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Inhibition of Total Gas and Methane Production in Anaerobic Digestion by Various Toxicants

Steven H. Malehorn

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INHIBITION OF TOTAL GAS AND METHANE PRODUCTION IN ANAEROBIC DIGESTION BY VARIOUS TOXICANTS

(TITLE)

BY

STEVEN H. MALEHORN

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ABSTRACT

Heavy metal inhibition of anaerobic digestion of municipal sewage sludge has been investigated over the past 50 years. Many assays began with steady-state conditions and observed the rate at which total gas or methane production decreased due to toxicant inhibition. These methods required considerable time and labor. Modified Warburg respirometers and serum bottle assays reduced much of the effort, but each method has limitations in terms of complexity, cost, and accuracy.

This series of experiments had two objectives: 1) develop a quick, inexpensive, and easy method to measure methanogenic activity in anaerobic digesters, and 2) determine median inhibitory concentrations (IC_{50}) of selenium, copper, chromium, zinc, mercury, lead, and cobalt to total gas and methane production in batch anaerobic digesters. Preliminary experiments indicated a linear increase in gas production during community recovery from oxygen inhibition. The rate of increase was relatively independent of substrate quality during the first 6 hr as long as no nutrients were limiting. Toxicants introduced immediately after reestablishment of anaerobic conditions reduced the rate of gas production recovery as a linear function of toxicant concentration.

Anaerobic sludge samples taken from the primary digester of the local wastewater treatment plant were supplemented with cow manure, dry dogfood, and ammonium acetate prior to immediate use. Five liter portions in 10 x 70 cm (4 x 28 in) PVC sewer pipe segments were agitated on a rotary shaker and toxicants were added immediately after reestablishment of anaerobic conditions. Total gas production rates (determined through displacement of 10% NaCl solution in 50 mL burets) and percent methane in headspace gases (determined by gas chromatography) were measured 6 hr later. IC_{50} values for total gas and methane production were determined from linear least squares line equations derived from inhibition as a percent of the control.

Results indicated that chromium, selenium, copper, and zinc were relatively equal in terms of toxicity to total gas production and methanogenesis. Calculated IC_{50} values for reduction of total gas production were 123, 133, 115, and 122 mgL^{-1}, respectively. IC_{50} values for reduction of methane in headspace gas were 102, 118, 114, and 170 mgL^{-1}, respectively. Correlation coefficients (r) for the line equations of these four toxicants ranged between 0.9733 and 0.9980. Mercury was slightly less toxic with IC_{50} values of 286 and 254 mgL^{-1} for reduction of total gas and methane production, respectively. Line equation correlation coefficients were 0.9677 and 0.9919 for these values. Lead and cobalt were the least inhibitory elements examined and median inhibitory concentrations were greater than the maximum concentration tested (400 mgL^{-1}). Extrapolated IC_{50} values for lead were 410 and 405 mgL^{-1} for total gas and methane production, respectively. Cobalt showed very little inhibition with IC_{50} values estimated at 551 and 1106 mgL^{-1} for total gas and methane production, respectively. Correlation coefficients for lead and cobalt line equations used to calculate IC_{50} values ranged between 0.8331 and 0.9609.

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INTRODUCTION

Methane produced during anaerobic digestion of municipal sludge is used by many treatment facilities as an energy source and can result in net energy savings of about 20 million Btu/ton of biochemical oxygen demand (BOD) removed (51). Unfortunately, anaerobic digestion is vulnerable to heavy metal inhibition and considerable research in heavy metal toxicity has been performed over the past 50 years (6, 52). Toxicity assays normally were performed as batch experiments, varying in size from 3 to 350 L (3, 5, 7, 25, 56). They were time and labor intensive, and many required several months to complete. Steady-state environmental conditions and microbial populations had to be established prior to experimentation; digesters had to be fed constantly to maintain consistent substrates; and gas production was measured on a daily basis. At times, toxicants were step-fed over extended periods. Digesters generally were monitored until failure occurred.

Several methods have been developed to reduce time and labor factors in determination of methanogenic activity in anaerobic digesters. These methods include modified Warburg respirometers (30, 38, 64) and serum bottle assays that require only 50-250 mL portions of substrate (13, 23, 27, 49, 64, 68). The modified Warburg respirometer has several limitations (49). It is expensive and requires some skill to operate, limited sample size makes gas and liquid analyses difficult, and extended incubation times produce inconsistent results. Respirometer and serum bottle samples are generally too small to produce sufficient gas for analysis of short-term (e.g. 5-30 minute) gas production rates.

This series of experiments had two objectives: 1) develop a quick, inexpensive, and easy method to measure methanogenic activity in anaerobic digesters; and 2) determine median inhibitory concentrations (IC_{50}) of chromium, cobalt, copper, lead, mercury, selenium, and zinc to total gas production and methanogenesis in batch anaerobic digesters.

LITERATURE REVIEW

Anaerobic digestion of municipal wastewater treatment plant sludge has been practiced since the turn of the century. Advantages (14, 50) of the anaerobic process over aerobic digestion include 1) low nutritional requirements, 2) no oxygen demands, thus eliminating energy intensive aeration equipment, 3) solids weight and volume reduction by >30%, 4) faster drying sludge, 5) destruction of pathogenic and parasitic organisms, and 6) production of fertilizer and methane as end products. Anaerobic digesters generate more energy in the form of methane gas than is required for their operation , producing approximately 11.2×10^6 Btu of energy per ton of BOD treated waste compared to approximately 8.5×10^6 Btu of energy per ton consumed by aeration equipment (49, 50). In a batch system, substrate utilization begins almost immediately and the rate remains constant until the substrate is exhausted and activity quickly declines (30). Gas production in a typical 3 L digester is shown in Figure 1. Average sludge retention intervals vary. Retention periods of 14, 21, 28, and 35 days are representative of those used in the United Kingdom for mesophilic digestion (3).

Efficient methanogenesis requires certain environmental conditions in anaerobic digesters. These include anaerobic conditions, the absence of light and nitrates, a pH range between 6 and 8, and methanogenic precursors (43) such as formate, acetate, hydrogen, and carbon dioxide which generally occur as metabolic byproducts from non-methanogenic organisms. Therefore, performance of anaerobic digestion depends on the coordinated actions of several populations of microorganisms as shown in Figure 2.

The overall microbiology of anaerobic digestion was extensively reviewed (65, 66) with isolation and characterization of 92 important bacteria from the non-methanogenic phase. Acid-forming bacteria convert complex organic compounds into acids that methanogens use to produce methane with acetic acid as the most prevalent volatile acid intermediate (15). About 70% of the methane produced in an anaerobic digester results from degradation of acetic acid (32). Methanogenic bacteria are morphologically diverse and exhibit varying biochemical natures (59). *Methanothrix* spp. are major acetate utilizing methanogens in anaerobic digesters (68).

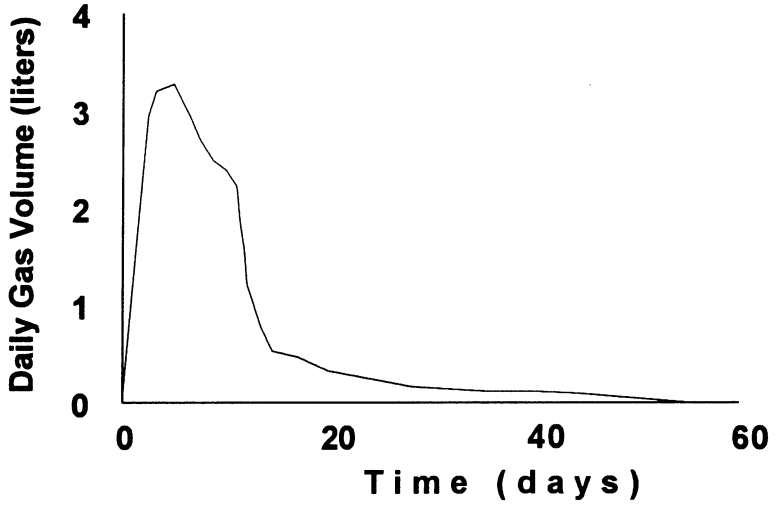

Figure 1. Gas production curve for a typical 3 L digester (42).

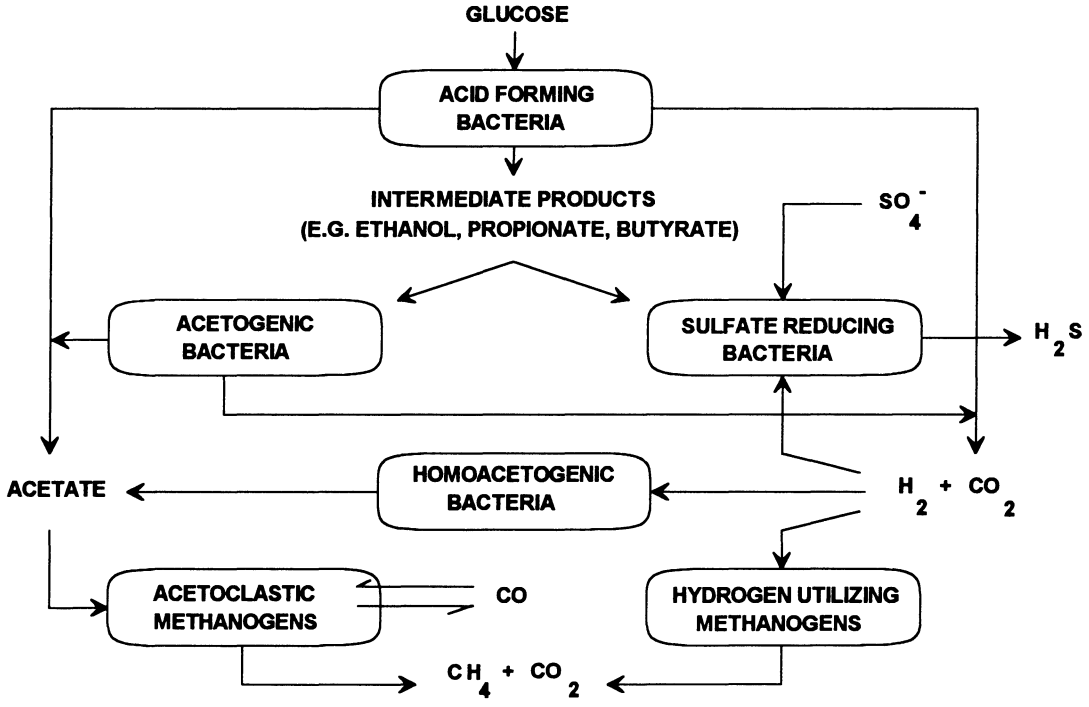

Figure 2. Potentially different communities in an anaerobic digester (23).

Methanosarcina spp. can utilize both acetate and H_2-CO_2 substrates. *Methanobacterium, Methanospirillum* and *Methanobrevibacter* species are the most frequently isolated formate and H_2-CO_2 utilizing methanogens and usually grow much faster (double every 6-12 hr) than acetotrophic methanogens (double every 1.5 to 7 d). Methanosarcinae usually predominate in digesters operated at short retention times due to their faster growth rates while *Methanothrix soehngenii* is usually more abundant in digesters operated for more than 30 days (43). Methanogen autotrophy is a newly recognized physiological type (34). Many of these organisms are capable of autotrophic growth on hydrogen and carbon dioxide, and all of them conduct the total synthesis of acetate from carbon dioxide.

The ecology of methanogens has been thoroughly reviewed (34) and many authors consider methane production to be the rate limiting step in anaerobic digestion (1). Methanogens seem to be more sensitive than acid-forming bacteria to changes in pH, temperature, and toxicants (2). Movement out of a pH tolerance range of approximately 6.5 to 7.6 (15) or a sudden drop of 2-5°C arrests methane production without affecting acid production (8, 9). Quality of methane produced generally ranges from 65-75% (15) with a 2:1 CH_4:CO_2 ratio considered normal (20). Gas composition and the rate of gas production have been used as indicators of digester performance for many years. Factors used to indicate onset of digester upset include decreasing gas production and/or percent methane, or an increase in hydrogen, carbon monoxide and/or volatile acids (9, 27).

Among other factors which can affect methanogenesis are heavy metals, an important class of anthropogenic pollutants derived from both point and non-point sources (13). Metals of most concern include Cr, Mn, Fe, Co, Ni, Zn, Cd, Hg, and Pb (17). For example, a study of trace metals in the influent to the Muncie Wastewater Treatment Plant found 0.27 ppm Cr, 0.25 ppm Cu, 0.79 ppm Zn, and 0.92 ppm Pb (16). Point sources such as paper, chemical, pesticide, petroleum, and metal plating industries contribute the greatest amount of heavy metals to sewage treatment systems, but residential contributions may be significant in large cities without industrial districts. Copper is added regularly to the New York City water supply to control algal growth

(35), and zinc is used to retard corrosion. As a result, the water supply contributes 67% more copper and 50% more zinc to sewage than all electroplating industries combined.

