Bull. Environ. Contam. Toxicol. (1996) 56:343-350

Bulletin of Environmental Contamination and Toxicology

A Study of Microbial Transformations of Trichloroaniline and p-Cresol Using Size-Fractionation Technique

H.-M. Hwang,[1] S. W. Maloney[2]

[1]Department of Biology, Jackson State University, Jackson, Mississippi 39217, USA
[2]U.S. Army Construction Engineering Research Laboratory, Champaign, Illinois 61826-9005, USA

Received: 13 February 1995/Accepted: 30 August 1995

Most aquatic environments are affected to some degree by past and ongoing inputs of contaminants, as the result of human activities such as agricultural practice, chemical control of pest organisms, and chemical use in coastal resorts or residential developments. A number of important agrochemicals are carbamates, ureas, and aromatic amides. Chloroanilines are formed from many of them as the initial microbial transformation products and may be substantially more resistant to further degradation (Neilson 1994). Eventually, many aniline and chlorinated anilines may enter the estuarine environments by a variety of routes (Bartha 1971; Kaufmann and Blake 1973). Cresol is used in disinfectants and fumigants, in the manufacture of synthetic resins, in photographic developers and explosives. Because of its widespread uses, cresol frequently occurs in a variety of aquatic environments and is listed as a priority pollutant by the U.S. EPA. Among the different isomers, p-cresol appears to be the most toxic form (Patnaik 1992). The fate of these xenobiotics is of our interest because of their presence and toxicity to aquatic organisms and their threat to human health (Messner et al. 1979).

In assessing the potential exposure of humans and other organisms to toxic chemical contaminants, it is necessary to first determine the toxicity and persistence of the pollutants in the environment. Microorganisms are important members of most aquatic environments, especially with respect to nutrient cycling, secondary productivity, and biodegradation processes. Therefore, to determine the toxicity of chemical contaminants to aquatic environments, microorganisms may serve as surrogates for the large body of organisms comprising the biotic component of a natural ecosystem. The major routes of degradation of xenobiotic organic contaminants in surface aquatic ecosystems include photodegradation and biodegradation (Francis 1994). Thus for assessment of persistence of chemical contaminants, it is essential that we understand the kinetics of photolysis and biodegradation of the contaminants in a given ecosystem. Photodegradation occurs primarily in the presence of UV light, and biodegradation is primarily conducted by microorganisms (Matsumura 1982; Hwang et al. 1986a). p-Chloroaniline was photolyzed to produce photoproducts such as 4-chloronitrosobenzene and 4-chloronitrobenze (Miille and Crosby 1983). The photoproducts are subject to further photolysis and biodegradation, via dechlorination and oxidative processes with ultimate mineralization to CO_2 (Matsumura 1982). In natural waters, algae may promote photosensitized reactions due to their cellular materials such as chlorophyll. As autotrophs, algae may not utilize xenobiotics as an energy source, whereas heterotrophic bacteria may be the major group of microbial degraders.

Correspondence to: H.-M. Hwang

One of the useful approaches to measuring microbial degradation rates of chemical pollutants is to determine the kinetics of heterotrophic potential for the utilization of the chemicals. One of the basic assumptions for measurement of heterotrophic potential is that all members of microbial consortia respond in the same way to variations in substrate concentration. This assumption may not be true because evidence exists for differential uptake rates by various bacterial populations, and by algal versus bacterial populations (Atlas and Bartha 1993). In this study, separation of microorganisms of different classes were achieved by size-fractionation with polycarbonate membrane filter. The contribution of microorganisms of different sizes to transformation of p-cresol and trichloroaniline photoproducts were determined.

