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Persistence and Mutagenic Potential of Herbicide-derived Aniline Residues in Pond Water*

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Herbicides that contain aniline or substituted aniline moieties are biodegraded with the eventual release of the anilines (Cripps and Roberts, 1978). A considerable amount of research has been conducted on the fate of herbicide-derived anilines in soil (Bartha, 1980) but their fate in aquatic environments, which the herbicides may contaminate due to drift or run-off, is less well explored (Lyons et al., 1984). The experiments presented here compare the persistence of aniline, 3-chloroaniline (3-CA), 4-chloroaniline (4-CA), 3,4-dichloroaniline (3,4-DCA), 2,6-diethylaniline (2,6-DEA) and 2,6-dinitro-4-trifluoromethylaniline (2,6-DNTFA) in pond water and pond water with a sewage sludge inoculum as models of unpolluted and sewage-polluted fresh-water environments. The kinetics of removal for the biodegradable compounds and the mutagenic potential of the anilines and their biodegradation intermediates are also explored. Additional information about the test compounds and some experimental techniques are presented in a related paper by Lyons et al. (1985).

MATERIALS AND METHODS

Aniline and substituted anilines were obtained from Aldrich Chemical Co. (Milwaukee, WI). Liquid anilines were purified by distillation, solid ones by recrystallizations from petroleum ether to constant melting point and/or single gas chromatographic (GC) peak. Water was freshly collected during the months of August and September from a shallow eutrophic pond (pH 6.9 - 7.1) located on the campus of Cook College, New Brunswick, NJ and was strained through cheesecloth to remove filamentous algae and large detritus. This pond water was used in biodegradation experiments alone or with a 0.1% inoculum of fresh settled activated sewage sludge (Raritan Valley Sewerage Authority Treatment Plant, Bridgewater, NJ). The test anilines were added in 0.5 mL acetone to 250 mL Erlenmeyer flasks with stainless steel cap closures. The acetone was allowed to evaporate, coating the

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anilines on the flask bottom. Pond water with or without sewage sludge inoculum was added (50 mL/flask), yielding aniline concentrations of 250 µg/mL. For sterile controls, the aniline solutions were filter-sterilized using 0.2 µm pore size Teflon membranes; the pond water was steam-sterilized. Incubation was at 20°C in the dark with rotary shaking (200 rpm). For the kinetic experiments, incubation conditions were the same; aniline concentations ranged from 2.5-500 µg/mL.

At each sampling time, each of the triplicate flasks was extracted three times with 50 mL portions of isooctane. The combined extracts were dried using anhydrous Na_2SO_4 prior to being evaporated to near-dryness at 45°C in a rotary evaporator and analyzed by GC as described by Lyons et al. (1984). In addition to the previously referenced conditions, quantitative analysis of aniline, 3-CA, 4-CA and 2,6-DEA was performed isothermally at 110°C where retention times were 0.97, 1.21, 1.27 and 1.10 min, respectively. A temperature of 130°C was used for the quantitative analysis of 3,4-DCA and 2,6-DNTFA whose respective retention times were 2.38 and 3.44 min.

The mutagenic potential of the aniline compounds and their metabolites was determined according to Maron and Ames (1983) using Salmonella typhimurium tester strains TA 98 and TA 100 for detection of frameshift and base-pair substitution mutations, respectively. The cultures were obtained from the laboratory of B. N. Ames, University of California, Berkeley. Upon receipt, 10 h cultures were grown in Oxoid Nutrient Broth No. 2, and were distributed into sterile cryogenic tubes with 10% dimethyl sulfoxide (DMSO) and preserved frozen at -80°C. The genotype of the two tester strains (his$^-$, rfa, uvrB, R-factor) were confirmed by appropriate tests before their use (Maron and Ames, 1983). For each assay, 10 h cultures were grown according to the recombinant procedures. Rat liver S-9, Aroclor 1254-induced and prepared in KCl (Litton Bionetics, Inc., Charlestown, SC) was received frozen and stored at -80°C. The protein content was 25 mg/mL and relative P448/P450 aryl hydrocarbon hydroxylase activity was 1.7 nmol hydroxybenzo(a)pyrene/20 min/mg protein.

The plate incorporation test was used. The test compound or sample (0.1 mL) plus 0.1 mL of the bacterial tester strain (TA 98 or TA 100) and 2.0 mL of soft agar with and without 0.5 mL of S-9 mix were mixed using a vortex apparatus and poured onto a minimal agar plate. Plates for spontaneous revertants and positive controls (5 µg benzo(a)pyrene) were included in each assay. The assays were performed in triplicates and all plates were incubated at 37°C for 48 h.