Soluble metals concentrate in sludge during clarification and aerobic digestion. Metals in secondary sludge concentrate to a greater extent than in primary sludge and can have more impact on anaerobic digestion than primary sludge alone (6). Anaerobic digester feed solids take up more than 99% of copper and lead, 90% of chromium, and 96% of mercury and zinc (6, 42, 53). Biological activity accounts for about 18% of copper and 8% of lead sedimentation during clarification, but does not apparently account for chromium and zinc in sediments (60, 61, 62, 63). Heavy metals present in sewage sludges are strongly associated with the solids fraction. More than 90% of solids in sludge are particulate in size (>1.1 mm). Supracolloidal fractions (5-8%), colloidal fractions (0.1-0.3%), and dissolved fractions (1-3%) make up the remainder of total solids (24). Particulate fractions hold more than 90% of metals in anaerobic digesters, while supra colloidal fractions contain 8-9%. The remainder are in colloidal and dissolved fractions (24).

These heavy metals can be toxic to microorganisms by altering cell morphology or metabolism, by bacteriostasis, or by cell death (19), but the mere presence of heavy metals in sludge does not mean toxic effects will occur. Several serve essential roles in life processes (e.g. cobalt, copper, and zinc) while others are considered to be poisons (e.g. mercury and lead). Cobalt and zinc serve as activators in some enzymes, but can substitute for magnesium in other enzymes, rendering them inactive. Many heavy metals physically denature enzymatic and structural proteins, making them non-functional (22). However, most adsorb onto microbial cells through nonspecific binding to cell surfaces, slime layers and extracellular matrices (22). Metabolism-dependent intracellular uptake is also important to the adsorption and absorption of some heavy metals. In this way, biological methylation of metals (e.g. mercury) to a volatile state or reduction to the elemental state may remove certain toxicants from the system (22). Biomass can take up a considerable amount of heavy metals without seriously hampering metabolism (10). In one study (25), 30-60% of the heavy metals were found in the intracellular fraction of sludge. Another study (55) determined the "optimal" contact time for removal of chromium and lead from the supernatant through biosorption was 2 to 4 hr.

In an anaerobic digester, total concentration of heavy metals is more important than concentration of each individual metal (41). Concentrations must reach a threshold level and be "available" to affect microorganisms (24). Bioavailability is determined by the total concentration of metals in the substrate (11) as well as various environmental factors (19). The presence of hydrous metal oxides, anions (e.g. sulfides, carbonates, and phosphates), competing cations, organic chelators, and clay materials modify metal availability. Factors such as pH, Eh, and temperature also can affect availability. Unfortunately, lack of data regarding the influence of environmental factors on the toxicity of heavy metals has hindered development of criteria for evaluating metal toxicity in various environments (21).

Dissolved heavy metals are considered inhibitory at concentrations in the 0.1 to 10 mgL^{-1} range (52). Precipitated metals are considered to be of little consequence to microorganisms in terms of toxicity (25), and conditions in an anaerobic digester efficiently convert more than 99% of metals to an insoluble form (6). Certain chemical reactions may reduce "available" heavy metal concentrations by a factor of over 1000. Thus, the ability of an anaerobic digester to tolerate heavy metals depends on the ability of sludge to precipitate or chelate metals, and is directly proportional to the dry solids concentration of digesting sludge (46). Substrate composition is therefore one of the major factors that determines digester ecosystem properties (36), and highly variable chemical and physical characteristics of sludges produce a wide variety of ecosystems.

Several methods exist to reduce metal availability in sludge. Hydrogen sulfide/sulfide and carbon dioxide/carbonate systems remove most heavy metal ions from solution (48). Sulfides play the most important role in preventing heavy metal toxicity in anaerobic digesters because metal sulfides are more insoluble than metal carbonates. All heavy metals (except chromium which precipitates as a hydroxide) can be removed from solution as insoluble sulfides (28). Relative concentrations of sulfides, carbonates, basic carbonates and hydroxides depend on pH, temperature, and soluble sulfide concentration. The major portion of sulfides in sewage sludge digestion occurs naturally through reduction of sulfates or other sulfur-containing inorganic compounds, or from the degradation of sulfur-containing proteins and amino acids (41). Metals continue to precipitate as sulfides as long at the total metal concentration does not exceed total

soluble sulfide ($H_2S + HS^- + S^{2-}$) levels. After soluble sulfides have been exhausted, metals will precipitate as carbonates and to a small degree as phosphates (12). Therefore, higher concentrations of heavy metals also can be tolerated if sufficient sulfide is present to act as a precipitant (39). However, concentrations of soluble sulfide above 200 mgL^{-1} as sulfur produce severe toxic effects and arrest gas production (40).

Soluble metals also adsorb onto hydrous metal oxides or negatively charged clay particles, and chelate or complex with inorganic species (ion pairs) . For example, nonalkali metals such as calcium and magnesium form soluble ion pairs with Se^{2-} (12). Heavy metals chelate, covalently bond, or cation exchange with organic matter due to their affinity for certain functional groups and locations within the compound (12). Chelating agents such as citrate, cysteine, glutamate, and EDTA result in large reductions in free metal ion concentrations (22). Humic and fulvic acids as well as proteins found in sewage sludge also bind strongly with metals (22). Cations such as magnesium and calcium compete with metals for binding sites on chelating agents and can increase toxicity in "hard" water.

Considerable effort has been devoted over the past 30 years to assessment of heavy metal toxicity in anaerobic digesters. Unfortunately, comparison of results is difficult due to variations in experimental methodologies. Investigators rarely used similar experimental procedures regarding batch reactor conditions, bacterial inocula, or substrates. Most metals investigated were less toxic at higher concentrations of biomass and substrate (26). Toxicants generally were introduced as a single pulse dose or step-fed over a time interval until digester failure occurred. In general, anaerobic digesters seemed to be more tolerant when step-fed than when pulse-fed (25). Most research in heavy metal toxicity in anaerobic digesters apparently has focused on chromium, copper, and zinc, and reported toxicities varied considerably between studies. Very few studies on methanogenic inhibition by lead, mercury, selenium, and cobalt have been conducted.

Toxicity varies with microbial species. Some may be stimulated by a heavy metal, while others may tolerate or be inhibited completely by the same metal concentration. For example, trace amounts of cobalt and selenium stimulated methanogenesis in some organisms. Addition of cobalt in trace amounts stimulated gas production by 20-133% in 17 of 60 digester samples tested

(57) and addition of 0.079 mgL^{-1} of selenium to synthetic medium stimulated growth of *Methanococcus vannielii* (33). Sodium selenate halted methane formation by *Methanobacterium* strain M.o.H. by serving as a methyl trap. It inhibited reductive demethylation of methylcobalamin to methane with an IC_{50} of approximately 2.8 mgL^{-1} of selenium (44). Copper at low concentrations increased the lag phase and at 300 mgL^{-1} was almost completely inhibitory to *Methanobacterium formicum* (28). *Methanobacterium formicicum, Methanosarcina barkeri* MS, *Methanospirillum hungatei* JF1, and *Methanobacterium thermoautotrophicum* strain Marburg in artificial substrates were exposed to copper chloride and zinc chloride. Very small metal concentrations were required for 50% reduction in methanogenesis (Table 1). Addition of sludge increased IC_{50} levels for *M. hungatei* to 65-275 mgL^{-1} for copper and 36 mgL^{-1} for zinc (31). Chromium, copper, lead, and zinc inhibition of gas production in anaerobic digesters has been demonstrated in many studies (Table 2). Chromium is reduced rapidly to the trivalent state under anaerobic conditions, and inhibition is a function of total concentration of chromium in the sludge regardless of the form in which it arrives (46). Anions also may play a role in reducing gas production. Highly oxygenated compounds (e.g. nitrates) interfere with methane and hydrogen sulfide production (29). Addition of metals as acids, sulfates, and nitrates may produce results different than those produced by chlorides. For example, dichromate caused digester failure at 450mgL^{-1} while chromium chloride caused failure at 530 mgL^{-1} (47), and copper nitrate produced an 80% reduction in gas production at less than 50 mgL^{-1} (25) while copper cloride reduced gas production by only 50% at 75-90 mgL^{-1} (27). Digesters also tolerated greater concentrations of chromium when introduced step-wise over a time interval than when added as a single pulse dose. This response is shown by a reduction in gas production of 80% with a pulse addition of 180mgL^{-1} of chromium nitrate, while 420 mgL^{-1} produced the same reduction when step-fed (25).

Retention time also is important to inhibitor tolerance (3), since methanogens have considerable potential for acclimation to toxicants. With continuous exposure, acclimation is possible if toxicants are added slowly to the system in a daily feed schedule. Methane bacteria can operate with no loss in efficiency at influent toxicant concentrations more than 12 times those causing inhibition to unacclimated systems (51). For example, shorter retention times (14 days)

Table 1. Concentrations (mgL^{-1}) of copper and zinc required to achieve a 50 percent reduction in methanogenesis by pure cultures of 4 methanogens (31).

Methanogen	$CuCl_2$	$ZnCl_2$
Methanobacterium formicicum	3.5	8.2
Methanosarcina barkeri MS	4.1	8.9
Methanospirillum hungatei JF1	2.3	1.3
Methanobacterium thermoautotrophicum	4.8	3.0

Table 2. Representative examples of chromium, copper, lead, and zinc concentrations required to inhibit digesters to varying degrees.

Metal	Anion	Dose	[C] (mgL^{-1})	Reduction in gas production (%)	Ref.
Cr	sulfate	pulse	200	80[a]	3
Cr	sulfate	pulse	300	95.6[a]	3
Cr	nitrate	pulse	<180	80	25
Cr	nitrate	step	420	80	25
Cr	dichromate	step	450	Digester failure	47
Cr	chloride	step	530	Digester failure	47
Cu	nitrate	pulse	<50	80	25
Cu	nitrate	step	70	80	25
Cu	chloride	pulse	75	50[b]	27
Cu	chloride	pulse	90	50[c]	27
Cu	acetate	step	190	Digester failure	47
Cu	unknown	step	200	Digester failure	56
Cu	unknown	pulse	250	Digester failure	56
Pb	acetate	pulse	106	33	48
Pb	acetate	pulse	212	71	48
Zn	acetate	pulse	65	36	48
Zn	acetate	pulse	130	92	48
Zn	acetate	step	163	50[d]	47

[a] 21 day solids retention time
[b] 20 day hydraulic retention time
[c] 10 day hydraulic retention time
[d] Digester failure

were impacted significantly by 50 mgL^{-1} of chromium while longer intervals (20 days) were unaffected by 300 mgL^{-1}. Older cells may be able to tolerate exposure to certain inhibitory materials better than younger cells, while younger cells may be more tolerant to others (50).

Though heavy metals are inhibitory, most toxicants exhibit reversible inhibition at concentrations normally encountered in municipal wastewater. One researcher found that methane production recovered over a 24 hr period at copper and zinc concentrations < 100 mgL^{-1} (27). Total recovery was observed at 25 mgL^{-1}, but no recovery was evident after 5 days at 300 mgL^{-1}. Another study found that introduction of 60 mgL^{-1} of copper caused methane production to cease for 22 days, but only 10 additional days were required to recover full methane production (58). Maximum continuous influent doses that allow satisfactory anaerobic digestion of sludge were determined to be 50 mgL^{-1} Cr, 5-10 mgL^{-1} Cu, and 10 mgL^{-1} Zn (6, 54). Safe limits for chromium in another study were determined to be 3-25 mgL^{-1} (4). Safe and toxic limits for heavy metals in anaerobic digesters must be determined for individual treatment facilities due to significant variability in sludge composition and retention time, environmental factors, and bacterial populations. Tolerance levels determined for one facility may not be suitable for all other facilities, therefore, each facility should determine its own thresholds through experimentation.

METHODS AND MATERIALS

Twelve 6.0 L digesters were made from 10 x 70 cm (4 x 28 in) thin-wall Genova-1 MS PVC sewer pipe (ASTM D2729) and installed on a plywood cradle on a New Brunswick Scientific Company Model S-3 Gyrotory shaker (Figure 3). The digesters were enclosed within a bag constructed from 3.5 mil black plastic sheet. Seven 75W incandescent lamps controlled by a Powerstat variable autotransformer were used to heat the digesters. Aluminum reflectors protected the bag from heat damage from the lamps. Air within the bag was circulated by a small squirrel cage fan. A threaded 1.25 cm (0.5 in) female PVC adapter served as a port at one end of each digester. A male 1.25 cm (0.5 in) PVC adapter with a rubber septum sealed with silicone rubber and a 0.62 cm (0.25 in) nylon hose barb vented gas into 1 m of 0.62 cm (0.25 in) Tygon R-3603 tube. The gas collection system included 1) a glass tee with a silicone grease coated water balloon for pressure relief, 2) a 3 cm silicone rubber hose segment for toxicant injection and gas sampling, 3) a glass wool filter for gas surge suppression, and 4) a bent Pasteur pipette which bubbled evolved gas into an inverted 50 mL buret filled with 10% NaCl solution. A copper/constantan thermocouple placed into one digester and a Wescor TH-65 digital thermocouple thermometer were used to monitor incubator temperature.

