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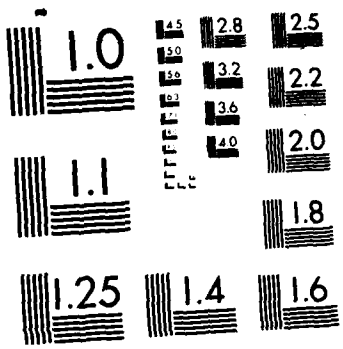

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BIODEGRADATION OF GUANIDINIUM BY AQUATIC MICROORGANISMS

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The microbial degradation of guanidinium was complete in surface water samples from various sites including two obtained in the vicinity of the Sunflower Army Ammunition Plant. Guanidinium degradation was characterized by lags prior to enhanced biodegradation and was considerably slower than degradation of the amino acid arginine. Microorganisms capable of utilizing guanidinium as a nitrogen source for growth and as a sole added carbon source could be generalized to all sites tested. The action of the microorganisms on

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20. Abstract (continued)

guanidinium resulted in the mineralization of its carbon. Microorganisms adapted to grow on guanidinium in laboratory cultures used it at a slower rate than two xenobiotic compounds considered to be readily metabolizable. In environmental water samples, short term guanidinium biodegradation was 20 times slower than that of urea. Following long-term incubation, the development of populations capable of enhanced guanidinium biodegradation was concentration dependent and could not be demonstrated at or below 0.01 µg/mL. The removal of guanidinium from surface waters by microbial action will likely be slow.

ACKNOWLEDGMENT

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INTRODUCTION

Manufacture of the military propellent nitroguanidine is carried out by a process involving the production of precursor guanidine nitrate from calcium cyanamide and ammonium nitrate, followed by dehydration of the precursor in strong acid to form nitroguanidine.[1] Sunflower Army Ammunition Plant (SFAAP), a 40 ton/day nitroguanidine production facility, was originally designed to operate with no wastewater discharges. It is now estimated that as much as 400,000 gallons of wastewater per day will be generated.[2-4] Wastewaters from the current nitroguanidine pilot facility ultimately enter the Kansas River via Hansen Creek and then Kill Creek adjacent to SFAAP.[5]

Analyses of production wastewaters indicate that in addition to inorganic ions and a diversity of low-level organic by-products, significant quantities of both nitroguanidine and guanidinium,* the guanidine species dominant at neutral pH, are present.[6] Guanidinium, measured as guanidine nitrate, has been reported to be approximately 100 mg/L in holding basins, 10 mg/L in outfalls, and 1 mg/L in Kill Creek.[3] In addition to direct discharge, anaerobic microbial action and photolysis of nitroguanidine to guanidinium also have the potential to contribute the pollutant to the environment.[4,7,8]

Little is known of the effect of microbial populations on guanidinium. One classical study has indicated that it can serve as a nitrogen source for growth of several bacterial and fungal species in pure cultures.[9] Soil microorganisms have been reported to degrade guanidine slowly under anaerobic conditions, but in the same study aerobic microflora from activated sludge did not degrade it at all, even as a nitrogen source for their cultivation on synthetic sewage.[10] Reports addressing the use of guanidinium as a sole carbon source for microbial growth do not appear in the literature. Nevertheless, microbial growth on other one-carbon compounds is well known and has been described for methane, methanol, formic acid, and formaldehyde.[11] Moreover, one-carbon compounds containing reduced nitrogen can be metabolized by microorganisms as is indicated by microbial growth on methylamine and hydrolysis of urea by a diversity of bacterial and fungal species.[12,13]

The objective of this study is to provide an integral part of the data base necessary to establish water quality criteria in a context of hazard assessment.[14,15] Toward that end, the study addresses the biodegradability of guanidinium by environmental microorganisms, including those obtained from the vicinity of SFAAP.

MATERIALS AND METHODS

BIODEGRADATION SCREENING AND DEVELOPMENT OF ENRICHMENT CULTURES

Biodegradation screening was carried out in shake flasks that contained 250 mL of water samples, 11 µg/mL guanidinium (20 µg/mL guanidine nitrate) or 17 µg/mL arginine, and buffer (0.2% potassium phosphate, pH 7). Flasks were incubated with agitation for 6 to 8 weeks at 25°C. Aliquots (2 mL) were

* Guanidinium is used to refer to guanidinium ion throughout.

withdrawn throughout the course of the experiment and were assayed for the test chemicals spectrofluorometrically as described below. Total bacteria were determined in triplicate following the plating of serial dilutions for 4 days at 25°C on Standard Methods Agar (SMA, Difco). Matched controls for the experimental flasks consisted of water samples that had been autoclaved for 15 minutes prior to the addition of test chemicals.

Aliquots (10 mL) from screening flasks showing guanidinium degradation were used to inoculate shake flasks containing 20 and 40 µg/mL guanidine nitrate in 90 mL buffered salts medium from which nitrogen containing compounds were omitted (BSM-N).[16] The flasks were incubated at 25°C and monitored for the disappearance of guanidinium spectrofluorometrically. Following its complete disappearance, the process was repeated stepwise using 20 to 40 µg/mL increments of guanidine nitrate until enrichment cultures were developed which were serially passed at 300 µg/mL (168 µg/mL guanidinium). Enrichment cultures developed in this manner could be maintained on either guanidine nitrate or hydrochloride as a sole added nutrient with little, if any, differences in times required for degradation.

CHEMICAL ANALYSES

Quantitation of guanidinium in surface water samples and laboratory cultures was conducted as described by Conn and Davis.[17] In all analyses, 2 mL of 0.5 percent ninhydrin and 2 mL of 1 N potassium hydroxide were added to 2 mL of centrifuged samples (10,000 XG, 10 min). Samples were incubated for 10 min prior to measurement of fluorescence at 495 nm using an excitation wavelength of 390 nm. Guanidinium was quantitated relative to standards consisting of guanidine nitrate or hydrochloride added to the natural water sample or culture matrix under study, and the percentage of guanidinium delivered upon dissociation of its salt (56 percent and 64 percent, respectively). Baseline settings were obtained from the same water sample or culture matrix without added guanidinium. Standard curves for guanidine nitrate or hydrochloride added to tap water, culture matrix, or surface water samples indicated that guanidinium could be assayed with a coefficient of correlation of 0.999 to a level of approximately 0.5 µg/L with less than 5 percent variation. Results for day-to-day analyses of experimental samples were somewhat more variable and are defined by heat killed controls in the studies. Arginine was assayed by the same method with essentially the same precision.

Adenosine triphosphate (ATP) levels in microorganisms concentrated from culture aliquots (100 mL) were determined by bioluminescence in a DuPont 760 Luminescence Biometer. Filtration of the aliquots, disruption of the microorganisms, elution of their ATP, and analysis of the samples in standard luciferin-luciferase reactions were conducted as previously reported.[18] Samples were assayed in quadruplicate, and the average ATP concentrations were converted to total ATP per original culture volume (300 mL). Operation of the biometer, including calibration, electronic stability checks, and inherent light determinations, were carried out as originally described.[19]

ISOTOPIC STUDIES

Scintillation counting was carried out in a Packard Tri-Carb Model 3385 scintillation spectrophotometer. Data were corrected for quench by the external standard channels ratio method and converted to disintegrations per

minute (DPM). All aqueous samples including traps, cultures, and culture fractions were counted in triplicate by adding 1 mL aliquots to 15 mL Instagel (Packard Instrument Co.). Filtered radioactivity was counted by complete immersion in 20 mL Instagel. Because of the wide range of guanidinium concentrations studied under differing experimental conditions, guanidine nitrate or hydrochloride was added to carbon-14 guanidine hydrochloride (22 mCi/mM, Amersham Corp.) to achieve the desired specific activities as described below.