Each of the six aniline compounds was assayed by the plate incorporation system in dose ranges 0.25, 2.5, 25, and 50 µg/plate. The concentration of S-9 per plate is critical, and can be variable from one compound to another. Too much S-9, as well as too little, can drastically lower the mutagenic response. Thus, standards for these six compounds were prepared in absolute ethanol and 0.1 mL volumes assayed with 0-50 µL of S-9 in 10-fold

serial concentrations. The mutagenic response to each compound is described at its respective optimized S-9 concentration. When evidence for metabolic transformation was observed at various sampling times, 0.1 mL amounts of the spiked pond water (with or without sewage sludge inoculum) were assayed in triplicate using the Ames plate incorporation assay. These tests were performed in conjunction with the kinetic studies over a concentration range of 2.5 to 500 µg/mL. Consequently, the amounts of aniline added to the Ames plates corresponded to 0.25, 2.5, 25 and 50 µg aniline (initial concentrations).

RESULTS AND DISCUSSION

Only 10% of the added unsubstituted aniline was recovered after 2 weeks of incubation in pond water; the sewage sludge inoculum greatly reduced persistence (Table 1). In sterile controls, 86% of the aniline persisted, indicating that most of the former removal was due to biodegradation. In contrast, the substituted anilines showed high persistence in pond water samples. The small losses observed were comparable to those in sterilized samples (data not presented), indicating that they were due to evaporation and autoxidation. In sewage sludge-supplemented samples, binding of the anilines to the humic components of the sludge may have contributed to the observed removal (Lyons et al., 1984).

Biodegradation of aniline in pond water, with or without sewage sludge, exhibited first order kinetics (Fig. 1). A V_{max} of 22.0 nmoles per L per day and an apparent K_m of 3.3 mM was measured in sewage sludge-supplemented pond water. The V_{max} was 9.1 nmoles per L per day and the apparent K_m was 5.0 mM in pond water without a sewage sludge inoculum. The above kinetic data were corrected for non-biodegradative loss.

As determined by the Ames test, the mutagenic potentials of aniline, 3-CA, 4-CA, 3,4-DCA and 2,6-DNTFA were negative or marginal. Without S-9 mix, they failed to increase the numbers of revertants. With optimized amounts of S-9 mix, and up to the maximum level of the anilines tested (50 µg per plate) the reversion rates of both test strains increased two to three times the spontaneous rate. No dose-response effect was observed within the range of the test. In the Ames test, results are not regarded positive unless the spontaneous reversion rate of the test strains is at least doubled. Because of the negative or marginal nature of the results, they are not presented here in detail. In contrast, 2,6-DEA in the presence of S-9 mix exerted a significant mutagenic effect on both TA 98 and TA 100 test strains and a definite positive correlation with increasing doses was evident (Table 2).

As stated earlier, unsubstituted aniline was essentially non-mutagenic. In the presence of an optimized amount of S-9 mix, aniline tripled the spontaneous reversion frequency of the TA 98 test strain (frame shift mutagenesis) but failed to affect TA 100 (base-pair substitution mutagenesis). However, aniline trans-

Table 1. Persistence of anilines in pond water with and without sewage sludge inoculum.[1]

Compound[2]	Pond water Percent remaining on day 4	7	14	Pond water + sewage sludge Percent remaining on day 4	7	14
Aniline	70	59	10	8	0	0
3-CA	97	95	93	95	93	91
4-CA	100	100	96	100	99	93
3,4-DCA	98	97	97	98	95	94
2,6-DEA	100	100	100	97	97	97
2,6-DNTFA	100	100	99	99	98	95

[1]Pond water contained 90-117 µg/mL microbial biomass, the sewage sludge inoculum added another 8 µg/mL biomass (Lyons et al., 1984). Data represent the averages of triplicate samples.

[2]Added at 250 µg/mL concentration.

Table 2. Mutagenic potential[1] of increasing quantities of 2,6-DEA as measured in the Ames test using Salmonella typhimurium mutants TA 98 and TA 100 with and without metabolic activation.

2,6-DEA µg/plate	Test strain and activation[2] TA 98	TA 98+S-9	TA 100	TA 100+S-9
0.00	25	59	145	170
0.25	29	686	147	672
2.50	21	808	171	750
25.00	30	956	251	838
50.00	29	977	257	1034

[1]Values indicate numbers of revertants per plate (average of triplicates). The spontaneous reversion frequencies of TA 98 and TA 100, with or without S-9 mix, are shown at 0.00 amount of 2,6-DEA.

