ASSESSMENT OF THE MICROTOX BIOASSAY AS A PREDICTOR FOR ANAEROBIC BACTERIAL TOXICITY

A Thesis Presented

by

DORIS SHEPHERD ATKINSON

Submitted to the Graduate School of the

University of Massachusetts in partial fulfillment

of the requirements for the degree of

MASTER OF SCIENCE

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ABSTRACT

This thesis addresses the following two questions: 1) can Microtox, a rapid biological toxicity assay, be used to predict toxicity to anaerobic methane producing bacteria, and 2) can Microtox be used to evaluate toxicity removal by anaerobic degredation. To answer these questions, literature and laboratory data from Microtox testing and anaerobic toxicity testing were compiled. Microtox tests were performed according to the manufacturer's directions, and toxicity to methanogens was evaluated using the anaerobic toxicity assay (ATA) developed by Owen et al. (1979). Additionally, data on hydrogen and carbon monoxide accumulation in toxified anaerobic reactors were used as indicators of anaerobic toxicity. The results showed no overall correlation between Microtox data and ATA data. There was also no correlation between Microtox and hydrogen and carbon monoxide accumulations. Laboratory studies on the use of Microtox to evaluate toxicity removal were discontinued due to high background toxicities. Microtox therefore does not appear to be useful as a predictor of anaerobic toxicity nor would it be useful as a tool to evaluate toxicity removal by anaerobic treatment.

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CHAPTER 1 - INTRODUCTION

Recently anaerobic treatment processes have been receiving increased attention owing to their advantages over aerobic biological treatment processes. These advantages include the production of smaller amounts of waste sludge and the production of methane gas as a useful by-product.

Anaerobic treatment processes convert complex organic material to carbon dioxide and methane in the absence of molecular oxygen. This process occurs through a series of biologically mediated steps, each involving different groups of microorganisms. The fundemental microbiological relationships have been investigated and reviewed by a number of authors (McCarty, 1964; Balch, 1979; Taylor, 1982). In the first step, polysaccarides are broken down to short chain fatty acids, acetate, carbon dioxide and hydrogen by fermentative bacteria. Another group of organisms, the acetogenic bacteria, break down fatty acids to acetate, carbon dioxide and hydrogen. The methanogenic bacteria then convert hydrogen and carbon dioxide to methane and also convert acetate to methane and carbon dioxide. The methanogens are generally slow growing organisms and represent the rate limiting step in anaerobic digestion.

There are a number of different process configurations used in anaerobic treatment, and these have been reviewed in the literature

(Speece, 1983; Switzenbum, 1983). Some of the types include: completely mixed systems, packed bed reactors, anaerobic filters, upflow anaerobic sludge blankets, and expanded or fluidized bed reactors. Recent developments in process technology have focused on techniques which minimize hydraulic retention times while maximizing solids retention times. This serves to provide the residence time necessary for the slow growing methanogens while reducing reactor volume.

One concern about the use of anaerobic treatment methods is the reliability of processes for wastes which may contain substances toxic to the methanogenic microorganisms. Another concern is the possible adverse or synergistic effects toxicants may have in combination with other toxicants. Anaerobic reactors which receive wastes containing more than one toxicant may experience upset conditions when each toxicant by itself may not cause significant toxicity.

Another interest in anaerobic treatment is as a biological treatment alternative for the purpose of toxicity removal. While anaerobic treatment processes are often thought of as a means to reduce levels of organic material, they also offer the opprtunity for biodegradation of toxic compounds, and there is a growing body of literature on toxicity reduction through anaerobic treatment (Healy and Young, 1979; Bouwer and McCarty, 1983; Boyd et al.,1983; Speece, 1983).

In response to the concern over the toxic effects of wastes on anaerobic treatment processes, bioassay methods for measuring toxicity

have been developed. The anaerobic toxicty assay (ATA) was initially developed by Owen et al. (1979) and is based on techniques developed by Hungate (1969) which were modified by Miller and Wolin (1974). It is currently one of the more widely used methods of determining anaerobic toxicity. The ATA is a batch method which measures the adverse effect of a substance or mixture on the rate of methane production from an easily degraded methanogenic substrate. While this method is simple and relatively inexpensive, it is time consuming, requiring up to two weeks before results are available.

Another important development in the monitoring of toxic conditions in anaerobic treatment processes has been the measurement of key intermediate gases. Hydrogen and carbon monoxide may accumulate in toxified reactors, and monitoring of these gases has been proposed as a basis for an early warning system for anaerobic digester upsets (Hickey, 1987). While hydrogen and carbon monoxide measurement may prove useful as a process control technique, it would be useful to have a quick, inexpensive, screening test for wastes entering a treatment unit to determine likely toxicity before upset occurs.

The Microtox toxicity analyzer, developed by Beckman Instruments and currently marketed by Microbics Corporation of Carlsbad, CA., provides a means for rapid, inexpensive assessment of toxicity of aqueous samples. The Microtox system is a relatively inexpensive test which employs aerobic bioluminescent marine bacteria (Photobacterium phosphoreum) and can yield reproducible results within one hour. Good

correlations between Microtox and rat, fish, daphnid, shrimp, algal, and other aerobic bacterial bioassays have been reported in the literature (e.g. Dutka and Kwan, 1981; Curtis et al.,1982; Ribo and Kaiser, 1983).

The primary objectives of this research were:

1) to determine whether the Microtox system could be used as a suitable surrogate for the longer ATA test,
2) to evaluate the interactive toxic effects of several known toxicants with a complex waste,
3) to compare Microtox response to toxicants to hydrogen and carbon monoxide accumulations in toxified reactors,

and 4) to determine if Microtox could be used to measure toxicity reduction by anaerobic biological treatment.

The study was conducted in five phases. In the first phase the available literature on both anaerobic toxicity and Microtox testing was reviewed and the reported toxcity levels for chemicals tested by both sytems compared. In the second phase further laboratory tests using both toxicty methods were performed. A study of the interactive effects of toxicants and a complex waste was done in the third phase of the project. In the fourth phase, Microtox data were compared to hydrogen and carbon monoxide data, and in the last phase, the

feasibility of using Microtox to evaluate toxicity removal by anaerobic treatment was investigated.

CHAPTER 2 - BACKGROUND

2.1 ANAEROBIC TOXICITY TESTING

Wastes entering an anaerobic treatment unit may contain materials which are potentially toxic to the organisms responsible for anaerobic degradation. There are several different classes of materials or conditions which may cause digester upset. These include: pH, oxygen contamination, inorganic toxicants, organic toxicants, and specific biological inhibitors (Speece and Parkin, 1983). Some examples of inorganic chemicals which are toxic to methane producing bacteria are: ammonia, cyanide, sulfide, and heavy metals (Mosey and Hughes, 1975; Yang et al.,1980; Parkin et al.,1983). Potentially toxic organics include: chloroform, formaldehyde, various phenolics and petrochemicals (Chou et al.,1978; Yang et al.,1980; Parkin et al.,1983; Benjamin et al.,1984). Specific biological inhibitors such as methane analogues and agricultural antibiotics may also cause toxic effects in anaerobic reactors (Thiel, 1969; Sykes and Kirsh, 1972; Varrel and Hashimoto, 1982; Jarrel and Hamilton, 1985). While there have been some attempts to produce predictive models for anaerobic toxicity (Parkin and Speece, 1982), direct laboratory testing is still required to determine the toxic effects of any given waste.

Over the last twenty or so years a number of different approaches to anaerobic toxicity testing have been used. Tests may be conducted

under batch or continuous flow conditions. The organisms may be in suspended culture or attached. The culture used may be a mixed cuture from a reactor, may be a washed cell suspension prepared from reactor contents, or may be a pure culture isolated in the laboratory. Tests may be conducted with non-acclimated organisms or organisms which have had some previous exposure to the toxicant being evaluated and a variety of different substrates may be used as the food source. These test parameters as well as other important factors such as solids retention time, temperature, pH, osmolarity, the presence or absence of antagonistic or synergistic substances will all bear on the test results.

The anaerobic toxicity assay (ATA) is a batch test, which uses suspended, mixed methanogenic cultures obtained from reseach reactors (Owen et al., 1979). The organisms are fed acetate and propionate as an easily degraded methanogenic substrate. The toxic substance to be tested may be either a pure substance or a complex mixture. While the test was designed to be used with non-acclimated organisms, it can be adapted to acclimation studies. The test is usually run under quiescent conditions, but continuous stirring may be used by adding magnetic stirrer bars to individual serum bottles. The pH of the test environment is held at or near 7.2 by means of a bicarbonate buffer. The incubation temperature is 35 C. The parameter measured in the ATA test is either the rate of total gas production or the rate of methane production. The volume of gas produced is measured using a

lubricated glass syringe. The suggested means of reporting data is to present the maximum rate ratio (MRR) which is the maximum rate of gas production of the toxified sample normalized to that of a non-toxified control.

The ATA is a simple, inexpensive test, which has advantages over more complicated continuous toxicity tests. The ATA test is a practical test which has been used sucessfully by a number of investigators to test a variety of substances such as pulp mill wastes (Benjamin et al., 1984), various priority organic pollutants (Johnson and Young, 1983), and industrial toxicants (Parkin et al.,1983). However, it is a time consuming test (requiring up to two weeks before test results are available) and is therefore not as practical for applications such as influent monitoring.

2.2 HYDROGEN AND CARBON MONOXIDE MONITORING

There are three key steps in the degradation of complex organic wastes to methane and carbon dioxide. In the first step, fermentative bacteria break down polysaccarides to short chain fatty acids, carbon dioxide and hydrogen. In the second step the fatty acids are further broken down to acetate, carbon dioxide and hydrogen. Methanogenic bacteria carry out the third step, which is to covert both acetate and carbon dioxide and hydrogen to methane (McCarty, 1964 ; Balch et al.,1979). In a well operating system, hydrogen should not accumulate, but should be used to produce methane as rapidly as it is formed. Its

accumulation in a reactor is an indication of upset conditions (Hickey, 1987). Carbon monoxide has also been found to accumulate under certain types of upset conditions, especially those caused by the presence of toxicants (Hickey, 1987). Measurement of these gases can be used as an early warning of reactor upset conditons.

2.3 MICROTOX TESTING

The Microtox test was originally developed by Beckman Instruments, but is now marketed by Microbics Corporation of Carlsbad, California. The Microtox test is a quick, relatively inexpensive bioassay which uses the aerobic, bioluminescent marine bacterium Photobacterium phosphoreum. The light emitting biochemical pathway used by the Microtox organism is known to be an electron transport pathway, providing the organism with an alternative to cytochrome electron transport. This pathway is thought to provide the organism with an adaptive advantage under microaerophilic conditions (Hastings and Nealson,1977; Hastings et al., 1985).

The principle of the Microtox test is that the intensity of bioluminescence is diminished in response to exposure to toxicants. This response is generally linear over some range of toxicant concentration. The light output at specified temperature, pH, and salinity is easily measured with the Microtox analyzer, which contains incubation wells, temperature controls and a photomultiplier tube connected to a digital output display. The analyzer can also be

connected to a strip chart recorder for a permanent record of the test data. Once the bacteria have been prepared for the test procedure, the initial light level is measured, a toxic challenge added, and the light level measured again after a specified period of exposure. Generally the concentration of toxicant causing a 50% decrease in light output relative to a reagent blank is the reported result (Beckman, 1982).

The Microtox test is a versatile test, which may be used to evaluate almost any aqueous sample. The test is short (one to two hours) and requires no laboratory culturing of test organisms. (The manufacturer supplies standard cultures of lyophilized bacteria in easy to handle single test reagent vials). It also has good reproducibility, with a coefficient of variation of 15 to 20% (Bulich et al., 1981). The Microtox system previously has been used for effluent monitoring (Bulich et al., 1981; Peltier and Weber, 1980; Samak and Noiseux, 1980; Vasseur et al.,1984) as well as wastewater treatability monitoring (Slattery,1985), studies of toxicity removal in activated sludge treatment plants (Neiheisel et al.,1982), evaluation of fossil fuel process waters (Lebsack et al.,1981), landfill leachate studies (Sheehan et al.,1984) and sediment toxicity studies (Atkinson et al.,1985).

Good correlations between Microtox tests and a number of other bioassays using organisms from a wide variety of phylogenetic groups have been found. Chang et al. (1981) found correlation coefficients (r) of 0.9 and 1.0 between Microtox results and rat and fish LD50's

respectivily for typical organic toxicants. Curtis et al. (1982) and Indorato et al. (1983) found correlation coeficients between Microtox and different species of fish between 0.80 and 0.95. Looking at 20 chlorophenols, 12 chlorobenzenes and 13 para-substituted phenols, Ribo and Kaiser (1983) compared results of 7 different bioassays with Microtox results. Two bacterial assays, three fish assays, and Daphnia and shrimp assays were included. Correlation coefficients for these studies all fell between 0.82 and 0.96. No examples of Microtox comparisons to anaerobic systems were found in the literature.

