PREDICTING THE PERSISTENCE OF ORGANIC POLLUTANTS IN NATURAL AQUATIC SYSTEMS BY USING KINETICS DATA OF MICROBIAL TRANSFORMATION PROCESSES

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INTRODUCTION

In assessing the potential exposure of humans and other organisms to toxic organic pollutants, it is necessary to first determine the persistence of the pollutants in the environment. This assessment, in turn, requires that we understand the kinetics of microbial transformation and complete mineralization (to CO_2) of the compounds in a given ecosystem. Traditionally, models of pollutant transformation in aquatic ecosystems have usually assumed that degradation followed simple, Michaelis-Menten, kinetics which can be described by a single hyperbolic relationship between substrate concentration and degradation rate; i.e., the kinetics are defined by a single set of kinetic parameters which include a K_t (the substrate concentration supporting half maximal degradation rate) and a V_{max} (the maximal rate of substrate degradation).

Wright and Hobbie (1965) used a modified Lineweaver-Burk linearization plot to examine microbial heterotrophic uptake of dissolved organic substrates. In addition to measuring the maximal uptake potential, this method can be used to calculate microbial turnover time for a given substrate and a transport constant that exhibits the affifinity of the uptake system(s) for the given substrate. Originally, this method only measured the incorporation into cells (i.e., uptake). Their approach was later modified to include corrections for losses of radiolabeled compound during respiration (Crawford et al. 1973). With this plot, [A] (the concentration of added substrate) is plotted against the quotient t/f (incubation time, t, divided by f, the fraction of added substrate transformed during incubation period). This type of plot is useful in ecological studies of natural waters for which direct kinetic plot of velocity, v, versus total substrate concentration, [S], are difficult to construct because of limitation of analytical sensitivity in determining the natural, ambient substrate concentration, S_n, in highly dilute natural waters; v can not be directly calculated if S_n is unknown.

The Wright-Hobbie plot eliminates the need to determine S_n and v and, graphically, yields the values of microbial maximum uptake rate V_{max} (from reciprocal of the slope), and $[K_t + S_n]$ (from the intercept on the x-axis), as well as the turnover time of the substrate at ambient concentration, T_t (from the intercept on the y-axis). A linear relationship between t/f and [A] indicates that the heterogeneous heterotrophic populations for transforming the compound possess uptake mechanisms with similar kinetic parameters. Later, this saturation-kinetics approach, based on the assumption of monophasic kinetics (linear relationship between t/f and [A]), was applied to determine the biodegradation rates of xenobiotic and naturally occurring toxic organic chemicals in aquatic environment (Button 1985; Pfaender and Bartholomew 1982). The biodegradation data are critical for the development of predictive models of chemical pollutants' persistence in natural aquatic environments.

The initial assumption that the degradation kinetics of an organic substrate by mixed microbial populations follow a monophasic kinetic pattern was drawn from the results of screening experiments conducted at low concentrations and over relatively narrow ranges of substrate concentrations (Pfaender and Bartholomew 1982; Wright and Hobbie 1965). For example, Pfaender and Bartholomew (1982) reported that linearity was assumed to be the valid relationship between turnover time and substrate concentration based on experiments in which pollutant concentration was varied by 10-fold or less. Indeed, linearity was usually a good approximation of the data fit. Thus, a single set of the kinetic constants, $[K_t + S_n]$ and V_{max}, was computed and used to calculate the persistence of the chemical pollutant in natural environment. The measured value of V_{max} is that it gives an estimate of *in situ* heterotrophic activities because V_{max} is a function of the indigenous population. It is subjected to environmental changes, even when the population size remains constant. Therefore, V_{max} is of ecological value when comparing the effects of temporal and spatial changes on the metabolic activities of indigenous microorganisms (Atlas and Bartha 1993).

There is no inherent reason to assume that degradation kinetics of an organic compound by a mixed population of microorganisms should behave in a manner identical to degradation mediated by a single enzymatic system. It had been assumed that this was the case, however, based on

early reports on microbial uptake of naturally occurring organic compounds, such as glucose, conducted over very narrow ranges of substrate concentration. One possible explanation was that ambient concentration of that natural substrate had been constant and low over long periods of time, resulting in selective pressure on all the microorganisms to evolve the similar enzymatic system (Azam and Hodson 1981). The concentrations of both chemical pollutants and naturally occurring compounds, however, can vary over wide ranges in natural environment spatially and temporally (Azam and Hodson 1981; Bartholomew and Pfaender 1983). Therefore, microbial degradation of organic compounds are likely to be influenced by such environmental heterogeneity in substrate concentrations. It then would seem reasonable to assume that microbial assemblages might possess mechanisms enabling them to effectively utilize low bulk substrate concentrations while retaining the capability to respond to locally high transient concentrations by increasing turnover rate (Azam and Hodson 1981).