Anaerobic sludge was supplemented with cow manure, dry dogfood, and ammonium acetate to ensure gas production was not limited by nutrient availability during the initial 6 hr interval. Cow manure was collected from a local pasture and dried overnight in a 105°C oven. Dry dogfood (Buyers Choice Chunk Style) was purchased from a local grocery. Both the manure and dogfood were ground up and passed through a 2 mm (#10) sieve (Fisher Scientific Company). An ammonium acetate solution was produced by titrating 10% acetic acid with 30% ammonium hydroxide to pH 7.00. Sludge was collected from the primary anaerobic digester of the Charleston Wastewater Treatment Plant. Samples were taken in the morning, prior to daily addition of sludge from primary and/or secondary clarifiers. For each experimental run, a 70 L portion of sludge was supplemented with 400 g of manure, 175 g of dog food, and 475 mL of ammonium acetate solution (to increase acetate concentration 500 ppm). Supplemented sludge

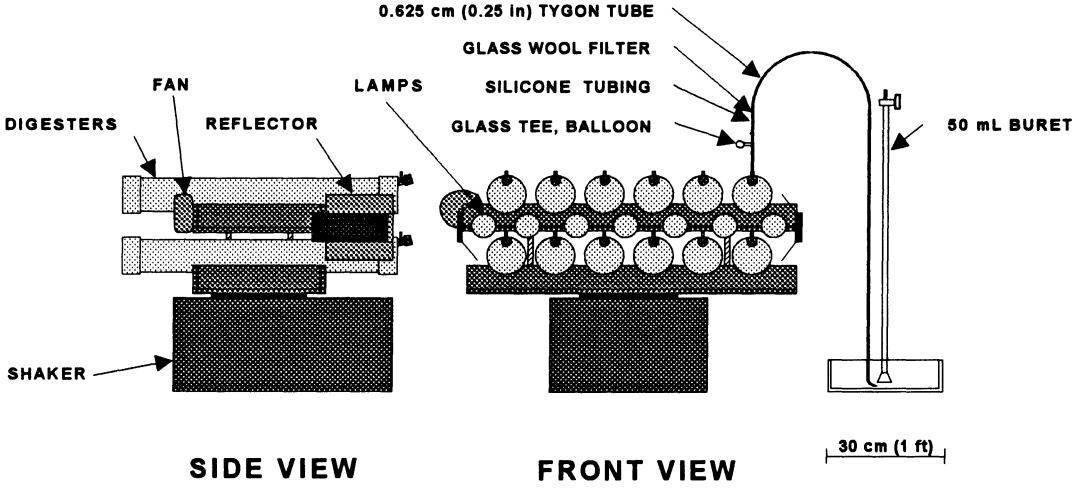

Figure 3. Schematic representation of the anaerobic digester system with the plastic bag removed. Digesters were connected to separate gas collection systems, only one of which is shown here.

was filtered through a 2 mm sieve and 5 L portions were randomly poured into each of the 12 digesters. Digesters were randomly positioned on the cradle and shaken at 150 rpm for 15 min while vented to the atmosphere to ensure all had achieved the same aerobic status. Digesters then were connected to the gas collection system, enclosed within the plastic bag, and heated to 36 +/- 1°C. Saline solution drawn into Tygon tubing indicated oxygen consumption and was used to determine establishment of anaerobic conditions and positive gas flow. Temperature equilibrium, anaerobic conditions, and positive gas flow were established within 45 to 60 min.

Selected concentrations (50-400 mgL^{-1}) of Co, Cr, Cu, Hg, Na, Pb, Se, or Zn as $CoCl_2$, CrO_3, $CuCl_2$, $HgCl_2$, NaCl, $(CH_3COO)_2Pb$, Na_2SeO_3, and $ZnCl_2$ were examined. HCl and NaOH also were examined to determine how total gas and methane production were affected by pH shifts due to toxicant addition. Concentrated doses were prepared as 20 mL portions in near-boiling Milli-Q (Millipore Corp.) water. All compounds except lead acetate were completely soluble in 20 mL of near-boiling water. The highest concentration of lead acetate was injected as a milky-white suspension with the assumption it would enter solution following dilution by sludge. Each concentration was tested in triplicate and injected via hypodermic syringes through silicone rubber tubing immediately after positive gas flow was observed. Digesters were shaken at 150 rpm for the duration of each experiment. Digesters and port fixtures were acid-washed with 500 mL of 2M HCl and rinsed with tap water between runs to prevent development of acclimated bacteria.

Total and suspended solids as well as pH shifts due to toxicant addition were determined for each supplemented sludge sample. Total solids were determined from 25 mL portions dried at 105°C for 2 hr. Dissolved solids were determined from sludge samples centrifuged at 10,000 rpm for 10 min. The supernatant was passed through glass filters and 25 mL portions were dried for 2 hr at 105°C. Initial suspended solids were determined from the difference between total and dissolved solids. Initial pH shifts due to toxicants were determined by dosing 500 mL of sludge with 2 mL of concentrated toxicant. Actual pH shifts under anaerobic conditions may have differed since determinations were performed under aerobic conditions.

Gas production rates and methane in headspace gases were determined every 2 hr for the first 24 hr and every 6 hr thereafter during the 72 hr copper chloride experiment. The parameters were determined 6 hr after toxicant addition for all other experiments. Gas production rates were determined through displacement of 10% saline solution in inverted burets over 5 to 30 min intervals. Methane concentrations were determined by flame ionization gas chromatography (Gow-Mac model 750, Bridgewater, N.J.) at room temperature with a Porapak R column and nitrogen as a carrier. Headspace gas samples (500 uL) were withdrawn from each digester via a gas-tight hypodermic syringe and diluted in 16 mm culture tubes sealed with rubber septa (Wheaton) prior to injection into the gas chromatograph. Each culture tube contained a small glass bead which was used to mix the gases. Various volumes (175-1000 uL) of diluted gas were injected into the gas chromatograph. An Apple II+ computer was interfaced with the gas chromatograph (Figure 11 in Appendix B) and custom software (Appendix C) was used to determine peak areas and methane concentrations from the signal.

RESULTS AND DISCUSSION

Preliminary experiments with sludge indicated total gas production rose within minutes after anaerobic conditions developed and peaked approximately 8 to10 hr later. A rapid decline or short plateau followed the peak within the next several hours, depending on substrate quality. Gas production then gradually decreased over several days. Total gas production rates integrated the output of all gases, including carbon dioxide, carbon monoxide, nitrogen, and hydrogen. Determination of methane in headspace gas provided a measure of methane production as a fraction of total gas production. Suspended solids, total gas production rates, and percent methane in headspace gases in 5 unsupplemented and 13 supplemented sludge samples were compared (Table 3). Supplementation with cow manure, dogfood, and ammonium acetate increased initial gas production rates by 40-50%, influenced non-methanogens more than methanogens, but did not significantly affect substrate variability.

Least squares analyses of measured parameters in enhanced sludge were used to determined whether concentration of suspended solids influenced gas production rates or methane in headspace gas. Correlation coefficients (r) between suspended solids and gas production (0.4060), as well as with methane in headspace gas (0.0458) indicated suspended solids concentrations did not influence results during the first 6 hr. Substrate variability was not a factor in this series of experiments because the same sludge sample was used for all concentrations of each toxicant examined. Determination of inhibition as a percent of the control allowed side-by-side toxicant comparisons.

A 72 hr assay provided a "long-term" look at how digesters responded to various concentrations of copper chloride. Copper concentrations up to 160 mgL^{-1} slightly inhibited gas production, but inhibited digesters eventually produced slightly higher rates than the control after 24 hr (Figure 4). This indicated that inhibition simply increased the lag phase of some organisms. The organisms may have required time to counter toxicant inhibition or remained inhibited until dissolved copper was removed by physiochemical and biological activity. Copper at 250 mgL^{-1} significantly inhibited gas production over the entire 72 hr interval (Figure 4). A single, copper

Table 3. Mean suspended solids, total gas production rates, percent methane in headspace gas, and coefficients of variation (Cv) for enhanced and unenhanced sludges after 6 hr.

	Susp. solids (gL^{-1})	Cv (%)	Rate ($ccmin^{-1}L^{-1}$)	Cv (%)	CH_4 (%)	Cv (%)
Unenhanced	28.7 +/- 3.4	11.8	0.75 +/- 0.24	32.0	55.0 +/- 4.9	8.9
Enhanced	26.4 +/- 2.6	9.8	1.07 +/- 0.34	31.8	44.7 +/- 2.8	6.3

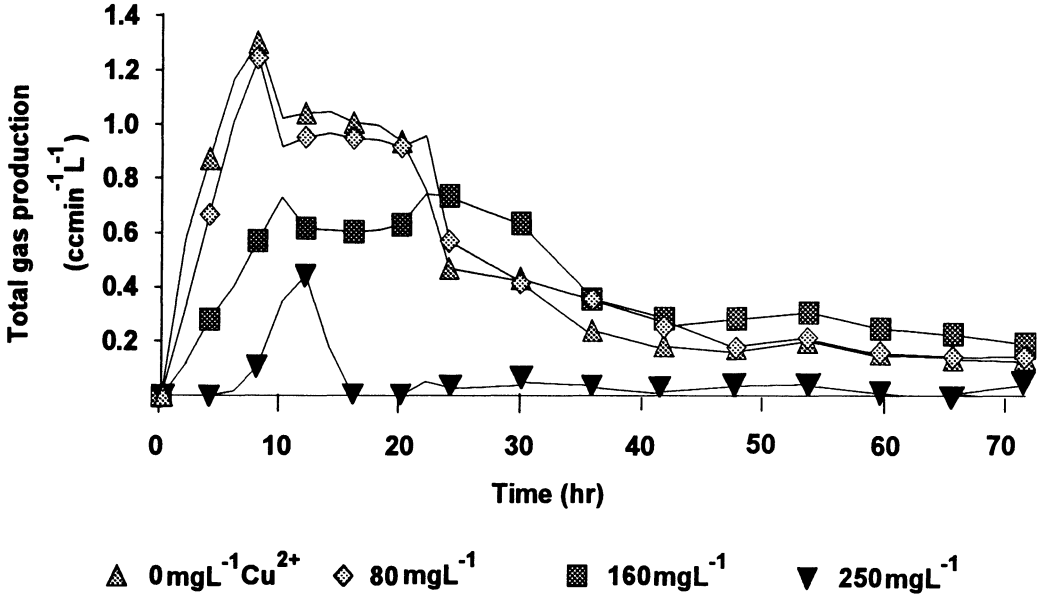

Figure 4. Comparison of total gas production rates in anaerobically digesting sewage sludge after addition of copper chloride.

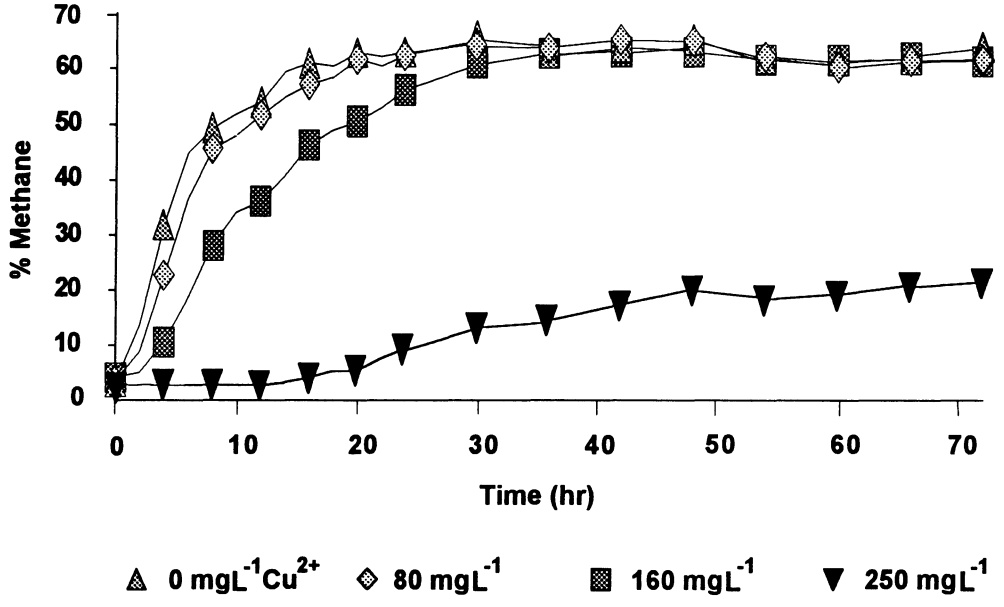

Figure 5. Comparison of percent methane in headspace gas over anaerobically digesting sewage sludge after addition of copper chloride.

resistant species that utilized a specific substrate probably produced the small peak at the 10 hr mark and died after all substrate was consumed. Gas resulting from this small peak did not contain methane (Figure 5). Copper concentrations up to 160 mgL^{-1} reduced methane production, but headspace gas eventually attained the same methane concentration as the control (Figure 5). Copper at 250 mgL^{-1} significantly inhibited methane production. Methane production remained arrested until 12 hr after toxicant addition when it appeared to begin slowly and maintain a constant rate for the next 60 hr (Figure 5). It is possible that copper tolerant methanogenic species were able to endure these conditions and produce this small quantity of methane. Methane content of the headspace gas reflected total gas production rates for the first 8 hr.