2.4 COMPLEX EFFLUENTS

When a biological system encounters more than one toxicant at a time, the response to those toxicants may be greater than or less than the sum of the observed effects of the toxicants acting individually. When the effects are greater than additive effects, the toxicants are said to act synergistically, and when the effects are less than additive effects, they act antagonistically. Complex effluents, such as pulp mill wastes may contain a wide variety of toxicants. Wastes with multiple toxicants pose a treatment problem in predicting the response to those toxicants even when the response to individual components may be known.

2.5 TOXICITY REMOVAL

Anaerobic treatment as a means to degrade toxic compounds has

recently been receiving greater attention. Several papers have been published presenting methods for determining anaerobic biodegradation potential (Shelton and Tiedje, 1984 ; Healy and Young, 1979; Owen et al., 1979). In general, these tests rely on measurement of gas production from the mineralization of organic pollutants. It should not be assumed, however, that these tests necessarily measure removal of toxicity. Intermediates formed during biological degradation may be as toxic, or in some cases more toxic than the compound from which they are derived (Bower and McCarty, 1983; Vogel and McCarty, 1985). Thus, some form of biological evaluation of anaerobic toxicity removal is warranted.

CHAPTER 3 - EXPERIMENTAL METHODS

3.1 OVERVIEW

The study was divided into 5 phases. In the first phase the available literature was searched for information on the toxicity of pure compounds to anaerobic methane-producing bacteria and to the Microtox organism. During the second stage further data on the toxicity of pure compounds were obtained in the laboratory using the Microtox bioassay and the anaerobic toxicty assay (ATA) with media-fed seed. The third phase was to study the toxic effects of a complex effluent using ATA's with sludge-fed seed and to compare the response to toxic chemicals of this seed to that of the media-fed seed. In the fourth stage Microtox data were compared to data on hydrogen and carbon monoxide accumulations in toxified anaerobic reactors. During the fifth stage of the study the feasibility of using the Microtox bioassay as an indicator of toxicity reduction by anaerobic treatment was briefly investigated.

3.2 LITERATURE REVIEW

Available literature was searched for information on the toxicity of pure chemicals to both Microtox and to anaerobic methane-producing bacteria. Side by side alphabetical lists by chemical of Microtox data and methanogen data were compiled. An initial assessment of the data was made by determining which chemicals were more toxic to Microtox,

which were more toxic to methanogens and which were essentially equally toxic to both. For chemicals for which appropriate data were found, the concentration causing 50% inhibition of methane production was plotted against the concentration causing 50% inhibition in the Microtox bioassay, and statistical relationships determined.

3.3 PURE CHEMICAL TOXICITY

In this phase of the study, additional information for the comparison of toxicity of chemicals to the Microtox bioassay and *methanogens was obtained in the laboratory. Anaerobic toxicity assays* (ATA's) as developed by Owen et al. (1979), as well as Microtox bioassays as developed by Beckman Instruments, were performed. In order to maximize data for comparison, tests were done with chemicals for which literature information on one or the other bioassay was available. As more literature data were available for Microtox tests, this study focused more heavily on anaerobic toxicity assays. Testing was also done for selected chemicals to assure that toxicity values generated in this laboratory were comparable to those found in the literature.

3.3.1 Anaerobic Toxicity Assays

Anaerobic toxicity assays (ATA's) as developed and described by Owen et al. (1979) were performed to determine the toxicity of various chemicals to methane-producing bacteria. This toxicity assay is a

batch test in which the inhibition of methane production from a readily digested substrate due to a toxicant is measured. An inoculum, or seed, of bacteria from a methane-producing reactor is added to anaerobically sealed serum bottles containing medium, substrate and toxicant. The bottles are incubated, and the gas production monitored over a period of two weeks.

The anaerobic seed for this phase of the study was obtained from a 15 L mixed culture anaerobic research reactor operated at 20 days solid retention time (SRT). The reactor was fed a sucrose based medium, containing bicarbonate buffer, nutrient salts and yeast extract. One test was also made to compare the seed from the 20 day SRT reactor to seed from an 8 day SRT reactor maintained on the same feed. The initial inoculum for starting both the 20 day and 8 day SRT reactors came from a mixture of several sources, including a dairy manure digester, a sewage digester and research reactors. Both reactors had been operating for approximately 15 months prior to this study. Gas production from each reactor was approximately 50% methane and 50% carbon dioxide, and both were maintaned at or near pH 7.0. A preliminary test was made to determine that 30% seed was an appropriate seed concentration to use for the anaerobic toxicity assays.

The defined medium used for the ATA's contains vitamins, nutrient salts, bicarbonate, sodium sulfide as a reducing agent, and resazurin to detect oxygen contamination. The medium and the method of preparation were the same as those described by Owen et al. (1979) with

minor modifications. Table 3-1 lists the stock solutions used and Table 3-2 lists the method of medium preparation. The medium was prepared fresh at the beginning of each ATA run from stored stock solutions.

125 mL glass serum bottles (actual volume approximately 164 mL) were used as the test vessels. All glassware was detergent washed, rinsed 5 times in tap water, soaked 5-10 minutes in 10% HCl, rinsed 5 times in distilled water and oven dried. Serum bottles were pre-purged with nitrogen gas then purged with a mixture of 70% nitrogen and 30% carbon dioxide gas.

ATA medium and seed were transferred to the serum bottles anaerobically. Volumes of 35 mL medium and 15 mL (30%) seed were used. The anaerobic transfers were accomplished using one of two different set-ups. The first set-up is descibed by Owen et al. (1979), and is depicted in Figure 3-1. After initial gassing, medium is pipetted by opening and closing pinch valves V1 and V2, while flushing with gas. After filling, serum caps are inserted while similtaneously removing the gas flushing needle. This first set-up proved to be somewhat awkward, and a second set-up was devised which proved to be equally reliable. This set-up is depicted in figure 3-2. The draw tube of a manual repipettor is inserted into the medium or seed bottle. Purge lines are inserted into the bottle and onto the suction vent of the repipettor. The repipettor is set to the desired volume, and medium or seed is pipetted into the serum bottles while flushing with the gassing

TABLE 3-1 STOCK SOLUTIONS FOR ATA MEDIUM *

Solution	Compound	Concentration (g/L)
1	Resazurin	1.0
2	$(NH_4)_2HPO_4$	26.7
3	$CaCl_2 \cdot 2H_2O$	16.7
	NH_4Cl	26.6
	$MgCl_2 \cdot 6H_2O$	120.0
	KCl	86.7
	$MnCl_2 \cdot 4H_2O$	1.33
	$CoCl_2 \cdot 2H_2O$	2.0
	H_3BO_3	0.38
	$CuCl_2 \cdot 2H_2O$	0.18
	$Na_2MoO_4 \cdot 2H_2O$	0.17
	$ZnCl_2$	0.14
4	$FeCl_2 \cdot 4H_2O$	370
5	$Na_2S \cdot 9H_2O$	500
6	Biotin	0.02
	Folic acid	0.02
	Pyridoxine hydrochloride	0.1
	Riboflavin	0.05
	Thiamin	0.05
	Nicotinic acid	0.05
	Pantothenic acid	0.05
	B_{12}	0.001
	p-Aminobenzoic acid	0.05
	Thioctic acid	0.05

* adapted from Owen et al. 1979

TABLE 3-2 ATA MEDIUM PREPARATION *

Step	Instruction
1	Add 1 liter distilled water to 2 liter volumetric flask
2	Add the following: 1.8 mL solution 1 5.4 mL solution 2 27 mL solution 3
3	Add distilled water to 1,800 mL mark
4	Boil for 20 min while flushing with N_2 gas
5	Cool to room temperature (continue flushing with N_2)
6	Add the following: 1.8 mL solution 6 1.8 mL solution 4 1.8 mL solution 5
7	Change gas to 30% CO_2 :70% N_2
8	Add 8.4 g $NaHCO_3$ as powder
9	Continue flushing with 30% CO_2: 70% N_2 until dipensed into serum bottles.

* adapted from Owen et al. 1979

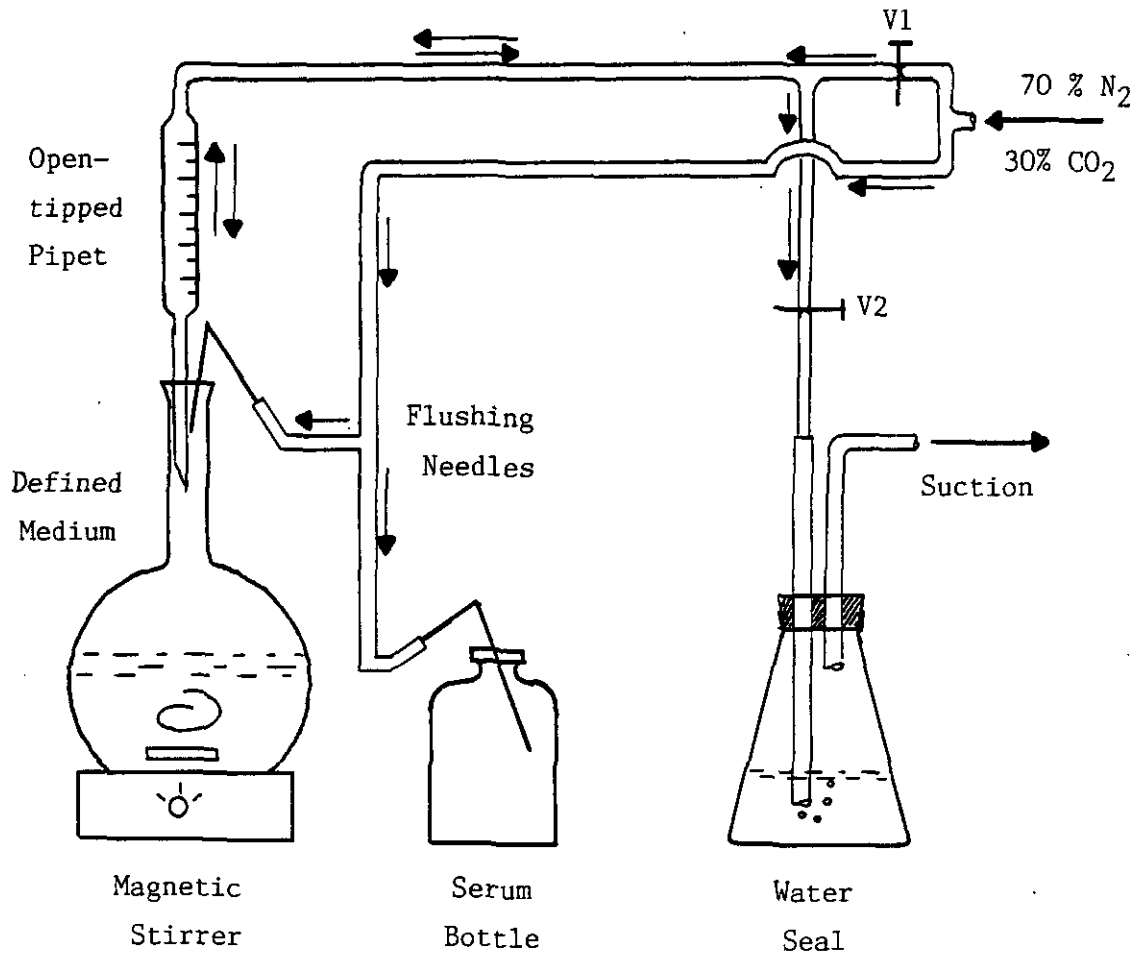

FIGURE 3-1 ANAEROBIC TRANSFER SET-UP A
(adapted from Owen et al.,1979)