MATERIALS AND METHODS

In 1993 and 1994, surface stream water samples (pH 5.5-6.2) were collected upslope (control site) and downslope (impacted site) of a hazardous landfill site in Northeast Georgia. Specific descriptions were given in Armstrong et al. (1991). Water samples were taken in acid-washed, 10-L polyethylene containers. Assays were initiated within 1 hr of collection. According to chemical analyses conducted in July 1987, the important chemical contaminants and their concentrations in the stream water of the impacted site were: benzene (1 µg/L), chloroform (0.1 mg/L), 1,2-dichloropropane (6 µg/L), ethyl benzene (68 µg/L), iron (200 mg/L), manganese (43 mg/L), methylene chloride (5 µg/L), naphthalene (1.6 µg/L), 2-nitrophenol (0.4 µg/L), 1,1,2,2-tetrachloroethane (7 µg/L), toluene (2 mg/L), trans-1,2-dichloroethylene (70 µg/L), 1,1,1-trichloroethane (0.2 mg/L), trichloroethylene (3 µg/L), and xylene (0.4 mg/L). In addition, surface estuarine water samples were collected in May from Skidaway River, an estuarine river located near Savannah, Georgia. At the time of sampling, the pH was 7.6, temperature was 29°C, and salinity was 23‰. U-^{14}C-p-cresol (10.3 mCi/mmol) and U-^{14}C-2,4,5-trichloroaniline (14.8 mCi/mmol) were obtained from Sigma Chemical Company, St. Louis, Missouri. ^{14}C- sodium bicarbonate (50 mCi/mmol) was obtained from DuPont NEN Research Products, Wilmington, Delaware. ^{3}H-labeled D-glucose (30 Ci/mmol) and [methyl-^{3}H]thymidine (74 Ci/mmol) were obtained from ICN Pharmaceuticals, Irvine, California.

Radiolabeled or unlabeled compounds were dissolved in acetone and added to 60 mL of water in 150-mL quartz flasks (for study of photochemical and microbiological degradation of trichloroaniline) or borosilicate glass bottles (for p-cresol degradation kinetics study). Quartz flasks allowed more than 85% transmission of light of wavelength ≤ 260 nm. Approximately 0.1 µCi of trichloroaniline or p-cresol was added to the flasks and incubated in triplicate under the conditions described in Hwang et al. (1986b) and in Hwang et al. (1989). Formaldehyde (final concentration 0.4%) was added to the water samples as poisoned/sterile control. Ultraviolet absorption by formaldehyde solution at this concentration was found to be negligible. Parent compound disappearance (transformation) and $^{14}CO_2$ appearance (mineralization) were determined for 2,4,5-trichloroaniline degradation study. The $^{14}CO_2$ produced was collected and radioassayed as described in Hwang et al. (1989). To determine disappearance rates of trichloroaniline, the samples containing parent compound were extracted three times with ethyl acetate after the pH was adjusted to 10 with 2N NaOH. Extracts were concentrated by evaporation under nitrogen. Analyses of the extracts were conducted by HPLC using a Waters (Milford, Massachusetts) 960 (UV-VIS

photodiode array detector) system equipped with a 4.6 x 250 mm reverse-phase octyl column of 5 µm ultrasphere (mobile phase: 50:50 acetonitrile:water; flow rate, 1.0 mL/min; detector wavelength, 254 nm). To verify microbial mineralization of the photoproducts of 2,4,5-trichloroaniline, filter-sterilized estuarine water with trichloroaniline (25 µg/L) was exposed to midday May sunlight for 1,2, and 3 hr. Equal amounts of unamended estuarine water or its filtrates (1.0-µm- and 3.0-µm-pored filters) were added to sterilized estuarine water containing photoproducts after exposure. Samples were incubated at 29°C (i.e., ambient temperature) in darkness for 48 hr and $^{14}CO_2$ production was measured.

Measurement of p-cresol mineralization kinetics followed the protocol as described in Hwang et al. (1994). Relative rates of bacterial heterotrophic activity were determined by measuring ^{3}H-glucose (8.3×10^{-4} pM) or ^{3}H-thymidine (5 nM) uptake. A correction factor of 0.5 for ^{3}H-D-glucose respiration to $^{3}H_2O$ was applied for final calculation of turnover rate (Hwang et al. 1986b). Similarly the uptake of $^{14}CO_2$ by algae under sunlight was determined by adding 2.7×10^{-4} µM of ^{14}C-sodium bicarbonate to the estuarine water and incubated in sunlight for 4 hr (Hwang et al. 1986b). Total bacterial numbers were determined by direct microscopic counting with epifluorescence microscopy of acridine orange-stained specimens (Hobbie et al. 1977). Total microbial biomass was determined with measurements of particulate adenosine triphosphate (ATP) (Hodson et al. 1981). Chlorophyll a, used as a measure of phytoplankton biomass, was determined fluorometrically using the method described in Strickland and Parsons (1972).