Production of labeled carbon dioxide by enrichment microorganisms growing on 300 µg/mL guanidine nitrate (168 µg/mL guanidinium, 0.01 µCi/mg) at 25°C was carried out as described by Gledhill.[20] In addition to the test chemical, culture flasks contained 300 mL BSM-N, a 5 percent (v/v) inoculum of a fully grown enrichment culture, and traps with 10 mL 0.5 N KOH. Mineralization of labeled guanidinium carbon in the presence of glucose was measured in the same manner, except that inoculum consisted of surface water microorganisms diluted to 10^4 CFU/mL in 0.2 percent phosphate buffer and held 24 hours to deplete nutrients, after which time it was made 500 µg/mL glucose, 23 µg/mL guanidine nitrate (13 µg/mL guanidinium, 0.7 µCi/mg), and reincubated. Both systems were incubated an additional 24 hours after the disappearance of guanidinium to facilitate maximum carbon dioxide trapping. For experiments conducted in the presence of glucose, levels of total carbon dioxide were determined by titrating 5 mL trap samples with 0.5 N HCl to the phenolphthalein end point after precipitation with 30 mL 0.3 N $Ba(OH)_2$.[20]

To assess the concentration dependence of guanidinium mineralization, series of the above trapping flasks containing 500 mL each of natural surface waters (Monocacy River or Carroll Creek, 0.2 percent potassium phosphate, pH 7) were incubated with 0.0005, 0.01, 0.1, 1.0, and 10 µg/mL guanidinium (added as 64 percent guanidine hydrochloride) and 1 µCi carbon-14 guanidinium. Incubation was carried out in the dark at 25°C, during which time the contents of each trap were removed for radioactivity determinations. Immediately after sampling, flasks were aerated for 30 sec by light sparging with 30 percent oxygen in nitrogen, fresh 0.5 N KOH was added, and incubation was continued. Controls consisted of water samples autoclaved prior to the addition of guanidinium. Percentages of guanidinium converted to carbon dioxide were determined from levels of trapped radioactivity in each flask, less abiotic background, and the measured amount of total carbon-14 guanidinium added. Cumulative percentages of guanidinium converted to carbon dioxide were then scaled for plotting by multiplying them by the original guanidinium concentration for the same flask.

Heterotrophic potential studies were conducted as described by Pfaender and Bartholomew with slight modification.[21] Each concentration of labeled guanidinium was tested in duplicate or triplicate by adding 100 mL samples of surface water (Monocacy River) to the substrate in three sets of 250 mL Erlenmeyer flasks. One set of flasks was sealed with rubber stoppers for measurement of substrate uptake by filtration. The remaining sets were sealed with rubber stoppers fitted with canulas and suspended traps containing 16-mm diameter filter papers (Whatman No. 1) to which was added 0.15 mL of 1 N KOH. One set of trapping flasks was amended with 0.1 percent $HgCl_2$ to measure abiotic uptake and carbon dioxide evolution. Following incubation, water in the first set of flasks for each concentration was filtered (47 mm, GS, Millipore), and the filters were washed with 50 mL of 50 percent ethanol and counted for isotopic uptake. The remaining flasks were each acidified by adding 0.15 mL 2N H_2SO_4 (final pH 2.3) through the canula, and incubation w..

continued for an additional 6 hours. Filter papers from traps were then removed and counted for respired isotope, and samples that had been amended with $HgCl_2$ were filtered and counted as before. Respiration data were corrected to 100 percent with the aid of trapping efficiency values from triplicate flasks containing 100 mL surface water and 0.1 μCi $NaH^{14}CO_3$ that had had the same treatment as the experimental flasks. The metabolism of guanidinium and urea were compared by use of 22 mCi/mM guanidinium hydrochloride and 9.8 mCi/mM urea (New England Nuclear) at equimolar concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0 nM/l and 4 hours incubation. Metabolic velocities (v) were calculated from the averaged data, less abiotic background, by means of Equation 1 below, in which T is the time in hours, and C is the concentration of substrate added:

$$v = \frac{C}{T} \left(\frac{\text{dpm uptake + dpm respired}}{\text{dpm subtrate added}} \right) \quad (1)$$

MOST PROBABLE NUMBER ESTIMATIONS

Most probable numbers (MPN) were measured for organisms capable of degrading guanidine nitrate alone or in the presence of glucose. Serial tenfold dilutions of local surface waters (Monocacy River and Carroll Creek) were incubated with 20 μg/mL guanidine nitrate (11 μg/mL guanidinium) alone or together with 500 μg/mL glucose in quintuplicate 10-mL final volumes (80 percent surface water dilutions, 20 percent sterile distilled water containing chemical additions including final 0.2 percent potassium phosphate, pH 7). Following 25°C incubation periods of 48 hours for tubes containing glucose and guanidinium or 7 weeks for guanidinium alone, the contents of each tube were centrifuged (10,000 XG, 10 min) and assayed for spectrofluorometrically guanidinium levels. Most probable numbers for tubes showing 100 percent and 40 percent or more guanidinium decreases were determined from statistical tables as previously described by Alexander.[22] The total bacteria in the water samples were measured by triplicate plating on SMA.

KINETICS OF GUANIDINIUM BIODEGRADATION BY ENRICHMENT MICROORGANISMS

Kinetic parameters for enrichment microorganisms developed from natural waters were determined by the graphical procedure, equations, and nomenclature of Stratton and McCarty.[23] Substrate utilization curves were generated at six different guanidinium concentrations (added as 56 percent guanidine nitrate) in flasks inoculated with identical seed of 2 percent (v/v) of a fully grown enrichment culture. Samples were removed throughout the course of the experiment and analyzed for guanidinium by spectrofluorometry. Following the complete disappearance of guanidinium from each flask, ATP levels in 100 mL samples were measured for yield estimations. The numerical value of the half velocity coefficient (K_s) was calculated by use of an incremental decrease of 11 μg/mL substrate (S) for two of the utilization curves, the instantaneous rates of change at points "m" and "n" on each of the two curves at the increment, and Equation 2:

$$K_s = \frac{dS_n/dt - dS_m/dt}{[(dS_n/dt)/S_n] - [(dS_m/dt)/S_m]} \quad (2)$$

Substrate utilization rate constants (k) were then determined by use of the calculated value of K_s, the growth yield coefficient (a) based on ATP determinations, and points "p" and "q" on each of the six substrate utilization curves with the aid of Equation 3:

$$k = \frac{(dS_p/dt)\left[\frac{K_s+S_p}{S_p}\right] - (dS_q/dt)\left[\frac{K_s+S_q}{S_q}\right]}{a(S_p-S_q)} \qquad (3)$$