Variability between digesters dropped significantly as gas production increased and remained below 3% for the duration of the experiment (Table 4). Gas production and methane increases were linear for the first 6 hr without a significant baseline lag phase for all copper concentrations. This linearity provided an excellent time interval to determine degrees of inhibition and the 6 hr mark proved optimal for maximum separation of measured values. Gas production rates and methane in headspace gas also showed a strong correlation. Least squares correlations between gas production rates and methane in headspace gas at the 6 hr mark were 0.9652, 0.9859, 0.9532, and -0.5556 for 0, 80, 160, and 250 mgL^{-1} Cu^{2+}, respectively (Table 5).

Addition of toxicants as pulse doses temporarily shifted the pH within each digester. Toxicant type and concentration influenced both the direction and degree of shifts. To determine if pH shifts biased parameter measurements, HCl and NaOH induced shifts were examined at the 6 hr mark (Figure 6). Gas production rates and methane concentrations increased by about 5% when the pH dropped by 0.30 units. Conversely, gas production rates and methane concentrations dropped by 10-15% when pH increased between 0.20-0.30 units. Sodium did not alter pH, whereas the majority of toxicants produced an initial negative shift ranging from -0.20 to -0.30 units (Table 6). Sodium selenite was the only toxicant that shifted pH toward greater alkalinity (Table 6). Smaller toxicant concentrations produced correspondingly smaller pH shifts. With the exception of selenium and copper, pH shifts due to toxicant introduction introduced no bias in the determination of toxicity. Inhibition due to pH accounted for less than 5-10% at the highest concentrations of selenium and copper added.

Table 4. Mean values and coefficients of variation (Cv) for measured parameters in the controls for the first 12 hr of the 72 hr copper chloride experiment.

	Total gas production rate		CH_4 in headspace gas	
Time (hr)	Mean ($ccmin^{-1}L^{-1}$)	Cv (%)	Mean (%)	Cv (%)
2	0.58 +/- 0.10	17.24	13.7 +/- 4.5	32.85
4	0.87 +/- 0.03	3.45	32.0 +/- 4.1	12.81
6	1.17 +/- 0.02	1.71	45.0 +/- 1.4	3.11
8	1.30 +/- 0.02	1.54	49.4 +/- 0.7	1.41
10	1.02 +/- 0.03	2.94	52.0 +/- 0.6	1.15
12	1.04 +/- 0.03	2.88	54.0 +/- 0.8	1.48

Table 5. Linear least squares line equations and correlation coefficients for parameters measured during the first 6 hr of the 72 hr copper chloride experiment.

Parameter	Cu^{2+} (mgL^{-1})	Slope	y-intercept	Corr. Coeff. (r)
Rate	0	0.190	0.085	0.9838
	80	0.169	-0.006	0.9999
	160	0.068	-0.005	0.9980
	250	0.002	-0.003	0.7746
CH_4	0	7.161	2.032	0.9949
	80	5.652	1.008	0.9834
	160	2.416	2.372	0.9517
	250	-0.004	2.856	-0.7746

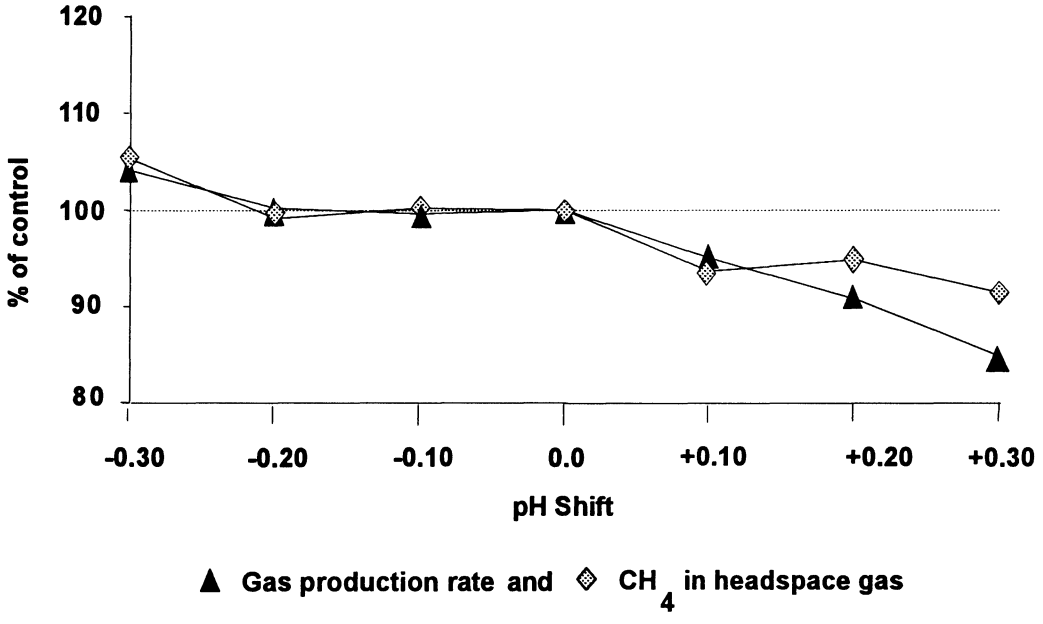

Figure 6. Comparison of total gas production rates and methane in headspace gas 6 hr after HCl and NaOH induced shifts from pH 7.10.

Table 6. Maximum pH shifts due to toxicant introduction.

	Toxicant							
pH	Zn^{2+}	Cu^{2+}	Hg^{2+}	Co^{2+}	Pb^{2+}	Cr^{3+}	Se^{2-}	Na^{+}
Initial	6.90	6.95	7.15	7.20	7.10	7.00	7.10	7.20
Final	6.70	6.65	6.95	7.00	6.90	6.75	7.30	7.20
Change	-0.20	-0.30	-0.20	-0.20	-0.20	-0.25	+0.20	0.00

Results cover a period of 4 months during which the experiments were performed, and it is important to know to what extent sludge variability influenced data. Statistical analyses were performed on controls of each toxicant assay to evaluate natural variability of gas production rates and methane present in headspace gas (Table 7). One-way analysis of variance (ANOVA) was performed to identify variations in controls of the various assays. Results show there are significant differences between the controls of different toxicant assays, but variation within the controls in each experimental run is rather small (Table 14).

Sodium chloride was used as a reference toxicant in this series of experiments because digesters are quite insensitive to sodium (Figures 7, 8). Sodium chloride up to 2 gL^{-1} stimulates digestion of domestic sludge (38), and the concentration limit for sodium is 4.6 gL^{-1} (37). Predictably, sodium was slightly stimulatory at all concentrations examined. Sodium concentrations of 200 mgL^{-1} stimulated total gas production and methane in headspace gas by 4-6%. Lead and cobalt were the least inhibitory elements examined (Figures 7, 8) and median inhibitory concentrations were greater than the maximum concentration tested (400 mgL^{-1}). This response suggests the substrate or environmental conditions efficiently removed the substances or they were less toxic than the others. Mercury produced moderate inhibition at the concentrations examined (Figures 7, 8). Selenium, chromium, copper, and zinc were the most inhibitory toxicants examined and produced almost complete inhibition at concentrations less than 200 mgL^{-1} (Figures 9, 10). Toxicant concentrations reduced gas production rates and methane in headspace gas in a linear fashion during the first 4-8 hr interval. Comparison of inhibition at 200 mgL^{-1} indicated relative toxicities were: $Na < Co < Pb < Hg < Zn = Cr = Se = Cu$. Linear least squares line equations based on percent inhibition were used to calculate median inhibitory concentrations (IC_{50}) for each toxicant (Table 8). Dosages necessary for 50% inhibition of total gas production differ only slightly from those required to reduce methane production by the same degree. The similarity suggests methanogenic populations are not generally more susceptible than other gas producers to the toxicants under these conditions.

Table 7. Mean values and coefficients of variation (Cv) for measured parameters in the controls at the 6 hr mark of each toxicant assay.

	Total gas production rate		CH_4 in headspace gas	
Toxicant	Mean ($ccmin^{-1}L^{-1}$)	Cv (%)	Mean (%)	Cv (%)
Chromium	1.07 +/- 0.02	1.87	46.8 +/- 0.7	1.50
Cobalt	1.25 +/- 0.05	4.00	45.7 +/- 5.2	11.38
Copper	1.32 +/- 0.04	3.03	47.0 +/- 1.8	3.83
Mercury	1.12 +/- 0.10	8.93	41.1 +/- 4.9	11.92
Lead	1.28 +/- 0.04	3.12	42.1 +/- 0.4	0.95
Selenium	0.98 +/- 0.01	1.02	39.1 +/- 0.4	1.02
Sodium	1.24 +/- 0.07	5.65	49.7 +/- 0.8	1.61
Zinc	1.12 +/- 0.01	0.89	46.6 +/- 2.5	5.36
Overall	1.17 +/- 0.12	10.26	44.8 +/- 4.1	9.15

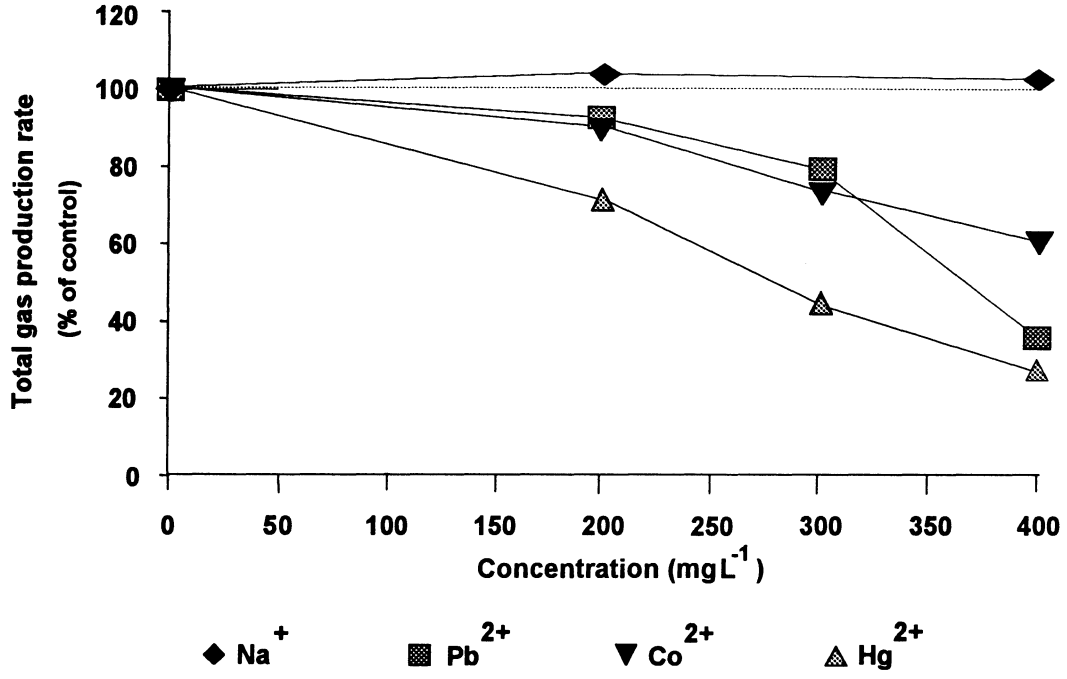

Figure 7. Comparison of total gas production rates 6 hr after addition of various doses of sodium, lead, cobalt, and mercury into anaerobically digesting sewage sludge.

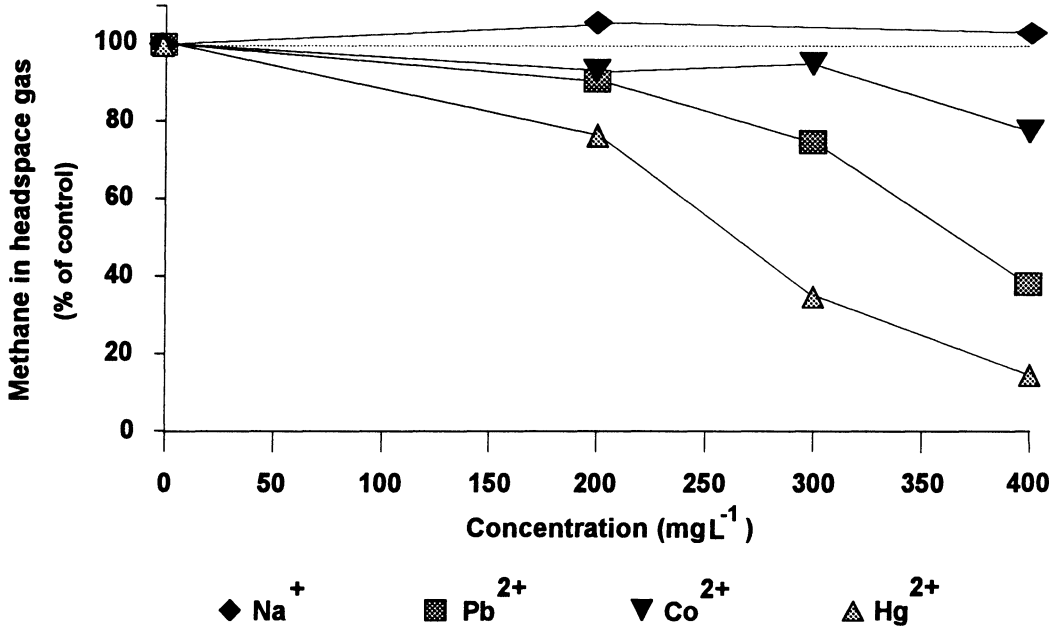

Figure 8. Comparison of methane in headspace gas 6 hr after addition of various doses of sodium, lead, cobalt,and mercury into anaerobically digesting sewage sludge.

