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Lack of Mutagenicity of Methanogenic Digester Effluent in the *Salmonella*/Microsome Test

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Energy is one of the most significant issues facing the world in the years ahead. For example, in 1980, the state of Florida consumed approximately 2.5 quadrillion BTu of primary energy and produced only 0.4 quadrillion BTu (State of Florida, Governor's Energy Office, 1981). This deficit situation between production and consumption has stimulated assessment of energy resources and their potential contributions to energy needs in the future. Among the several valuable resources studied is the use of animal waste for energy generation. Useable energy in the form of methane can be produced by anaerobic microbial digestion of manure from farms and feedlots.

Kaplan Industries (Bartow, FL) has initiated a large-scale waste utilization program to dispose 500 tons/day of manure (50 tons dry matter, at 10% solids) from a 20,000 head cattle feed lot. About 30% of the solid material in the manure is separated and recycled as an organic fertilizer. The liquid portion, with some suspended solids, is transferred to methane digesters (320,000 gallons or 2,100 cubic meters) which provide fuel in the form of methane for the adjacent slaughterhouse through the action of anaerobic methanogenic bacteria. Methanogenic digester effluent is centrifuged to yield a 25% protein product that can be used as an animal feed supplement; the liquid effluent is used for agricultural irrigation.

Studies on the use of municipal sewage sludge as fertilizer on farmlands have demonstrated that high levels of PCB's have been found in crops grown on PCB-contaminated sludge-amended soils. Animals fed these crops also showed high levels of PCB's in their tissues (Babish et al. 1979). In addition, increased blood levels of PCB's have been detected in gardeners using this sludge (Baker et al. 1980). Other toxic compounds such as nitrosamines have been shown to occur in municipal sewage in the presence of high concentrations of secondary amines and nitrite (Pancholy 1978). These N-nitroso compounds are highly toxic and carcinogenic to laboratory animals at mg/kg levels (Wolf & Wasser 1972). They are mutagenic to many *in vitro* assay systems, including the Ames *Salmonella*/mammalian microsome assay. Since similar reaction products, or their metabolites may be produced from heavy metals,

pesticide residues, antibiotics or residues from feed present in animal manure during anaerobic conversion of this biomass to methane and find their way into biological systems, groundwater and the environment, better assessment of the potential hazard of digester effluent is needed.

The Ames Salmonella/microsome assay for mutagenicity has been widely applied in in vitro tests for potential carcinogenicity and mutagenicity of environmental chemicals or mixtures (Ames 1979). In this study, we used the three most sensitive strains of Salmonella typhimurium, i.e., TA 97, TA 98 and TA 100, to evaluate the possible presence of genotoxic compounds in the methanogenic digester effluent. Strain TA 97 is a new tester strain recently developed by Levin et al. (1983). Its mutagenic specificity has been found to be similar to that of the frameshift mutagen tester strain TA 1537 but with higher sensitivity. It is thus suggested that TA 1537 be replaced by TA 97 for general screening of mutagenicity.

MATERIALS AND METHODS

Three samples were collected in July 1982, from Kaplan Industries located in Bartow, FL. Samples included raw cattle manure and mesophilic and thermophilic digester effluents. Raw manure was sampled from a valve in the loading line to the digester opened momentarily. The digester tank was loaded at a rate of 1200ℓ/hr. For the month of July 1982, the manure averaged 5% solids. Dilution is common during the summer due to both incident rainfall and increased ingestion of water by the cattle in the feed lot. The cattle were on a ration consisting of 90% wheat and 10% corn silage with a protein level of 12%.

Digester effluent was sampled from ports in the digesters. The contents of the digesters were agitated continuously. Operating parameters of the digesters were as follows:

	Mesophilic	Thermophic
Temperature	38°C	45°C
pH	7.1	7.3
Retention time	11 days	12 days
Alkalinity (CO_3)	6200 ppm	6500 ppm
Volatile acidity	4700 ppm	2500 ppm
Dry matter	2.6%	2.7%
N (dry)	1.0-1.5	1.0-1.5
P (dry)	1.0-1.7	1.0-1.7

The samples were collected in Erlenmeyer flasks precleaned with acetone and deionized water, kept in Gas Pak anerobic system (BBL, Cockeysville, MD), and transported back to the laboratory at Gainesville for testing. The samples were stored at approximately 2°C for 2 days before they were subjected to extraction with dichloromethane. This extraction procedure has been widely used to extract mutagens from complex mixtures, such as municipal

sewage sludge and diesel particles (Babish et al. 1983; King et al. 1981). After an aliquot of the sample was extracted 5 times in a separatory funnel with equal volume of dichloromethane; the five filtrates were combined, concentrated under reduced pressure, and transferred to preweighed vials. The concentrated samples were then blown dry with a stream of nitrogen gas, dissolved and serially diluted with spectrophotometric grade dimethyl sulfoxide (DMSO, Schwartz/Mann, Orangeburg, NY). Samples were refrigerated until tested.

Mutagenicity was tested by plate incorporation assay and preincubation assay of the Salmonella/microsome test described by Ames et al. (1975) and Yahagi et al. (1977). Strains TA 97, TA98 and TA 100 were obtained from Dr. Bruce N. Ames, Biochemistry Department, University of California at Berkeley. The bacteria were maintained and their genotypic characteristics checked following the procedures outlined by Ames et al. (1975). When spontaneous mutability of these strains was checked, the number of spontaneous revertants fell within the range of spontaneous revertant number of each tester strain reported by deSerres & Shelby (1979) and Levin et al. (1983).