Subsequent studies of microbial degradation of naturally occurring chemicals measured over wide ranges of concentration revealed that kinetic patterns were multiphasic; i.e., the microbial populations in natural marine and freshwater environments possess kinetic parameters that change with substrate concentration. This phenomenon has been termed kinetic diversity, and it is indicative of the simultaneous presence and activity of multiple uptake/degradation systems for a given compound in a natural water sample. Relative to what is known about the kinetics of the turnover of naturally occurring dissolved organic compounds, little is known about the importance of kinetic diversity in the biodegradation of xenobiotic or other toxic organic compounds (Lewis et al. 1985). If degradation of these compounds follows simple Michaelis-Menten kinetics (or first order kinetics), calculation of substrate persistence will be a simple procedure. However, if multiphasic degradation patterns predominate for some or all anthropogenic compounds, predictive models of pollutant persistence and risk of environmental exposure will need to incorporate multiple kinetic parameters corresponding to changing pollutant concentrations. In this study, a series of experiments were conducted to examine the kinetic patterns of microbial degradation of several toxic organic pollutants in a variety of natural aquatic ecosystems.

MATERIALS AND METHODS

Sampling

In 1986 and 1987, Lakewater samples (top 0.5 m depth, pH 6.6-8.9) were collected from Lago Lake, a shallow, moderately eutrophic lake near Athens, Georgia. Okefenokee Swamp surface water samples (pH 3.1-3.4) were collected from Mizell Prairie, a grass and sedge prairie (Murray and Hodson 1984). During October 1986, on a research oceanographic cruise aboard the O.R.V. 'Columbus Iselin', seawater samples (pH 8.0-8.4, sanility 34-35.5 $^{o}/_{oo}$) were collected during high tide periods from mangrove stands at Mamma Rhoda Channel near Chub Cay, Bahamas. All water samples were taken in acid-washed, 10-liter polyethylene containers. Assays were initiated within 1 h of collection for the lakewater and seawater samples and within 6 h of collection for the swampwater. Between 1989 and 1991, ground water samples were collected from a landfill site in Northeast Georgia as that 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^{-1}), iron (200 mg L^{-1}), naphthalene (150 µg L^{-1}), toluene (6.9 mg L^{-1}), trichloroethylene (490 µg L^{-1}), xylene (2.3 mg L^{-1}), methylene chloride (28 mg L^{-1}), 1,1,2,2-tetrachloroethane (1.7 mg L^{-1}), formaldehyde (41 mg L^{-1}), benzene (15 mg L^{-1}), and chloroform (40 mg L^{-1}).

Samples of Georgia ground water were obtained from a control well (upslope) and impacted wells (downslope) from the landfill according to procedures described in Hwang et al. (1993) and Armstrong et al. (1991). Temperature and dissolved oxygen levels were measured in the wells using portable monitors (Models 33 and 57; Yellow Springs Instrument Co., Yellow Springs, OH). *In situ* dissolved oxygen concentrations ranged from 11.2 to 11.5 mg L^{-1} for control water samples and 2.8 to 3.5 mg L^{-1} for impacted ground water samples. For control well samples and impacted well samples, pH ranged from 5.2 to 6.2 and 6.1 to 6.5, respectively. Temperature of the ground water samples ranged from 10.5 to 11.0°C.

Chemicals

U-^{14}C-labeled phenol (118 mCi/mmol) was obtained from Amersham Company. U-^{14}C-labeled p-cresol (10.33 mCi/mmol), methanol (8.7 mCi/mmol), p-chlorophenol (8.9 mCi/mmol), 1,3-[^{14}C]acetone and toluene (56.3 mCi/mmol) were obtained from Sigma Chemical Company and D-[U-^{14}C]glucose (257.7 mCi/mmol) was obtained from DuPont NEN Research Products. Unlabeled analytical reagent-grade methanol and acetone were obtained from J.T. Baker Chemical Company. Other unlabeled organic chemicals were obtained from Aldrich Chemical Company.