RESULTS

BIODEGRADATION SCREENING

The effects of microorganisms from Hansen Creek (SFAAP) on guanidinium are shown in Figure 1. With no additional carbon source, little if any decrease in guanidinium was detectable for the first 11 days of incubation, after which time its disappearance became linear, and it could no longer be detected by the 20th day. Results for an identical water sample incubated with the amino acid arginine are also shown in Figure 1 (insert). A slight disappearance of the amino acid could be detected within 5 hours of its addition, and its disappearance was 90 percent complete within 24 hours. Decreases in guanidinium or arginine concentrations were not noted in controls consisting of sterilized water samples (Figure 1). Thus, while microorganisms mediated the disappearance of guanidinium from the environmental sample, the times required for the initiation of a rapid phase of degradation and complete degradation of guanidinium were considerably slower than required for a natural compound bearing the guanidine group. The results of Figure 1 also show that the addition of glucose to Hansen Creek water led to a much more rapid disappearance of guanidinium than in the sample that contained guanidine nitrate alone. In the presence of 500 µg/mL glucose, a substantial guanidinium decrease was detectable after the first day of incubation, and the cation decreased to below detectable levels by 2 days. In addition, turbidity increases were observed in flasks containing glucose and guanidine nitrate and suggests that guanidine nitrate stimulates microbial growth in the presence of glucose.

The results of degradation studies conducted with eight different water samples from six sites, including Hansen and Kill Creeks, at SFAAP, are summarized in Table 1. In all cases tested, the patterns for guanidinium and arginine disappearances (without an added carbon source) were similar to those of Figure 1, although times required for microbial activity on guanidinium were variable. Significant decreases in guanidinium were not detectable during an initial period of from 11 to 52 days, after which time its concentration decreased to below detectable levels in from 6 to 15 days. Thus, microorganisms capable of degrading guanidinium can be generalized to a diversity of geographical sites, but in all cases, its disappearance was considerably slower than that of the amino acid arginine. From the data of Table 1, neither the time required for detectable guanidinium decreases nor intervals required for complete degradation uniformly correlate with initial bacterial levels. Thus, the fraction of natural populations capable of degrading the cation can vary from site to site and from time to time at a given site. Periods prior to significant degradation further suggest that the initial number of such organisms is small, even in water samples that resulted in the most rapid degradation of guanidinium (Kill Creek and Hansen Creek).

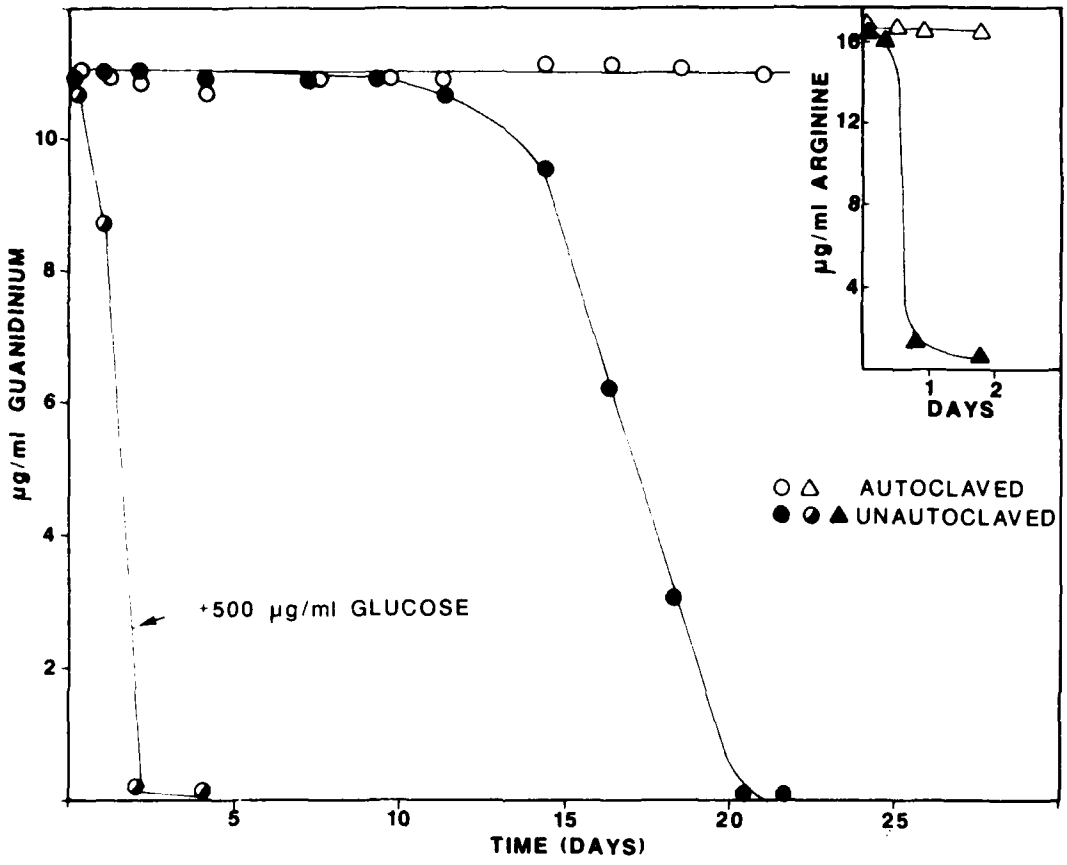

Figure 1. Incubation of guanidinium with Hansen Creek water.

TABLE 1. SUMMARY OF GUANIDINIUM AND ARGININE MICROBIAL TRANSFORMATIONS IN SURFACE WATERS

Site	Compound*	Days Prior to Detectable Disappearance	Days for Complete Disappearance	Initial Bacterial Titer (CFU/mL)
Carroll Creek West of Frederick, MD (Dec 83)	Arginine	<1	<2	2.4×10^4
	Guanidine nitrate	52	68	2.4×10^4
Carroll Creek Central Frederick, MD (Dec 83)	Arginine	<1	<2	7×10^4
	Guanidine nitrate	28	34	7×10^4
Monocacy River North of Frederick, MD (Dec 83)	Arginine	<1	<2	1.9×10^5
	Guanidine nitrate	22	30	1.9×10^5
Monocacy River South of Frederick, MD (Dec 83)	Arginine	<1	<2	2×10^5
	Guanidine nitrate	20	28	2×10^5
Kill Creek SFAAP, KS (Jan 84)	Arginine	<1	<2	1.2×10^5
	Guanidine nitrate	16	26	1.2×10^5
Hansen's Creek SFAAP, KS (Jan 84)	Arginine	<1	<2	1.3×10^5
	Guanidine nitrate	11	20	1.3×10^5
Carroll Creek Central Frederick, MD (Jul 84)	Guanidine nitrate	50	65	2×10^5
Monocacy River South of Frederick, MD (Jul 84)	Guanidine nitrate	40	52	3×10^5

* Guanidinium was 11 µg/mL and arginine was 17 µg/mL.

