The Use of Aniline as an Indicator of Persistence in Environmental Studies

Richard M. Kline and Christopher A. Schmalz
Lilly Research Laboratories
Greenfield, Indiana 46140

Introduction

Biodegradability is suggested by many regulatory agencies as one of the tests to evaluate environmental persistence. The Environmental Protection Agency (EPA) lists several methods in their Chemical Fate Guidelines (3) and similar tests are recommended by the Organization for Economic Cooperation Development (OECD) in their OECD Guidelines for Testing of Chemicals (5).

Aniline is one of the control compounds proposed as a standard for biodegradability in sewage, water, and soil systems. It was suggested because of its aromatic structure which is common to several pesticides.

According to the EPA Guidelines (3), the positive controls must show a removal of 70% of the dissolved oxidizable carbon in the test system within 28 days or 60% of the theoretical carbon dioxide must be produced. A 14-day acclimation period is recommended for these tests. Numerous other investigators also used acclimated inocula in their experiments. Most of these investigators used pure cultures and were interested in metabolic pathways of aromatic compound degradation. Baumgarten, et. al. (2) studied a strain of *Alcaligenes faecalis* which used aniline as the sole source of carbon and nitrogen. A *Moraxella* sp., also capable of mineralizing aniline using it both as a source of carbon and nitrogen, was studied by Zeyer et. al. (8).

Experiments reported here were designed to determine the feasibility of using non-acclimated sewage organisms as an indicator of relative persistence within a group of experimental pesticides.

Materials and Methods

Sewage inocula were obtained from an aerated lagoon of an industrial sewage disposal plant. The plant was constructed to specifications of a municipal waste treatment facility and handles an average of 500,000 to 600,000 gallons of sanitary waste per day. A quantity of sewage was removed, allowed to settle, and filtered through glass wool to obtain a clear, amber liquid. The filtrate was sparged overnight with laboratory air, then fortified with 10 ml/L of synthetic sewage (7). The composition of the synthetic sewage solution is shown in Table 1. Twenty ml quantities of the fortified sewage filtrate were then distributed into 50 ml erlenmeyer flasks.

Table 1. Synthetic Sewage

Ingredient	Quantity (g)
Glucose	13.0
Nutrient broth	13.0
Beef extract	13.0
Dipotassium hydrogen phosphate	13.0
Ammonium sulfate	2.5
Tap water	1000 ml

An aniline (Aldrich Chemical Co., Inc., 99% pure) solution was prepared in acetone at a concentration of 1 mg/ml and added to the flasks prior to adding sewage. The acetone was allowed to evaporate and the aniline remained as a film on the glass surface.

One-half of the flasks were aerated on a gyratory shaker at 250 rpm and the remaining flasks were allowed to stand in the laboratory undisturbed. Incubation was accomplished at ambient temperature, approximately 25 °C.

At each sampling interval, representative flasks were removed and 20 ml high-performance liquid chromatographic (HPLC) grade methanol was added to inhibit growth. Flasks were then stored in the freezer, −5 °C, until the experiment was terminated. Before analyses were performed, the contents of each flask and rinses were transferred to 50 ml volumetric flasks. Final volumes were achieved with a methanol water solution (1:1).

At the time of assay, a 5 ml aliquot was removed with a syringe and the contents filtered through a 0.45 micron Acrodisc (Gelman sciences, Ann Arbor, MI). Analyses were performed on an HPLC system composed of a Waters Associates chromatographic pump and a Waters μBondapak C-18 column, 250 × 4.5 mm ID connected to a Model 450 variable wavelength detector set at 235 mm. Volumes of 50 μL were injected and peak heights were measured on chromatograms produced on a Houston Instrument Omniscribe Recorder.

Dissolved oxygen utilization experiments were performed in standard 60 ml biochemical oxygen demand (BOD) bottles. Air saturated dilution water was prepared according to Standard Methods for the Examination of Water and Wastewater (4). Aniline and inoculum were added to bottles as previously described and dilution water was siphoned to volume.