Incubation and Degradation Kinetics Measurements

^{14}C-labeled pollutant compounds such as 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 sample of water in 160-ml Pyrex bottles. To adjust the concentrations of dissolved oxygen during sampling ground water samples, head space of the sampling 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 25±1°C with gentle shaking (100 rpm). Killed controls contained formaldehyde (final concentration of 1.9%). Except for time course and toluene degradation experiments, samples of degradation experiment were incubated for various periods of up to 24 h.

Microbial Biomass and Activity Measurements

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). During the incubation periods, total bacterial numbers were monitored and were found to be constant. Final substrate concentrations for degradation kinetics measurements ranged from 0.1 µg L^{-1} to 1g L^{-1}, depending on the compound. The $^{14}CO_2$ produced was collected with the two-trap method of Hodson et al. (1977), and radioactivity was measured with a liquid scintillation counter (Beckman LS 9000). 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^{-1} [^{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 h. After incubation, water samples were filtered through 0.22-µm pore-size filters (Millipore). 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^{-1}. Filters then were placed in 10-ml Scintiverse I counting cocktail and radioassayed.

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 h 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 $^{14}CO_2$ [10].

To determine disappearance rates for p-cresol and p-chlorophenol, the samples containing parent compounds and degradation product(s) were extracted twice with ethyl acetate after acidification. Extracts were concentrated by evaporation under nitrogen, applied to silica gel TLC plates (E. Merck) and developed with a solvent system of hexane:acetone (1:1, v/v). Spots containing parent compound and degradation products were scraped from the thin-layer plates and their radioactivity determined.

Differentiation between Multiphasic and Monophasic Kinetics

Wright-Hobbie plot of the degradation kinetic data were examined using both linear and nonlinear regression analysis. The F test was used to determine the relative appropriateness of the two alternative models, ie., linear regression (monophasic) and nonlinear regression (multiphasic) represented by Equation 1, to describe the degradation kinetic data (Robinson 1985). This test was carried out at the 5% level of statistical significance. The resulting equations for the best fit to the data were subsequently used to calculate values of the kinetic parameters V_{max} and $[K_t + S_n]$ at different substrate concentrations. Standard nonlinear regression methods (NLIN procedure) (SAS Institute 1982) were used to develop best-fit curves for the multiphasic kinetic data. Analyses were begun with the assumption that the relationship between t/f and [A] was roughly hyperbolic. The first derivative of the regression equation is the slope of the line tangent to the curve at a particular value of [A]. The slope, together with the solution for the original regression equation for a given substrate concentration, [A], can then be used to calculate V_{max} and $[K_t + S_n]$ at that substrate concentration. The equation for the nonlinear regression is

$$t/f = (pA)/(q + A) \qquad (1)$$

where t is incubation time, f is the fraction of added compound transformed in time t, A is the substrate concentration added, p is the maximum value of t/f at the asymptote of multiphasic Wright-Hobbie plots, q is the value of A at t/f equals 1/2p.

RESULTS AND DISCUSSION

Microbial Degradation Kinetics of Candidate Chemicals in Surface Waters

Wright-Hobbie Plot of microbial mineralization of acetone added at various concentrations in a freshwater (Lago Lake) near Athens, Georgia, followed complex, multiphasic patterns in both January and November, although the rate of degradation was faster in January water sample (Figure 1). The slopes of the curves decreased with increasing [A], indicating that V_{max} increased progressively with substrate concentrations. Approximation of the slopes and the resulting x- and y-intercepts for various ranges of A can be obtained by connecting with straight lines any two or three adjacent data points (Atlas and Bartha 1993). Precise

determination of the slope and intercepts, and consequently of the effective values of V_{max} and $[K_t + S_n]$, can be made from the derivative of the equation for the nonlinear regression fit of the data. The kinetic parameters can then be calculated from the slope and intercepts of the line tangent to the curve at any given value of [A]. Table 1 indicates the changes in $[K_t + S_n]$ and V_{max} for microbial mineralization of acetone in Lago Lake. For example, values of V_{max} and $[K_t + S_n]$ increase from 0.2 to 844 µg L^{-1} and 0.1 to 3.4 x 10^4 µg L^{-1} respectively when [A] increases from 1 to 500 µg L^{-1}. S_n, by definition, does not change with added substrate (i.e., [A]); therefore, changes in $[K_t + S_n]$ are due to changes in effective values of K_t. S_n, therefore, must be equal to or less than the value of $[K_t + S_n]$ for the lowest value of [A] examined.