GUANIDINIUM AS A NITROGEN SOURCE FOR MICROBIAL GROWTH

To test the possibility that guanidinium was being used as a nitrogen source for growth in the presence of a carbon source, Hansen Creek water samples were diluted to deplete nutrients as described in Methods and were then amended with 500 µg/mL glucose and equal molar concentrations (0.16 mM) of guanidine nitrate, guanidine hydrochloride, urea, or ammonium chloride. Controls consisted of water samples which contained glucose and no added nitrogen compound or the nitrogen compounds alone. Results are shown in Figure 2. Only samples to which both glucose and the nitrogen compounds were added showed growth. Since no other nitrogen was added in the case of guanidine hydrochloride, guanidinium was utilized as a nitrogen source for microbial growth. Moreover, peak growth ratios measured by turbidity increases relative to ammonium ion were: 1-ammonium chloride, 2.5-urea, 3.5-guanidine hydrochloride, and 4.2-guanidine nitrate. When corrected for their molar content of nitrogen, the ratios are 1/1.25/1.2/1.1 respectively for the four compounds. Thus guanidinium is at least as effective in promoting microbial growth as a nitrogen source as is ammonium ion and is nearly as effective as urea. Results for guanidine chloride like those shown in Figure 2 could be obtained from all source waters listed in Table 1; thus, organisms capable of using guanidinium as a nitrogen source are widespread in their distribution.

The degree to which environmental microorganisms degrade guanidinium in the presence of glucose was measured for Hansen Creek, Kill Creek, and Monocacy River populations as described in Methods. Results for the three populations are shown in Table 2. Levels of growth equivalent to those of Figure 2 (guanidine nitrate) were accompanied by decreases in guanidinium to below levels of spectrofluorometric detection and by carbon-14 reductions of 93 percent or more. For the three populations, 90 percent or more of the labeled guanidinium carbon could be recovered as carbon dioxide following its metabolism. Conversion of total carbon to carbon dioxide, 99 percent of which was derived from added glucose, varied from 67 percent to 77 percent of the theoretical maximum for the three populations. Thus, the result of the metabolic process is the mineralization of most guanidinium carbon. Under conditions in which guanidinium will serve as a nitrogen source for growth, its carbon is more completely converted to carbon dioxide than is that of the carbon source glucose; and only 1 to 2% of the initial guanidinium carbon remains associated with washed microbial cells generated during growth (Table 2, cell pellet), suggesting different catabolic fates for the two.

BIODEGRADATION OF GUANIDINIUM BY ENRICHMENT MICROORGANISMS

Enrichment cultures capable of growing on guanidinium with no additional carbon source could be developed from all sites listed in Table 1, as described in Methods. At concentrations higher than 300 µg/mL guanidine nitrate (168 µg/mL guanidinium) growth rates were slower than at lower concentrations. Once established, enrichment cultures from all sites degraded guanidinium at approximately the same overall rates: 10 percent (v/v) inocula of the cultures completely degraded 168 µg/mL guanidinium in from 7 to 10 days. Enrichment microorganisms did not grow as uniformly turbid suspensions but appeared as clumps dispersed throughout the medium, which enlarged as guanidinium degradation proceeded. Because of their clumped nature, enumeration of the organisms by plate counting was variable, and ATP measurements were used for biomass estimations. Plating of the enrichment culture developed from Hansen

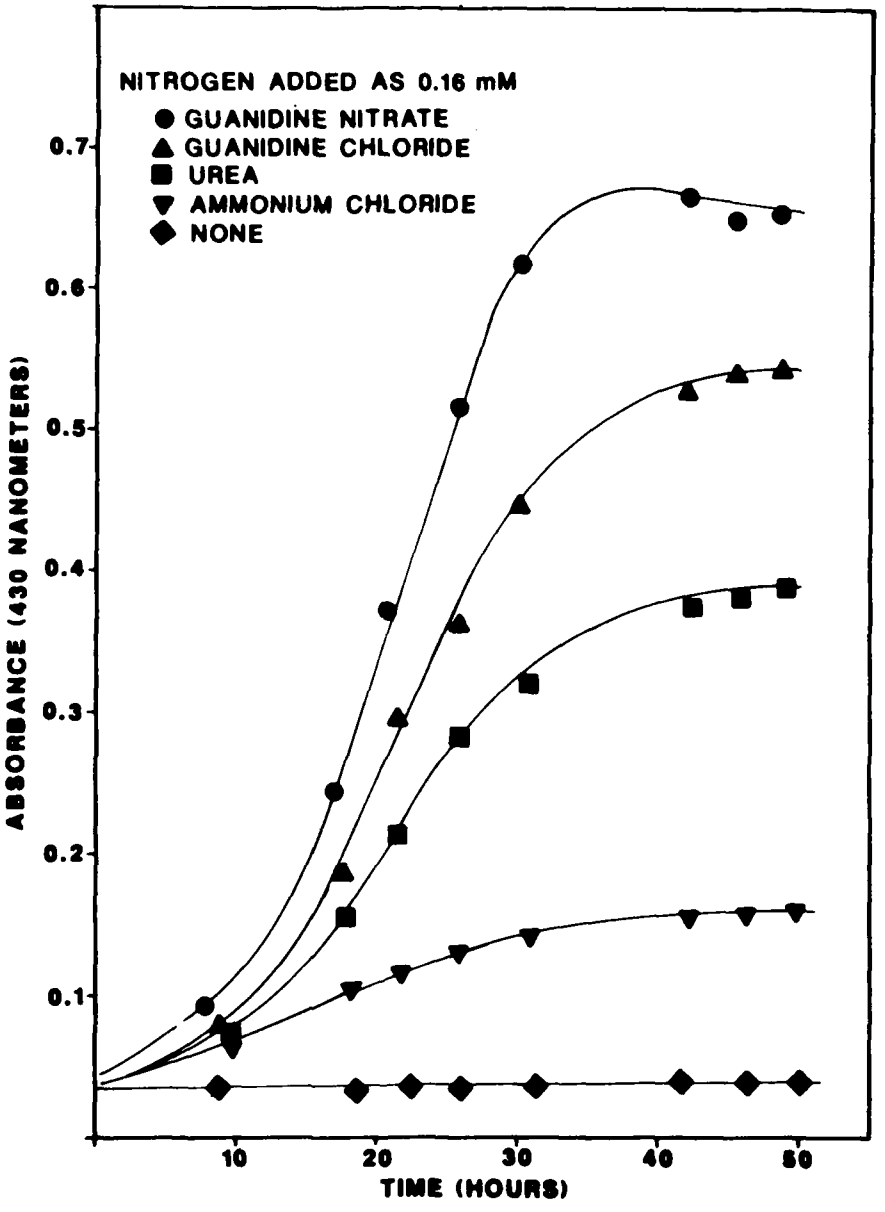

Figure 2. Microbial growth in the presence of 500 µg/mL glucose and various nitrogen compounds.