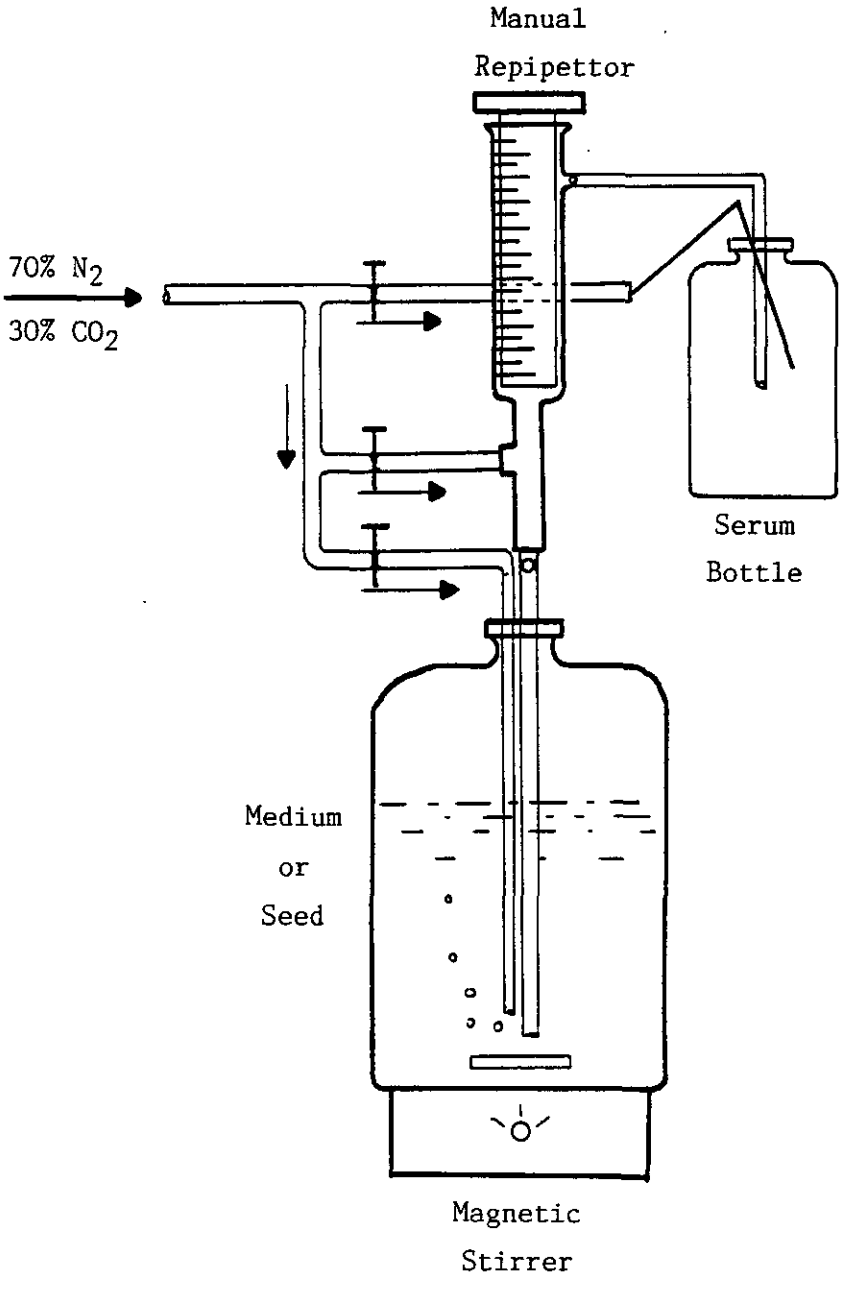

FIGURE 3-2 ANAEROBIC TRANSFER SET-UP B

needle. The serum bottles are then stoppered.

After filling with medium and seed, crimped aluminum seals were placed on the stoppered serum bottles, and the bottles allowed to equilibrate to 35° C in an incubator. After equilibration, a substrate spike and varying amounts of toxicant were added to the serum bottles via syringe. The substrate spike contained 75 mg sodium acetate and 26.5 mg propionic acid sodium salt in a 2 mL volume of distilled water. Toxicants were dissolved in distilled water, and varying volumes, up to 2 mL, were added to achieve the desired final toxicant concentrations. In general, controls containing substrate but no toxicant were run in triplicate, and duplicates of each toxicant concentration were made. Also, for each run, at least one control without substrate spike was prepared to assure that excessive methanogenic substrate was not carried over into the experiment from the anaerobic reactors.

Once the substrate spike and toxicant had been added, the head space pressure of each serum bottle was equilibrated to atmosphereic pressure through a well lubricated glass syringe. Gas inside the bottle is allowed to expand and push the plunger of the syringe outward until equilibrium is reached with atmosphereic pressure on the outside of the syringe. The syringe is then withdrawn and the gas within discarded. After zeroing, the serum bottles were incubated at 35° C +/- 1.5° C in a Thelco Precision incubator.

For each ATA run, total gas production as well as methane production was monitored on a periodic basis for up to two weeks.

Total gas production was measured using the glass syringe method. The percentage of methane in the head space gas at each sampling time was determined by gas chromatography. 1 mL samples of the head space gases were injected into a Gow Mac thermal conductivity gas chromatograph, using helium as the carrier gas and operating at a detector temperature of approximately 70° C. A calibrated Hewlett-Packard integrator, connected to the gas chromatograph, provided the output data of methane as a percentage of the injected sample. The incremental methane production was calculated as the measured percentage of methane times the volume of total gas produced plus the change in the stored volume of methane in the head space of the serum bottles. Cumulative total gas production and cumulative methane production for controls and each toxicant concentration were plotted, and the maximum rates of gas production at each concentration calculated. The ratio of the maximum rate of gas production to the maximum rate of gas production of the controls was calculated for each toxicant concentration. The maximum rate ratios (MRR's) were used to determine the concentration of toxicant causing 50% inhibition of the maximum rate of gas production relative to the controls (MRR = 0.5). These values were then compared to the concentrations of toxicant causing 50% inhibition in the Microtox bioassay.

3.3.2 Microtox Bioassays

Microtox toxicity assays were performed following the procedures

described by the manufacturer (Beckman, 1982). The Microtox system employs lyophilized aerobic marine bacteria which, upon reconstitution, emit light. The initial light level is measured; a toxic challenge is added and the percent light loss due to the toxicant is determined. Up to four concentrations may be tested at once.

Microtox reagent (lyophilized bacteria) supplied by the manufacturer, was reconstituted with a buffered saline reconstitution solution (also supplied by the manufacturer). The reconstituted bacteria were allowed to equilibrate for at least 15 minutes at the test temperature (15° C), after which 10 microliter aliquots were pipetted into 10 vials, each containing 0.5 mL of 2.2% saline dilution fluid. After 15-20 minutes the initial light level of each vial was measured and recorded. Then either 0.5 mL dilution fluid (blanks) or 0.5 mL of one of four serial dilutions of toxicant in dilution fluid was added to each of 2 of the 10 test vials. After 5 minutes and after 30 minutes exposure time the light level of each vial was again measured, and the percent light loss relative to the average of the reagent blanks was calculated. The percent light loss was plotted against toxicant concentration, and the concentration resulting in 50% light loss determined by interpolation. This value is referred to as the 5 minute or 30 minute 50% effective concentration (5EC50 or 30EC50).

3.4 COMPLEX EFFLUENT TOXICITY

The toxic effects of a complex effluent and its interactive effects with various toxicants were studied using anarobic toxicity assays as previously described. ATA's of a pulp mill waste (PMW) alone, PMW combined with various toxicants, and toxicants in distilled water were performed. A different seed inoculum was used in this phase of the study (sludge-fed seed), and comparisons of the toxicity of pure chemicals to this seed and to the medium-fed seed used earlier were made.

The seed used for this phase of the study came from a laboratory anaerobic sludge digester. This reactor was fed a mixture of waste activated sludge (WAS) brought to 1.5% total solids with dissolved air floats (DAF). The initial inoculum for this reactor came from a municipal anaerobic sludge digester. The laboratory reactor was operated at 20 days SRT, produced approximately 67% methane and 26% carbon dioxide, and had been in operation for approximately 18 months prior to this study. An initial study indicated that a 38% seed concentration would be appropriate for toxicity testing with the ATA.

The complex effluent used was obtained from the waste stream of an integrated pulp mill plant. The waste stream contained combined wastes from the main pulping and milling operations as well as waste from bleaching operations. Portions of the same effluent sample were used for each ATA run. A preliminary test of the PMW was made to determine an appropriate concentration to use for the combined chemical plus PMW toxicity tests. A final concentration of 3.8% PMW by volume was used

for each of the combined tests.

The inhibition caused by toxicant plus PMW was compared to that caused by toxicant alone and PMW alone. The interactive effects were determined by assessing whether toxicity due to the mixture was greater than or less than the toxicity predicted by simple additive effects of the PMW and the toxicant. Finally, toxicity of pure compounds to the sludge-fed seed was compared to toxicity to the medium-fed seed which had been determined earlier.

3.5 HYDROGEN AND CARBON MONOXIDE DATA

Certain toxic upset conditions in anarobic digesters are characterized by accumulations of hydrogen and carbon monoxide gas. In this phase of the study, Microtox data were compared to hydrogen and carbon monoxide accumulations in toxified reactors to determine if Microtox could be used as a predictor of these upset conditions.

Microtox data were taken from the literature and previously gathered laboratory results. Hydrogen and carbon monoxide data were taken from a study conducted at the University of Massachusetts, Amherst, in which toxicity assays similar to the ATA's described in this report were performd (Hickey, 1987). In these studies, waste activated sludge, seed from sludge-fed digesters and toxicant were combined in closed serum bottles, and hydrogen and carbon monoxide as well as methane and carbon dioxide measured over one day. Gas measurements were made with a trace analytical mecury reduction

detector gas chromatograph.

3.6 TOXICITY REDUCTION

The feasibility of using the Microtox bioassay as a measure of toxicity removal by anaerobic treatment was investigated. It was proposed to measure the toxicity of compounds to the Microtox organism both before and after treatment by anaerobic biodegradation in batch mode. Microtox bioassays were performed on the ATA defined medium and supernatant from both medium-fed and sludge-fed seed. Supernatant from the anaerobic seed souces was obtained by centrifugation in a table-top centrifuge for approximately 10 minutes. Dissolved oxygen measurements were made using an electronic dissolved oxygen probe in order to assure that the Microtox tests were not oxygen limited. Additionally, Microtox tests of air-purged supernatant from the sludge-fed seed and supernatant of the sludge used to feed this reactor were made. After reviewing the results of these initial tests, it was decided to discontinue work on this phase of the study.

CHAPTER 4 - RESULTS

4.1 LITERATURE REVIEW

Table 4-1 presents the results of the initial literature review. Literature data for both Microtox and anaerobe toxicity were found for a total of 39 chemicals. Of these, 24 (62%) were found to be more toxic to Microtox. Eleven (28%) were of the same order of magnitude toxicity to both Microtox and methanogens, and the remaining 4 (10%) were more toxic to methanogens. Considerably more data are available for Microtox testing than for anaerobe testing, and are reported in a more consistant manner. Many different types of anaerobic toxicity data were included in the initial review. Some of the data are based on mixed culture studies while others are based on enriched or pure cultures of methanogens. Many procedural differences were found in the anaerobic literature. For some chemicals, reports were found simply stating that a certain chemical was not toxic at a given concentration. When this value was appreciably above the reported Microtox 5EC50, the chemical was listed as more toxic to Microtox.