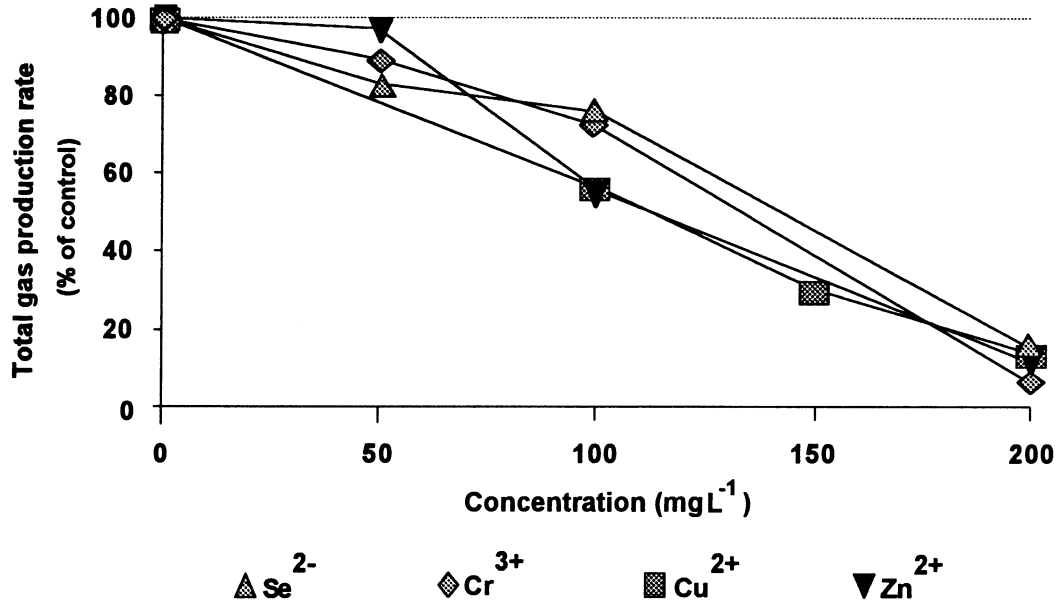

Figure 9. Comparison of total gas production rates 6 hr after addition of various doses of selenium, chromium , copper, and zinc into anaerobically digesting sewage sludge.

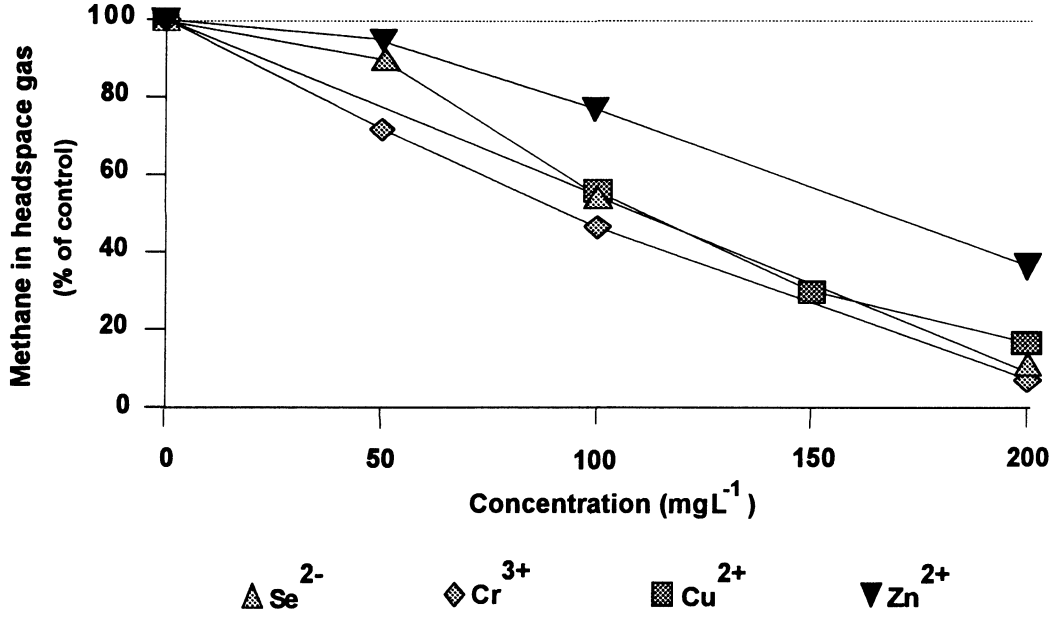

Figure 10. Comparison of methane in headspace gas 6 hr after addition of various doses of selenium, chromium, copper and zinc into anaerobically digesting sewage sludge.

Table 8. Linear least squares line equations and correlation coefficients between toxicant concentration and reductions in gas production and methane. Equations were derived from inhibitions as a percent of the control and used to calculate IC_{50} values for each toxicant.

		Line Equation			
		----------			IC_{50}
Toxicant	Parameter	Slope	y-intercept	Corr. Coeff.	(mgL^{-1})
Cobalt	Rate	0.097	-3.38	0.9609	*551
	CH_4	0.047	-2.04	0.8331	*1,106
Lead	Rate	0.145	-9.48	0.8556	*410
	CH_4	0.143	-7.96	0.8957	*405
Mercury	Rate	0.183	-2.36	0.9919	286
	CH_4	0.220	-5.96	0.9677	254
Selenium	Rate	0.413	-5.08	0.9733	133
	CH_4	0.466	-4.80	0.9891	118
Chromium	Rate	0.481	-9.00	0.9738	123
	CH_4	0.459	3.38	0.9961	102
Zinc	Rate	0.473	-7.62	0.9753	122
	CH_4	0.328	-5.84	0.9821	170
Copper	Rate	0.439	0.41	0.9980	115
	CH_4	0.428	1.19	0.9956	114

* Extrapolated values.

Lower IC_{50} values for methane in headspace gas than total gas production would result if methanogens were more sensitive to a toxicant than other gas producers. Conversely, higher IC_{50} values for methane in headspace gas than total gas production would indicate methanogens were less sensitive than other gas producers. Methane producing organisms apparently were more sensitive than other gas producers to selenium, mercury, and chromium by 12, 21, and 13% respectively. Copper and lead appeared to affect both populations equally. Zinc was 39% more toxic to non-methanogenic gas producers than to methanogens. Based on extrapolated IC_{50} values, cobalt was 200% more toxic to non-methane gas producers than methane producers. Cobalt reduced gas production by 40% and methane in headspace gas by 22% at 400 mgL^{-1}. It is highly unlikely that commercial digesters will experience cobalt at this concentration, therefore, cobalt cannot be considered significant as a toxicant in anaerobic digesters.

Amended sludge showed considerable tolerance to lead when compared with other treatments. Digester failure occurred at less than 200 mgL^{-1} in one study (48), while in this study it occured at more than 300 mgL^{-1}. Extrapolated results obtained for cobalt and lead may not accurately reflect actual inhibitory concentrations. Lead forms a variety of organometallics. Both lead and mercury show strong affinities for ligands such as phosphates, cysteinyl, and histidyl side chains of proteins, purines, pteridines, and porphyrins. Both inhibit a large number of enzymes having functional sulfhydryl groups, and lead inhibits most enzymes less readily than mercury (67).

Mercury and selenium toxicity in anaerobic digesters has not been explored to the same extent as that of copper, chromium, and zinc. Neither are major chronic pollutants in municipal sewage sludge and do not threaten anaerobic digesters often. Mercury readily forms complexes with ammonia, amines, halides, and cyanide, and reacts with sulfur. Methylation of mercury by methanogenic bacteria is considered a detoxification reaction, freeing them of mercury (67). Since both mercury and selenium undergo methylation, a portion of their toxicity to methanogens may have been due to metal methylation which consumed methyl free radicals.

Interpretation of the effects of metal salts on microorganisms is complicated by the different types of media used (18). However, inhibitory concentrations of chromium, copper, and zinc determined under these experimental conditions were consistent with several other studies. After 6 hr, 200 mgL^{-1} of chromium reduced gas production by 94%, compared with 80%

reduction in sludge solids retained for 21 days (3). Likewise, 100 mgL^{-1} of copper reduced gas production by 43.5% while 90 mgL^{-1} reduced it by 50% under a 10 day hydraulic retention time (27). It is difficult to compare zinc toxicity values with other studies because it affected non-methanogens much more than methanogens. Digester failure generally occurred between about 130-163 mgL^{-1} (47, 48) compared with 100-200 mgL^{-1} in this study.

Chromium is not used as a catalytic cofactor by bacteria due to its apparent substitution inertness in the normal biological redox states. However, highly oxygenated compounds (e.g. nitrates) interfere with methane and hydrogen sulfide production (29). This implies the oxide components of chromium trioxide and sodium selenite may have influenced methanogenesis more than other gas producing processes.

Most batch experiments begin with steady-state conditions and observe the rate at which total gas or methane production in anaerobic digesters decrease over time due to toxicants. This series of experiments shows that a linear interval of gas production during recovery from oxygen inhibition can be used to determine inhibition by other toxicants. Median inhibitory concentrations for sodium, chromium, copper, and zinc were consistent with results determined by other studies using batch analysis. This finding means toxicant assays can be determined quickly, with simple methods and inexpensive materials.

CONCLUSIONS

1. Sodium chloride, used as the reference toxicant, was slightly stimulatory (3-6%) at all concentrations tested. Cobalt and lead IC_{50} values were greater than the maximum concentrations tested (400 mgL^{-1}). Median inhibitory concentrations based on total gas production rates were cobalt ,551 mgL^{-1}; lead, 410 mgL^{-1}; mercury, 286 mgL^{-1}; chromium, 123 mgL^{-1}; zinc, 122 mgL^{-1}; copper, 115 mgL^{-1}; and selenium, 133 mgL^{-1}. Median inhibitory concentrations based on reductions in methane in headspace gas were cobalt, 1106 mgL^{-1}; lead, 405 mgL^{-1}; mercury, 254 mgL^{-1}; chromium, 102 mgL^{-1}; zinc, 170 mgL^{-1}; copper, 114 mgL^{-1}; and selenium, 118 mgL^{-1}. Comparison of inhibition at 200 mgL^{-1} indicated relative toxicities were:

$$Na < Co < Pb < Hg < Cr = Zn = Cu = Se.$$

2. Results for chromium, zinc, and copper were consistent with other studies using modified Warburg respirometers and serum bottle analysis. Therefore, this is an inexpensive, quick, and easy method to determine inhibition of total gas and methane production in anaerobic digesters.

3. The first 6 hr of recovery from oxygen inhibition showed a linear increase in total gas production rates and methane in headspace gas. The rate was relatively independent of substrate quality as long as no nutrients were limiting. Additionally, toxicants reduced the rate of recovery as a linear function of toxicant concentration during this time interval.

4. Digester hardware and test equipment used in this series of experiments were off-the-shelf items that cost less than $500 (excluding the rotory shaker, gas chromatograph, and computer system). Tests were completed quickly in 8 hr with parameter measurements taking less than 20 min. Measurement of total gas production by liquid displacement and percent methane by computer assisted gas chromatography provided an easy method for determination of IC_{50} values.

LITERATURE CITED

1. Andrews, J.F. 1968. Chromatographic analysis of gaseous products and reactants for biological processes. Water Sew. Works. 115(2):54-59.

2. Andrews, J.F. 1975. Control strategies for the anaerobic digestion process, part I. Water Sew. Works 122(3):62-65.

3. Bailey, D.A., J.J. Dorrelll, and K.S. Robinson. 1970. The influence of trivalent chromium on the biological treatment of domestic sewage. Water Poll. Control. 69:100-110.