In order to determine whether or not degradation of other candidate pollutants in natural surface waters also are regulated by multiphasic kinetic parameters, similar experiments were conducted using a variety of radiolabeled compounds in water samples from various habitats including Lago Lake, the Okefenokee Swamp, and seawater samples from a mangrove stand in the Bahamas (Figure 2; Table 2). The majority of the samples taken from these sites degraded the pollutants according to multiphasic kinetics. However, some habitats showed multiphasic kinetics on some sampling days/seasons and monophasic kinetics on other sampling days/seasons (Table 2). We speculate that changes in the diversity of microbial assemblages and/or enzymatic systems caused the shifts between monophasic and multiphasic patterns, with monophasic patterns being typical of populations having lower metabolic or species diversity. Surprisingly, kinetic diversity was also observed in the Bahamas for microbial degradation of pollutants such as methanol [Table 2], although methanol degradative activity was at least an order of magnitude lower in Bahamas seawater than in the lake water. The difference could be related to the large differences in bacterial numbers between two sites. Bacterial numbers averaged (1.5 ± 0.4) x 10^7 ml^{-1} in lake water in July and (8.1 ± 2.0)x 10^5 ml^{-1} in Bahamas seawater in October.

Table 2 is a summary of $[K_t + S_n]$ and V_{max} values observed for microbial mineralization of five toxic organic chemicals in several surface aquatic environments during different dates. Note the large changes in values for these parameters when the substrate cocentrations were varied. The predominance of kinetic diversity for degradation of these pollutants indicated the changes in the parameters must be taken into account in calculating their persistence if reasonable estimates are to be made. Multiphasic kinetic patterns were apparent for methanol degradation in Lago Lake water samples collected in both summer and winter (Figure 2; Table 2). If examined over a narrow substrate concentration range, however, the degradation kinetics can appear artifactually to be monophasic. For example, between the concentrations of 27.5 and 300 µg L-1, methanol mineralization kinetics data for a January water sample (Figure 2) were described well by linear regression (r = 1.00, n = 5). Only when the range of concentrations has been expanded, does the multiphasic pattern become apparent. The traditional monophasic kinetic model was based on the assumption that all the degradation rate is determined by only a set of kinetic parameters, $[K_t + S_n]$ and V_{max}, then degradation rate, v, follows the equation: $v = (V_{max}.A)/(K_t + A)$ and that these values hold true for all substrate concentrations. If multiphasicity is taken into account, then the values of V_{max} and $[K_t + S_n]$ will change with the substrate concentration. According to our experimental data, using the two alternative models, differences as large as 40,000 -fold were observed for the calculated values for v.

In addition to mineralization measurement, microbial degradation kinetics of two compounds, p-cresol and p-chlorophenol, were also determined by direct measurement of the disappearance of parent compound. Again, multiphasic kinetics were observed for their degradation in Lago Lake water samples collected in May and September, respectively (Figure not shown). Concurrent measurement of $^{14}CO_2$ mineralization kinetics for these same samples indicated similar multiphasic patterns (not shown), although the ratios of disappearance rate to mineralization rate ranged from 1:3 to 1:6.

Microbial Degradation Kinetics of p-cresol and Toluene in Ground Water

Microbial toluene degradation in samples amended with 40 µg L^{-1} toluene was neglible in the control well, whereas rates of degradation were higher up to 20 fold in the impacted well (Armstrong et al. 1991; Hwang et al. 1993), suggesting microbial adaptation for enhanced degradation of toluene. Indeed, this was verified by the result of enumeration of toluene degraders with most probable number technique (Armstrong et al. 1991). When microbial degradation kinetics data were linearlized according to the method of Wright and Hobbie, multiphasic kinetic patterns were observed for p-cresol degradation in Georgia control ground water (Figure 3) and for toluene degradation in impacted ground water (Figure 4) from the landfill site. This is indicative of the existence of multiple uptake/degradative systems therein and such kinetic diversity had only begun to be found in ground water microbial assemblages (Hwang et al. 1993). The finding of this subsurface kinetic diversity is interesting because it suggests bioremediation potential for bioengineering with subsurface indigenous microorganisms. In addition, such kinetics information has significant implications for modeling pollutant persistence and exposure in risk assessment.

