USE OF RIBOFLAVIN AS A PHOTO-SENSITIZER FOR ENHANCING ATRAZINE DEGRADATION IN A FRESHWATER ENVIRONMENT

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INTRODUCTION

Atrazine is a herbicide used to selectively control broadleaf weeds in agricultural fields. Most atrazine is released to the environment through agricultural use as a herbicide and in effluents from manufacturing facilities. Current U.S. estimations of application are 80 million pounds per year. Generally, annual runoff losses of atrazine are 2% to 5% of the total applied. Atrazine is toxic, often bioaccumulative and persistent (Fernando et al. 1992). Consequently, the search for effective remediation methods of removing atrazine from water is important.

Photolysis and microbial degradation have been recognized as important removal forces of many organic pollutants in natural surface waters (Hwang et al. 1986; Hwang et al. 1998; Hwang et al. 2000). Photodegradation of triazine herbicides under direct solar light occurs very slowly (Trotter et al. 1990). In natural aquatic environments, photodegradation of organic pollutants can be strongly enhanced by photosensitizers (Tsao and Eto 1994). Riboflavin as a photosensitizer has been reported to enhance the photochemical degradation of many compounds in aqueous solutions (Mopper and Zika, 1987; Hwang et al. 2000; Cui et al. 2001). Riboflavin is easy to handle, economical, and environmentally benign, which makes it attractive for treatment of environmentally contaminated water; however, the photoinduced toxicity to microbes by organic contaminants and riboflavin has been reported (Hoffmann and Meneghini, 1979; Khan et al. 1973). The wide range of reports on this problem suggests that these compounds may affect environmental health.

In this work, microbial bioassays were used to measure toxicity of the study chemicals based on the assumption that microorganisms can act as surrogates for higher organisms in the ecosystem. The relative contribution of microbial assemblages to the riboflavin-sensitized phototransformation of atrazine in a natural water environment was explored using high performance liquid chromatography. The effect of the metabolite mixture on microbial populations during phototransformation was examined using spread plate analysis and radiotracer mineralizationtechniques.

MATERIALS AND METHODS

Sampling, Chemicals, and Solutions

Surface freshwater samples were collected from the Ross Barnett Reservoir near Jackson, MS. The pH of the water samples ranged between 6.5-7.5, and the temperature ranged between 26°C and 29°C. Assays were initiated within one hour of collection.

Atrazine (ATR), desisopropylatrazine (DIA), desethylatrazine (DEA), 2-hydroxy-atrazine (HA), **desethyl-2-hydroxyatrazine** (DEHA), and the chemical desethyldesisopropyl-2-hydroxyatrazine (DEDIHA) were obtained from ChemService (West Chester, PA). UL-^{14}C-Atrazine (s.a. 20.1 mCi/mmol; purity 98.4%) was obtained from Sigma Chemical Company (St. Louis, MO). Riboflavin was purchased from Aldrich (Milwaukee, WI). Individual standard solutions of atrazine, DEA and DIA were prepared by dissolving the required amount in acetonitrile. Individual standard solutions of HA, DEHA and DEDIHA were prepared by dissolving the required amount in water (HPLC grade) acidified with 5 mmol/L HCl. Unused portions were stored in the dark at 4°C.

Atrazine Degradation

To 150-mL quartz flasks, 50 ml of freshwater containing 10 mg/L atrazine and 100 µM concentration of riboflavin was added and incubated in triplicates. The flasks were suspended in an outdoor tub which contained running water for maintaining the water temperature at 25±3°C. Dark groups were in 160-mL Pyrex bottles wrapped in aluminum foil. The water level in the flask was about 3 cm below the surface of the cooling water. Killed group was accomplished with the water samples

being autoclaved at 121.1°C at 15 psi for 20 minutes. All bottles were capped with silicon stoppers.