4. Barash, V.A. 1955. The influence of some mineral and organic substances on methane formation in sewage sludge, p.105-114. *In* Vsesoyuz. Nauch.-Issledovatel. Inst. Vodosnabzhen., Kanalizats., Gidrotekh. Sooruzhenii i Inzhener. Gidrogeol., Materialy Soveshchaniya (Pub. 1957). (Chem. Abstr.52:7583)

5. Barth, E.F., B.V. Salotto, G.N. McDermott, J.N. English, and M.B. Ettinger. 1963. Effects of a mixture of heavy metals on sewage treatment processes, p. 616-635. *In* Proc. 18th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

6. Barth, E.F., M.B. Ettinger, B.V. Salotto, and G.N. McDermott. 1965. Summary report on the effects of heavy metals on the biological treatment processes. J. Water Poll. Control Fed. 37(1):86-96.

7. Blanc, F.C., J.C. O'Shaughnessy, D. Walker, and S. Miller. 1987. Toxicity studies of combined metal finishing/municipal wastewaters, p. 853-862. *In* Proc. 42nd Ind. Waste Conf. Purdue Univ., Lafayette, IN.

8. Buswell, A.M. 1939. Anaerobic Fermentations. Bulletin no. 32, Illinois State Water Survey, Urbana, Illinois.

9. Buswell, A.M. 1964. Methane fermentation, p. 508-517. *In* Proc. 19th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

10. Button, M.P., and A.F. Gaudy, Jr. 1981. A process for removal of heavy metals from secondary sludge, p.509-518. *In* Proc. 36th Ind. Waste Conf. PurdueUniv., Lafayette, IN.

11. Callander, I.J. and J.P. Barford. 1983. Precipitation, chelation, and the availability of metals as nutrients in anaerobic digestion. I. Methodology. Biotech. Bioeng. 25:1947-1957.

12. Callander, I.J. and J.P. Barford. 1983. Precipitation, chelation, and the availability of metals as nutrients in anaerobic digestion II. Application. Biotech. Bioeng. 25:1959-1972.

13. Capone, D.G., D.D. Reese, and RP. Kiene. 1983. Effects of metals on methanogenesis, sulfate reduction, carbon dioxide evolotion, and microbial biomass in anoxic salt marsh sediments. Appl. and Environ. Microbiol. 45:1586-1591.

14. Cillie, G.G., M.R. Henzen, G.J. Stander, and R.D. Baillie. 1969. Anaerobic digestion IV. The application of the process in waste purification. Water Res. 3:623-643.

15. Dague, R.R. 1968. Application of digestion theory to digester control. J. Water Poll. Control Fed. 40:2021-2032.

16. Davis, J.A III and J. Jacknow. 1975. Heavy metals in wastewater in three urban areas. J. Water Poll. Control Fed. 47:2292-2297.

17. Dean, J.G., F.L. Bosqui, and K.H. Lanouette. 1972. Removing heavy metals from wastewater. Environ. Sci. Technol. 6:518-522.

18. Duxbury, T. 1981. Toxicity of heavy metals to soil bacteria. FEMS Microbiol. Lett. 11:217-220.

19. Duxbury, T. 1985. Ecological aspects of heavy metal responses in microorganisms, p.185-235. *In* Advances in Microbial Ecology, K.C. Marshal ed., Plenum Press, N.Y.

20. Ellerker, R., H.J. Dee, F.G.I. Lax, and D.A. Sargent. 1968. Application of chromatography in the examination of sewage, sewage sludge and sewage irrigated land. Water Poll. Control 67:542-553.

21. Erten, M., J.L. Pitt, N.L. Gale, B.G. Wixson, and R.Unal. 1990. Evaluating toxicity of speciated metals from metal discharging industries, p. 739-750. *In* Proc. 45th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

22. Gadd, G. M. and A.J. Griffiths. 1978. Microorganisms and heavy metal toxicity. Microb. Ecol. 4:303-317

23. Giraldo, E., K. Norgren, M.S. Switzenbaum, and R. F. Hickey. 1989. Hydrogen and carbon monoxide as early warning indicators of toxic upsets in anaerobic digestion, p. 247-256. *In* Proc. 44th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

24. Gould, M.S. and E.J. Genetelli. 1975. Heavy metal distribution in anaerobically digested sludges, p. 689-699. *In* Proc. 30th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

25. Hayes, T.D. and T.L. Theis. 1976. Effects and fate of selected heavy metals in anaerobic digestion, p. 157-173. *In* Proc.31st Ind. Waste Conf. Purdue Univ., Lafayette, IN.

26. Heck,R.P. III., D. J. Schaezler, and G.R. Kramer. 1972. Effects of heavy metals on microorganisms. Application to process design, p. 350-368. *In* Proc. 27th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

27. Hickey, R.F., J. Vanderwellen, and M.S. Switzenbaum. 1989. The effect of heavy metals on methane production and hydrogen and carbon monoxide levels during batch anaerobic sludge digestion. Water Res. 23:207-218.

28. Hobson, P.N. and B.G. Shaw. 1976. Inhibition of methane production by *Methanobacterium formicum*. Water Res. 10:849-852.

29. Ingols, R.S. 1953. The toxicity of chromium, p. 86-95. *In* Proc. 8th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

30. James, A., C.A. Chernicharo, and C.M.M. Campos. 1990. The development of a new methodology for the assessment of specific methanogenic activity. Water Res. 24:813-825.

31. Jarrell, K.F., M. Saulnier, and A. Ley. 1987. Inhibition of methanogenesis in pure cultures by ammonia, fatty acids, and heavy metals, and protection against heavy metal toxicity by sewage sludge. Can. J. Microbiol. 33:551-554.

32. Jeris, J.S. and P.L. McCarty. 1965. The biochemistry of methane fermentation using C^{14} tracers. J. Water Poll. Control Fed. 37:178-192.

33. Jones, J.B. and T.C. Stadtman. 1977. *Methanococcus vannielii*: Culture and effects of selenium and tungsten on growth. J. Bacteriol. 130:1404-1406.

34. Jones, W. J., D.P. Nagle Jr., and W.B. Whitman. 1987. Methanogens and the diversity of archaebacteria. Microbiol. Rev. 51:135-177.

35. Klein, L.A., M. Lang, N.Nash, and S.L. Kirschner. 1974. Sources of metals in New York City wastewater. J. Water Poll. Control Fed. 46:2653-2656.

36. Kotze, J.P., P.G. Thiel, and W.H.J. Hattingh. 1969. Anaerobic digestion II. The characterization and control of anaerobic digestion. Water Res. 3:459-494.

37. Kugelman, I.J. and P.L. McCarty. 1964. Cation toxicity and stimulation in anaerobic waste treatment II. daily feed studies, p.667-686. *In* Proc. 19th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

38. Kugelman, I.J. and P.L. McCarty. 1965. Cation toxicity and stimulation in anaerobic waste treatment. J. Water Poll. Control. Fed. 37(1):97-116.

39. Kugelman, I.J. and K.K. Chin. 1971. Toxicity, synergism and antagonism in anaerobic waste treatment processes. Anaerobic biological treatment processes. Adv. Chem. Ser.105:55-90. (Chem. Abs. 75:132739f)

40. Lawrence, A.W., P.L. McCarty, and F.J.A. Guerin. 1964. The effects of sulfides on anaerobic treatment, p.343-357. *In* Proc. 19th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

41. Lawrence, A.W. and P.L. McCarty. 1965. The role of sulfide in preventing heavy metal toxicity in anaerobic treatment. J. Water Poll. Control Fed. 37(3):392-406.

42. Lingle, J.W. and E.R. Hermann. 1975. Mercury in anaerobic sludge digestion. J. Water Poll. Control Fed. 47:466-471.

43. Mah, R.A. 1983. Interactions of methanogens and non-methanogens in microbial ecosystems, p. 13-22. *In* Proc. 3rd Intnl. Symp. Anaerobic Digestion.

44. McBride, B.C. and R.S. Wolfe. 1971. Biosynthesis of dimethylarsine by *Methanobacterium*. Biochemistry 10(23):4312-4317.

45. Meyers, R.E. 1982. Microcomputer graphics. Addison-Wesley Publishing Co., MA .

46. Mosey, F.E. 1976. Assessment of the maximum concentration of heavy metals in crude sewage which will not inhibit the anaerobic digestion of sludge. Water Poll.Control 75(1):10-17.

47. Mosey, F.E. and D.A. Hughes. 1975. The toxicity of heavy metal ions to anaerobic digestion. Water Poll. Control 74(1):18.

48. Mosey, F.E., J.D. Swanwick, and D.A. Hughes. 1971. Factors affecting the availability of heavy metals to inhibit anaerobic digestion. Wat. Poll. Control. 70:668-678.

49. Owen, W.F., D.C. Stuckey, J.B. Healy, Jr., L.Y. Young, and P.L. McCarty. 1979. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res. 13:485-492.

50. Parkin, G.F. and S.W. Miller. 1982. Response of methane fermentation to continuous addition of selected industrial toxicants, p.729-743. *In* Proc. 37th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

51. Parkin, G.F., R.E. Speece, C.H.J. Yang, and W.M. Kocher. 1983. Response of methane fermentation systems to industrial toxicants. J. Water Poll. Control Fed. 55:44-53.

52. Parkin, G.F. and W.F. Owen. 1986. Fundamentals of anaerobic digestion of wastewater sludges. J. Environ. Eng. 112:867-920.

53. Patterson, J.W. and S-S. Hao. 1979. Heavy metals interactions in the anaerobic digestion system, p.544-555. *In* Proc. 34th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

54. Reid, G.W., R.Y. Nelson, C. Hall, U. Bonilla, and B. Reid. 1968. Effects of metallic ions on biological waste treatment processes. Water Sew. Works. 115:320-325.

55. Roman-Seda, R.A. 1985. Adsorption of heavy metals with inert biological sludges at neutral pHs, p.451-458. *In* Proc. 40th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

56. Rudgal, H.T. 1941. Bottle experiments as guide in operation of digesters receiving copper-sludge mixtures. Sewage Wks. J. 13:1248.

57. Speece, R.E. 1988. A survey of municipal anaerobic sludge digesters and diagnostic activity assays. Water Res. 22:365-372.

58. Speece, R.E. and G.F. Parkin. 1983. The response of methane bacteria to toxicity, p.23-35. *In* Proc.3rd Intnl. Symp. Anaerobic Digestion..

59. Stafford, D.A. 1974. Methane production from waste. Effl. Water Treat. J. 14(2):73-79.

60. Stones, T. 1955. The fate of chromium during the treatment of sewage, p.345-347. *In* Jour. and Proc. Inst. Sew. Purif., Part 4.

61. Stones, T. 1958. The fate of copper during the treatment of sewage, p. 82-83. *In* Jour. and Proc. Inst. Sew. Purif., Part 1.

62. Stones, T. 1959. The fate of zinc during the treatment of sewage, p.254-257. *In* Jour. and Proc. Inst. Sew. Purif., Part 2.

63. Stones, T. 1960. The fate of lead during the treatment of sewage, p.221-223. *In* Jour. and Proc. Inst. Sew. Purif., Part 2.

64. Stuckey, D.C., W.F. Owen, P.L. McCarty, and G.F. Parkin. 1980. Anaerobic toxicity evaluation by batch and semicontinuous assays. J. Water Poll. Control Fed. 55:720-729.

65. Toerien, D.F. 1970. Population description of the non-methanogenic phase of anaerobic digestion I. Isolation, characterization and identification of numerically important bacteria. Water Res. 4:129-148.

66. Toerien, D.F. and W.H.J. Hatting. 1969. Anaerobic digestion I. The microbiology of anaerobic digestion. Water Res. 3:385-416.

67. Vallee, B.L. and D.D. Ulmer. 1972. Biochemical effects of mercury, cadmium, and lead. Ann. Rev. Biochem. 41:91-128.

68. Wu, W-M., R.F. Hickey, L. Bhatnagar, M.K. Jain, and J.G. Zeikus. 1989. Fatty acid degradation as a tool to monitor anaerobic sludge activity and toxicity, p. 225-233. *In* Proc. 44th Ind. Waste Conf. Purdue Univ., Lafayette, IN.

APPENDIX A

Table 9. Unenhanced sludge suspended solids, gas production rates, and percent methane in headspace gas after 6 hr.

Susp. Solids (gL^{-1})	Rate ($ccmin^{-1}L^{-1}$)	CH_4 (%)
23.20	0.53 +/- 0.03	58.9 +/- 3.8
32.26	0.71 +/- 0.07	53.7 +/- 8.9
29.40	0.51 +/- 0.08	47.4 +/- 5.0
30.07	0.97 +/- 0.06	55.4 +/- 3.4
28.92	1.02 +/- 0.04	59.7 +/- 0.0

Table 10. Enhanced sludge suspended solids, gas production rates, and percent methane in headspace gas after 6 hr.

Susp. Solids (gL^{-1})	Rate ($ccmin^{-1}L^{-1}$)	CH_4 (%)
22.24	1.12 +/- 0.01	46.6 +/- 2.5
23.54	1.32 +/- 0.06	47.0 +/- 1.8
23.69	1.12 +/- 0.10	41.1 +/-4.9
26.36	1.25 +/- 0.52	45.7 +/- 5.2
25.77	1.28 +/- 0.04	42.1 +/- 0.4
24.78	1.07 +/- 0.02	46.8 +/- 0.7
28.86	0.98 +/- 0.01	39.1 +/- 0.4
29.24	1.24 +/- 0.07	49.7 +/- 0.8
24.52	1.29 +/- 0.04	45.0 +/- 1.7
28.22	1.02 +/- 0.01	45.2 +/- 2.2
30.61	1.03 +/- 0.01	45.5 +/-4.3
27.98	1.17 +/- 0.02	45.0+/- 1.4
27.36	1.04 +/- 0.05	42.3 +/- 2.0

Table 11. Average gas production rates and percent methane in headspace gas over a 72 hr interval in enhanced sewage sludge containing copper.