TABLE 2. FATE OF GUANIDINIUM CARBON IN THE PRESENCE OF THREE NATURAL MICROBIAL POPULATIONS AND 500 µg/mL GLUCOSE

	Hansen Creek		Kill Creek		Monocacy River	
	0 Hours	72 Hours	0 Hours	72 Hours	0 Hours	72 Hours
Absorbance (420 nm)	0.03	0.66	0.02	0.6	0.05	0.55
Guanidinium (µg/mL)	13	<0.5	12.6	<0.5	12.1	<0.5
Guanidinium Carbon-14[a]	2.14×10^6	0.138×10^6	2.05×10^6	0.15×10^6	2.07×10^6	0.14×10^6
supernatant	--	0.11×10^6	--	0.11×10^6	--	0.12×10^6
cell pellet	--	0.025×10^6	--	0.05×10^6	--	0.03×10^6
Trapped $^{14}CO_2$[b] (% maximum)	--	1.92×10^6 (89.7%)	--	1.96×10^6 (95.6%)	--	2.02×10^6 (97.5%)
Trapped Total CO_2 (mMoles)[c] (% maximum)	--	1.29 (77%)	--	1.12 (67%)	--	1.27 (76%)

a. Total DPM/100 mL culture based on triplicate 1 mL determinations ($1\delta < 5\%$).
b. Total DPM/10 mL trap based on triplicate 1 mL determinations ($1\delta < 5\%$).
c. Each flask initially contained glucose and guanidine carbon sufficient for the production of 1.68 mMoles CO_2.

Creek water (SMA) indicated the presence of a consortium of at least seven different bacterial strains based on differences in colony morphology. All were Gram negative rods.

To demonstrate that guanidinium will serve as a carbon source for microbial growth, Hansen Creek enrichment microorganisms were incubated in trapping flasks containing buffer with and without carbon-14 labeled guanidinium. A separate flask contained the substrate alone in buffer to measure background levels of radioactivity and bioluminescence. Results are shown in Table 3. Following the incubation period, guanidinium was no longer detectable in the inoculated flask and levels of carbon-14 decreased by more than 90 percent of the initial value. Carbon dioxide evolution accompanied guanidinium degradation, and 86 percent of the degraded guanidinium carbon could be accounted for as labeled carbon dioxide. Concomitant with the degradation of the cation, ATP levels were nine times that of the original inoculum. Neither significant increases in ATP and carbon dioxide nor decreases in guanidinium occurred in the controls. Carbon dioxide and ATP production accompanying guanidinium degradation indicate that the cation serves as a substrate for growth and that it is ultimately biodegradable. The conversion of most guanidinium carbon to carbon dioxide and the low ATP yield (0.041 µg ATP/mg guanidinium) suggest that it is a poor nutrient. Over the course of six similar determinations where ATP yields were measured immediately following the complete disappearance of from 14 to 168 µg/mL guanidinium, the average ATP yield for Hansen Creek microorganisms growing on the cation was 0.068 µg ATP/mg guanidinium degraded (1σ = 0.027). Based on this average and the reported range of ATP levels in bacteria,[19] yields of from 0.7×10^8 to 3×10^8 cells/mg guanidinium degraded can be estimated for Hansen Creek enrichment microorganisms growing on guanidinium.

The rate at which Hansen Creek enrichment microorganisms were capable of degrading guanidinium was estimated graphically as described by Stratton and McCarty.[23] Substrate utilization curves for the enrichment microorganisms at six different guanidinium concentrations are shown in Figure 3. Using Equation 2 (Methods) and the derivatives of the two lowest substrate utilization curves at points "n" and "m" (-0.159 and -0.143 mg/L respectively) at an integral substrate decrease (ΔS) of 11 mg/L, the half velocity coefficient (K_s) was calculated to be 0.33 mg/L. From this value of K_s and the average ATP yield of 0.068 µg ATP/mg guanidinium described above, utilization rate constants were calculated by means of Equation 3 (Methods) and the derivatives at points "p" and "q" in Figure 3. Kinetic parameters for the six substrate utilization curves and calculated values of the utilization rate constants for each curve are shown in Table 4. Rate utilization constants for the degradation of guanidinium showed no clear-cut concentration dependence and were averaged to be 0.14 (1σ=0.03) mg guanidinium degraded/hour-µg ATP. Again over the ranges of ATP concentrations reported for bacteria,[19] the average rate of guanidinium utilization can be estimated as from 0.3 to 1.4×10^{-10} mg guanidinium degraded/hour-cell. Utilization rate constants of approximately 5×10^{-10} mg/hour-cell have been reported for p-cresol and quinoline enrichment cultures and have been used to support the contention that the compounds will not persist in the environment.[24]

TABLE 3. ULTIMATE BIODEGRADATION OF GUANIDINIUM AS A SOLE CARBON SOURCE BY HANSEN CREEK ENRICHMENT MICROORGANISMS[a]

Test System	Day 0	Day 28
Enrichment Microorganisms plus Guanidinium		
Guanidinium (µg/mL)	165	<0.5
Guanidinium Carbon-14 (DPM/% total)	2.1×10^6/100%	1.9×10^5/9%
Trapped $^{14}CO_2$ (DPM/% total)	--	1.8×10^6/86%
ATP (µg/300 mL)	0.25	2.325
Enrichment Microorganisms plus Buffer		
Guanidinium (µg/mL)	<0.5	<0.5
ATP (µg/300 mL)	0.197	0.128
Buffer plus Guanidinium		
Guanidinium (µg/mL)	167	165
Guanidinium Carbon-14 (DPM/% total)	2.09×10^6 100%	2.2×10^6/100%
Trapped $^{14}CO_2$ (DPM/% total)	--	1.32×10^3/<0.1%
ATP (µg/300 mL	0.001	0.001

a. Values are averages of triplicate determinations for guanidinium and carbon-14 (1δ <5%). Quadruplicate bioluminescence determinations were made for ATP (1δ <10%). Measured carbon-14 and ATP levels were corrected to totals based on culture (300 mL) or trap (10 mL) volumes as described in Methods.

MOST PROBABLE NUMBERS OF GUANIDINIUM DEGRADING MICROORGANISMS

All water samples tested contained microorganisms capable of mediating the degradation of guanidinium slowly alone (Table 1) and rapidly in the presence of glucose (Table 2). To compare levels of indigenous guanidinium biodegrading microorganisms in natural waters, samples from the Monocacy River and Carroll Creek were diluted, amended with guanidine nitrate either alone or with glucose, and MPN estimates were conducted as described in Methods. Results are shown in Table 5. For both water sources, depending on whether complete (100 percent or partial (40 percent guanidinium biodegradation is used as an end point, microorganisms active on the cation in the presence of glucose are present in from 20- to 100-fold higher numbers than those capable of degrading it when added alone. Since both samples contained more than 10^5 CFU/mL total bacteria, microorganisms capable of significant guanidinium degradation, either alone or in the presence of glucose, constitute small fractions of the total natural populations. For microorganisms from both sites, MPNs for partial guanidinium degradation in the presence of glucose are from more than 10-fold (Carroll Creek) to 20-fold (Monocacy River) higher than MPNs for complete degradation. These differences likely indicate population heterogeneity in which the majority of microorganisms capable of transforming guanidinium in the presence of glucose do so at a slower rate than less numerous but more rapid degraders.