HPLC Analysis

After the degradation, the samples were filtered through an Autovial® 0.45-µm nylon membrane filter (Whatman, USA). The separation, quantitation, and identification of atrazine and its degradation products were performed with a Waters 996 HPLC system equipped with a Waters 717 plus autosampler, a photodiode array detector, a pre-column filter (Sigma-Aldrich, USA) and a Hypersil ODS column (250 x 4.6 mm I.D., 5 µm) (Supelco Inc., USA). Mixtures of acetonitrile (A) and water (B) were used as the mobile phase. The following gradient elution was applied: 20 to 80% A from 0 to 8 min, held at 80% A from 8 to 20 min, 80 to 20% A from 20 to 22 min. The flow rate was 1.0 mL/min. The detection wavelength was 223 nm. The temperature of the autosampler was controlled at 7°C. The identification of the degradation products of atrazine was carried out by comparison of the retention time and UV-visible absorption spectra with that of standards.

Mineralization of Atrazine

UL-^{14}C-Atrazine (100 µg/L) was added to 50 ml of freshwater with and without 10 and 100 µM riboflavin. The samples were placed in quartz flasks (light exposures) or aluminum covered pyrex bottles (darkness). Plastic center wells (Kontes Scientific) were trimmed and inserted into the bottom of the silicone stoppers used for flasks and bottles. Filter paper squares (2.5 x 2.5 inches) were folded and placed inside the center wells. The samples were exposed for up to three days in running water. To trap $^{14}CO_2$ produced from the parent compound, a two-trap method was used where initial trapping is done with 0.8 ml 1N NaOH. After this step, the filter papers are placed within another bottle, acidified with 2N H_2SO_4, and re-trapped using 0.8 ml β-phenylethylamine. Following the trapping, the filter papers were removed and placed in polyethylene scintillation vials with 8 ml of UltimaGold scintillation fluid and counted by liquid scintillation spectroscopy (LSS, Packard 1600TR). Results were compared to the percentage of labeled compound in the standards.

Activity During Phototransformation

Five hundred (500) ml working solutions of fresh live river water were prepared containing: 10 mg/L atrazine, 100 µM riboflavin, and 10 mg/L atrazine + 100 µM riboflavin. 50 ml aliquots of the solutions were added to the foil covered pyrex bottles (abiotic) or quartz flasks (phototransformation). The sample bottles were inoculated with ^{14}C-UL-glucose (1 µg/L) and incubated in triplicate for the desired experimental time. Trapping of $^{14}CO_2$ was achieved using a single trap with 0.8 ml β-phenylethylamine. The metabolic activity was stopped at the desired time of 6 hours using 2N H_2SO_4. Trapping was continued overnight for efficiency and measured using LSS to determine the riboflavin sensitized phototransformation effect on microbial assemblages.

Photoproduct Effects

Five hundred (500) ml working solutions of autoclaved river water containing atrazine (10 mg/L) and the desired environmental conditions of unamended or 100 µM riboflavin addition was prepared. 25 ml aliquots of the experimental solutions were added to the bottles/flasks to simulate dark and light reactions. The sample bottles were incubated for six hours exposure time in running water. 25 ml of fresh live river water was added to each bottle and allowed to incubate over night (>12 hrs.) in darkness. Following incubation, one (1) ml was taken from each bottle/flask for spread plate analysis. The remaining sample portions were inoculated with ^{14}C-UL-glucose (1 µg/L) and allowed to sit for 1 hour. Before processing, metabolic activity was stopped using 2N H_2SO_4. Trapping of $^{14}CO_2$ produced from glucose was achieved using a single trap with 0.8 ml β-phenylethylamine as a trapping fluid. Trapping was done overnight to increase efficiency, and measurements were conducted as mentioned in the previous section. These results are listed in Table 2.

RESULTS AND DISCUSSION

Effect of Biotic Components on Riboflavin-sensitized Atrazine Photo-transformation

Direct photolysis had no effect on atrazine degradation over the three day period of these studies; however, riboflavin was successful in sensitizing photodegradation. With the addition of riboflavin at 100 µM concentration, loss of parent compound reached levels above 80% in three days. Comparing the strict photochemical (killed) with the combined biotic and photochemical (live) results, there is an apparent

enhancement in photodegradation in living samples (Table 1).

The riboflavin sensitized degradation was found to be primarily photochemical by analyzing the loss of parent compound immediately following photodegradation, placing the sample in darkness and measuring the concentration of parent compound remaining after greater than 12 hours. No additional loss of parent compound was observed in darkness following photoexposure (Table 2).