For chemicals for which there were more detailed literature data, the concentration of toxicant causing 50% anaerobic inhibition was determined. The reported values, or in some cases estimates, were plotted against the Microtox 5EC50 values on logarithmic axes. This information is presented graphically in Figure 4-1 and in tabular form in Table 4-2. In cases where more than one Microtox value was

TABLE 4-1 RELATIVE TOXICITY - LITERATURE SURVEY

More Toxic to Microtox	Equally Toxic (Same Order of Magnitude)	More Toxic to Methanogens
Acrolein	Ammonium (Total)	Acrylonitrile
Ammonia (Free)	Cadmium	Chloroform
Arochor 1242	Carbon tetrachloride	1,2-Dichloroethane
Benzyl Alcohol	Copper	Methanol
Catechol	m-Cresol	
4-Chloro-3-methylphenol	Ethylacetate	
2-Chlorophenol	Nickel Chloride	
p-Cresol	Nitrite	
Cyanide	Nitrobenzene	
2,4-Dicholorophenol	4-Nitrophenol	
2,4-Dimethylphenol	2-Propanol	
Dimethylphthalate		
2,4-Dinitrophenol		
Formaldehyde		
Mercuric Chloride		
m-Methoxyphenol		
Naphthalene		
1-Octanol		
Pentachlorophenol		
Phenol		
Resorcinol		
1,1,2,2-Tetrachloroethane		
2,4,6-Trichlorophenol		
Zinc Sulfate		

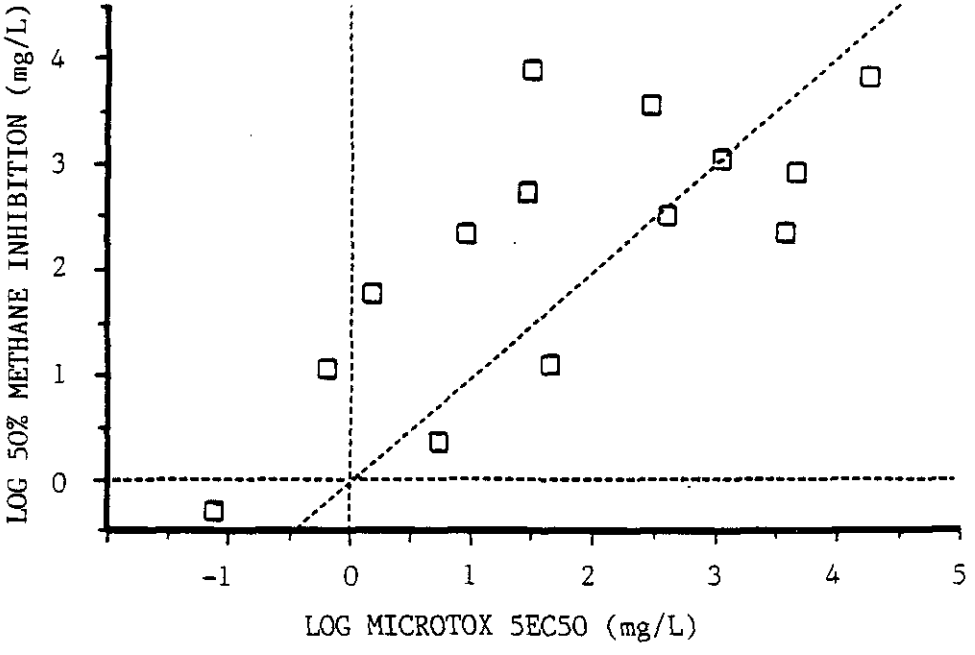

FIGURE 4-1 MICROTOX 5EC50 vs. METHANE INHIBITION
LITERATURE DATA

TABLE 4-2 LITERATURE TOXICITY VALUES

Chemical	Anaerobe 50% Inhibition (mg/L)	Microtox 5EC50 (mg/L)
Acrolein	11.2	0.67
Acrylonitrile	212	3,910
Ammonia (Free)	59	1.56
Ammonium (Total)	750	4,833
Carbon Tetrachloride	2.2	5.6
Catechol	6,966	32
Ethyl acetate	969	1,180
Formaldehyde	200	9.1
Nickel Chloride	300	410
Nitrobenzene	12.3	46.2
Pentachlorophenol	0.5	0.08
2-Propanol	5,408	17,700
Resorcinol	3,193	310
Zinc Sulfate	400	29.7

available, the average value was plotted. Statistical analysis of the data yielded a correlation coefficient of 0.74. The solid line shown on Figure 4-1 is the line of equal value. Ten out of 14 data points (71%) lie on or above this line. These represent chemicals which are either more toxic or equally toxic to Microtox.

4.2 PURE CHEMICAL LABORATORY RESULTS

4.2.1 Anaerobic Toxicity Assays

An initial test was made to determine an appropriate concentration of medium-fed seed to use in the ATA's. Four seed concentrations were tested. Figure 4-2 shows the cumulative methane production for non-toxified controls of each seed concentration. Based on this test it was decided to use 30% seed for the various ATA's using medium-fed seed. The production of methane was compared to the production of total gas. Figure 4-3 shows that for the controls, methane and total gas production were very similar, but that at later points total gas production did not level off as flatly as did methane production.

The results of a typical ATA run are shown graphically in Figures 4-4 and 4-5. Figure 4-4 shows the cumulative methane production for controls and three concentrations of chloroform. This is very similar to the pattern of total gas production shown in Figure 4-5. The higher initial gas reading for total gas is most likely due to improper temperature equilibration prior to zeroing the serum bottles. These

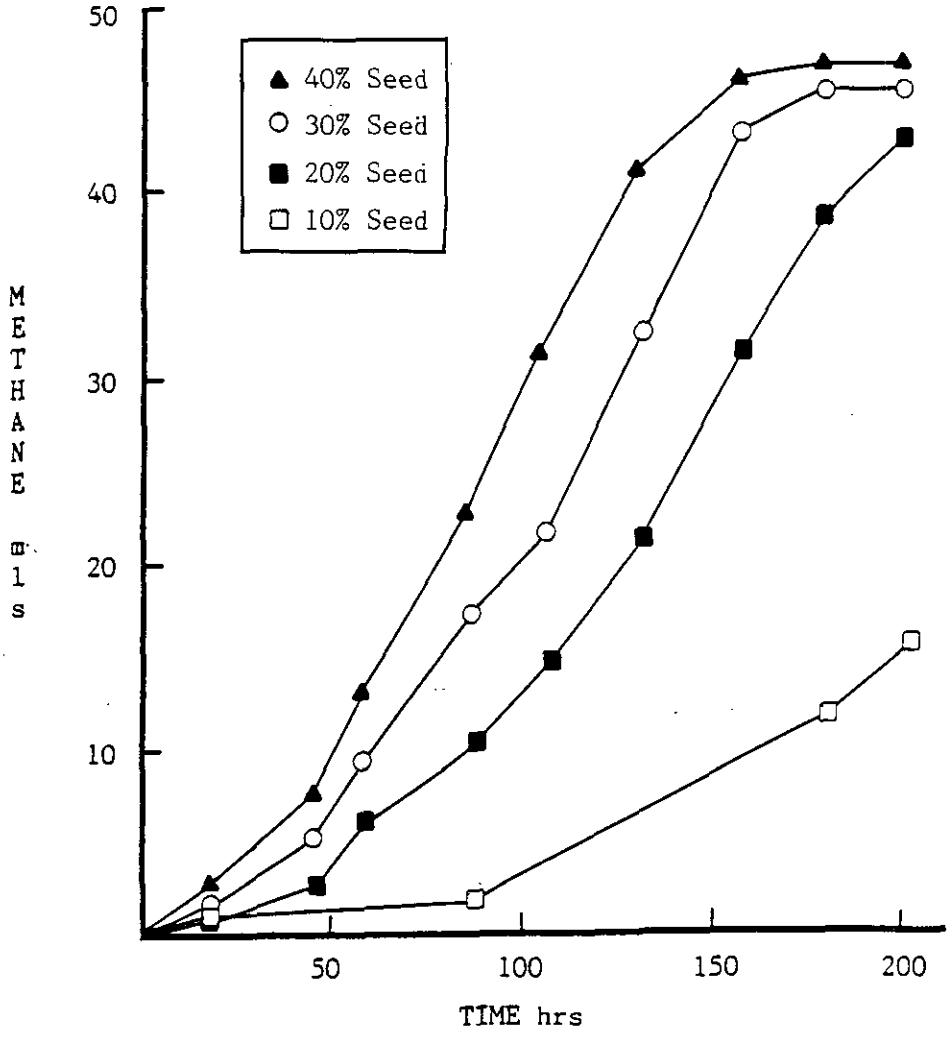

FIGURE 4-2 CUMULATIVE METHANE PRODUCTION FOR NON-TOXIFIED CONTROLS (Medium-Fed Seed)

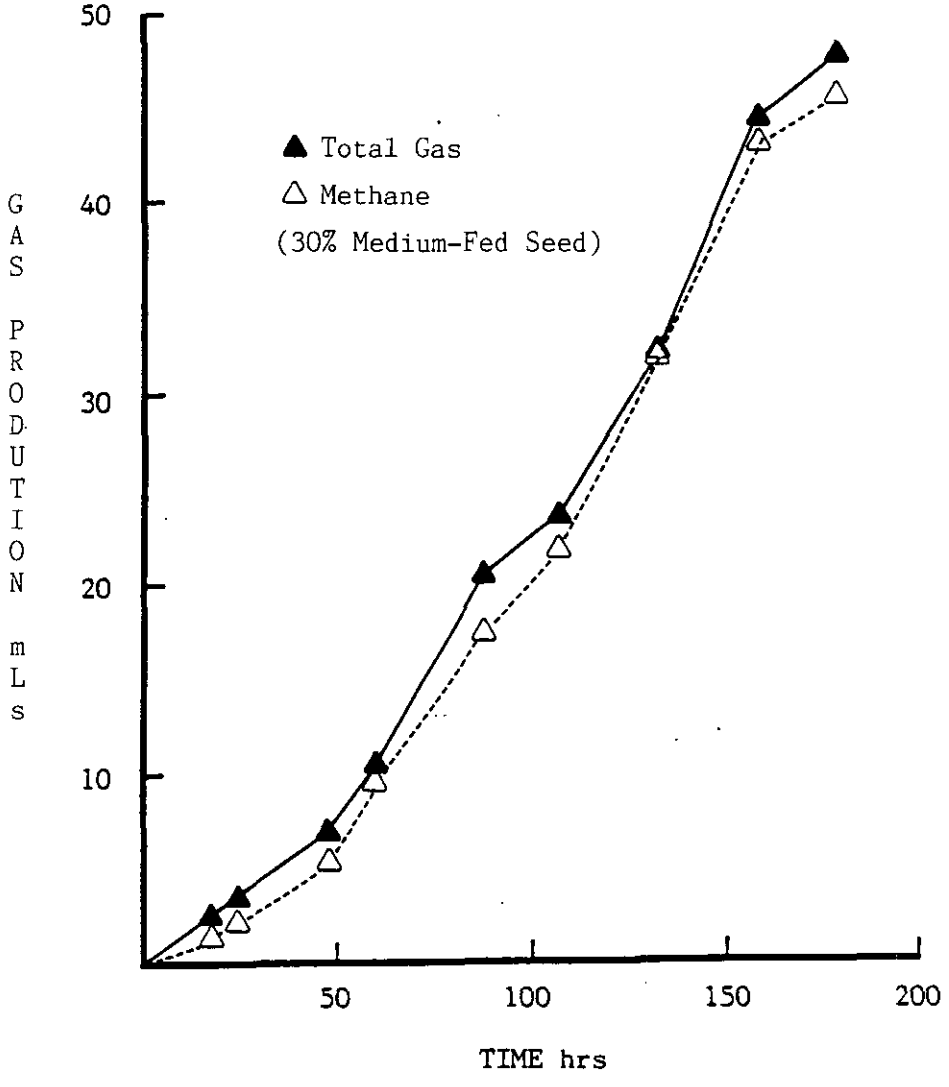

FIGURE 4-3 COMPARISON OF METHANE AND TOTAL GAS PRODUCTION
(Non-Toxified Controls)

