Bull. Environ. Contam. Toxicol. (1993) 50:856–863

Environmental Contamination and Toxicology

Microbial Degradative Activity in Ground Water at a Chemical Waste Disposal Site

Huey-Min Hwang,[1] Robert E. Hodson,[2] David L. Lewis,[3] and Richard Scholze[4]

[1]Department of Biology, Jackson State University, Jackson, Mississippi 39217, USA; [2]Institute of Ecology, Department of Marine Sciences, and Department of Microbiology, University of Georgia, Athens, Georgia 30602, USA; [3]Environmental Research Laboratory, Environmental Protection Agency, Athens, Georgia 30613, USA; [4]U.S. Army Construction Engineering Research Laboratory, Champaign, Illinois 61824-4005, USA

Ground water supplies the drinking water for more than 50% of the total U.S. population and nourishes many natural ecosystems valued for providing food resources, wildlife habitat, and recreation opportunities (The Conservation Foundation 1987). Most ground water supplies in the United States are of good quality. Increasing discoveries of ground water contamination resulting from the use, transportation, storage, and disposal of pesticides and hazardous chemicals, however, underline the need for studies of the fate and effects of these chemical pollutants in the subsurface.

Among the principal pollutant transformation processes, microbial degradation may be the only one force that can completely remove chemical pollutants from subsurface waters. Although not as thoroughly studied as in surface waters, the biodegradation of organic pollutants by indigenous subsurface bacteria is assumed to be affected by physical, chemical and biological factors such as temperature, concentration of inorganic nutrients, hydrogen ion activity, levels of dissolved oxygen or other terminal electron acceptors, and the presence of other toxic substances. Among these factors, concentrations of inorganic nutrients and potential electron acceptors present the potential to be manipulated to formulate bioremediation techniques. Length of adaptation lag periods prior to microbial transformation of organic chemicals at low concentrations was correlated with limiting nutrient (N or P) concentrations for samples from surface (Lewis et al. 1986) and subsurface water (Swindoll et al. 1988), respectively. Transformations of certain halogenated compounds are known to occur in anoxic environments only in the presence of suitable electron acceptors (Bouwer and McCarty 1983).

This study was designed to examine the microbiological fate and effects of toxic organic chemicals at the ambient concentration in leachates derived from a waste disposal landfill site. Analyses revealed that ground water downslope from the burial site contained high levels of certain dissolved hazardous chemicals such as toluene, xylene, benzene, and methylene chloride. To study the fate of these compounds in such systems, the use of simplified laboratory studies of the biodegradation of individual compounds is often inappropriate in that complex interactions between and among the various chemicals can result in either enhancement or inhibition of the biodegradation of particular compounds. Moreover, the kinetics of the persistence of a compound based on degradation studies conducted over a narrow concentration range can be misleading and result

---

Send reprint request to Huey-Min Hwang at the above address.

in large errors if extrapolated to higher or lower concentrations (Hwang et al. 1989). In this study, we used two compounds, p-cresol and toluene, as model pollutants and investigated the kinetics of their microbiological dissimilation over a wide range of substrate concentrations. In addition, the effects of inorganic nutrients, presence of other chemical pollutants, and alternative electron acceptors (sulfate and nitrate) on microbial degradative activity were assessed.

MATERIALS AND METHODS

The study site was a landfill in Northeast Georgia which was previously described in Armstrong et al. (1991). According to chemical analyses conducted in November 1986, the dominant chemical species and their maximum concentrations in the ground water of the most contaminated sites were: manganese (43 mg/L), iron (200 mg/L), naphthalene (150 µg/L), toluene (6.9 mg/L), trichloroethylene (490 µg/L), xylene (2.3 mg/L), methylene chloride (28 mg/L), 1,1,2,2-tetrachloroethane (1.7 mg/L), formaldehyde (41 mg/L), benzene (15 mg/L), and chloroform (40 mg/L).