[2]S-9 mix added at optimal (50 µL per plate) level.

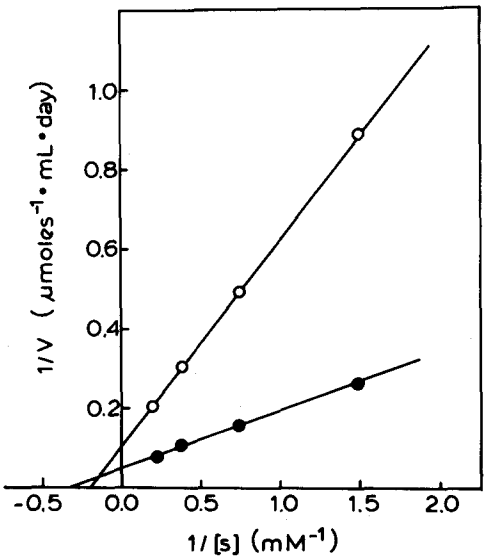

Figure 1. Lineweaver-Burk plot of aniline biodegradation rates in pond water (○) and pond water with sewage sludge inoculum (●). Rates were measured over a one-week time period and at concentrations ranging from 2.5 to 500 μg/mL (0.27-5.37 mM).

formation products generated during incubation of pond water with sewage sludge inoculum had definite mutagenic potential both towards TA 98 and TA 100 test strains (Table 3). Addition of S-9 mix was not required for detection of mutagenesis. In sterile, sewage sludge-amended pond water, no evidence was obtained for formation of any mutagenic aniline transformation products at 0, 2 and 4 days. On day 7, a moderate increase in revertants of both TA 98 and TA 100 tester strains was observed at all aniline concentrations, but only in the presence of S-9 mix.

Because of a lack of evidence for their metabolic transformation in pond water, no Ames tests were performed after various incubation times on the five substituted anilines.

The pathways and mechanisms of unsubstituted aniline removal from pond water were described earlier (Lyons et al., 1984). Recent work by Aoki et al. (1983) indicates that aniline-degrading bacteria are widespread even in environments without previous pollution history. This present paper adds some kinetic data on aniline biodegradation in pond water and confirms that biological removal of aniline can occur over a relatively wide concentration range. The high persistence of the substituted anilines in pond water, as compared to unsubstituted aniline, was unexpected since the same compounds were extensively transformed during silage fermentation (Lyons et al., 1985) and the use of 4-CA as a sole carbon and nitrogen source was previously reported (Briggs and Walker, 1973). Other reports indicate that 3-CA, 4-CA and 3,4-DCA can be extensively metabolized by Pseudomonas strains when

Table 3. Metabolic conversion of aniline to biodegradation intermediates with mutagenic potential in pond water with sewage sludge inoculum (upper half of the Table) and autoxidation of aniline to products with mutagenic potential in sterile controls (lower half of the Table) as measured by the Ames test using S. typhimurium TA 98 and TA 100 test strains.[1]

		Incubation time (days)							
	Metabolites	0		2		4		7	
Strain	(µg/plate)[2]	(-)S-9	(+)	(-)S-9	(+)	(-)S-9	(+)	(-)S-9	(+)
TA 98	0.00	53	26	56	66	34	47	56	78
	0.25	41	84	56	52	99	176	168	325
	2.50	62	100	60	49	200	268	378	284
	25.00	48	104	130	106	449	437	1810	582
	50.00	56	96	140	148	433	397	1792	756
TA 100	0.00	106	117	116	136	142	151	109	170
	0.25	93	161	116	132	190	284	2267	438
	2.50	113	158	127	124	301	407	630	488
	25.00	106	167	176	170	324	369	1254	887
	50.00	149	205	177	207	500	468	1389	972
TA 98	0.00	50	53	28	51	72	25	25	25
	0.25	26	27	34	25	51	16	299	26
	2.50	18	20	31	24	46	22	253	52
	25.00	26	19	36	36	46	23	331	29
	50.00	30	21	37	37	50	28	440	15
TA 100	0.00	125	106	136	147	136	145	145	131
	0.25	117	106	118	137	147	177	341	196
	2.50	110	112	112	136	167	138	409	195
	25.00	106	109	124	118	177	170	374	181
	50.00	118	119	131	159	177	173	600	188

[1]Values indicate numbers of revertants per plate (average of triplicates). The spontaneous reversion frequencies of TA 98 and TA 100, without or with 20 µL of S-9 mix, are shown on the lines at 0.00 amount of aniline addition.