The plate incorporation assay was performed by adding to 2ml of top agar 0.1 ml of culture of S. typhimurium, 25 µl of the test solution and 0.5 ml of the 10% or 0% S-9 mix. The mixture was poured onto a bottom agar plate. Rat liver S-9 preparation was obtained from male Sprague-Dawley rats pretreated with Aroclor 1254 and the S-9 mix was prepared as outlined by Ames et al. (1975). In the 0% S-9 mix, rat liver was replaced by 0.25 M potassium phosphate buffer (pH 7.4). For the preincubation assay, 25 ml of the test solution and 0.5 ml of the 10% or 0% S-9 mix were added to test tubes containing 0.1 ml of culture of S. typhimurium. The mixture was preincubated at 37°C for 20 min in a water bath, mixed with 2 ml top agar at 45°C and poured onto a bottom agar plate. The plates were incubated at 37°C for 2 days before colonies were counted. Microscopic examination of the background bacterial lawn also was performed to check the bacterial toxicity of the compounds, i.e., a sparse bacterial lawn with pin-point colonies indicated toxic levels.

Known mutagens were tested concurrently to confirm the revertant properties of the tester strains used in each experiment (Ames et al. 1975; deSerres & Shelby 1979, Levin et al. 1983); 2-aminofluorene was used for all strains in plates containing S-9 mix. For those plates containing no S-9 mix, 2-nitrofluorene was used for TA 98, methylmethane-sulfonate for TA 100, and ICR-191 for TA 97.

RESULTS AND DISCUSSION

Mutagenic compounds are produced through microbial action in municipal sewage sludges. Teleford et al. (1982) demonstrated the presence of mutagenic activity in dichloromethane extracts of sludge from the Syracuse Ley Creek plant. In a survey study for

Sample	Test Concentration (µg/plate)	Number of Revertants/Plate[a]					
		TA 100		TA 98		TA 97	
		- (S9)	+ (S9)	- (S9)	+ (S9)	- (S9)	+ (S9)
SR		95±10	114±8	23±3	28±4	117±17	121±14
+ DMSO[b]		100±14	111±3	25±1	28±4	99±12	124±6
	1880	T[c]	92±5	T	33±4	T	T
Thermophilic	188	96±4	93±3	13±3	30±7	T	108±14
Digester Effluent	18.8	117±11	107±11	27±2	30±2	103±11	124±11
	1.88	104±13	109±16	24±4	30±5	124±10	123±10
	635	T	99±14	25±2	30±6	112±12	120±12
Mesophilic	63.5	101±7	111±6	31±6	32±3	104±17	124±10
Digester Effluent	6.35	99±14	108±18	28±3	31±4	100±8	123±13
	0.635	114±13	108±17	32±6	29±2	105±13	121±8
	3880	T	109±14	26±2	31±4	T	T
Raw Manure	388	111±13	113±15	24±3	29±2	111±16	95±12
	38.8	92±4	111±14	35±4	31±3	133±6	118±14
	3.88	99±4	98±11	30±4	32±5	129±13	127±10
2-Aminofluorene	5	-[d]	-	-	-	-	726±102
	2.5	-	318±31	-	538±21	-	-
2-Nitrofluorene	2.5	-	-	203±17	-	-	-
ICR 191	5	-	-	-	-	1548±57	-
Methylmethane sulfonate	0.25 µl	356±36	-	-	-	-	-

[a] Mean ± standard deviation from quadruplicate plates.

[b] SR = Spontaneous revertant colonies. DMSO = Dimethyl sulfoxide.

[c] T = Toxic.

[d] Analysis not performed.

Table 2. Mutagenicity of Dichloromethane Extracts of Methanogenic Digester Effluents in Preincubation Assay

Sample	Test Concentration (µg/plate)	Number of Revertants/Plate[a] TA 100 - (S9)	TA 100 + (S9)	TA 98 - (S9)	TA 98 + (S9)
SR		107±9	102±10	28±3	27±5
+ DMSO[b]		107±14	108±14	29±5	30±3
	1880	T[c]	T	T	T
Thermophilic	188	T	106±13	T	29±4
Digester Effluent	18.8	100±12	99±13	28±3	30±4
	1.88	104±17	108±8	29±3	29±6
	635	T	T	T	T
Mesophilic	63.5	93±7	96±6	31±5	32±2
Digester Effluent	6.35	115±15	116±10	27±2	31±3
	0.635	122±4	110±13	29±1	29±3
	3880	T	T	T	T
Raw Manure	388	92±3	98±17	T	33±3
	38.8	95±8	114±11	29±5	30±2
	3.88	96±11	116±9	28±4	29±5
2-Aminofluorene	2.5	-[d]	342±23	-	331±23
2-Nitrofluorene	2.5	-	-	345±42	-
Methylmethane sulfonate	0.25 µl	421±32	-	-	-

[a]Mean ± standard deviation from quadruplicate plates.

[b]SR = Spontaneous revertant colonies. DMSO = Dimethyl sulfoxide.

[c]T= Toxic.

[d]Analysis not performed.

mutagenicity of municipal sewage sludges from 34 American cities, Babish et al. (1983) found mutagenic activity in 33 samples. This mutagenic activity can migrate from the sludge to vegetables grown in the sludge-treated soil and finally to the urine of rats or sheep fed the vegetables (Boyd et al. 1982; Teleford et al. 1982). It is, therefore, of obvious concern to assess the mutagenic activity of methanogenic digester effluent which can also be used for agricultural purposes.

Our mutagenicity studies of the raw cattle manure and the mesophilic and thermophilic digester effluents showed that none of three dichloromethane extracts showed any mutagenic activity toward any of the three tester strains (TA 97, TA 98 and TA 100) with or without a metabolic activation system in either the plate incorporation or preincubation assay systems (Tables 1 & 2). Thus, bacterial mutagens were not present in raw cattle manure nor were they produced during the methanogenic fermentation process.

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