Sampling procedures followed those described in Armstrong et al. (1991). Samples of ground water were obtained from a control well (upslope) and experimental wells (downslope) from the landfill, using 3-ft-long (1 L) ethanol-sterilized teflon bailers. Standing water was removed from the wells by collecting and discarding three-bailer-volumes of water and sampling with a different bailer after the well had refilled from surrounding ground water. Test wells were drilled according to EPA standards for research and monitoring wells (Barcelona et al. 1985). Ground water samples were transferred to argon-filled, 2.5-L, acid-washed glass bottles as aseptically as possible to prevent contamination. The bottles were completely filled according to the zero-headspace method to minimize degassing of volatiles. Temperature and dissolved oxygen levels were measured in the wells using portable monitors (Models 33 and 57; Yellow Springs Instrument Co., Yellow Springs, Ohio).

(U-^{14}C)-labeled p-cresol (10.33 mCi/mmol) and toluene (56.3 mCi/mmol) were obtained from Sigma Chemical Co. (St. Louis, Missouri) and D-[U-^{14}C]glucose (257.7 mCi/mmol) was obtained from DuPont NEN Research Products (Wilmington, Delaware). Unlabeled p-cresol and toluene were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). ^{14}C-labeled p-cresol and toluene (dissolved in acetone) were mixed with varying concentrations of the unlabeled form of the chemical and added over a wide range of concentrations (acetone volumes ranged from 2 µL to 50 µL) to 50 mL samples of water in 160-mL Pyrex bottles. To adjust the concentrations of dissolved oxygen, head space of the bottles was filled with argon for water samples from the impacted well to simulate the in situ dissolved oxygen concentrations. Triplicate samples were incubated in the dark at 24±1°C with gentle shaking (100 rpm). Killed controls contained formaldehyde (final concentration of 1.9%). Except for time course experiments, samples for toluene degradation experiments were incubated for various periods of up to 24 hr.

During the incubation periods, bacterial numbers were monitored and were found to be constant. Final substrate concentrations for degradation kinetics measurements ranged from 10 to 300 µg/L. The $^{14}CO_2$ produced was collected with the two-trap method of Hodson et al. (1977; Hwang et al. 1989), and radioactivity was measured with a liquid scintillation counter (Beckman, LS 9000;

Fullerton, California). Quench corrections were made using the sample channels ratio method. Rates of toluene utilization were measured by [^{14}C]- toluene uptake and mineralization. For uptake experiment, 40 µg/L [^{14}C]toluene was added to 50-ml ground water samples and formalin-killed controls in 160-mL Pyrex bottles and incubated for various times up to 60 hr. After incubation, water samples were filtered through 0.22-µm pore-size filters (Millipore, Bedford, Massachusettes). Filters then were washed three times with 10-ml aliquots consisting of distilled water (pH 6.5):ethanol (80:20) and unlabeled toluene added to a concentration of 6 mg/L. Filters then were placed in 10-mL Scintiverse I counting cocktail and radioassayed.

Bacterial numbers in the ground water samples were determined by direct microscopic counting with epifluorescence microscopy of acridine orange-stained specimens (Hobbie et al. 1977). Relative rates of bacterial heterotrophic activity were determined by measuring [^{14}C]-D-glucose uptake. [^{14}C]glucose (less than 5 nM) was added to the 50-mL water samples and formalin-killed controls in 160-mL Pyrex bottles and incubated for various periods of up to 24 hr at 24°C. After incubation, ground water samples were filtered through 0.22-µm pore-size filters (GS type, Millipore). Filters then were washed with prefiltered ground water from the control site, dissolved in 1 mL of ethyl acetate and radioassayed. Glucose mineralization rates were measured by collection of evolved $^{14}CO_2$ (Hwang et al. 1989).

