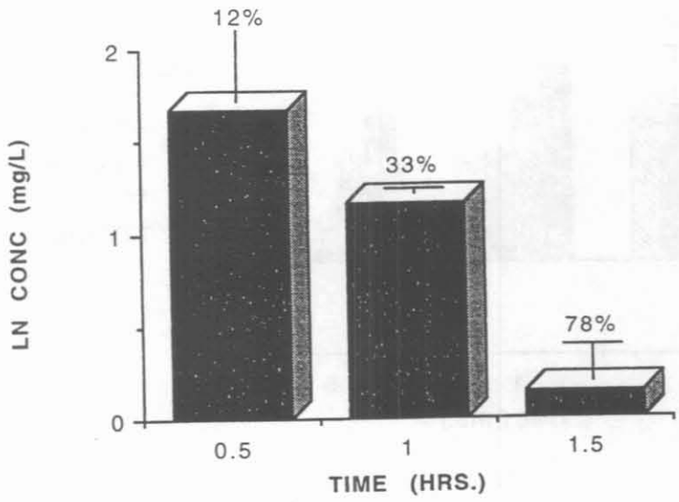

Figure 2. Percent degradation of 2,4,6-Trinitrotoluene in river water after 1.5 hours.

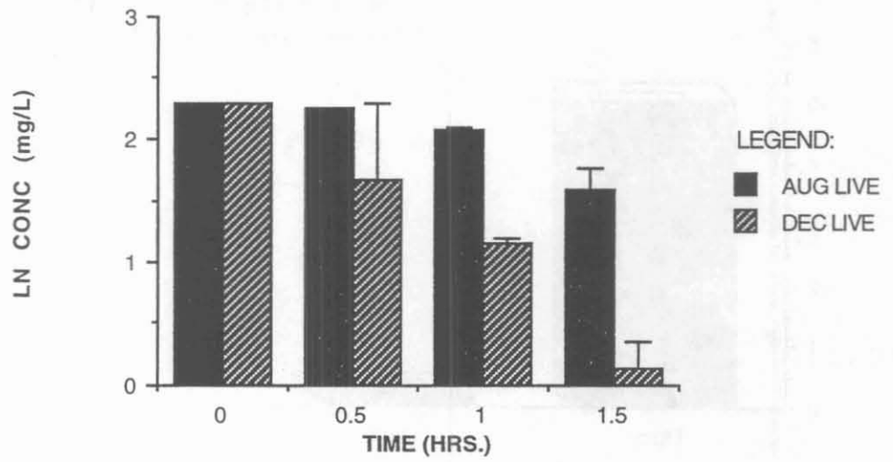

Figure 3. Comparison of the degradation of 2,4,6-Trinitrotoluene in river water samples from different seasons (summer and winter).

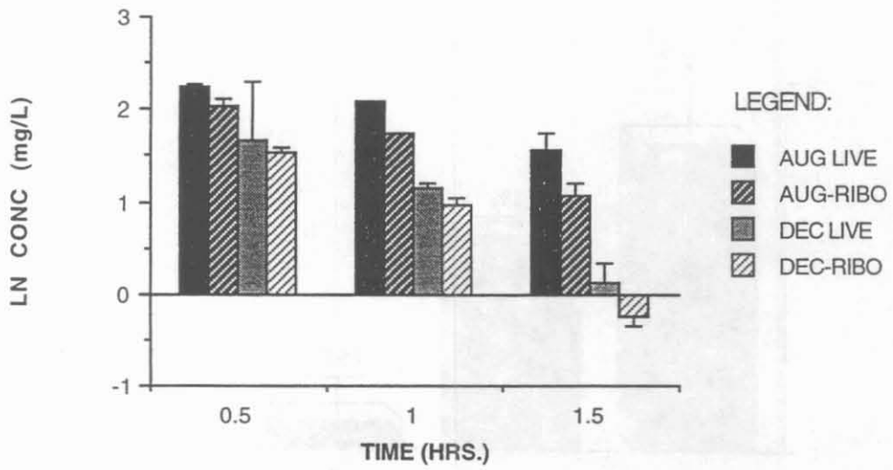

FIGURE 4. Comparison of the remaining concentration of 2,4,6-Trinitrotoluene in river water with and without riboflavin (August and December).

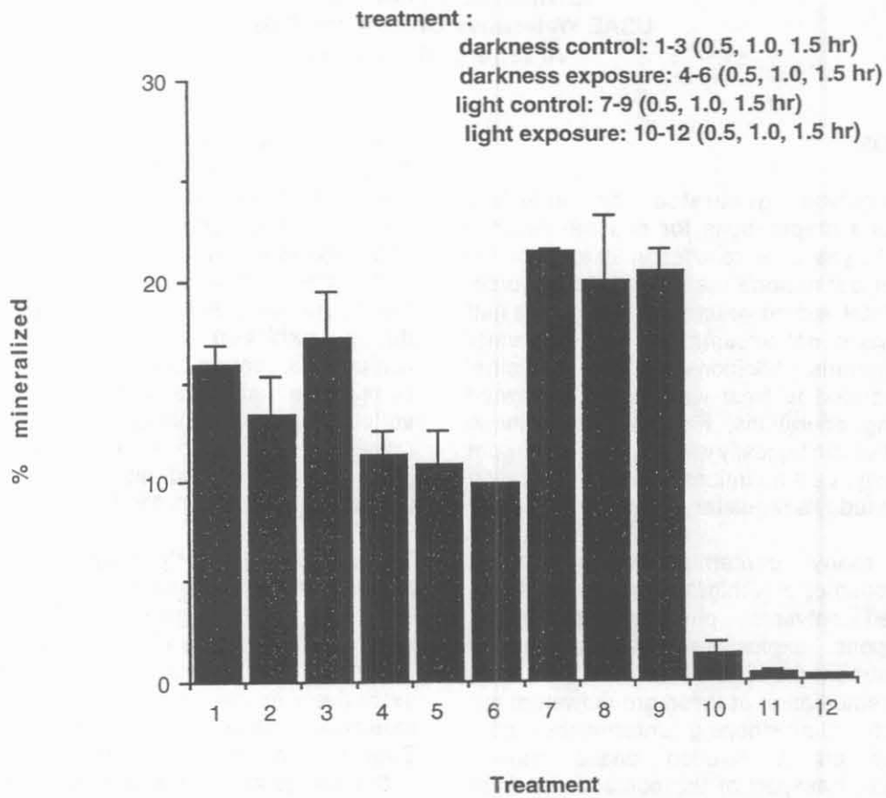

Figure 5. Effect of TNT and/or its photoproducts (initial total concentration: 10 mg/L) on bacterial mineralization of glucose (1 μg/L). Numbers are expressed as mean ± 1 standard deviation (n=3). Darkness control and light control refer to the groups incubated in darkness and in sunlight respectively without adding TNT.