RESULTS AND DISCUSSION

In May, Skidaway River water samples were incubated with 2,4,5-trichloroaniline (2.5 µg/L) at 29°C under 24 hr of sunlight and 24 hr of darkness. After the incubation, 75.8 and 71.6% of the compound was transformed (i.e., indicated by the loss of parent compound) in poisoned and unamended water samples, respectively (Table 1). Since no degradation was observed in darkness and no significant difference in transformation rate was seen among different water samples (i.e., untreated vs. poisoned), transformation process appeared to be initiated by photolysis. After the initial phototransformation process, the photoproducts were further mineralized to CO_2 by both photochemical and microbial degradative processes, with 3.1 and 8.3% of the compound mineralized in poisoned and untreated water, respectively (Table 1). Thus, 63% of the total mineralization was attributable to microbial degradation. In addition, the amount of trichloroaniline mineralized after 48 hr in water filtered through 3.0- and 1.0-µm filters increased to 9.2 and 10.4%, respectively. The 3.0- and 1.0-µm-pore filters excluded most of the photosynthetic activity (> 90%) and chlorophyll a (> 85%), while the 3.0- and 1.0-µm-pore filtrates retained 67% and 45% of heterotrophic activity, respectively, as indicated by uptake of ^{3}H-glucose (Table 2). The higher trichloroaniline mineralization activity in 1.0-µm- and 3.0-µm- pore estuarine filtrate relative to unfiltered water was presumably due to the lack of inhibition by the secreted organic compounds from algae, removal of algae that bioaccumulate trichloroaniline protecting against photodegradation, adsorption processes affecting bioavailability of transformation products, and algal refixation of $^{14}CO_2$ in unfiltered water (Hwang et al. 1986a). Moreover, our data also indicated that after photolysis trichloroaniline solutions enhanced microbial activities such as

mineralisation of glucose (not shown). Therefore, the direct enhancement of bacterial activity by the photoproducts may also account for increased trichloroaniline degradation activity in estuarine water filtrates.

Table s1. Degradation of 2,4,5-trichloroaniline by different size fractions of Skidaway River water[a].

Sample	Mineralization ($^{14}CO_2$ production)		Transformation[b]	
	Poisoned[c]	Untreated	Poisoned[c]	Untreated
River Water (unamended)	0.49±0.03 (3.1±0.2)	1.36±0.01[d] (8.3±0.1)	14.2±0.6 (75.8±3.2)	13.2±0.7 (71.6±3.8)
River water filtrate				
3.0-µm	0.51±0.01 (3.2±0.1)	1.5±0.02[d] (9.2±0.1)	13.6±0.1 (72.5±0.5)	13.5±0.5 (72.2±2.7)
1.0-µm	0.50±0.04 (3.2±0.3)	1.6±0.01[d] (10.4±0.1)	13.9±0.3 (74.0±1.6)	13.4±0.5 (71.6±2.7)

[a] pH: 7.6; temperature: 29°C. Trichloroaniline was added at a final concentration of 25 µg/L. The samples were incubated for 24 hr in sunlight and 24 hr in darkness in May. No degradation was observed in darkness. Degradation is in 10^3dpm ± standard deviation (n=3). The % of trichloroaniline degraded ± standard deviation (n=3) is given in parentheses.

[b] Refers to loss of parent compound, including mineralization.

[c] River water was treated with formaldehyde (final concentration 0.4%).

[d] Significantly different from poisoned sample (t-test; $p \leq 0.05$).