Riboflavin's Effect on Mineralization of Atrazine

Table 3 lists the effect of riboflavin on atrazine mineralization at 1 and 3 day time periods. Riboflavin at 10 and 100 μM concentrations slightly reduced the amount mineralized relative to the control (no riboflavin) group, however mineralization was enhanced in the dark (microbial degradation) samples. Since the 10 μM riboflavin treatment is larger than the control, and the 100 μM treatment is more than the both the dark control and 10 μM, it was assumed that the enhancement here is dose dependent and attributed to the added nutrient increasing microbial growth.

Riboflavin's Effect on Viability and Heterotrophic Activity During Degradation

Riboflavin acts as a nutrient source and increases the growth of microbes in dark exposures, but this trend is not seen when riboflavin is photoexposed. Table 4 illustrates riboflavin's photoinduced toxicity. During photoexposure to riboflavin, the heterotrophic activity decreased relative to the control group. The viability began to decrease after one hour and was noticeable at both the 3 and 6 hour marks as well. The addition of riboflavin initially increases microbial growth, but after the riboflavin is degraded, the numbers continued to decrease until no visible growth was observed. This may be due to single strand DNA damage caused by free radicals formed from photoexposed riboflavin (Hoffman and Meneghini 1979).

Riboflavin-sensitized Photoproduct Effects on Viability and Heterotrophic Activity

Table 5 lists the effect of atrazine and riboflavin's photoproducts on microbial viability and heterotrophic activity. The spread plate count shows decreased viability in the samples photoexposed to riboflavin. The sample group containing only riboflavin was not significantly different from the sample group containing both riboflavin and atrazine. In darkness, the presence of riboflavin stimulated growth relative to the controls as expected. Glucose mineralization results from this experiment vary slightly from spread plate results. Although riboflavin greatly reduced uptake in light exposures as in the spread plates, the stimulation was not observed in dark exposures and may be due to microbial use of riboflavin as a growth substrate rather than glucose.

Trends in the spread plate data indicate that atrazine (10 mg/L) is tolerated by microbial assemblages. Changes to the environment through the addition of nutrient sources such as riboflavin may cause the microbial assemblages to react differently, and subsequently appear as stimulation or inhibition of viability.

When riboflavin was photoexposed regardless of the presence of atrazine, there was a dramatic difference in glucose mineralization relative to the control group. This data support the spread plate results, and further supports the conclusion that riboflavin photoproducts or the free radicals produced photochemically are toxic to microbial assemblages when exposure surpasses one hour.

CONCLUSIONS

Riboflavin facilitated atrazine phototransformation more with live freshwater microbes than killed freshwater. Atrazine was tolerated by the microbial assemblages after both photoexposure and darkness exposure; however, when the microbial assemblages were exposed to the riboflavin photoexposure mixture and the riboflavin and atrazine photoexposure mixture, the trend was reversed. The photoexposed riboflavin was found to be harmful to the microbial assemblages in both groups. This leaves the question of whether the active oxygen species created by the photodegradation of riboflavin is the cause of microbial damage. In both the spread plate count and the glucose mineralization analysis, dramatic differences were observed between riboflavin containing groups and those not containing riboflavin. Because of the economical advantage of riboflavin use, further investigation is necessary to determine why the biotic components enhance riboflavin-phototransformation of atrazine while losing both viability and activity.

Such work may lead to cost effective surface water treatment systems involving combined photochemical and microbial degradation processes.

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Table 1. Effect of biotic components on percentage of 10 mg/l atrazine remaining after photodegradation with 100 μM riboflavin.

TREATMENT	LIGHT LIVE (%)	LIGHT KILLED (%)
Time Zero	100	100
1 hr	75.7 ± 5.5	85.0 ± 2.5
2 hrs	65.9 ± 5.3	78.3 ± 8.4
4 hrs	48.2 ± 6.8	59.3 ± 9.2
6 hrs	42.0 ± 1.9	59.3 ± 7.7
24 hrs	43.9 ± 5.0	42.1 ± 5.0
48 hrs	30.5 ± 4.3	42.5 ± 7.8
72 hrs	17.1 ± 6.8	27.2 ± 4.9

Table 2. Effect of darkness on riboflavin-sensitized transformation and heterotrophic activity.