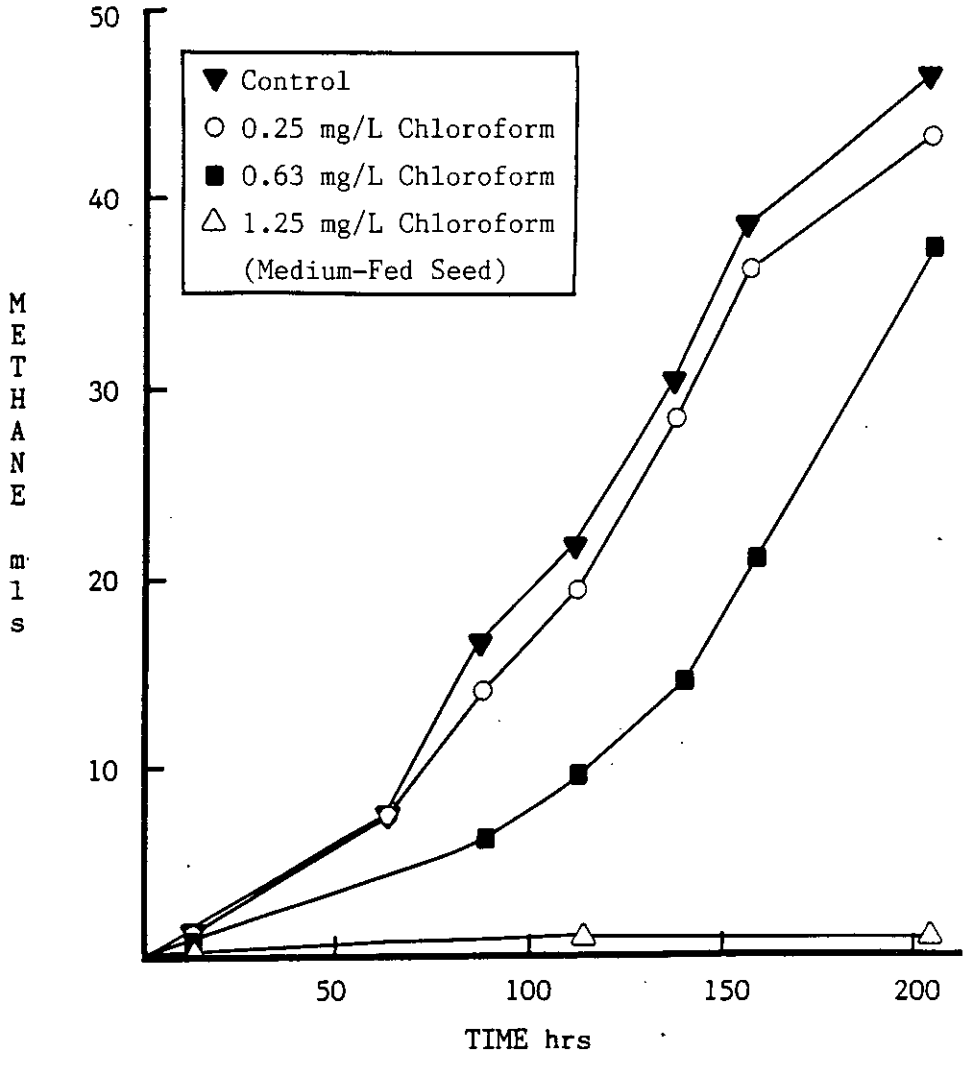

FIGURE 4-4 CHLOROFORM ATA - METHANE PRODUCTION

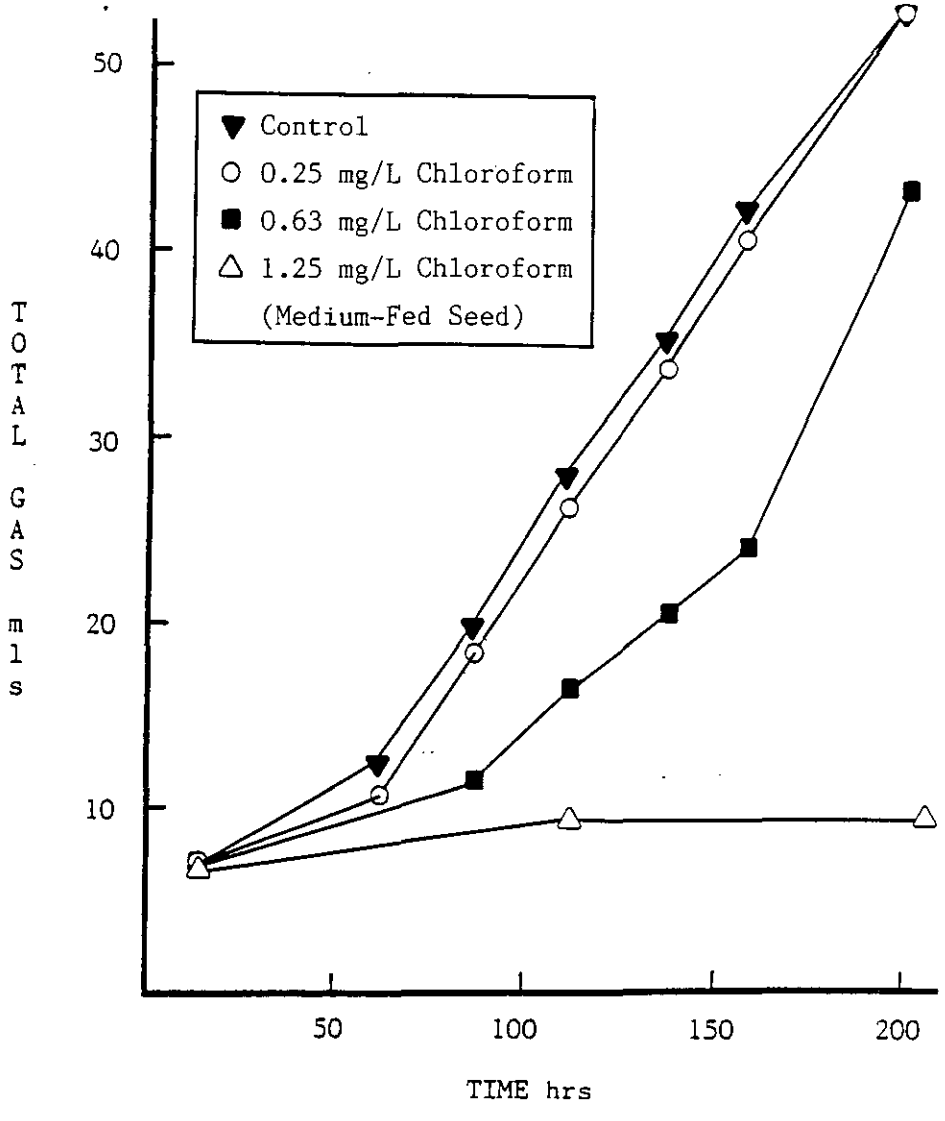

FIGURE 4-5 CHLOROFORM ATA - TOTAL GAS PRODUCTION

results are used to calculate the concentration of toxicant which will result in a 50% inhibition of the maximum rate of methane or total gas production.

In order to assure that our laboratory results could be compared reasonably to results reported in the literature, ATA assays were performed using chemicals for which there were previously reported literature data. Also, duplicate ATA's of two chemicals were run. Table 4-3 presents these results. The reproducibility between laboratories appears to be exceptionally good (1 and 5.5% differences in values). Intra-laboratory reproducibility, however, showed much higher differences in values (28 and 34% differences). Replicates of controls and toxified samples were generally well matched. Figure 4-6 shows the cumulative methane production for three replicate controls. The coefficient of variation between the three replicates (standard deviation / mean) averages around 3.1% for measurements at different time points.

A brief study was made to investigate potential differences in toxicity of chemicals to two anaerobic seeds from reactors which differed only in their solids retention times. Table 4-4 presents the comparison of toxicity to 20 day and 8 day SRT medium-fed seed. The 8 day seed appeared to be slightly more resistant to the toxic effects of mercuric chloride and sodium arsenate. For the rest of the study only the 20 day SRT seed was used.

A summary of ATA results is presented in Table 4-5. The

TABLE 4-3 REPRODUCIBILITY OF ATA RESULTS

Chemical		Concentration Causing 50% Inhibition (mg/L) Laboratory	Literature
Chloroform		0.91	0.96 Thiel et al. (1969)
Mercuric Chloride	(1)	241	
	(2)	333	
Phenol		505	500 Pearson et al. (1980)
Sodium Arsenate	(1)	10.4	
	(2)	6.9	

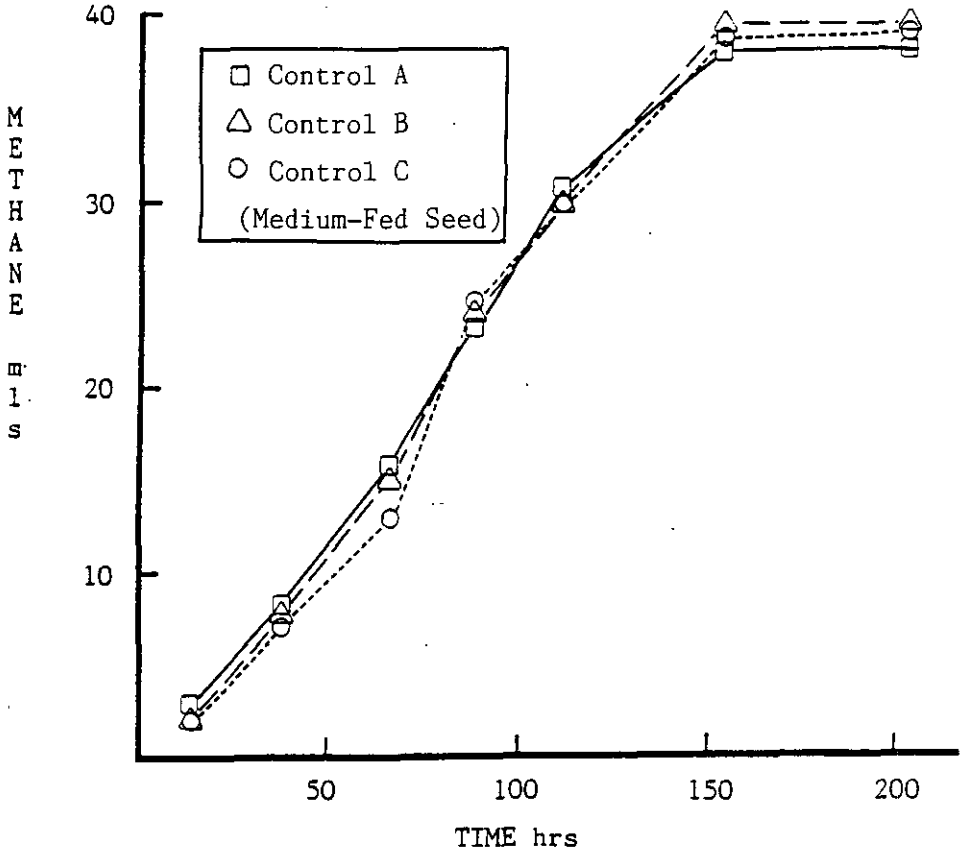

FIGURE 4-6 REPLICATE CONTROLS - METHANE PRODUCTION

TABLE 4-4 COMPARISON OF 20 AND 8 DAY SRT MEDIUM-FED SEED

Chemical		Concentration Causing 50% Inhibition (mg/L) 20 Day Seed	8 Day Seed
Mercuric Chloride	(1)	241	466
	(2)	333	
Sodium Arsenate	(1)	10.4	11.2
	(2)	6.9	

TABLE 4-5 SUMMARY OF ATA RESULTS

Chemical	Concentration Causing 50% Inhibition (mg/L) Methane Production	Total Gas Production
Acetone	31,200	35,700
Beryllium Sulfate	3,340	6,440
n-Butyl Alcohol	6,690	6,730
Chloroform	0.91	1.00
Isopropyl Alcohol	31,200	26,100
Mercuric Chloride	287	287
Methyl Isobutyl Ketone	12,900	36,400
Phenol	505	500
Potassium Chromate	337	251
Sodium Arsenate	8.65	6.71

concentrations of various toxicants causing 50% inhibition of the maximum rate of methane and total gas production are reported. Where more than one ATA was performed, the average of computed values is reported. In general, the concentration causing 50% inhbition of total gas was close to the concentration causing 50% inhibition of the maximum rate of methane production. The average percent difference between the two values is 20%. The number of chemicals which showed apparently greater toxicity based on total gas measurements is approximately the same as those which showed greater toxicity based on methane data.

4.2.2 Microtox Bioassays

Microtox bioassays were performed following the procedures described by the manufacturer. The results of individual tests were plotted to determine the concentration of toxicant causing 50% inhibition of light output after 5 and 30 minutes exposure. Figure 4-7 shows typical results for a 5 minute Microtox bioassay of phenol. The Microtox test usually provides data which show strong linearity when plotted on semi-log graphs. The correlation coefficient for nearly all tests was well above 0.97.

Microtox tests also show good reproducibility both between tests and between laboratories. Table 4-6 shows the range of Microtox 5EC50's for mercuric chloride and phenol determined in this study and reported in the literature. Table 4-7 provides a summary of the

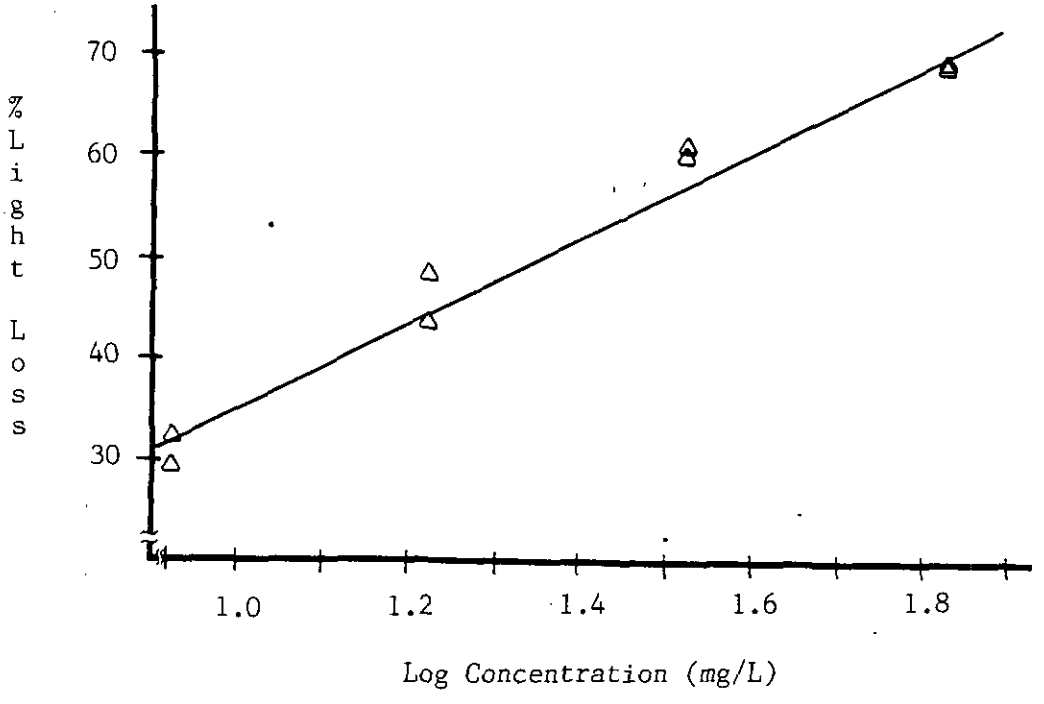

FIGURE 4-7 MICROTOX BIOASSAY - PHENOL

TABLE 4-6 REPRODUCIBILITY OF MICROTOX RESULTS

Mercuric Chloride

Laboratory Data 5EC50's (mg/L)	Literature Data 5EC50's (mg/L)	
0.051	0.064	Dutka & Kwan (1981)
0.045	0.065	Bulich & Isenberg (1981)
0.055	0.08	Qureshi et al. (1982)
	0.07	Beckman (1983)
(n=3 ave=0.055 s.d.=.0041)	(n=4 ave=0.069 s.d=.0006)	