The indirect evidence for bacterial mineralization of the photoproducts of trichloroaniline is shown in Figure 1. Trichloroaniline was mineralized in unamended water to the extent of 1.6, 5.8, and 10.7% after 1, 2, and 3 hr of exposure to midday May sunlight, respectively. Trichloroaniline was reported to photodegrade via dechlorination and hydroxylation to phenols and catechols (Matsumura 1982; Miller et al. 1980). We did not identify the photoproducts in this study. The additional CO_2 production in un-poisoned water was speculated to derive from microbial mineralization of photoproducts. Microbial mineralization rates of the photoproducts were the same in unamended and 3.0-µm-pore filtered estuarine water. The 3.0-µm-pore filtrate contained primarily bacteria (Table 2); therefore, we assume that heterotrophic bacteria were responsible for the total mineralization activity. The mineralization rate of 2 hr- and 3 hr- photoproducts in 1.0-µm-pore filtrate was significantly lower than in unfiltered samples, presumably this was due to the lower bacteria numbers in the filtrate. In a similar experiment, bacterial consortia from a freshwater reservoir were incubated with 1-hr trichloroaniline photoproducts for 2 d in darkness. Heterotrophic bacteria and total bacteria populations increased from 4.4 x 10^5 to 1.5 x 10^6 c.f.u./mL and from 2.5 x 10^6 to 8.1 x 10^6 /mL, respectively, while the

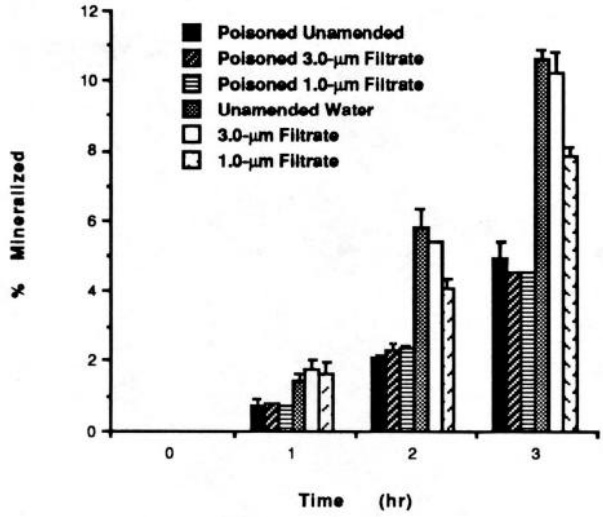

Figure 1. Microbial mineralization of the photoproducts of 2,4,5-trichloroaniline (25 µg / L.

Table 2. Biomass and uptake of ^{3}H-glucose or ^{14}C-bicarbonate by different fractions of Skidaway River water[a].

Samples	Acridine orange direct count (#/L)	ATP (µg/L)	Chlorophyll a (µg/L)	Glucose uptake[b] (%)	Bicarbonate uptake[c] (%)
River water	8.2×10^{9}	1.87	6.71	30.6 (100%)	1.7 (100%)
3.0-µm filtrate	5.2×10^{9}	0.44	1.07	20.4 (66.7%)	0.2 (9.1%)
1.0-µm filtrate	3.6×10^{9}	0.31	0.47	13.9 (45.4%)	0.1 (4.1%)

[a] The physical and chemical conditions are the same as those in Table 1.

[b] Water samples were incubated with 8.3×10^{-4} pM ^{3}H-glucose for 1 hr. Values are means of triplicate samples. The average of difference in triplicate samples was < 5%. The proportion of total uptake by each fraction is given in parenthesis. For example, in the 3.0-µm-pore filtrate, the proportion was (20.4 / 30.6) x 100%= 66.7%.

[c] Water samples were incubated with 2.7 x 10^{-4} µM ^{14}C- bicarbonate in sunlight for 4 hr.

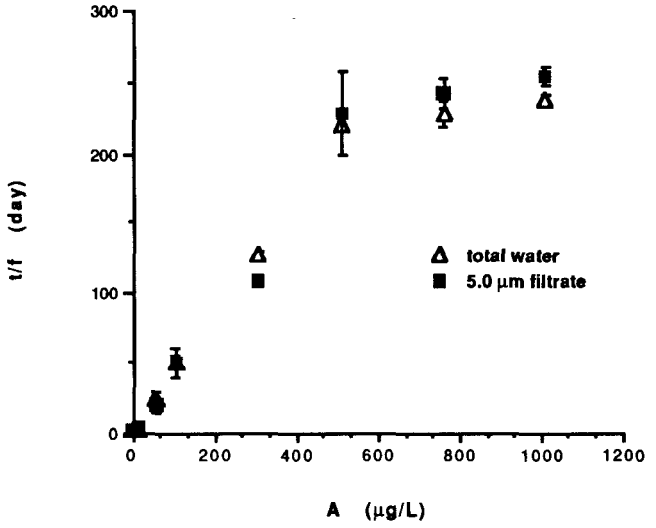

Figure 2. Wright-Hobbie plots for p-cresol mineralization in an April water sample collected from the control stream of a landfill site near Athens, Georgia. Incubation time was 9 hr and temperature was 24°C.

bacterial numbers remained constant in the dark control. Therefore, trichloroaniline photoproducts appeared to be utilized by the bacterial consortia to increase their population sizes.