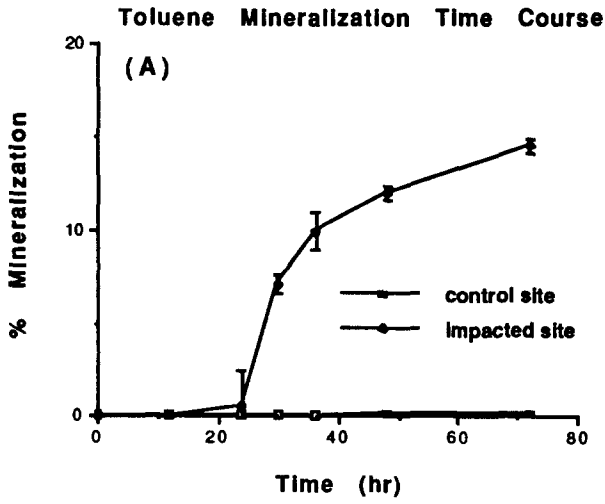
To assess the effect of impacted ground water, bacteria from ground water of the control site were collected on 0.22-µm-pore-size Nuclepore filters (Solution Consultants, Atlanta, Georgia) and resuspended in filter-sterilized control-well water or unamended impacted-well water. Water from the impacted well was not filtered to preclude possible artificial removal of pollutants by adsorption to filters. UL-^{14}C-p-cresol was added to the water samples at a final concentration of 10 µg/L and incubated at 24±1°C for 24 hr. Microbial mineralization rates were measured and expressed as µg/L hr^{-1}.

Varying amounts of chemical pollutants identified in water from the impacted wells (such as methylene chloride and toluene), of inorganic nutrients such as NH_4NO_3 and K_2HPO_4, and of potential electron acceptors for anaerobic respiration (such as sulfate and nitrate) were added to water from either the control well or the impacted wells to assess their effects on microbial mineralization of p-cresol (10 µg/L).

RESULTS AND DISCUSSION

Biodegradation of a specific pollutant by indigenous subsurface microorganisms can be affected by exposure to chemical pollutants, depending on their concentrations and duration of exposure. The effects of ground water from the impacted site containing its complex mixture of pollutants on microbial degradation of a model organic chemical pollutant (i.e., toluene) were determined (Figure 1).

Microbial degradation rates of p-cresol in control-well and impacted-well water at 24°C were 0.1 and 0.01 µg/L hr^{-1}, respectively. Degradation rates supported by the microbial populations in the control well decreased significantly, however, if the populations from the control well were collected and resuspended in unaltered



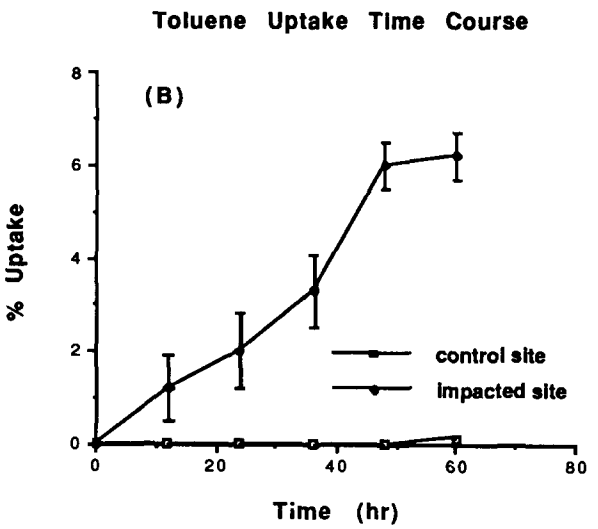


Figure 1. Microbial utilization of [U-^{14}C]toluene in ground water samples collected from the control well and the impacted well. (A) Mineralization of toluene (20 µg/L) in ground water samples collected in October. (B) Uptake of toluene (40 µg/L) in ground water samples collected in November.

water from the impacted well. Those samples contained the natural populations from the impacted well plus populations from the control well. Controls were run to rule out effects of filtration and resuspension of control-well bacteria. Assuming microbial degradative rates are additive, the observed total degradation rate (i.e., 0.03 µg/Lhr^{-1}) by mixed microbial populations of the control and impacted sites indicated that microbial degradative activity of the control site was inhibited by 73% by exposure to ground water of the impacted site. Moreover, microbial utilization of glucose was 2-to 5-fold slower in the impacted

well than that in the control well (Armstrong et al. 1991).

Data from preliminary experiments revealed that seven of the seventeen chemical species in the contaminated ground water exhibited significant effects on microbial mineralization of p-cresol. Therefore, these seven chemicals were chosen for further studies. At the concentration reported for the impacted well, each of the chemical species inhibited microbial degradation of p-cresol, from 20% to 100% (Table 1). Among the candidate chemicals, toluene, xylene, methylene chloride, and tetrachloroethane were the most inhibitory, blocking microbial degradation of p-cresol by virtually 100% (Table 1).