Positive controls containing glucose and glutamic acid, 3 ppm each, were tested with and without aniline to demonstrate toxicity to waste treatment organisms. Inoculum controls, dilution water controls, and aniline controls were also analyzed with each test.

Dissolved oxygen concentrations were determined with a Wheaton 60 Second BOD System. To eliminate contamination, it was necessary to prepare several replicates of each treatment and make only one reading per bottle.

Results and Discussion

Aniline, sodium citrate, dextrose, phthalic acid, and trimellitic acid are proposed as reference compounds for environmental experiments by the Office of Toxic Substances (3). The object is to check the activity of the inoculum to determine unrelated toxicity in test systems. Aniline was selected in these tests because the aromatic structure is basic to several pesticides. It was anticipated that the compound may also be used to predict relative persistence of experimental pesticides under field conditions.

In four different experiments run during the spring season, inocula were taken from the aerated lagoon of the sewage disposal system and fortified with aniline at 10 ppm. Between 5 and 8 days were required to show complete loss of the compound. These data appear in Table 2. Both the mechanically aerated flasks and the non-agitated systems produced similar results, indicating that air was not limiting in the non-agitated flasks

TABLE 2. Effect of Inoculum on Aniline Degradation

Experiment	Degradation[1] Time (Days)	
	Aerated	Non-Aerated
I	—	6
II	7	7[2]
III	8	8[3]
IV	7	7

[1] Aniline concentration below the .156 ppm limit of detection

[2] Did not run 6-day samples

[3] Did not run 7-day samples

and excessive aeration was not inhibitory to sewage organisms. Differences in experiments were anticipated because Jones and Alexander (1985) reported that kinetics were altered by uncharacterized organic substrates, but perhaps the rapid decline observed in these tests made the tests insensitive to the expected environmental changes. When the time intervals were reduced to daily samples, the gradual decrease in aniline content can be seen. As shown in Table 3, the data confirm previous experiments that both methods

TABLE 3. Effect of Aeration on Aniline Degradation by Sewage Microorganisms

	Aniline Concentration (ppm)			
Time (days)	Aerated	S.D.	Non-Aerated	S.D.
0	8.3	1.0	8.9	1.0
3	7.3	0.1	8.0	0.4
4	5.9	1.7	8.0	0.4
5	2.8	3.0	7.0	0.8
6	0.1	0.2	2.8	2.8
7	0	—	0	—

produced similar results. Large standard deviations at the lower values were the result of one or two replicates with no detectable aniline.

In two experiments, aniline degradation was followed in a restricted oxygen environment. Aniline concentrations ranged from 1.25 to 10 μg per ml in the two studies, which extended for one month. As previously noted, the mineral salts medium was saturated with air to a concentration approximating 9.0 ppm and measured at several intervals during the test duration.

Each of the aniline-treated systems utilized dissolved oxygen at a rate dependent upon initial chemical concentration. Anson and MacKinnon (1) also found that adapted microorganisms degraded aniline under aerations at a rate dependent upon initial concentration. The two experiments were run with different inoculum concentrations, but the higher the aniline concentration, the more oxygen was utilized. These data appear in Tables 4 and 5. When aniline was analyzed in the high level experiment, it was noted

TABLE 4. Effect of Aniline on Dissolved Oxygen Utilization

	Dissolved Oxygen Concentration (ppm)				
Identity	Day 0	Day 5	Day 15	Day 20	Day 30
Inoculum Control[1]	9.1	7.8	7.5	5.0	6.3
Aniline (10 μg/ml)	9.0	7.1	0.3	0.2	0.4
Aniline (5 μg/ml)	9.1	7.5	1.2	0.3	0.4
Aniline (10 μg/ml) Cont.	9.2	7.7	0.6	0.4	0.4