Wright-Hobbie plots of microbial mineralization of p-cresol in an April water sample from the impacted stream (downslope) of a landfill site near Athens, Georgia, followed complex, multiphasic patterns (Figure 2). The slopes of the data points decreased with increasing [A] (i.e., substrate added), indicating that V_{max} increased progressively with substrate concentrations. This is indicative of the existence of multiple uptake/degradative systems within the water samples (Hwang et al. 1994). Size-fractionation of the water sample was used to obtain 5.0-µm filtrates which mainly consisted of heterotrophic microorganisms. The concurrent measurements of microbial activities indicated that the filtrates retained 9%, 67% and 53% of the autotrophic light-uptake of bicarbonate, heterotrophic uptake of glucose, and heterotrophic uptake of thymidine, respectively, of the total (i.e., unamended water) water samples (results not shown). Indistinguishable multiphasic degradation kinetics (t-test; $p < 0.05$) were observed for unamended water samples and 5.0-µm-pore filtrates (Figure 2). Thus, heterotrophic microbial populations (≤ 5.0 µm) mediated the mineralization process of p-cresol with multiphasic kinetics. Lewis et al. (1935) reported the occurrence of multiphasicity of microbial degradation of methyl parathion by a single species of bacterium Flavobacterium sp. Our data cannot specify whether or not the multiphasicity was attributed to a single species or multiple species of bacteria in the samples. Microbial uptake of ^{14}C-glucose and ^{3}H-thymidine in control and impacted streamwaters were also measured to characterize general metabolic activities of the indigenous microbes therein. Microbial uptake of glucose

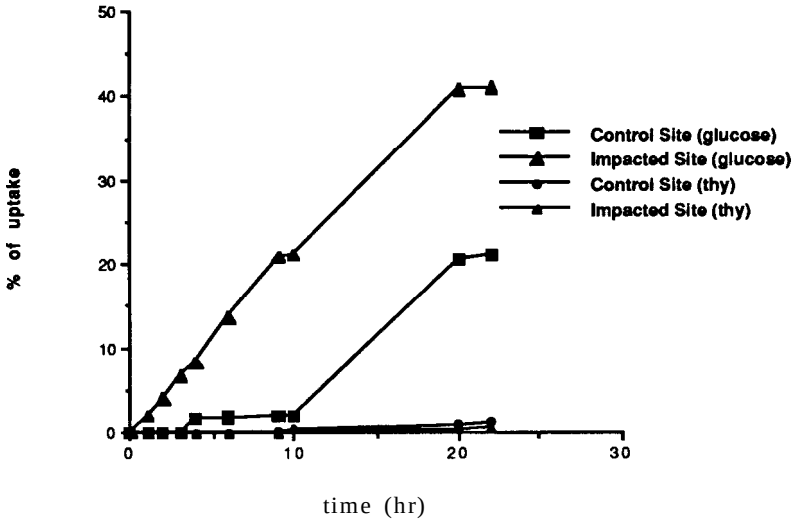

Figure 3. Microbial uptake of glucose and thymidine in streamwater samples collected from control site and impacted site at a hazardous landfill site near Athens, Georgia in May. The average of difference in triplicate samples was < 5%.

was faster (2-8 fold) in the impacted site than in the control site (Figure 3), while thymidine uptake activity was slightly faster in the control site (up to 2 fold after 24 hr) than in the impacted site. The total bacterial number was 2 x 10^5/mL and 6 x 10^5/mL in the control water and impacted water, respectively. Apparently, microbial consortia in the impacted water utilized more substrate (e.g., glucose) for maintenance purpose and less substrate (e.g., thymidine) was converted to biomass in the incubation period. The occurrence of kinetic diversity for p-cresol degradation also has been found in other aquatic environments (Hwang et al. 1994). These findings indicate that changes in the kinetic parameters with substrate concentrations should be taken into account in calculating pollutants' persistence if reasonable estimates are to be made.