Assessment of risk of exposure to toxic pollutants in natural environment is a complex task. The finding of multiphasicity for pollutant degradation further complicates the calculation processes. Failure to take into consideration the existence of kinetic diversity of degradative/uptake systems simultaneously present in natural aquatic systems, however, can result in significant errors in degradation rate estimations and, consequently, in pollutant persistence. Some questions regarding the performance and interpretation of heterotrophic potential measurements, however, 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. In addition, the assumptions that a unique uptake system exists for each substrate being measured and 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). However, the kinetics approach remains as one of the most useful techniques in the study of microbial transformation of a particular organic pollutant in natural environments. Measurement of kinetic parameters of heterotrophic potentials permits studies on the effects of environmental factors on microbial activities and estimations on pollutant persistence.

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Table 1. Kinetic Parameters of Acetone Mineralization by Micribial Assemblages in January Lake Water from Lago Lake near Athens, Georgia.

[A] ($\mu g\ L^{-1}$)	[$K_t + S_n$] ($\mu g\ L^{-1}$)	Vmax ($\mu g\ L^{-1}\ d^{-1}$)
1	0.1	0.24
2	0.5	0.30
8	7.9	0.77
10	12.3	0.97
50	307.9	10.0
100	1,231.7	34.5
105	1,357.6	37.7
500	33,742.0	843.7

Table 2. Summary Table of Sampling Sites, Dates and Kinetics Data

					[Kt + Sn]		Vmax	
Compound	Date	Site	Kinetic Type[a]	[A]	Low	High	Low	High
Acetone	11/86	Lake	Multiphasic*	1-500	2.3	7.3×10^4	0.07	1,000
	1/87	Lake	Multiphasic**	0.5-500	0.03	3.7×10^4	0.2	887
Methanol	7/86	Lake	Multiphasic**	0.5-100	0.01	23	0.4	11
	1/87	Lake	Multiphasic**	1-500	0.6	4,155	0.6	45
	10/86	Bahamas	Multiphasic**	1-500	1,845	1.3×10^4	0.03	17
p-Cresol	6/86	Lake	Monophasic	$1\text{-}10^4$		45		11
	12/86	Lake	Monophasic	$1\text{-}10^5$		12,184		36
	2/87	Lake	Multiphasic***	$1\text{-}10^4$	1.6	1.1×10^5	1.3	176
	10/86	Bahamas	Multiphasic***	1-500	1.2	1,300	0.06	0.8
	12/86	Swamp	Monophasic	$1\text{-}10^5$		3,933		40
Phenol	4/86	Lake	Multiphasic***	$0.1\text{-}10^6$	0.01	3.6×10^7	0.5	4,082
	2/87	Lake	Monophasic	0.3-2,000		520		3
p-Chlorophenol	11/86	Lake	Monophasic	$1\text{-}10^5$		2.8		4
	1/87	Lake	Monophasic	1-2,000		58		6
	10/86	Bahamas	Multiphasic*	8-1,000	0.7	1.3×10^4	0.02	2.6

Units: [A], $\mu g\ L^{-1}$; V_{max}, $\mu g\ L^{-1}\ d^{-1}$; [$K_t + S_n$], $\mu g\ L^{-1}$.

[a] Level of siginificance of the multiphasic (nonlinear) curve fit: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

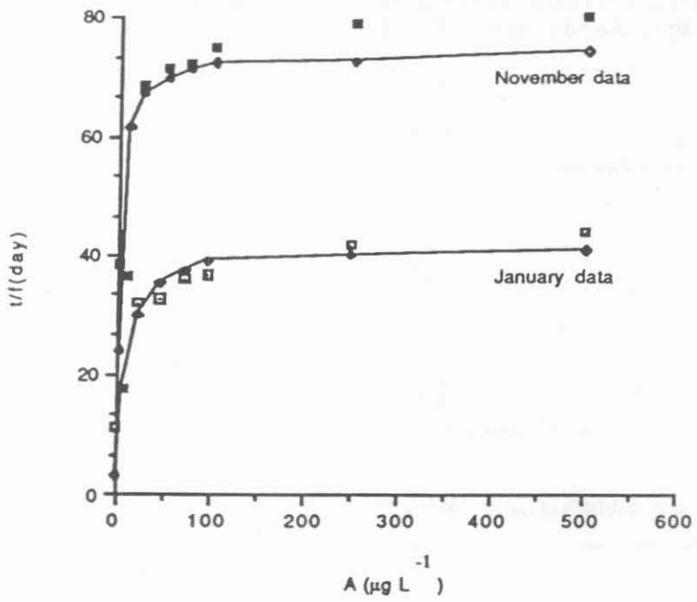

Figure 1. Wright-Hobbie plots for acetone mineralization in water samples from Lago Lake near Athens, Georgia. The average difference in triplicate samples was less than 4%.