[1] 1.0 ml inoculum in 30 ml medium

TABLE 5. Effect of Aniline on Dissolved Oxygen Utilization

	Dissolved Oxygen Concentration (ppm)				
Identity	Day 0	Day 5	Day 15	Day 20	Day 29
Inoculum Control[1]	9.2	8.1	6.7	8.2	5.9
Aniline (1.25 μg/ml)	9.4	7.2	5.3	6.7	5.1
Aniline (2.5 μg/ml)	9.3	5.4	4.3	4.4	4.3
Aniline (2.5 μg/ml) Cont.	9.3	7.2	4.9	5.9	5.5

[1] 0.5 ml inoculum in 30 ml medium

that the low concentrations, 5 μg/ml was depleted in 15-20 days (Table 6). However, there were very small quantities of dissolved oxygen remaining after 30 days. This would

TABLE 6. Aniline Degradation Under Limited Oxygen

Identity	Aniline Concentration (ppm)				
	Day 0	Day 5	Day 15	Day 20	Day 30
Inoculum Control	0	0	0	0	0
Aniline (10 μg/ml)	10.8	9.8	3.8	2.2	3.6
Aniline (5 μg/ml)	5.6	4.1	0.4	0	0
Aniline (10 μg/ml) Cont.	11	10.1	2.7	2.4	3.3

suggest that in this study, there was insufficient oxygen to completely degrade 10 ppm aniline in the closed container.

Graphic representation of the effect of concentration of aniline on oxygen consumption is shown in Figure 1. As the concentration of aniline was increased, more dissolved

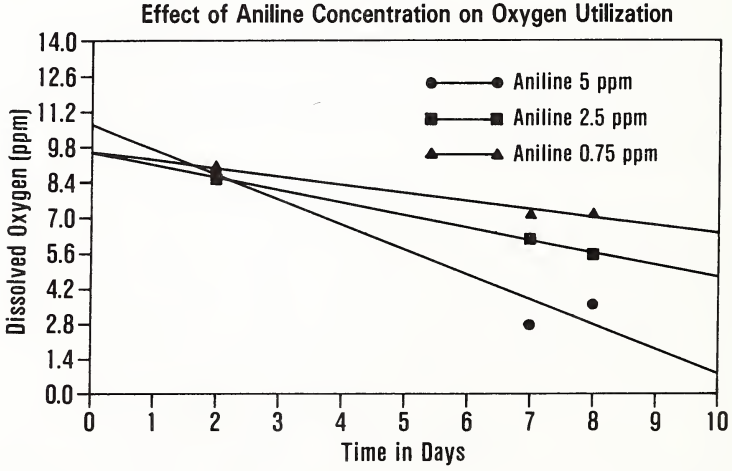

FIGURE 1.

oxygen was utilized. Only 36% of the original oxygen content was present after 8 days when the medium contained 5 ppm aniline. At 0.75 ppm aniline, 72% of the dissolved oxygen was present at the same time interval.

It was also noted in these experiments that the aniline controls, i.e. uninoculated systems, were also showing a loss of oxygen and aniline indicating chemical or physical oxidation, contaminated compound or other system components. To eliminate the possibility that the degradation of aniline was not biological, an experiment was run with sterile controls and aniline content determined at weekly intervals. As shown in Table 7, only one-third of the compound was lost in the sterile flask during a 25-day incubation period. The non-sterile aniline solution showed complete depletion of aniline within one week. To confirm the sterility, a sample of the sterile medium was subcultured into brain heart infusion broth with no apparent growth appearing through 23 days. These

data would indicate that the loss of aniline was predominantly associated with biological systems.