[2]Based on the initial aniline concentrations added to pond water.

aniline is present as the principal growth substrate (Reber et al., 1979; You and Bartha, 1982a). The latter condition did not exist in these studies and microorganisms capable of utilizing the substituted anilines as sole substrates were apparently not present in either the pond water or the domestic sewage sludge. Acclimation of an aquatic environment to aniline wastes may alter this situation. Also, in some industrial effluents, substituted aniline metabolism may be facilitated by the presence of unsubstituted aniline in the same effluent. Such stimulation was previously observed in culture medium (Reber et al., 1979; You and Bartha, 1982a) and in soil (You and Bartha, 1982b).

The relatively high mutagenic potential of 2,6-DEA, as compared to the rest of the anilines tested, is probably related to the respective electron densities on the amino groups. Both halogens and nitro groups have an electron withdrawing effect, while the ethyl groups, especially in the 2,6-positions act as donors and increase the electron density on the amino nitrogen. This electron density was found to correlate with the mutagenic-carcinogenic potential of aromatic amines (Weinberg and Sahini, 1981). Both 3-CA and 4-CA were previously found to be non-mutagenic in the mouse testicular DNA synthesis test (Seiler, 1978; 1979); aniline was found to be non-mutagenic in the Ames test (Tanaka et al., 1980). However, in analogy to our aniline results, the mammalian urinary metabolites of aniline were found to be mutagenic by Tanaka et al. (1980).

The main pathway of aniline biodegradation in pond water involves a dioxygenase attack resulting in catechol. Catechol is degraded further by ortho-cleavage with ultimate oxidation to CO_2 (Lyons et al., 1984). A side reaction involves a reversible acylation to acetanilide. None of the intermediates in the above pathways are suspected mutagens. Another side reaction, that appeared to be associated with sewage sludge bacteria and was not detected in pond water alone, involved N-oxidation to phenylhydroxylamine. Phenylhydroxylamine or its further oxidation and condensation products such as nitrosobenzene and azo products are postulated as the probable causative agents of the observed mutagenic potential associated with aniline biodegradation products in sewage sludge-inoculated pond water. Autoxidation in sterile pond water may have generated similar products but much more slowly and in lower concentrations.

REFERENCES

Aoki K, Ohtsuka K, Shinke R, Nishira H (1983) Isolation of aniline assimilating bacteria and physiological characterization of aniline biodegradation in Rhodococcus erythropolis AN-13. Agric Biol Chem 47:2569-2575

Bartha R (1980) Pesticide residues in humus. ASM News 46:356-360

Briggs GC, Walker N (1973) Microbial metabolism of 4-chloroaniline. Soil Biol Biochem 5:695-697

Cripps RE, Roberts TR (1978) Microbial degradation of herbicides. In: Hill JR, Wright SJL (eds) Pesticide microbiology. Academic Press, New York, p 670

Lyons CD, Katz SE, Bartha R (1984) Mechanisms and pathways of aniline elimination from aquatic environments. Appl Environ Microbiol 48:491-496

Lyons CD, Katz SE, Bartha R (1985) Fate of herbicide-derived anilines during ensilage. Bull Environ Contam Toxicol this issue

Maron DM, Ames BN (1983) Revised methods for the Salmonella mutagenicity test. Mutat Res 113:173-215

Reber H, Helm V, Karanth NGK (1979) Comparative studies of the metabolism of aniline and chloroanilines by Pseudomonas multivorans strain An 1. Eur J Appl Microbiol Biotechnol 7:181-189

Seiler JP (1978) Herbicidal phenylalkylureas as possible mutagens. I. Mutagenicity tests with some urea herbicides. Mutat Res 58: 353-359

Seiler JP (1979) Herbicidal phenylalkylureas as possible mutagens. II. Chemical basis of mutagenic activity. Pest Biochem Physiol 12:183-190
Tanaka KI, Marui S, Mu T (1980) Mutagenicity of extracts of urine from rats treated with aromatic amines. Mutat Res 79:173-176
Weinberg JH, Sahini VE (1981) Xylidine isomers. VII. Structure hepatotoxic activity relationships of some xylidines. J Theor Biol 88:153-159
You IS, Bartha R (1982a) Metabolism of 3,4-dichloroaniline by Pseudomonas putida. J Agric Food Chem 30:274-277
You IS, Bartha R (1982b) Stimulation of 3,4-dichloroaniline mineralization by aniline. Appl Environ Microbiol 44:678-681

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