Phenol

Laboratory Data 5EC50's (mg/L)	Literature Data 5EC50's (mg/L)	
22.0	25	Lebsack (1980)
28.8	42	Samak & Noiseux (1980)
32.7	25	Bulich & Isenberg (1981)
33.5	26	Chang (1981)
	28	Dutka & Kwan (1981)
	40.2	Curtis (1982)
	22.0	Qureshi et at. (1982)
	40.7	Beckman (1983)
	25	Indorato (1983)
(n=4 ave=29.3 s.d.=4.55)	(n=9 ave=31.1 s.d=7.80)	

TABLE 4-7 SUMMARY OF MICROTOX RESULTS

Chemical	5EC50 (mg/L)	30EC50 (mg/L)
Bacitracin	3,940 5,556	14,200 15,503
BES *	55,500	---
1,2-Dichloroethylene	994 1,450	1,290 ---
Lasalocid	N.T. **	---
Mercuric Chloride	0.051 0.045 0.055	0.038 0.031 0.039
Monensin	N.T.	---
Phenol	22.0 28.8 32.7 33.5	30.4 99.9 69.7 58.6
Spiramysin	N.T.	---
Sulfamethazine	779 977 706	672 621 590
Trichloroacetic acid	9,860 10,600	3,303 6,250
Vinyl Acetate	2,140 1,760 2,170	2,420 2,190 2,640

* 2-Bromoethanesulfonic acid
** Not toxic at limit of solubility

Microtox results for this study.

4.2.3 Combined Results

Literature and laboratory results were combined, and are presented in Table 4-8. The concentrations causing 50% inhibition in the 5 minute Microtox test and 50% inhibition of methane production were compared. Figure 4-8 shows very slight correlation between these values ($r = 0.32$). Approximately 45% of the chemicals were more toxic to Microtox; 38% were more toxic to methanogens, and 17% were equally toxic to Microtox and methanogens.

The difference between using the 5 minute and the 30 minute Microtox results for comparison to anaerobe data was examined. Table 4-9 presents Microtox 5EC50's, 30EC50's as well as anaerobe data for ten chemicals. The correlation coefficent for anaerobe vs. 5EC50 was essentially the same as for anaerobe vs. 30EC50 ($r= 0.41$ and 0.42 respectively). These values are statistically insignificant in each case.

4.3 COMPLEX EFFLUENTS

An initial test was made to determine the appropriate amount of sludge-fed seed to use for ATA's. Based on the results, a 38% seed concentration was chosen for ATA's with sludge-fed seed.

The effect of stirring on gas production was studied by comparing stirred and non-stirred controls. Three controls with stirring bars on

TABLE 4-8 COMBINED DATA

Chemical	Anaerobe 50% Inhibition (mg/L)	Microtox 5EC50 (mg/L)
Acetone	31,200	21,750
Acrolein	11.2	0.67
Acrylonitrile	212	3,910
Ammonia (Free)	59	1.56
Ammonium (Total)	750	4,833
Bacitracin	160	4,750
Beryllium Sulfate	3,340	15
BES	10.5	55,500
n-Butyl Alcohol	6,690	2,690
Carbon Tetrachloride	2.2	5.6
Catechol	6,966	32
Chloroform	0.91	678
1,2-Dichloroethylene	77.5	1,220
Ethyl acetate	969	1,180
Formaldehyde	200	9.1
Isopropyl Alcohol	31,200	42,000
Mercuric Chloride	287	0.050
Methyl Isobutyl Ketone	12,900	0.08
Nickel Chloride	300	410
Nitrobenzene	12.3	46.2
Pentachlorophenol	0.5	0.08
Phenol	505	29.3
Potassium Chromate	337	22
2-Propanol	5,408	17,700
Resorcinol	3,193	310
Sodium Arsenate	8.65	64.5
Trichloroacetic acid	340	10,200
Vinyl Acetate	920	2,030
Zinc Sulfate	400	29.7

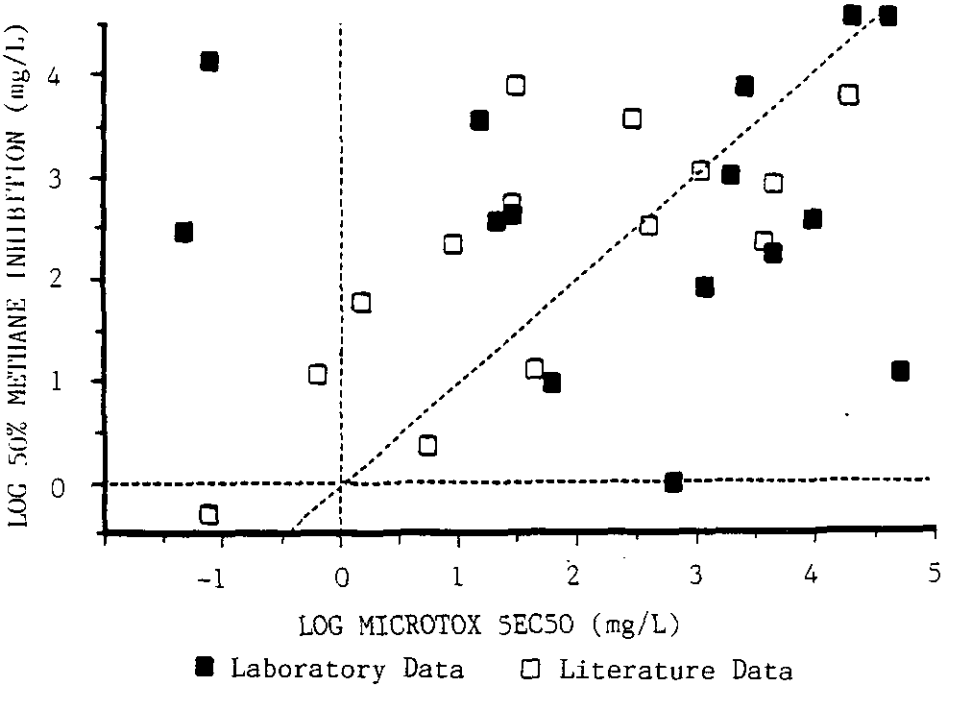

FIGURE 4-8 MICROTOX 5EC50 vs. METHANE INHIBITION
COMBINED DATA

TABLE 4-9 COMPARISON OF 5 MINUTE AND 30 MINUTE MICROTOX VALUES

Chemical	Anaerobe 50% Inhibition (mg/L)	Microtox 5EC50 (mg/L)	Microtox 30EC50 (mg/L)
Amonia (free)	59	1.56	2.0
Bacitracin	160	4,750	14,800
1,2-Dichloroethylene	77.5	1,220	1,290
Formaldehyde	200	9.1	3.0
Mercuric Chloride	287	0.050	0.036
Nickle Chloride	300	410	13.9
Phenol	505	29.3	64.7
2-Propanol	5,408	17,700	35,000
Trichloroacetic Acid	340	10,200	4,780
Vinyl Acetate	920	2,030	2,410

continuously operating stirring plates were compared to regular controls which were stirred only by shaking each time the bottles were read. Figure 4-9 shows cumulative total gas and methane production for the stirred and non-stirred controls. This figure shows that stirred and non-stirred controls have similar gas production for the first few days, after which stirred controls show both higher methane and total gas production. The maximum rate of total gas production was 10% higher for stirred controls. However, the maximum rate of methane production was only 4% higher than the non-stirred controls.

The three replicates of both stirred and non-stirred controls showed good reproducibility. Coefficients of variation were larger for the later time points for the stirred controls, but in general ranged between 1 and 3% for both stirred and non-stirred controls. Duplicate ATA's of phenol and chloroform were performed. The results are in fairly close agreement (16 and 45% differences respectivly). This is similar to the degree of reproducibility observed for ATA's with medium-fed sludge.

Five concentrations (2.0, 3.8, 5.7, 7.4, and 9.1%) of pulp mill waste were tested for toxicity to methane producing bacteria using the ATA with sludge-fed seed. Based on the results of this preliminary test, a concentration of 3.8% PMW was chosen for evaluating combined effects of toxicants and the pulp mill waste.

Figure 4-10 shows the results of a typical ATA run for mercuric chloride with and without PMW. A summary of the results for complex

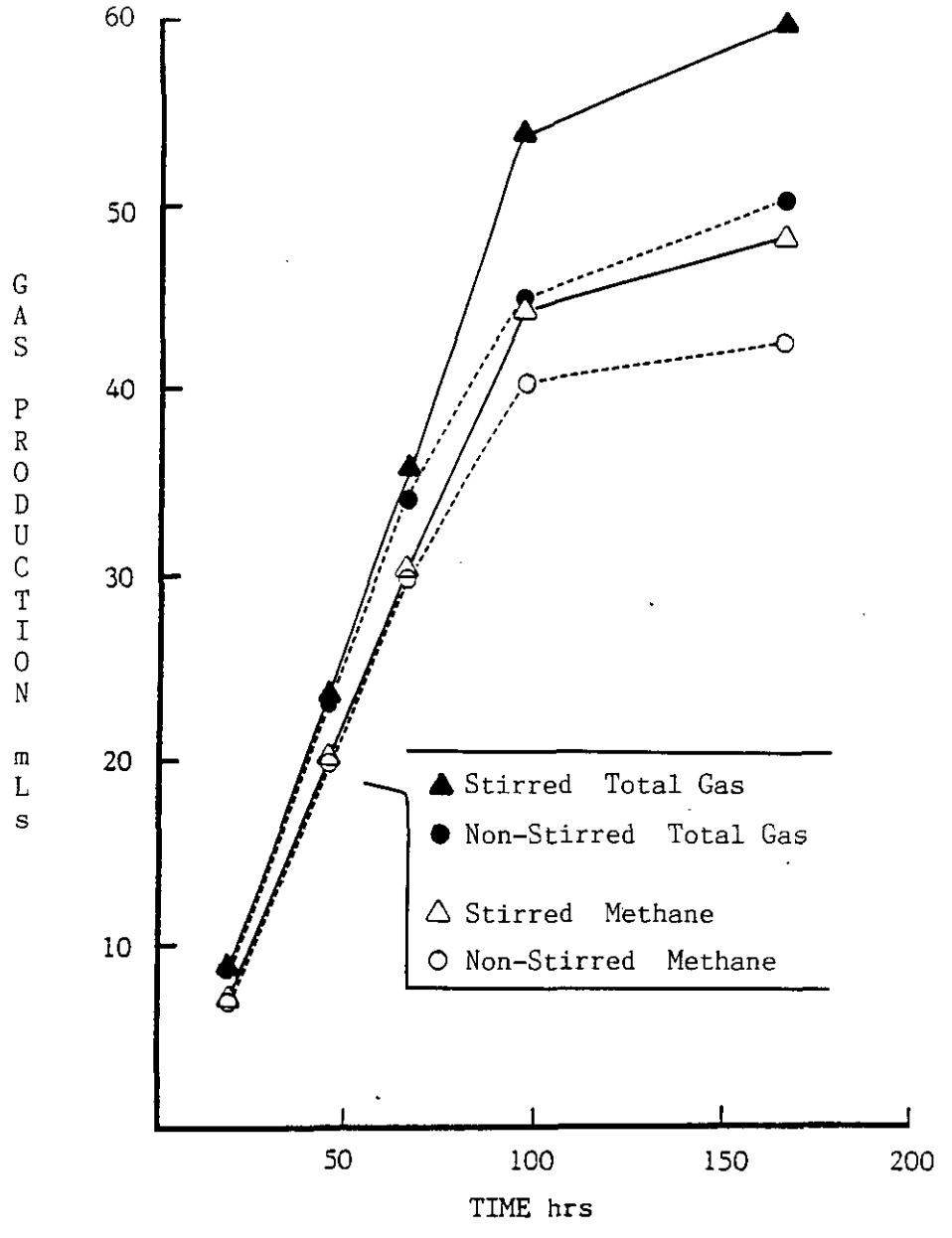

FIGURE 4-9 STIRRED AND NON-STIRRED CONTROLS (Sludge-Fed Seed)