Assessment of risk of exposure to toxic pollutants in natural environments is a complex task. Failure to take consideration of the existence of kinetic diversity of degradative/uptake systems simultaneously present in natural aquatic systems can result in significant errors in degradation rate estimations, and consequently, in pollutant persistence (Hwang et al. 1989). In addition, some questions regarding the performance and interpretation of heterotrophic potential measurement need to be cautioned. For example, more refined mathematical transformation is required to account for competitive and noncompetitive inhibitory effects by substrates other than the candidate compound present in the system. The assumptions that a unique uptake system exists for each substrate being measured and that all members of the microbial population respond in the same way to the substrate at various concentrations may not be valid (Atlas and Bartha 1993). Nevertheless, the kinetics approach remains as one of the most useful techniques for the study of microbial transformation of organic pollutants in natural environments. During this study, size-fractionation technique

was also proven to be useful for determining the roles of microorganisms in transforming organic contaminants.

Acknowledgments. This research was supported by: US Army Construction Engineering Research Lab., under contract #DACABB-91-D-0008 (to Clark Atlanta University); Subcontract #OSP-92-10-668-026 to Jackson State University, and NIH MBRS Program Grant SO6GM08047 (to JSU).

REFERENCE

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

Atlas RM, Bartha R (1993) Microbial Ecology: Fundamentals and Applications, 3rd ed. The Benjamin/Cummings Publishing Company, Inc, Redwood City, California.

Bartha R (1971) Fate of herbicide-derived chloroanilines in soil. J Agric Fed Chem 19: 385-387

Francis BM (1994) Toxic Substances in the Environment. John Wiley & Sons, Inc., New York

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, Maccubbin AE, Pomeroy LR (1981) Dissolved adenosine tiphosphate utilization by free-living and attached bacterioplankton. Mar Biol 64: 43-51

Hwang H-M, Hodson RE, Lee RF (1986a) Photochemical and microbial degradation of 2,4,5-trichloroaniline in a freshwater lake. Appl Environ Microbiol 50: 1177-1180

Hwang H-M, Hodson RE, Lee RF (1986b) Degradation of phenol and chlorophenols by sunlight and microbes in estuarine water. Environ Sci Technol 20: 1002-1007

Hwang H-M, Hodson RE, Lewis DL (1989) Microbial degradation kinetics of toxic organic chemicals over a wide range of concentrations in natural systems. Environ Toxicol Chem 8: 6574

Hwang H-M, Loya JA, Perry DL, Scholze R (1994) Interactions between subsurface microbial assemblages and mixed organic and inorganic contaminant systems. Bull Environ Contam Toxicol 53:771-778

Kaufmann DD, Blake J (1973) Microbial degradation of several acetamide, acylanilide, carbamate, toluidine and urea pesticides. Soil Biol Biochem 5: 297-308

Lewis DL, Hodson RE, Freeman LF III (1985) Multiphasic kinetics for transformation of methyl parathion by Flavobacterium species. Appl Environ Microbiol 50: 553-557

Matsumura F (1982) Degradation of pesticide s in the environment by microorganisms and sunlight. In: Matsumura F, Murt i CRK (eds) Biodegradation of Pesticides. Plenum Press, New York

Messner B, Berndt J, Baum I (1979) Changes in fatty acid and cholesterol synthesis in rat liver slices due to aromatic amines formed during the degradation of some herbicides. Bull Environ Contam Toxicol 21: 831-836

Miille MJ, Crosby DG (1983) Pentachlorophenol and 3,4-dichloroaniline as models for photochemical reactions in seawater. Mar Chem 14: 111-120

Neilson, AH (1994) Organic Chemicals in the Aquatic Environment: Distribution, Persistence, and Toxicity. Lewis Publishers, Boca Raton, FL

Patnaik P (1992) A Comprehensive Guide to the Hazardous Properties of Chemical Substances. Van Nostrand Reinhold, New York

Strickland JD, Parsons TR (1972) A Practical Handbook of Seawater Analysis. Fisheries Research Board of Canada, Ottawa