Table 1. Effects of the chemical pollutants of the landfill on microbial metabolism of ^{14}C- p-cresol and D-glucose.

water samples\pollutants*	a	b	c	d	e	f	g
1. p-cresol mineralization							
A.unimpacted site							
(May 1988)	57%	56%	0%	0%	0.4%	40%	0%
(June 1988)	46%	22%	n.d.	n.d.	2%	24%	n.d.
(November 1988)	7%	80%	0.3%	0.2%	0.5%	80%	0%
B. impacted site							
(November 1988)**	6%	105%	0.7%	0.4%	0.8%	164%	0.4%
2. D-glucose metabolism							
glucose uptake							
A. unimpacted site							
(May 1988)	50%	83%	1%	0.5%	0.4%	82%	2%
(June 1988)	82%	96%	n.d.	n.d.	5%	95%	n.d.
B. impacted site							
(November 1988)**	0%	86%	1%	0%	0%	67%	0%
glucose mineralization							
(November 1988)**	0.9%	96%	5%	4%	3%	94%	2%

*Symbols and concentrations of the chemical pollutants are as follows: a. Fe^{3+} (200 mg/L), b. Mn^{2+} (43 mg/L), c. toluene (6.9 mg/L), d. methylene chloride (28 mg/L), e. xylene (1.1 mg/L), f. trichloroethylene (0.19 mg/L), g. tetrachloroethane (1.7 mg/L). n.d. not determined. All numbers reported represent the relative values (%) of the exposure groups to the control (i.e., adding no pollutants).

** Bacteria of the impacted site were collected on 0.22-μm-pore-size Nuclepore filter and resuspended in filter-sterilized unimpacted ground water before they were exposed to the chemical pollutants and incubated under argon. Bacterial numbers of the exposure groups as % of the control were as follows: a. 43%, b. 67%, c. 49%, d. 55%, e. 41%, f. 81%, g. 42%.

The magnitude of inhibition by a given chemical varied with sampling date, probably reflecting temporal changes in microbial populations in the subsurface and dilution of pollutants by changes in ground water flow. For example, microbial degradation of p-cresol in the control well was inhibited by Fe^{3+} by 43% in May. The magnitude of the inhibition, however, drastically increased to 93% in November (Table 1). Likewise, rates of microbial heterotrophic uptake of glucose as well as bacterial numbers (not shown) generally decreased in the presence of toluene, xylene, and tetrachloroethane, suggesting that the observed

inhibition by these three compounds was due to general toxicity to the bacterial populations. Nevertheless, for the remaining four test chemicals, the magnitude of the inhibition of heterotrophic activity did not correlate with that of inhibition of p-cresol degradation. Interestingly, p-cresol degradation at the control site was inhibited by exposure to trichloroethylene, whereas the degradation rates at the impacted site were actually enhanced by 64% (Table 1). Because general microbial activity parameters such as the total bacterial numbers and heterotrophic uptake of glucose did not increase after exposure to trichloroethylene, the enhancement in p-cresol degradation can be assumed to be the result of a specific enhancement in degradation, possibly the induction of degradative enzyme(s) or a change in the community that allowed the number of degraders to increase. Trichloroethylene or p-cresol degrader organisms were not determined in this study; however, a relevant study of the same site indicated that toluene degraders in the impacted site averaged $2.2x10^2$ cells/mL, whereas no toluene degraders were detected in samples from the control site (Armstrong et al. 1991).

Inorganic nutrients and alternative electron acceptors were recently reported to influence microbial degradation of organic pollutants (Lewis et al. 1986; Bouwer et al. 1983). In June 1990, nitrogen and phosphorus (0.14 mg/L PO_4^{-3}-P and 11.5 mg/L NO_3^--N) were added to ground water from both the control and the impacted site to assess possible effects. No significant effect was observed, however, even when the concentrations of N and P were doubled (data not shown). Incubations in April 1991 for up to 4 d with the additions of ammonium nitrate, ammonium chloride, potassium nitrate and potassium chloride (100 mg/L each) to the impacted ground water also failed to stimulate microbial degradation of p-cresol. Therefore, we assume that microbial degradative activities at the landfill were not limited by depletion of these inorganic nutrients in short-term incubations.