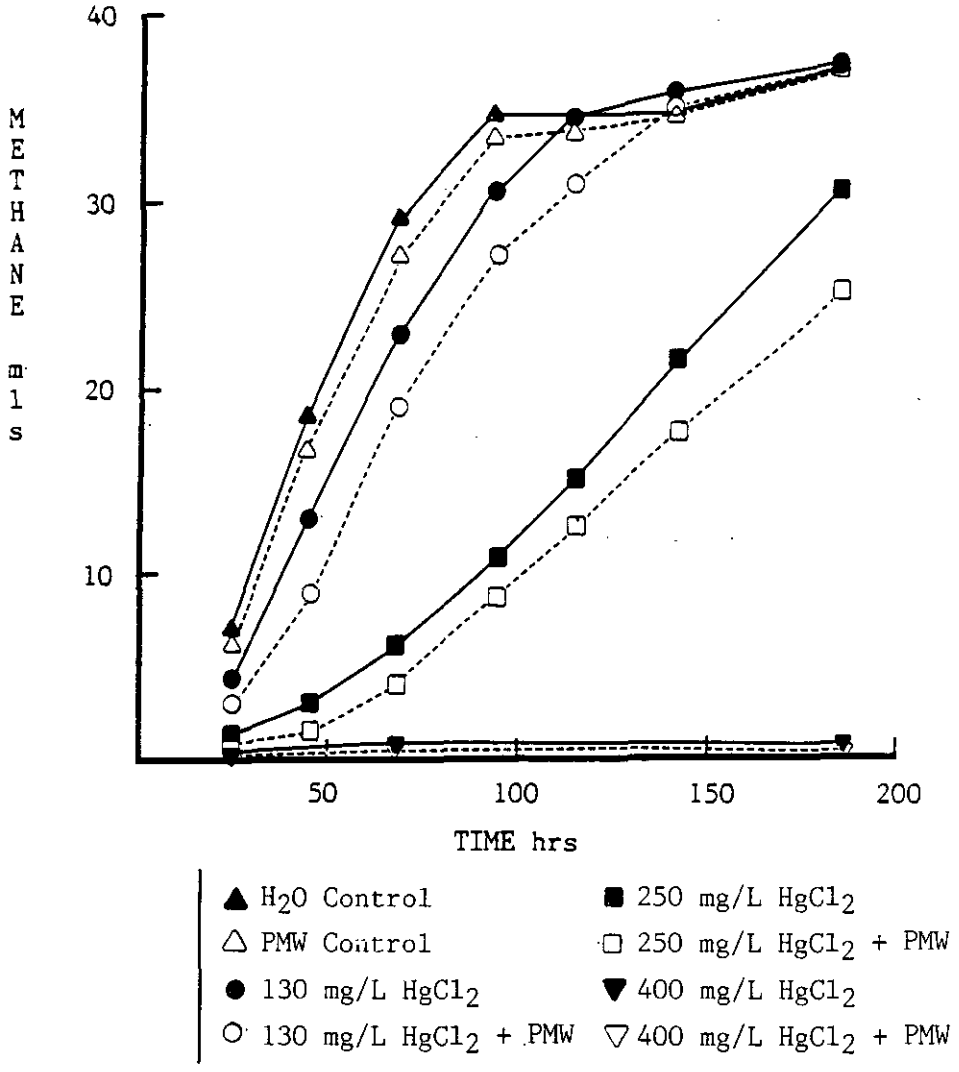

FIGURE 4-10 MERCURIC CHLORIDE ATA - SLUDGE-FED SEED

effluent studies is presented in Table 4-10. This table lists the concentration of toxicant which results in 50% inhibition of total gas and methane production for toxicants with and without PMW. The concentration of toxicant predicted to cause 50% inhibition when combined with PMW is also presented. This value is based on purely additive effects of PMW and toxicant, and is used to determine whether the observed combined effects are antagonistic, synergistic, or additive. The results show that for all but one of the six tests, the combined effect of PMW and toxicant was antagonistic based on methane production. For total gas production, 3 tests showed additive interaction (observed = predicted +/- 10%); 2 showed antagonistic interactions and one was synergistic.

The data from ATA's on chemicals without PMW using the sludge-fed seed were compared to data using the medium-fed seed. Table 4-11 presents this information. Chloroform and mercuric chloride showed toxicity in the same concentration range for both medium and sludge-fed seed. Phenol and sodium arsenate were less toxic to the sludge-fed seed.

4.4 HYDROGEN AND CARBON MONOXIDE DATA

Data on hydrogen and carbon monoxide accumulations in toxified anaerobic reactors were compared to Microtox data. The response pattern of hydrogen production versus concentration depends on the particular toxicant. For some toxicants, hydrogen increases with

TABLE 4-10 SUMMARY OF COMPLEX EFFLUENT RESULTS

Concentration Causing 50% Inhibition of Gas Production (mg/L)

Toxicant	Total Gas without	Total Gas with PMW	Methane without	Methane with PMW
Chloroform (1)	0.67	0.62 (0.44)*	0.52	0.50 (0.43)
Chloroform (2)	1.03	0.94 (1.03)	0.94	0.94 (0.83)
Mercuric Chloride	255	182 (186)	223	209 (187)
Phenol (1)	1472	1237 (1488)	1774	1598 (1790)
Phenol (2)	1790	1861 (1783)	2120	2015 (1589)
Sodium Arsenate	82.8	83.2 (53.2)	76.6	71.4 (59.2)

* Numbers in parentheses are values predicted based on additive effects of PMW and toxicant.

TABLE 4-11 COMPARISON OF MEDIUM-FED AND SLUDGE-FED SEED

Toxicant	Concentration Causing 50% Inhibition of Methane Production (mg/L)		
	20 day SRT Sludge-Fed	20 day SRT Medium-Fed	8 day SRT Medium-Fed
Chloroform	0.73 (ave)	0.91	---
Mercuric Chloride	223	287 (ave)	466
Phenol	1947 (ave)	505	---
Sodium Arsenate	76.6	8.65 (ave)	11.2

increasing toxicant concentration, and for others it decreases. The carbon monoxide response pattern generally is increasing carbon monoxide production with increasing toxicant concentration (Hickey,1987). Table 4-12 summarizes the findings of this phase of the study. Statistical analysis of the data shows no correlation between Microtox toxicity and hydrogen or carbon monoxide accumulations in toxified reactors.

4.5 TOXICITY REDUCTION

Prior to running tests to determine if Microtox could be used to measure toxicity reduction by anaerobic treatment, preliminary tests were made to determine background toxicity. Microtox tests were performed on supernatant from anaerobic seed, air-purged supernatant, ATA medium, and the sludge used for feed. The results of these tests are presented in Table 4-13. These results indicate that the background toxicity measured by Microtox is high. Air purging of the sludge-fed seed supernatant indicated that a considerable portion of the toxicity due to the supernatant is volatile. The sludge used for feed showed no toxicity, suggesting that the toxicity is due to changes that occur in the anaerobic reactor. Based on the high background toxicities the decision was made to discontinue this phase of the study.

4.6 SUMMARY OF RESULTS

TABLE 4-12 COMPARISON OF MICROTOX WITH HYDROGEN AND CARBON MONOXIDE DATA

Chemical	Microtox 5EC50 (mg/L)	Concentration Causing 2-Fold Change in 12 Hour Gas Production (mg/L) * Hydrogen		Carbon Monoxide	
2-Bromoethane-sulfonic Acid	55,500	1,055	+	5,250	+
Chloroform	677	0.51	+	0.33	+
Copper	12.2	87.5	-	175	+
Formaldehyde	7.1	50	+	5	+
Trichloroacetic Acid	10,200	---		3	+
Zinc	2.5	600	-	50	+

* - indicates 2-fold reduction ; + indicates doubling of gas production.

TABLE 4-13 BACKGROUND MICROTOX TOXICITY

		5EC50	30EC50
Medium-Fed Seed (Supernatant)		4.9%	---
Sludge-Fed Seed (Supernatant)	(1)	24.0%	23.4%
	(2)	12.9%	20.3%
Sludge-Fed Seed (Air-Purged Supernatant)		86.8%	65.7%
ATA Medium		26.3%	25.8%
WAS Sludge (Supernatant)		Not Toxic	Not Toxic

The initial literature review indicated that there was a fairly good correlation between Microtox 5EC50's and concentration of toxicant causing 50% inhibition of methane production ($r = 0.74$; $n = 14$). Laboratory tests using Microtox and ATA's showed good reproducibility. The combined laboratory and literature data showed only a slightly significant correlation between Microtox and methane inhibition ($r = 0.32$; $n = 29$). The results were not changed when 30 minute Microtox data were used instead of 5 minute data.

Comparision of ATA data using different seed for inocula showed that sludge-fed seed was less susceptible to toxicity from certain chemicals. For other chemicals toxicity to sludge-fed and medium-fed seed was approximately equal. Medium-fed seed grown at 8 days SRT instead of 20 days SRT also appeared to be somewhat more resistant to toxicity.

Studies with a complex effluent showed predominantly antagonistic interactions with four different chemicals. There was no statistical relationship between Microtox data and hydrogen and carbon monoxide accumulations in toxified anaerobic reactors. Toxicity reduction studies were not feasible due to high background toxicity.

CHAPTER 5 -DISCUSSION

5.1 EVALUATION OF MICROTOX AS A SURROGATE FOR ATA's

A toxicity test may be useful as a surrogate for another test if it can be shown to satisfy at least one of two criteria. The first criterion is that a good surrogate test should have some predictable, quantifiable relationship to the test it is to replace. The second criterion is that the test should be as sensitive or more sensitive to toxicants and act as a conservative estimator of toxicity. The first criterion is best judged by statistical relationships while the second may be judged by the percentage of toxic cases the test sucessfully identifies, or screens out.

The initial literature survey looked promising in meeting the second criterion. Based on the initial survey, it would seem that the Microtox test should be able to identify methanogenic toxicants with approximately 90% reliability. (Only 10% of the chemicals were more toxic to methanogens.) Further study of literature values through regression analysis also looked promising, yielding a correlation coefficient of 0.74. This value is close to some of the values reported in studies comparing Microtox to other bioassays.

The laboratory data, however, did not yield such positive support for the use of Microtox as a surrogate for the ATA test. The correlation coeffient for this set of data was too low to reject the null hypothesis at any level of significance (Sharp, 1979). The

laboratory data also did not indicate that Microtox would be a good screening tool (43 % of chemicals were more toxic to methanogens). One reason for such a discrepancy between literature and laboratory results may be the method of choosing chemicals to test. In the literature study, data were gathered for each test without presupposition of the toxicity of chemicals to the other test. In the laboratory study, an attempt was made to look at some chemicals known to be specifically toxic to each system, some chemicals with very little toxicity to one of the tests, and at some which were thought likely to fall in some middle range. This deliberate testing of extremes may account for the lack of correlation of the data. The combined data show a slight correlation, and taken as a whole, 59 % of the chemicals studies were of equal or greater toxicity to Microtox.