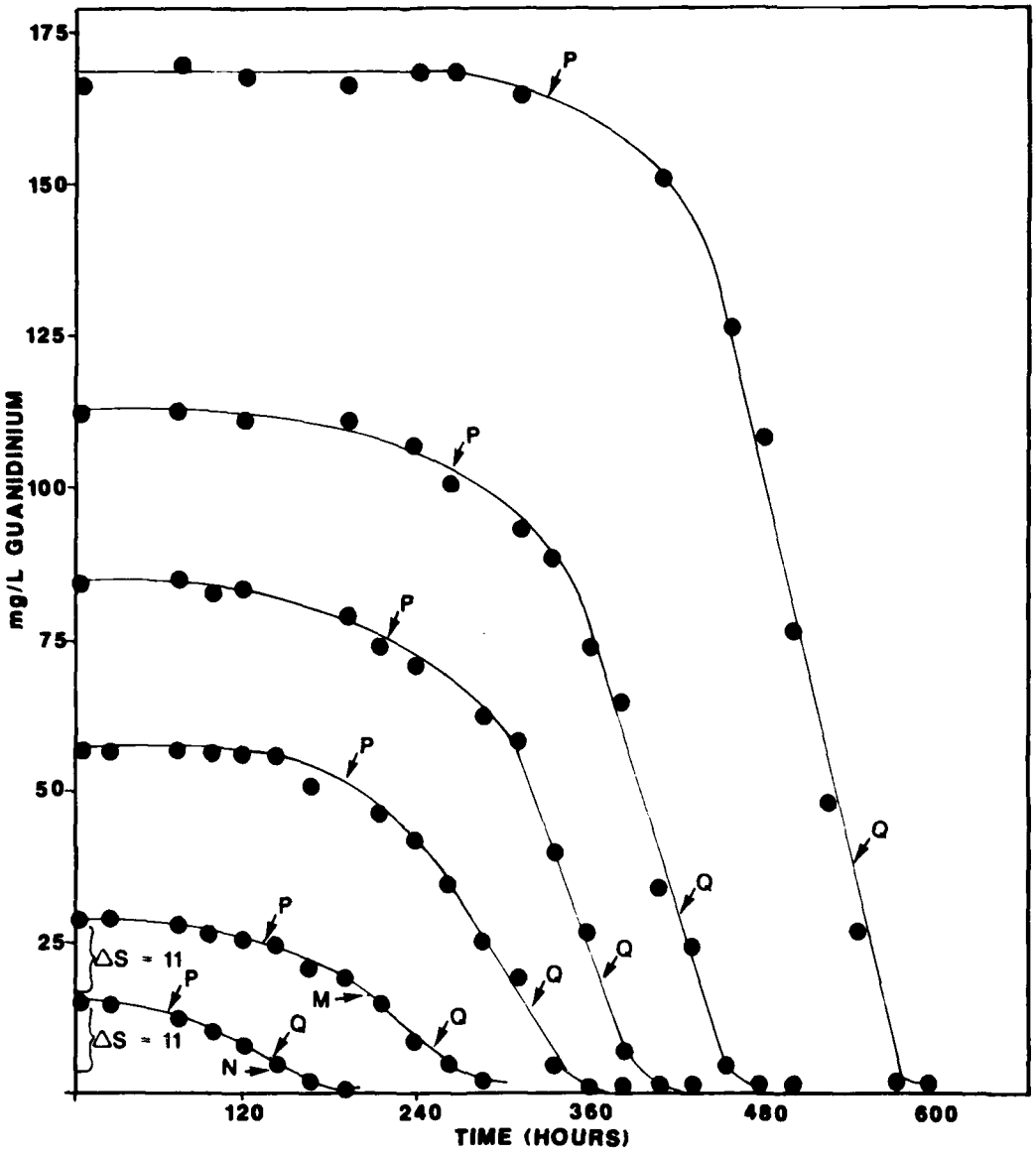

Figure 3. Guanidinium utilization curves for Hansen Creek enrichment microorganisms.

TABLE 4. KINETIC PARAMETERS FOR HANSEN CREEK ENRICHMENT MICROORGANISMS

Guanidinium[a] (mg/L)	S_p (mg/L)	dS_p/dt (mg/L-hour)	S_q (mg/L)	dS_q/dt (mg/L-hour)	Utilization Constant[b] (mg guanidinium degraded/hour-μg ATP)
14	13	-0.07	5.5	-0.15	0.17
28	24	-0.07	6.5	-0.25	0.16
56	50.5	-0.11	13	-0.39	0.12
84	75	-0.12	18	-0.67	0.15
112	102.5	-0.12	29	-0.75	0.13
168	162.5	-0.12	37.5	-0.97	0.10
					Average = 0.14 (1δ = 0.03)

a. Added as 25, 50, 100, 150, 200, and 300 mg/L guanidine nitrate.
b. Calculated from Equation 3 (Methods) using the tabulated data and the average yield coefficient 0.068 μg ATP/mg guanidinine degraded.

TABLE 5. MOST PROBABLE NUMBERS (MPN) OF GUANIDINIUM DEGRADING MICROORGANISMS IN TWO NATURAL POPULATIONS

% Degradation[a] Dilution	Carroll Creek		Monocacy River	
	Glucose Plus Guanidinium[b]	Guanidinium[c]	Glucose Plus Guanidinium[b]	Guanidinium[c]
100%				
10^0	5/5	3/5	5/5	1/5
10^{-1}	5/5	1/5	5/5	0/5
10^{-2}	0/5	0/5	0/5	0/5
$\frac{MPN^d}{8\ mL}$ =	23(76-7)	1.1(4-0.3)	23(76-7)	0.2(0.7-0.06)
40%				
10^0	5/5	5/5	5/5	1/5
10^{-1}	5/5	1/5	5/5	1/5
10^{-2}	4/5	0/5	5/5	0/5
10^{-3}	2/5	0/5	2/5	0/5
10^{-4}	1/5	-	0/5	-
10^{-5}	0/5	-	0/5	-
$\frac{MPN^d}{8\ mL}$ =	260(858-79)	3.3(10-1)	490(1620-148)	0.4(1.3-0.1)

a. Number of samples showing 100% or 40% degradation per five replicate samples.
b. 500 µg/mL glucose and 20 µg/mL guanidine nitrate (11.2 µg/mL guanidinium) for 48 hours incubation.
c. 20 µg/mL guanidine nitrate (11.2 µg/mL guanidinium) for 7 weeks incubation.
d. From most probable number tables for use with tenfold dilutions and five samples per dilution in reference 22. Confidence intervals of 95% are in parentheses.

METABOLISM OF GUANIDINIUM AT ENVIRONMENTAL CONCENTRATIONS

Although guanidinium degradation was not seen in environmental water samples following short periods of incubation without the addition of a carbon source, decreases of less than 0.5 µg/mL would have been difficult to detect by fluorescence spectrophotometry. Since natural nutrients and metabolically active microorganisms can be anticipated in fresh natural waters, at least some guanidinium degradation should occur after short periods of incubation by organisms capable of metabolizing it in the presence of the nutrients and by the few organisms present capable of using it as a carbon source for growth. Short term incubation (4 hour) heterotrophic potential studies were conducted to assess degradation at very low guanidinium levels and to compare the rate of degradation to that of the natural analogue urea. Figure 4 shows the rate of labeled substrate uptake, respiration, and the two combined for equal molar concentrations of guanidinium and urea in identical Monocacy River water samples (10^5 CFU/mL). Results confirm that short term guanidinium degradation does occur in environmental samples, albeit at an extremely low level. Less than 0.1 percent of the labeled guanidinium carbon at any of the five concentrations tested could be accounted for as respired and cell associated during the 4-hour incubation period. As can be seen in the figure, the rate of urea degradation was from 15 to more than 20 times faster than that of guanidinium, depending on whether total respiration and uptake or just respiration rates are compared. In the course of four determinations conducted over a period of 8 weeks with Monocacy River water, results for total guanidinium degradation like those in Figure 4 did not vary by more than 30 percent.