To assess the potential effects of alternative electron acceptors on anaerobic microbial degradation of the test compound, concentrations of sulfate and nitrate were increased. Previous investigations had revealed that anaerobic metabolism of aromatic compounds occurs by sulfate reduction and/or denitrification in subsurface waters (Bouwer and McCarty 1983; Evans and Fuchs 1988). Results of sulfate ammendment on p-cresol degradation in this study were inconsistent. When sodium sulfate was added at concentrations ranging from 1 mg/L to 100 mg/L in August, microbial mineralization of p-cresol was enhanced by 100 to 300%. Addition of molybdate (a competitive inhibitor of sulfate reduction [Evans and Fuchs 1988]) at concentrations ranging from 1 to 100 mg/L decreased microbial mineralization of p-cresol by 18 to 64%. This inhibition, moreover, was reversible by the addition of sulfate. The results of this experiment, therefore, suggest that the sulfate reducers contributed to the total microbial degradation of p-cresol. The results differed, however, when the experiment was repeated in September. At that time, addition of sodium sulfate at a concentration of 100 mg/L actually inhibited p-cresol degradation by 25%, but the addition of molybdate (200 mg/L) or the addition of a combination of sulfate and molybdate did not affect the degradation rate (data not shown). One possible explanation is that dominant microbial populations shifted temporally between sulfate reducing bacteria and bacteria of other metabolic type(s), with sulfate reducers being dominant in August and other metabolic type(s) in September.

In September when ammonium nitrate was added at concentrations between 10 and 100 mg/L, p-cresol mineralization was enhanced by 20% to 50% while total bacterial numbers remained constant. Enhancement of the degradation rate was

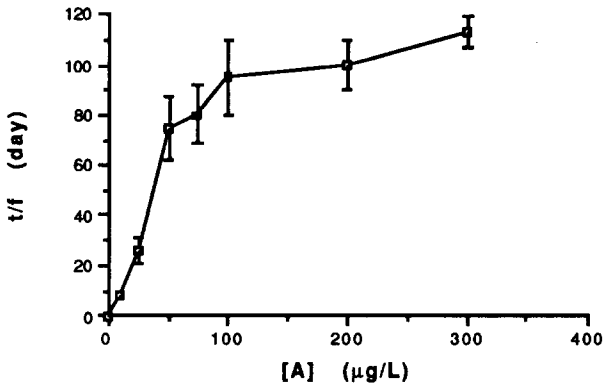


Figure 2. Wright-Hobbie plot for the mineralization of toluene in ground water samples collected in November from the impacted well. Toluene concentrations added, [A], are plotted against t/f (t, incubation time; f, fraction mineralized).

more significant (up to 230%), with the addition of potassium nitrate in the same month (i.e., September). The total bacterial numbers, however, sometimes increased with the addition. Nitrate respiration and concommitant transformation of toxic organic chemicals have been reported under conditions favoring denitrification where the redox condition was between that for aerobic and methanogenic transformation (Bouwer and McCarty 1983). Ground water from the impacted site was not anaerobic (concentration of bulk dissolved oxygen averaged 3 mg/L), denitrification could occur in micro-anaerobic zones within the impacted ground water, however. In addition, not all of denitrification processes require totally anaerobic conditions (personal communication, W. J. Payne, University of Georgia). We assume that nitrate might serve as either a nutrient or an alternative electron acceptor or both for the microbial community of the impacted site. Thus, we can not differentiate from these experiments stimulatory effect of NO_3^- due to its serving as a nutrient from an effect owing to its functioning as an electron acceptor.