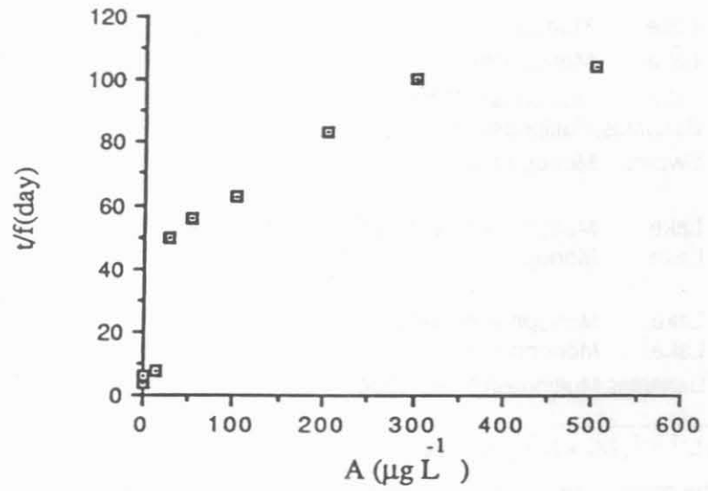

Figure 2. Wright-Hobbie plot for microbial mineralization of methanol in water samples from Lago Lake near Athens, Georgia in January.

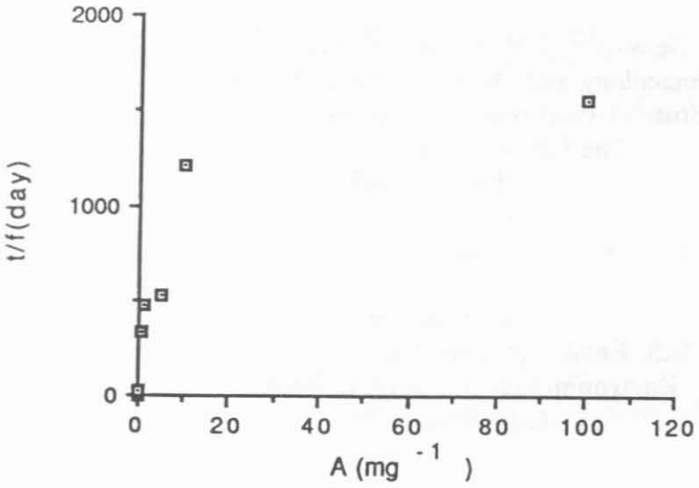

Figure 3. Wright-Hobbie plot for microbial mineralization of p-cresol in Georgia control ground water.

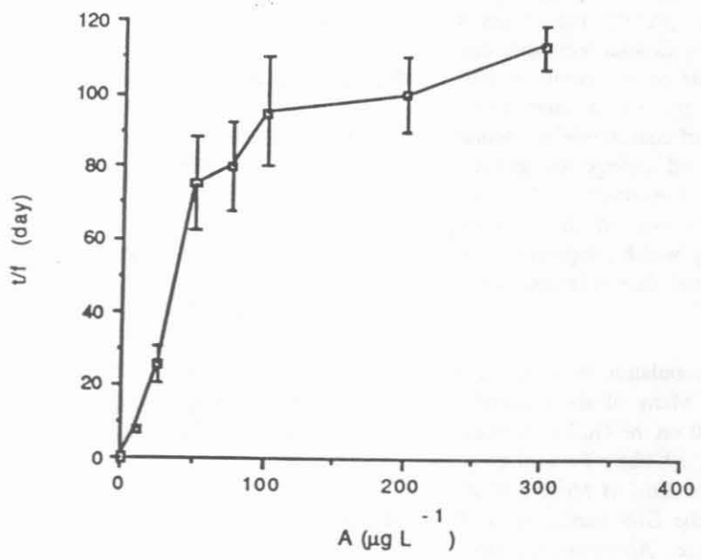

Figure 4. Wright-Hobbie plot for microbial mineralization of toluene in Georgia impacted ground water.