Microorganisms in a diversity of surface water samples are capable of enhanced guanidinium degradation without a carbon source following varying intervals of long term incubation (Table 1). To study the concentration dependence of guanidinium degradation, Monocacy River water samples ($3x10^5$ CFU/mL) were incubated with 10, 1.0, 0.1, 0.01, and 0.0005 µg/mL carbon-14 labeled guanidinium, and levels of radiolabeled carbon dioxide produced were measured. Because of the wide range of concentrations tested, results are plotted logarithmically in Figure 5. Levels of carbon dioxide production after 1 and 10 days did not exceed 1 and 5 percent respectively for any of the concentrations tested and likely correspond to the low level metabolism of guanidinium reported in Figure 4. At the three higher concentrations, inflections in carbon dioxide production occurred with continued incubation such that by the end of the experiment 85, 80, and 77 percent, respectively of the guanidinium carbon was converted to carbon dioxide. The same experiment conducted with water obtained at a second site, Carroll Creek ($2x10^5$ CFU mL), led to results almost identical with those of Figure 5. Thus the development of populations capable of enhanced guanidinium degradation is related to its concentration, and such populations do not develop following exposure to low levels of the pollutant.

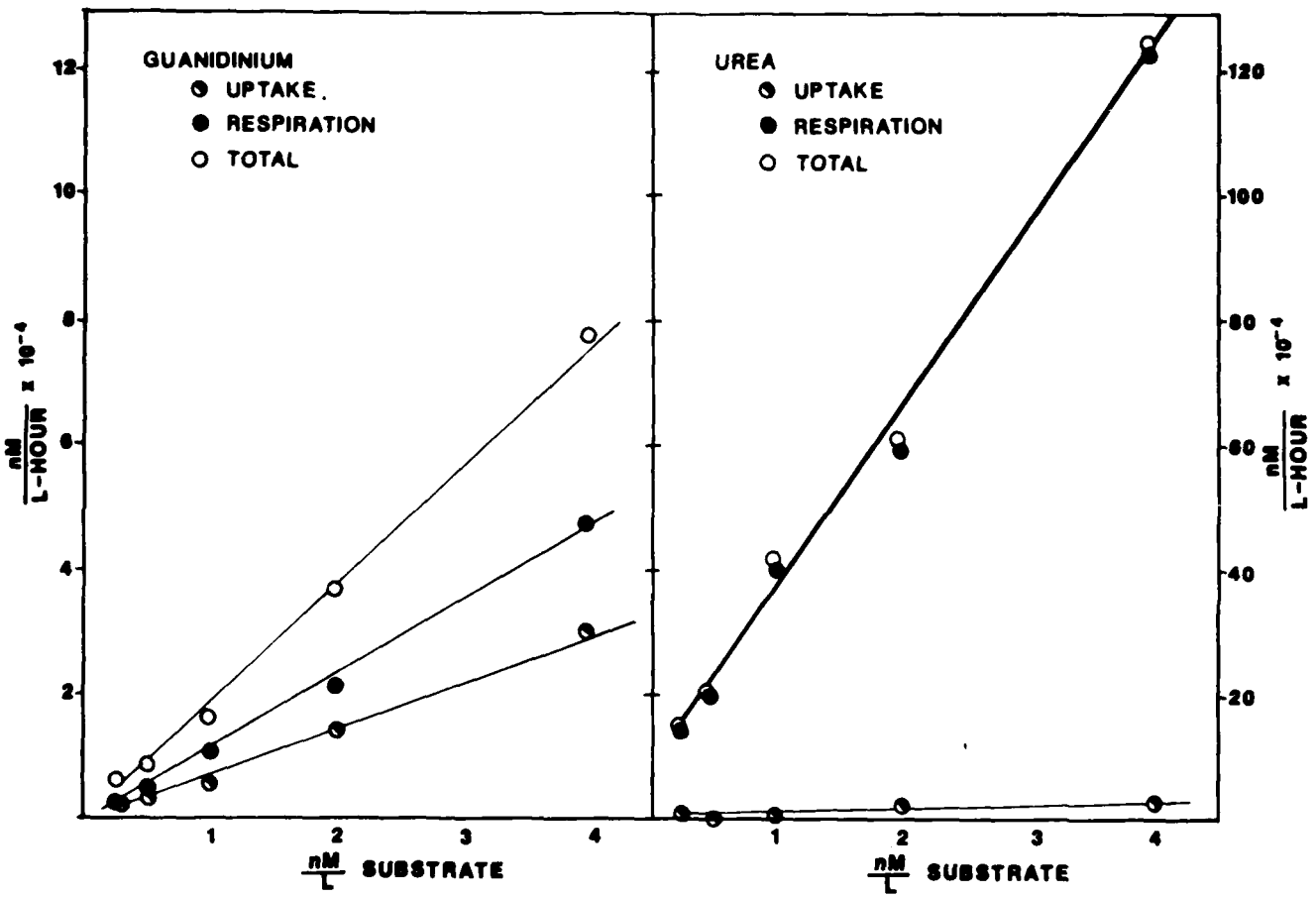

Figure 4. Short-term metabolism of guanidinium and urea by Monocacy River microorganisms.