Time	Total gas production ($ccmin^{-1}L^{-1}$)				% Methane in headspace gas			
(hr)	0 mgL^{-1}	80	160	250	0 mgL^{-1}	80	160	250
0	0.00	0.00	0.00	0.00	3.4	3.6	4.2	2.8
2	0.58	0.32	0.12	0.00	13.7	8.9	5.2	2.9
4	0.87	0.67	0.28	0.00	32.0	22.8	10.6	2.8
6	1.17	1.01	0.40	0.02	45.0	36.6	18.5	2.8
8	1.30	1.24	0.57	0.11	49.4	45.7	28.4	2.8
10	1.02	0.92	0.73	0.35	52.0	48.1	34.2	2.7
12	1.04	0.95	0.62	0.44	54.0	51.7	36.2	2.7
14	1.04	0.96	0.61	0.18	59.4	54.9	40.8	3.2
16	1.00	0.95	0.60	0.00	61.0	57.1	46.4	4.0
18	1.00	0.94	0.61	0.00	60.4	58.5	49.0	5.4
20	0.94	0.92	0.63	0.00	62.9	61.7	50.6	5.4
22	0.76	0.96	0.74	0.05	62.2	60.4	53.0	7.6
24	0.47	0.57	0.74	0.03	63.0	62.4	56.2	9.2
30	0.43	0.41	0.63	0.06	66.2	64.2	61.1	13.1
36	0.24	0.36	0.36	0.03	62.8	63.8	62.8	14.5
42	0.19	0.25	0.28	0.02	64.0	65.3	63.0	17.3
48	0.17	0.19	0.28	0.04	65.4	65.0	63.0	19.9
54	0.20	0.21	0.31	0.05	61.9	62.2	61.6	18.5
60	0.16	0.16	0.25	0.01	61.6	60.3	61.4	19.2
66	0.13	0.14	0.23	0.01	61.8	61.5	62.3	20.8
72	0.14	0.14	0.19	0.05	64.0	61.6	61.4	21.3

Table 12. Changes in parameters after 6 hr due to shifts from pH 7.10.

Compound	pH shift	Rate ($ccmin^{-1}L^{-1}$)	CH_4 (%)
HCl	0.00	1.03 +/- 0.01	45.5 +/- 4.3
	-0.10	1.03 +/- 0.01	45.6 +/- 3.2
	-0.20	1.04 +/- 0.02	45.1 +/- 1.7
	-0.30	1.08 +/- 0.02	47.9 +/- 4.3
NaOH	0.00	1.04 +/- 0.05	42.3 +/- 2.0
	0.10	0.99 +/- 0.11	39.6 +/- 2.0
	0.20	0.94 +/- 0.44	40.1 +/- 3.3
	0.30	0.88 +/- 0.08	38.7 +/- 3.3

Table 13. Parameter changes as a percent of the control after 6 hr due to HCl and NaOH induced shifts from pH 7.10 (control - toxicant)/control .

	pH						
Parameter	6.80	6.90	7.00	7.01	7.20	7.30	7.40
Rate	4.1	0.2	-0.4	0.0	-5.0	-9.2	-15.2
% CH_4	5.3	-0.9	0.2	0.0	-6.4	-5.2	-8.5

Table 14. Completely randomized one-way ANOVA for gas production rates and methane in headspace gas in controls of all toxicants after 6 hr ($F_{(7, 16, 0.05)} = 3.22$).

		Gas production rates				Methane in headspace gas			
Source	DF	SS	MS	F_{calc}	P	SS	MS	F_{calc}	P
Group	7	0.280	0.040	9.14	0.0001	271	38.7	5.03	0.0036
Error	16	0.070	0.004			123	7.7		
Total	23	0.350				394			

Table 15. Mean data and coefficients of variability (Cv) for each toxicant assay.

Toxicant	[C] (mgL^{-1})	Rate ($ccmin^{-1}L^{-1}$)	Cv (%)	CH_4 (%)	Cv (%)
Zinc	0	1.12 +/- 0.01	0.89	46.6 +/- 2.5	5.36
	50	1.08 +/- 0.06	5.55	44.1 +/- 1.0	2.27
	100	0.62 +/- 0.08	12.90	35.7 +/- 6.2	17.37
	200	0.13 +/- 0.02	15.38	17.2 +/- 1.5	8.72
Copper	0	1.32 +/- 0.04	3.03	47.0 +/- 1.8	3.83
	100	0.75 +/- 0.03	4.00	26.3 +/- 1.5	5.70
	150	0.40 +/- 0.04	10.00	14.1 +/- 5.2	36.88
	200	0.18 +/- 0.01	5.55	7.9 +/- 1.4	17.72
Mercury	0	1.12 +/- 0.10	8.93	41.1 +/- 4.9	11.92
	200	0.80 +/- 0.06	7.50	31.5 +/- 2.6	8.25
	300	0.49 +/- 0.13	26.53	14.3 +/-3.7	23.77
	400	0.31 +/- 0.05	16.13	6.1 +/- 0.5	8.20
Cobalt	0	1.25 +/- 0.05	4.00	45.7 +/- 5.2	11.38
	200	1.14 +/- 0.05	4.39	42.6 +/- 5.0	11.74
	300	0.93 +/- 0.03	3.23	43.4 +/- 1.7	3.92
	400	0.77 +/- 0.04	5.19	35.5 +/- 2.6	7.32
Lead	0	1.28 +/- 0.04	3.12	42.1 +/- 0.4	0.95
	200	1.19 +/- 0.03	2.52	38.1 +/- 3.2	8.40
	300	1.01 +/- 0.04	3.96	31.4 +/- 0.4	1.27
	400	0.45 +/- 0.02	4.44	16.0 +/- 1.7	10.62
Chromium	0	1.07 +/- 0.02	1.87	46.8 +/- 0.7	1.50
	50	0.96 +/- 0.05	5.21	33.6 +/- 3.1	9.23
	100	0.68 +/- 0.02	2.94	21.9 +/- 1.6	7.31
	200	0.07 +/- 0.02	28.57	3.4 +/- 0.9	26.47
Selenium	0	0.98 +/- 0.01	1.02	39.1 +/- 0.4	1.02
	50	0.81 +/- 0.05	5.10	35.2 +/- 3.7	10.51
	100	0.74 +/- 0.01	1.35	21.5 +/- 1.5	6.98
	200	0.16 +/- 0.05	31.25	4.3 +/- 0.3	6.98
Sodium	0	1.24 +/- 0.07	5.65	49.7 +/- 0.8	1.61
	200	1.29 +/- 0.02	1.55	52.5 +/- 0.2	0.38
	400	1.27 +/- 0.08	6.30	51.3 +/- 3.1	6.04

Table 16. Inhibition of gas production rates after 6 hr as a percent of control. [(control - toxicant)/control](100%)

	Concentration (mgL^{-1})						
Toxicant	50	100	150	200	250	300	400
Se	16.9	24.1		83.3			
Cr	10.6	27.7		93.9			
Cu		43.5	69.7	86.2	98.9		
Zn	2.9	44.1		88.0		96.6	
Hg				28.3		55.7	72.0
Pb				7.2		20.9	64.7
Co				9.3		25.8	38.9
Na				-4.4			-2.7

Table 17. Reduction of methane in headspace gas after 6 hr as a percent of controls [(control - toxicant)/control](100%)

	Concentration (mgL^{-1})						
Toxicant	50	100	150	200	250	300	400
Se	10.0	45.0		89.0			
Cr	28.2	53.2		92.7			
Cu		44.0	70.0	83.2	87.7		
Zn	5.4	23.1		63.1		83.7	
Hg				23.4		65.2	85.2
Pb				9.5		25.4	62.0
Co				6.8		5.0	22.3
Na				-5.6			3.2

APPENDIX B

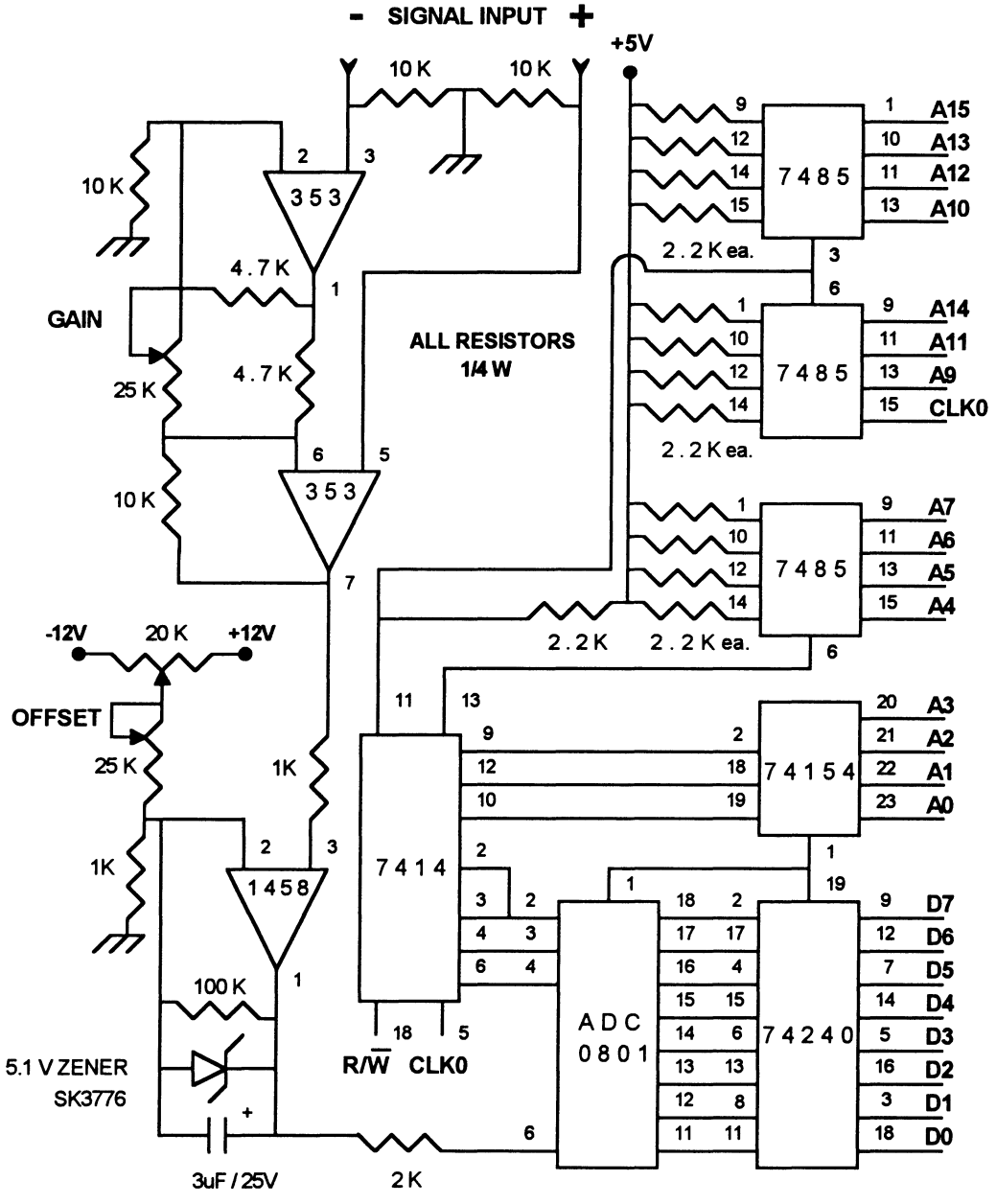

Figure 11. Schematic diagram of the analog to digital interface between the gas chromatograph and an Apple II+ computer.

APPENDIX C

COMPUTER PROGRAM ADDRESSES

(Data addresses used by the computer program)

232	Low byte of shape alphabet address (186)
233	High byte of shape alphabet address (144)
16350	Baseline offset (O)
24577	Top cursor position (-)
24578	Bottom cursor position (+)
24579	Multiplier (AB)
37030	Low byte of last data point entered (Z)
37031	High byte of last data point entered (Z)
37032	Screen line position of last data point entered (T)
37033	Last data position within screen line (S)
49152	Keyboard input
6300-16349	Last 10050 data points
16351-16375	Peak pointer (maximum of 25)
16384-24576	Screen graphics
24580-37029	First 12450 data points
37050-37843	Shape alphabet

COMPUTER PROGRAM AND FILE NAMES

BLANK1,A24580,L12450	Null file for erasing first part of data field
BLANK2,A6300,L10050	Null file for erasing second part of data field
BLNKSCRN,A16350,L8230	Null file for erasing screen
PLAYBACK	Data manipulation program
RECORD	Data aquisition program
SCREEN,A16350,L8230	Temporary disk file containing screen graphics
SHAPE ALPHABET,A37050,L793	Shape alphabet for graphics
TEMPA,A24580,L12450	Temporary disk file for first part of data field
TEMPB,A6300,L10050	Temporary disk file for second part of data field
"BSAVE _____,A6300,L3070"	Saves data and screen graphics as a single unit
"BSAVE _____,A16350,L8230"	Saves only the screen graphics

Substitute "BLOAD" for "BSAVE" to load the data and/or screen from the disk.
Only four data fields will fit on a disk.