	MEASUREMENTS		
TREATMENT	A	B	C
Light w/ Atrazine	95.2 ± 0.33	93.8 ± 9.6	7 ± 1.0
Light w/ Atrazine &Riboflavin	51.2 ± 8.8	51.5 ± 5.9	0.3 ± 0.2
Dark w/ Atrazine	99.2 ± 3.5	97.1 ± 2.2	4.4 ± 1.1
Light Control			3.4 ± 1.0
Light w/ Riboflavin			0.3 ± 0.1
Dark Control			3.6 ± 0.8

A - % Atrazine remaining after 6 hours photoexposure.
B - % Atrazine remaining in A following additional >12 hours dark exposure
C - % Glucose mineralization of live freshwater (25 ml) added to sample water B and incubated for 1 hour

Table 3. The effect of atrazine and riboflavin on atrazine mineralization.

TREATMENT RIVER WATER + ^{14}C-ATRAZINE	1 DAY	3 DAYS
LL Atrazine	0.05 ± 0.003	0.14 ± 0.08
LK Atrazine	0.02 ± 0.01	0.04 ± 0.001
LL Atr + 10 μM Riboflavin	0.03 ± 0.002	0.09 ± 0.01
LK Atr + 10 μM Riboflavin	0.02 ± 0.002	0.02 ± 0.01
LL Atr + 100 μM Riboflavin	0.04 ± 0.02	0.04*
LK Atr + 100 μM Riboflavin	0.02 ± 0.01	0.08*
DL Atr	0.13 ± 0.016	0.13 ± 0.05
DK Atr	0.03 ± 0.002	0.12 ± 0.04
DL Atr + 10 μM Riboflavin	0.12 ± 0.04	0.6 ± 0.2
DK Atr + 10 μM Riboflavin	0.05 ± 0.01	0.11 ± 0.07
DL Atr + 100 μM Riboflavin	0.27 ± 0.11	1.7 ± 0.7
DK Atr + 100 μM Riboflavin	0.03*	0.09*

*- No standard deviation due to loss of replicates.
♦ **Legend:** L- Light, D- Dark, L- Live, K- Killed, Atr- Atrazine

Table 4. Time course effect of riboflavin sensitized atrazine phototransformation on microbial viability.

TREATMENT ♦	30 MIN	1 HR	2 HRS	6 HRS
Control	5467 ± 808.3	6867 ± 1858	5467 ± 723.4	6200 ± 1905
Atrazine	6367 ± 1222	6000 ± 1510	4233 ± 945.1	5100 ± 953.9
100 μM Riboflavin	6600 ± 2030	2467 ± 1750^	6333 ± 230.9	966 ± 814^
Atr+100μM Riboflavin	5900 ± 608.3	1533 ± 1101^	1250 ± 700.0^	400 ± 100^

TREATMENT	24 HRS	48 HRS	72 HRS
Control	36033 ± 9203	50000+ (TNC)	50000+ (TNC)
Atr	39100 ± 9051	50000 +(TNC)	50000+ (TNC)
100 μM Riboflavin	200 ^	NVG ^	NVG ^
Atr+100μM Riboflavin	200 ^	NVG ^	NVG ^

♦ Legend: All treatments contained fresh river water.
Numbers reflect Colony Forming Units/mL
^- significantly different from control by t-test ($p \leq 0.05$) & ANOVA
TNC- Too Numerous to Count
NVG- No Visible Growth

Table 5. The effect of riboflavin-sensitized photodegradation on microbial viability and heterotrophic activity in unamended freshwater after 6 hours of photoexposure.

TREATMENT	SPREADPLATE (C.F.U./ML)	% GLUCOSE MINERALIZATION
Light Control (w/o Atr)	381000 ± 33420	11.1 ± 0.9
L Atr	220000 ± 24020	11.9 ± 1.3
L 100μM Riboflavin (w/o Atr)	37000 ± 8480	0.2 ± 0.01
L Atr + 100μM Riboflavin	50330 ± 10690	0.2 ± 0.02
Dark Control (w/o Atr)	215500 ± 53030	8.1 ± 0.5
D Atr	239000 ± 46670	5.9 ± 1.0
D 100 μM Riboflavin (w/o Atr)	467000 ± 23000	4.9 ± 0.6
D Atr + 100 μM Riboflavin	471670 ± 13800	4.1 ± 0.5