TABLE 7. Stability of Aniline Following Sterilization

Identity	Aniline Concentration (μg/ml)				
	Day 0	Day 6	Day 9	Day 16	Day 25
Sterile Cont.	0	0	0	0	0
Sterile Aniline[1,2]	9.0	8.4	6.2	7.6	6.1
Non-Sterile Cont.	0	0	0	0	0
Non-Sterile Aniline[1]	11.0	0	0	0	0

[1] Fortified @ 10 μg/ml

[2] Sterility test - BHI - No apparent growth after 23 days

The addition of glucose-glutamic acid to the sewage increased the rate of oxygen utilization, but the addition of the relatively small quantity of aniline did not alter the rate as shown in Figure 2. Aniline analyses indicated 79% of the original concentration

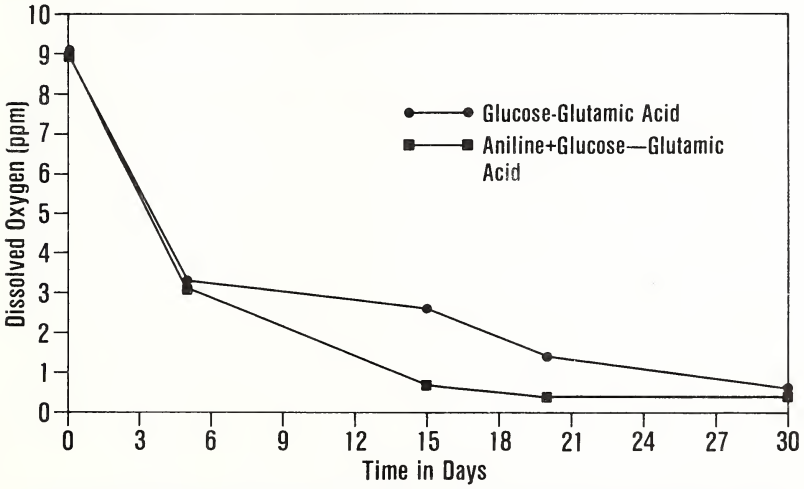

FIGURE 2.

was present after 30 days. Thus, the oxygen consumption was almost entirely attributed to the more easily utilizable glucose-glutamic acid substrate.

Conclusion

Aniline was shown to rapidly degrade in sewage without an acclimation period. The test compound was added at concentrations equal to or less than 10 ppm which would approximate the concentration of pesticides used on agronomic cropland. The compound as suggested by the OECD (5) and the EPA (3) would appear to be a good indicator of environmental degradation in soil. The rapid degradation of aniline in our test systems prevents its use as a comparative reference to measure the relative persistence of slowly degrading pesticides.

Acknowledgments

The authors wish to thank Mr. J. Peloso and Ms. Theresa Wagner for their assistance in preparation of this manuscript.

Literature Cited

1. Anson, J.G. and G. MacKinnon. 1984. Novel *Pseudomonas* plasmid involved in aniline degradation. *App. Environ. Microbiol.* 48:868-869.
2. Baumgarten, J., T. Mann, and F. Schmidt. 1983. Microorganism cultures decomposing polysubstituted aromatic hydrocarbons and their use in biological sewage plants. Fed. Rep. Ger. Patent 322588J. C.A. 100(14) 108668W.
3. Environmental Protection Agency. 1982. Chemical fate guidelines. Office of Pesticides and Toxic Substances. U.S.E.P.A. Washington, D.C.
4. Greenberg, A.E., R.R. Trussell and L.S. Clesceri. 1985. Standard methods for the examination of water and wastewater. Amer. Public Health Assoc. Washington, D.C.
5. Jones, S.H. and M. Alexander. 1985. Kinetics of phenol and p-nitrophenol mineralization at low concentrations in lake water. Abstracts of the annual meeting of the American Society for Microbiology.
6. Organization for Economic Cooperation and Development. 1982. Guidelines for testing chemicals. O.E.C.D. Publ. Office. Paris.
7. Soap and Detergent Association. 1966. Test procedures and standards. Scientific and technical report No. 3.
8. Zeyer, J., A. Wasserfallen and K.N. Timmin. 1985. Microbial mineralization of ring substituted anilines through an ortho-cleavage pathway. Appl. Envirion. Microbiol. 50:447-453.