Four subsets of the combined data were evaluated: organics, inorganics, priority pollutants and organic priority pollutants. Plots of each data subset are presented in Figures 5-1 to 5-4. Each subset showed insignificant correlation. Microtox seemed to be more suitable as a screening tool for inorganic toxicants than for other the other three groups. Only 1 of 8 inorganics (13%) was more toxic to methanogens, compared to 29% for organics, 35% for priority pollutants and 45% for organic priority pollutants (see Table 5-1). When considering the types of toxicants most likely to enter an anaerobic treatment unit, this observation is of interest. The 1980 EPA study of priority pollutants in publicly owned treatment works (U.S. EPA, 1980)

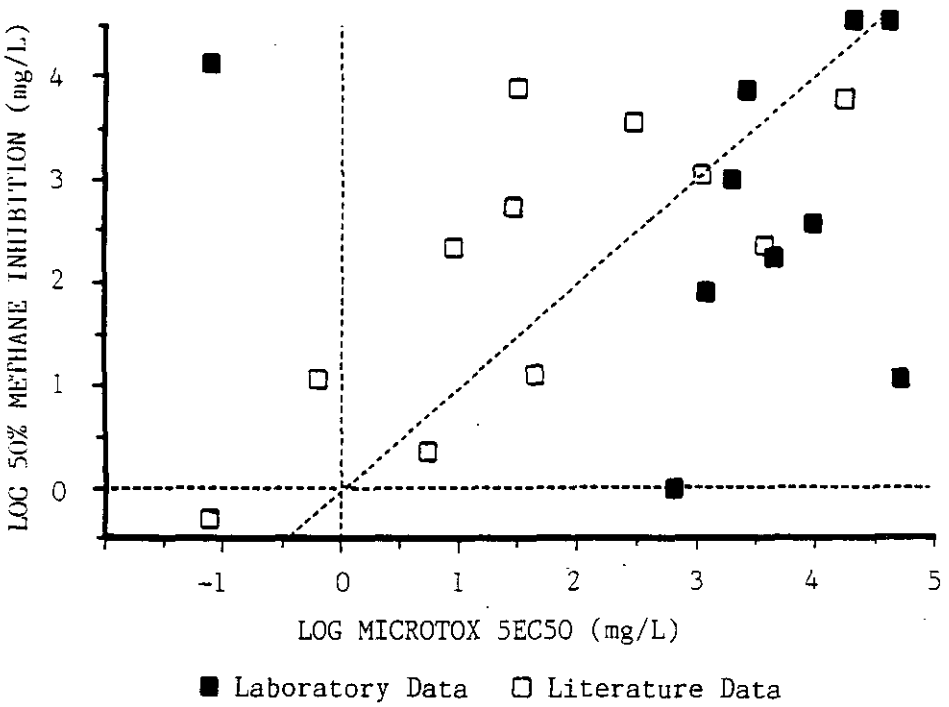

FIGURE 5-1 MICROTOX 5EC50 vs. METHANE INHIBITION
ORGANICS

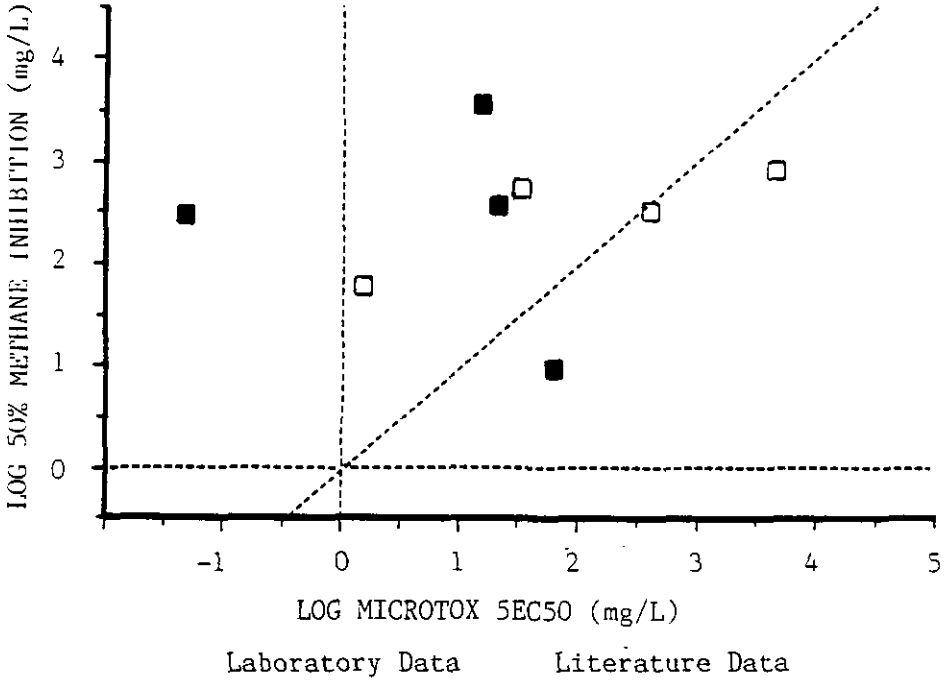

FIGURE 5-2 MICROTOX 5EC50 vs. METHANE INHIBITION
INORGANICS

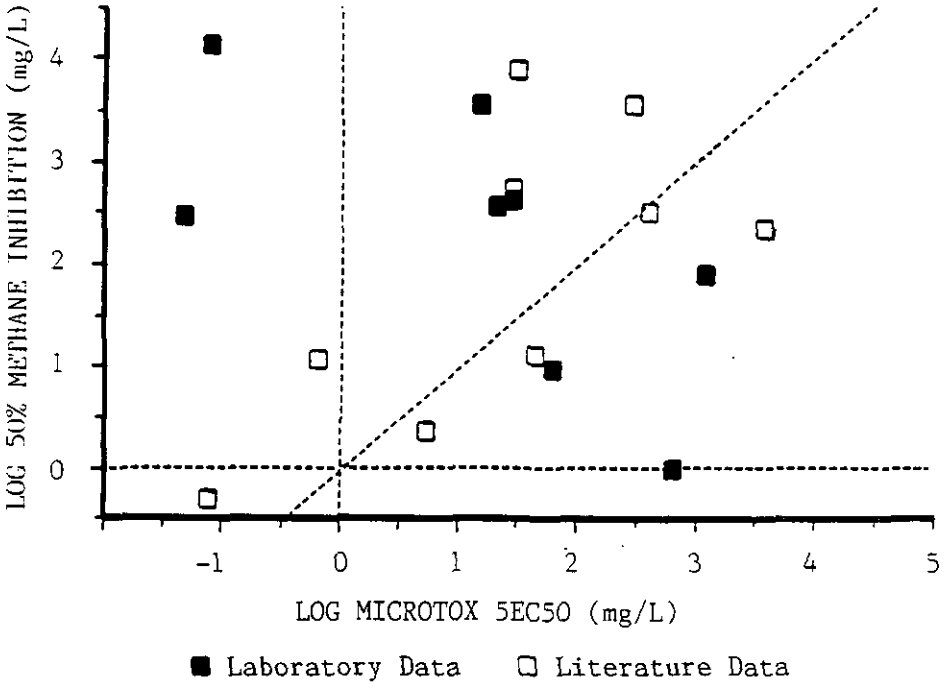

FIGURE 5-3 MICROTOX 5EC50 vs. METHANE INHIBITION
PRIORITY POLLUTANTS

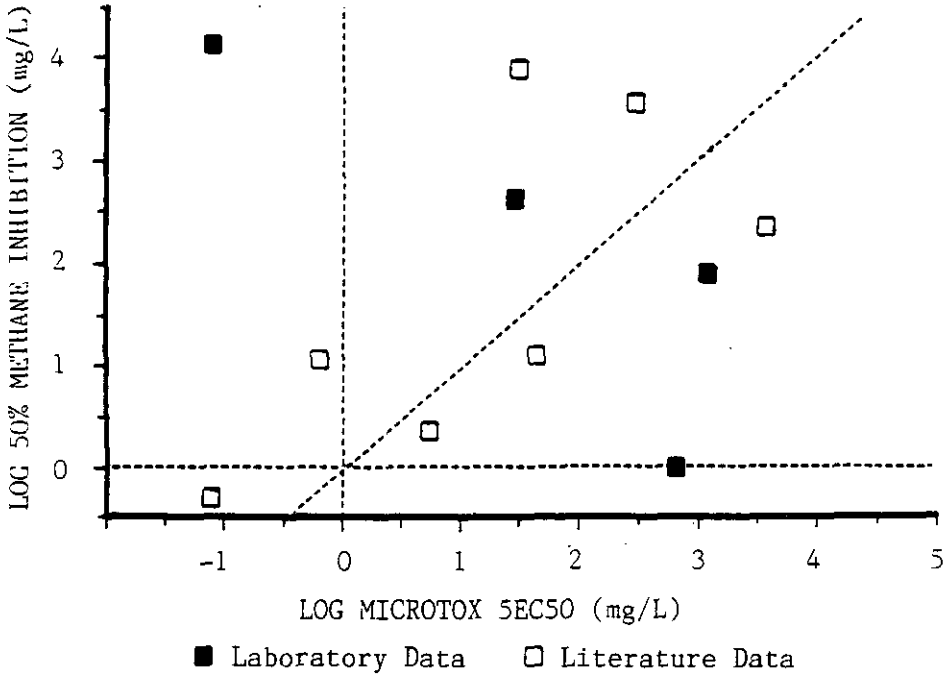

FIGURE 5-4 MICROTOX 5EC50 vs. METHANE INHIBITION
PRIORITY ORGANICS

TABLE 5-1 STATISTICAL SUMMARY

Data Set	n	r	% Data Points More Toxic to Anaerobes
Literature Review	39	---	10
Literature Data	14	.74	29
Laboratory Data	15	insig.	43
Combined Data	29	.32	41
Organics	21	insig.	29
Inorganics	8	insig.	13
Priority Pollutants	17	insig.	35
Priority Organics	11	insig.	45

lists the occurence of priority pollutants in raw sludge samples as a percentage of times detected. Of the inorganic toxicants reported on in both this study and the EPA study, the range of percent times detected was from 32 to 98% with an average detection rate of 82%. Organic toxicants, on the other hand, had a range of 2-62% with an average detection rate of 24%.

Because the Microtox test is so short compared to the ATA, the question arose whether a longer Microtox test might have greater correlation to the ATA test. Ten chemicals were studied to see if 30 minute Microtox data compared more favorably than 5 minute data. Statistical analysis showed that the two sets of data did not have any meaningful differences.

Differences in how anaerobe toxicity was computed were also studied. The difference in results between using methane data and total gas data was small (20%). Based on the small difference in results, and the increased amount of time nessecary to gather daily methane data, using total gas as a surrogate for methane production seems to be a reasonable alternative.

The results for this phase of the study showed unusually high reproducibility between labs for ATA's (very much higher than within labs). Only 2 data points were available to evaluate inter-lab reproducibility, and due to the large number of confounding variables expected between labs, it seems most likely that the closeness of results is more a matter of luck than anything else.

5.2 COMPLEX WASTE

For the most part, the pulp mill waste showed antagonistic interactions with the various chemicals tested. It is very difficult to generalize from such limited data, especially when working with poorly defined toxicants such as PMW. What can be said is that for an anaerobic treatment unit already under stress from wastes similar to the PMW used in this study, the additional toxic burden on the chemicals tested would not be as great as one would otherwise suspect. This kind of testing may have more application in a situation in which wastes are to be combined prior to anaerobic treatment. In a case such as this, testing for synergistic effects could help predict reactor performance while treating mixed wastes.

5.3 HYDROGEN AND CARBON MONOXIDE DATA

No correlation could be found between Microtox 5EC50 values and hydrogen and carbon monoxide accumulations in toxified anaerobic reactors. The use of hydrogen and carbon monoxide monitoring is still in the early stages of development, and the significance of these gases as indicators of anaerobic toxicity is still being studied. The comparison of data was furthur complicated by the variability in response pattern seen for hydrogen accumulation for different toxicants. While it is not the intent of this study to explore the reasons behind this variable response, it does serve to point out the

complex nature of toxicity in a mixed culture such as is found in an anaerobic reactor.

5.4 TOXICITY REDUCTION

Although the toxicity reduction studies did not procede as far as was hoped, the results do help point out an important consideration for any treatment process. The sludge used to feed the reactor was not toxic to Microtox. However, the effluent from the reactor (anaerobic seed for ATA's) was toxic to Microtox, indicating that changes which occured due to treatment resulted in toxicty. It is very likely that the observed effect was a result of sulfate conversion to sulfide, as evidenced in part by the volatile nature of at least some of the toxicity. This helps to point out the sometimes conflicting goals of waste stabilization and toxicity removal.

CHAPTER 6 - CONCLUSIONS

Overall, the results of this study do not indicate that Microtox would be expected to serve as a particularly good surrogate for the ATA in monitoring potentially toxic wastes entering an anaerobic treatment unit. The initial literature review looked promising, but furthur laboratory work reversed initial impressions. The Microtox may have an application for monitoring waste streams which may be subject to inorganic toxicants but are unlikely to be contaminated with organic toxicants.

The interactive effects of a pulp mill waste and several toxicants were evaluated. For the most part, the interactions were antagonistic. However, generalizations to other wastes and their possible interactive effects can not be made from the limited data available.

No correlation was observed between Microtox data and hydrogen and carbon monoxide accumulations in toxified anaerobic reactors.

The background toxicity of the anaerobic seed used in this study was too high to warrant continuing toxicity removal studies. It was observed that the anaerobic treatment process itself was responsible for some toxicity.

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