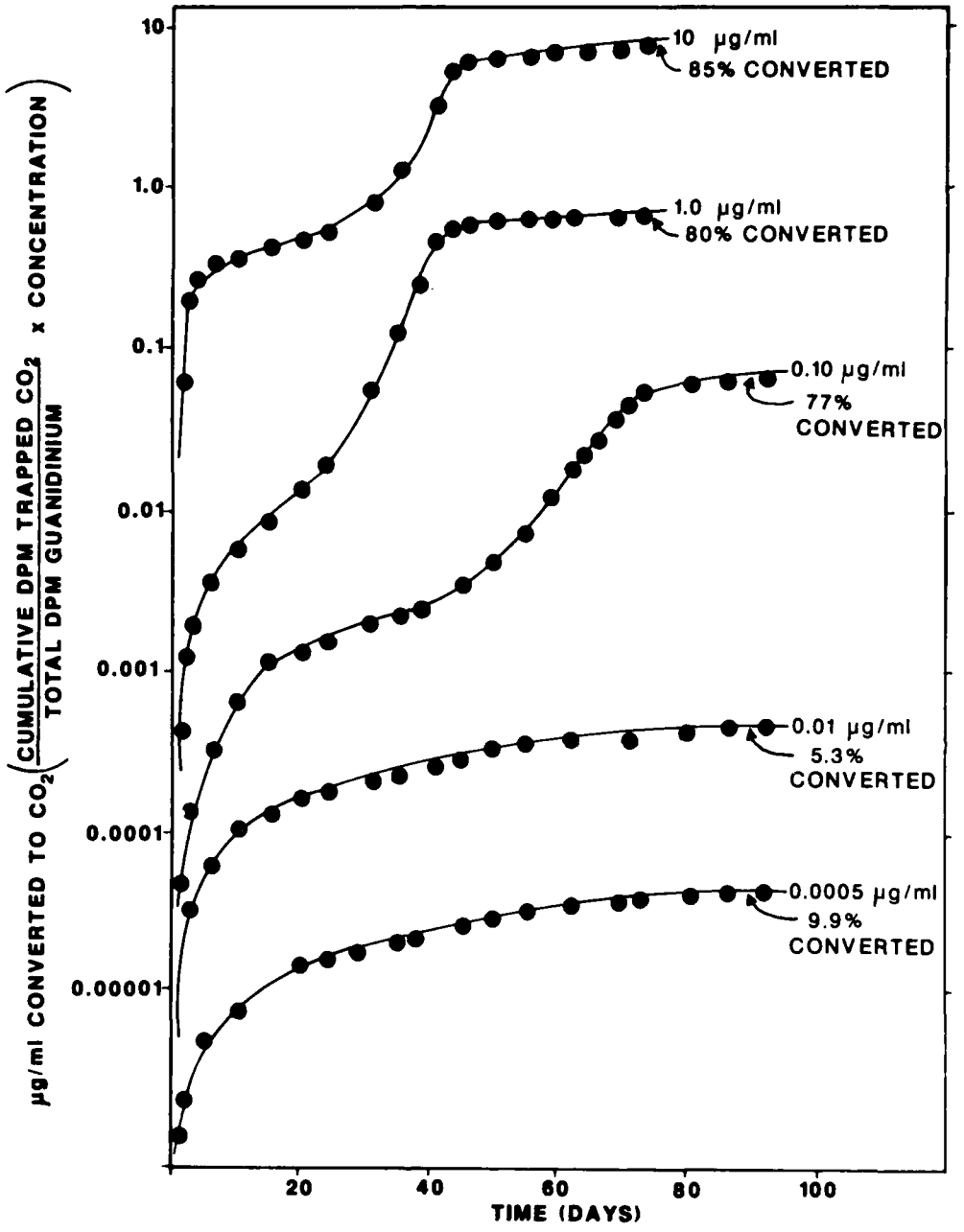

Figure 5. Formation of carbon dioxide from guanidinium in Monocacy River water.

DISCUSSION

Microorganisms in samples from the vicinity of SFAAP (Hansen Creek and Kill Creek), as well as in various local (Frederick, MD area) water samples, are capable of mediating the degradation of guanidinium following prolonged periods of incubation. Initial periods prior to detectable degradation indicate that such microorganisms, although widely distributed, are present in variable but low numbers. Most probable number estimations with local water samples support such a conclusion. Although still relatively long, lag times prior to enhanced guanidinium degradation tended to be shorter in waters obtained in the vicinity of SFAAP than in local samples, and could result from a slight adaption of those microbial populations to pilot facility discharges containing the cation. In contrast to guanidinium, the amino acid arginine was degraded in all samples tested without an apparent lag and was complete within 24 to 48 hours. Thus compared to results with a natural compound bearing a guanidine group, the microbially mediated degradation of guanidinium is extremely slow.

Microorganisms capable of growing on guanidinium as a sole added nutrient could be enriched from all natural water sites showing degradation. The continued maintenance of these microorganisms on guanidinium, as well as carbon dioxide evolution and ATP increases concomitant with its degradation shown for Hansen Creek microorganisms, are evidence that the disappearances described above are biodegradation rather than just microbial transformation. The biodegradability of guanidinium is confirmed by its mineralization at higher concentrations in local water samples. Thus, environmental microorganisms have the capability to adapt to and biodegrade guanidinium.

Cell yield estimates of from 0.7 to 3×10^8 bacteria per mg guanidinium degraded indicated that it is a relatively poor substrate for growth.[18] Microbial enrichment cultures developed from xenobiotic compounds such as p-cresol, quinoline, and malathion have all given yields of from nearly 10 (p-cresol and quinoline) to almost 300 (malathion) times higher than the upper value of the estimate.[24,25] From the estimated yields and kinetic data for Hansen Creek enrichment microorganisms, guanidinium can be degraded at a rate of from 0.3 to 1.4×10^{-10} mg/hour-cell. Faster rates of approximately 5×10^{-10} mg/hour-cell were reported for similar enrichment cultures developed with p-cresol and quinoline and were interpreted as indications that the compounds were readily metabolizable by microorganisms.[24] Like guanidinium, microbial degradation of p-cresol and quinoline could be demonstrated in a variety of natural waters. In contrast to guanidinium, lengthy lags prior to their degradation were not generally observed, indicating that populations capable of degrading them were either already present or developed rapidly after their addition. As reviewed by Alexander,[26] threshold concentrations exist for some xenobiotics below which acclimated populations needed for enhanced biodegradation do not develop. As demonstrated with local water samples, enhanced guanidinium biodegradation is concentration dependent. Its enhanced mineralization did not occur at or below 0.01 µg/mL and developed more slowly at 0.1 µg/mL than at higher concentrations. Thus, the rate described above for guanidinium degradation by enrichment microorganisms would be irrelevant for unadapted populations exposed to low levels of guanidinium. Taken together, lags prior to degradation, rate of degradation, and the concentration dependence of degradation suggest that guanidinium biodegradation will be slow compared to readily metabolizable xenobiotics.

In contrast to guanidinium biodegradation by microorganisms capable of growing on it, the addition of glucose to surface waters resulted in the rapid disappearance of the cation. As demonstrated in local water samples, organisms present capable of degrading guanidinium in the presence of glucose were in higher numbers than those which degraded it alone, but still constituted a small fraction of the total population. Under such conditions, guanidinium could serve as a nitrogen source for microbial growth, in which case it was very nearly as effective as the fertilizer urea. Moreover, the fate of most guanidinium carbon was the same as that reported for urea carbon following microbial action: it was converted to carbon dioxide.[12] Since rapid degradation was not detected spectrofluorometrically in environmental water samples without the added carbon source, the process is likely of little significance in removing high concentrations of guanidinium from surface waters. Such a conclusion is supported by sensitive heterotrophic potential studies with local water samples in which rapid guanidinium metabolism was detected, but resulted in extremely low levels of guanidinium degradation and was from 15 to 20 times slower than urea metabolism. However, the capability of microorganisms to biodegrade guanidinium in the presence of a carbon source could be of significance in environments such as soils and sediments where levels of organic matter and microorganisms are higher than in surface waters. The possibility also exists that the capability could be exploited in the development of a biological treatment technology for waste streams containing guanidinium.

SUMMARY AND CONCLUSION

I. Microorganisms are capable of adapting to and degrading guanidinium in environmental water samples.

II. The action of microorganisms on guanidinium results in its ultimate biodegradation.

III. Biodegradation of guanidinium is extremely slow compared to that of two natural compounds and slower than that of readily metabolizable xenobiotics.

IV. The development of microbial populations capable of enhanced guanidinium degradation did not occur at low concentrations.

V. A carbon source can facilitate rapid guanidinium metabolism and could increase the rate of its degradation in some microbial systems.

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