Microbial toluene degradation in samples amended with 40 µg/L toluene was negligible in the control well, whereas rates of degradation were more than 20 fold higher in the contaminated well (Figure 1), suggesting adaptation for enhanced degradation of toluene. Indeed, most probable number (MPN) techniques indicated toluene degraders significantly increased in the impacted site due to adaptation processes (Armstrong et al. 1991). In Figure 2, degradation kinetics data have been plotted according to the method of Wright and Hobbie 1965, in which concentration of added substrate [A] is plotted against t/f (incubation time, t, divided by f, the fraction of substrate utilized in time t). With this type of plot, if the data fall along a straight line, degradation is assumed to be mediated by a single saturable system with a single K_t (intercept of line on the x-axis) and V_{max} (inverse of the slope). If the data fall along a curved line with slope decreasing as [A] increases, we may assume that the microbial population degrading the compounds is kinetically diverse with multiple uptake/degradative systems (Azam and Hodson 1981; Hwang et al. 1989). This latter type of kinetic pattern was seen repeatedly for toluene degradation in water from the impacted well (Figure 2). Such kinetic diversity has not been reported previously for ground water microbial populations and has significant implications for modeling pollutant persistence and exposure in risk assessment. Efforts were made during this study to minimize "well effects" such as sample contamination from soil surface and

errors in calculating bacterial biomass and activity, due to sampling standing well water. Our data reflected those bacterial assemblages in the water that flowed into the well after the purging procedures. Bacterial samples in wells do not always represent those of the surrounding formation unless the well is treated before sampling (Thomas et al. 1989). Methods such as exhaustive pumping and treatment of the wells with disinfectants prior to sampling have been proposed to minimize possible artifacts (Thomas et al. 1989). During this study, the concurrent samplings by other investigators (including state and federal agencies) evaluating this land fill site for restoration prevented us from treating the wells prior to sampling.

Acknowledgments- This research was funded in part by US Army Construction Engineering Research Lab., under contract #DACW8891MO321. Additional funds were provided by US Environmental Protection Agency-University of Georgia Cooperative Research Agreement No. CR812638-01-2 and LBL/JSU/AGMUS Science Consortium Program awarded by US Department of Energy.

REFERENCES

Armstrong AQ, Hodson RE, Hwang H.-M., Lewis DL (1991) Environmental factors affecting toluene degradation in ground water at a hazardous waste site. Environ Toxicol Chem 10:147-158

Azam F, Hodson RE (1981) Multiphasic kinetics for D-glucose uptake by assemblages of natural marine bacteria. Mar Ecol Prog Ser 6:213-222

Barcelona, MJ, Gibb JP, Helfrich JA, Garski ED (1985) Practical Guide for Groundwater Sampling, EPA 600/S2-85/104, Robert S. Kerr Environ Laboratory, Ada, Oklahoma

Bouwer EJ, McCarty PL (1983) Transformations of halogenated organic compounds under denitrification conditions. Appl Environ Microbiol 45:1295-1299

Evans WC, Fuchs G (1988) Anaerobic degradation of aromatic compounds. In: Ornston LN, Balows A, Baumann P (eds) Annual Review of Mcrobiology, vol 42. Annual Reviews, Inc, Palo Alto, California, p 289

Hobbie JE, Daley RJ, Jasper S (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225-1228

Hodson RE, Azam F, Lee RF. (1977) Effects of four oils on natural marine bacterial populations: a controlled ecosystem pollution experiment. Bull Mar Sci 27:119-126

Hwang H-M, Hodson RE, Lewis DL (1989) Microbial degradation kinetics of toxic organic chemicals over a wide range of concentartions in natural aquatic systems. Environ Toxicol Chem 8:65-74

Lewis DL, Kollig HP, Hodson RE (1986) Nutrient limitations and adaptation of microbial populations to chemical transformations. Appl Environ Microbiol 51:598-603

Swindoll CM, Aelion CM, Pfaender FK (1988) Influence of inorganic and organic nutrients on aerobic biodegradation and on the adaptation response of subsurface microbial communities. Appl Environ Microbiol 54:212-217

The Conservation Foundation (1987) Groundwater Protection. The Conservation Foundation, Washington, DC

Thomas JM, Lee MD, Scott MJ, Ward CH (1989) Microbial ecology of the subsurface at an abandoned creosote waste site. J Ind Microbiol 4:109-120

Wright RT, Hobbie JE (1965) The uptake of organic solutes in lake water. Limnol Oceanogr 10:22-28

Received June 23, 1992; accepted December 2, 1992.