COMPUTER PROGRAMS

DISK INITIALIZATION

(Records BLANK1, BLANK2, AND BLNKSCRN programs on the floppy disk)

```
1       REM         DISK INITIALIZATION
5       HIMEM:6299
10      FOR N = 0 TO 12450: POKE (24580 + N),255: NEXT N
20      FOR N = 0 TO 10050: POKE (6300 + N),255: NEXT N
30      PRINT CHR$ (4);"BSAVE BLANK1,A24580,L12450"
40      PRINT CHR$ (4);"BSAVE BLANK2,A6300,L10050"
5000    PRINT CHR$ (4);"BLOAD SHAPE ALPHABET,A37050": POKE 232,186:
        POKE 233,144: HGR2: HCOLOR = 3: ROT = 0: SCALE = 1
5005    FOR Y = 9 TO 159 STEP 10: HPLOT 16,Y TO 18,Y: HPLOT 279,Y TO 277,Y:
        NEXTY: FOR X = 25 TO 275 STEP 25: HPLOT X,7 TO X,9: HPLOT X,160 TO
        X,162: NEXT X
5020    ROT = 16: DRAW 52 AT 0,26: DRAW 41 AT 0,51: DRAW 45 AT 0,76: DRAW
        37 AT 0,101: DRAW 11 AT 0,126: ROT = 32: DRAW 62 AT 28,0: O = 5: ROT
        = 0: FOR NN = 1 TO 18: READ N: READ X: READ Y: DRAW N AT X,Y:
        NEXT NN: RESTORE
5050    PRINT CHR$ (4);"BSAVE BLNKSCRN,A16350,L8230"
9000    DATA 54, 85, 8, 47, 110, 8, 44, 135, 8, 52, 160, 8, 51, 185, 8, 11, 2, 10, 8, 8, 0,
        170, 13, 8, 170, 9, 16, 170
9100    DATA 8, 0, 180, 11, 8, 180, 9, 16, 180, 33, 0, 190, 50, 8, 190, 37, 16, 190, 33,
        24, 190, 13, 9, 13, 11, 9, 162
```

RECORD

(Keyboard commands for the data aquisition program)

Note: enter "RUN 4500" after loading the program

[Ctrl]	S [RETURN]	Run the record subroutine	
		[RETURN]	Begin data aquisition (19 samples per second)
		Esc	Pause from data aquisition, but monitor signal
		E [RETURN]	Exit recording subroutine
		A [RETURN]	Determine area under the last methane peak
			Enter microliters of sample and press return
	P [RETURN]	Run playback program	
	L [RETURN]	Load data and/or screen graphics from disk	
		Enter loading instructions at the question mark	
	S [RETURN]	Save data and/or screen to disk	
		Enter saving instructions at the question mark	

```
1       REM                 DATA AQUISITION PROGRAM
10      FOR S= 1 TO 150: POKE 65520,0: POKE Z,PEEK (65520):
        HPLOT (275 - PEEK (65520)),T: Z = Z - 1
20      IF Z = 24579 THEN Z = 16349
30      IF Z = 6299 THEN 1000
35      IF PEEK (49152) = 155 THEN 60
40      NEXT S: T = T + 1: GOTO 10
50      FOR S = PEEK (37033) TO 150
60      IF PEEK (49152) = 141 THEN 40
65      IF PEEK (49152) = 193 THEN GOSUB 6500
70      IF PEEK (49152) = 197 THEN A = 13: B = 162: AB = 150: AX = 0:
        BX = 0: GOTO 5500
80      POKE 65520,0: HPLOT 25,161 TO (275 - PEEK (65520)),161:
        HCOLOR = 0: HPLOT 25,161 TO (275 - PEEK (65520)),161: HCOLOR = 3
90      GOTO 60
1000    POKE 24577,13: POKE 24578,162: POKE 24579,AB: POKE 16350,5
1010    PRINT CHR$ (4);"BSAVETEMPA,A24580,L12450": PRINT CHR$
        (4);"BSAVETEMPB,A6300,L10050"
1015    PRINT CHR$ (4);"BSAVESCREEN,A16350,L8230": GOTO 5500
4500    HIMEM:6299
5000    PRINT CHR$ (4);"BLOAD SHAPE ALPHABET,A37050": POKE 232,186:
        POKE 233,144: HGR2: HCOLOR = 3: ROT = 0: SCALE = 1
5005    FOR Y = 9 TO 159 STEP 10: HPLOT 16,Y TO 18,Y: HPLOT 279,Y TO 277,Y:
        NEXTY: FOR X = 25 TO 275 STEP 25: HPLOT X,7 TO X,9: HPLOT X,160 TO
        X,162: NEXT X
5020    ROT = 16: DRAW 52 AT 0,26: DRAW 41 AT 0,51: DRAW 45 AT 0,76: DRAW
        37 AT 0,101: DRAW 11 AT 0,126: ROT = 32: DRAW 62 AT 28,0: O = 5: ROT
        = 0: FOR NN = 1 TO 18: READ N: READ X: READ Y: DRAW N AT X,Y:
        NEXT NN: RESTORE
5500    GET N$: N = ASC (N$)
5510    Q = FRE (0)
5575    IF N = 19 THEN T = 10: Z = 37029: GOTO 6000
5580    IF N = 80 THEN 6050
5585    IF N = 76 THEN 6200
5590    IF N = 83 THEN 6350
5599    GOTO 5500
6000    INPUT N$
6005    PRINT CHR$ (4);"BLOADBLANK1"
6010    PRINT CHR$ (4);"BLOADBLANK2"
6020    PRINT CHR$ (4);"BLOADBLNKSCRN"
6030    INPUT N$: GOTO 10
6050    INPUT N$
6100    PRINT CHR$ (4);"RUNPLAYBACK"
6200    INPUT N$
6205    POKE -16303,0: POKE -16300,0: INPUT N$: PRINT CHR$ (4),N$:
        POKE -16297,0: POKE -16304,0: POKE -16299,0: POKE -16302,0
6210    Z = PEEK (37030) + (PEEK (37031) * 256): T = PEEK (37032): GOTO 50
6250    INPUT N$
6255    POKE 37030,(Z - 256 * INT (Z / 256)): POKE 37031,(INT (Z / 256)): POKE
        37032,T: POKE 37033,S
```

```
6260    POKE 24577,13: POKE 24578,162: POKE 24579,AB: POKE 16350,5:
        POKE -16303,0: POKE -16300,0: INPUT N$
6270    PRINT CHR$ (4);N$: POKE -16297,0: POKE -16304,0:
        POKE -16299,0: POKE -16302,0: GOTO 5500
6500    INPUT N$
6505    HCOLOR = 0: FOR Y = 164 TO 190: HPLOT 30,Y TO 279,Y: NEXT Y:
        HCOLOR = 3: MM = 30
6510    C$ = "MICROLITERS=": FOR N = 1 TO LEN (C$): N$ = MID$ (C$,N,1): M =
        ASC (N$) - 32: DRAW M AT (130 + (8 * N)),170: NEXT N: INPUT X
6521    A$ = STR$ (X): FOR N = 1 TO LEN (A$): N$ = MID$ (A$,N,1): M = 16 + VAL
        (N$): DRAW M AT (235 + (8 * N)),170: NEXT N
7005    F = Z
7010    F = F + 1
7015    IF F = 16350 THEN F = 24580
7020    IF F > 37029 THEN RETURN
7025    IF PEEK (F) < 240 THEN 7100
7040    GOTO 7010
7100    FOR N = 1 TO 25: F = F - 1
7110    IF F = 24579 THEN F = 16349
7120    NEXT N
7130    BB = 37029 - F: SUM = 0
7140    IF F < 16350 THEN BB = BB + 8231
7150    GOSUB 8120
7235    FOR N = 1 TO LEN (S$): N$ = MID$ (S$,N,1): M = 16 + VAL (N$): DRAW M
        AT (MM + (8 * N)),190: NEXT N
7240    A$ = STR$ (AA): B$ = STR$ (BB-1): MM = 30: FOR N = 1 TO LEN (A$): N$
        = MID$ (A$,N,1): M = 16 + VAL (N$):
        DRAW M AT (MM + (8 * N)),170: NEXT N
7245    FOR N = 1 TO LEN (B$): N$ = MID$ (B$,N,1): M = 16 + VAL (N$):
        DRAW M AT (MM + (8 * N)),180: NEXT N
7250    C$ = "%METHANE=" + LEFT$ (C$,5): FOR N = 1 TO LEN (C$): N$ = MID$
        (C$,N,1): M = ASC (N$) - 32:
        DRAW M AT (130 + (8 * N)),190: NEXT N
7299    RETURN
8120    AA = BB - 150
8200    FOR A = F TO F + 149
8210    IF A = 16350 THEN A = 24580
8220    SUM = SUM + (250 - PEEK (A)): NEXT A: S$ = STR$ (SUM)
8221    C = ((SUM + 969) * (0.9203))/X: C$ = STR$ (C)
8225    Q = FRE (0)
8230    RETURN
9000    DATA 54, 85, 8, 47, 110, 8, 44, 135, 8, 52, 160, 8, 51, 185, 8, 11, 2, 10, 8, 8, 0,
        170, 13, 8, 170, 9, 16, 170
9100    DATA 8, 0, 180, 11, 8, 180, 9, 16, 180, 33, 0, 190, 50, 8, 190, 37, 16, 190, 33,
        24, 190, 13, 9, 13, 11, 9, 162
```

PLAYBACK

(Keyboard commands for the data manipulation program)

L [RETURN]	Load the screen from disk	
S [RETURN]	Save the screen to disk	
F [RETURN]	Load the temporary data files from disk	
O	Move the baseline offset cursor	
	Esc	Escape from cursor control
	<	Move the offset cursor up
	>	Move the offset cursor down
B	Move the bottom cursor	
T	Move the top cursor	
	Esc	Escape from cursor control
	T	Change to the top cursor
	B	Change to the bottom cursor
	<	Move the cursor up one step
	>	Move the cursor down one step
	←	Move cursor up ten steps
	→	Move cursor down ten steps
P	Display the cursor positions	
V	Enter peak vertical expansion value (1 to 99)	
X	Expand the area between the cursors to fill the screen	
A	Determine and display the area between cursors T and B	
M	Move the peak	
	Esc	Escape from peak movement subroutine
		Redraw the peak
	←	Move the peak up ten steps
	→	Move the peak down ten steps
N [RETURN]	Enter notes on the bottom of the screen (3 lines and a maximum of 16 characters per line)	
[Ctrl] X	Select a maximum of 25 peaks for automated expansion	
[RETURN]	Sequence through the peaks selected for expansion	

[Ctrl] P [RETURN] print the graphics screen

```
1       REM             DATA MANIPULATION PROGRAM
5       GOTO 4500
100     N = F:XB = 0: FORY = 10 TO 159: FOR YY = 1 TO AB
130     IF N < 24580 THEN P = 8231
140     V = (VV * (250 - PEEK (N-P))) + 25
150     IF V > 275 THEN V = 275
151     IF V < 21 THEN V = 21
155     IF PEEK (49152) = 155 THEN BB = BX: RETURN
160     HPLOT V,Y: N = N - 1: XB = XB + 1: NEXT YY: NEXT Y: BB = AA + XB:
        DRAW 13 AT 9,13: DRAW 11 AT 9,162
197     A = 13: B = 162: GOSUB 5909: RETURN
4500    HIMEM:6299: N = 10: Z = 37029: AB = 150: S = 0: VV = 1: A = 13: B = 162: HOME
5000    PRINT CHR$ (4);"BLOAD SHAPE ALPHABET,A37050": POKE 232,186:
        POKE 233,144: HGR2: HCOLOR = 3: ROT = 0: SCALE = 1
5005    FOR Y = 9 TO 159 STEP 10: HPLOT 16,Y TO 18,Y: HPLOT 279,Y TO 277,Y: NEXTY:
        FOR X = 25 TO 275 STEP 25: HPLOT X,7 TO X,9: HPLOT X,160 TO X,162: NEXT X
5020    ROT = 16: DRAW 52 AT 0,26: DRAW 41 AT 0,51: DRAW 45 AT 0,76: DRAW 37 AT
        0,101: DRAW 11 AT 0,126: ROT = 32: DRAW 62 AT 28,0: O = 5: ROT = 0: FOR NN =
        1 TO 18: READ N: READ X: READ Y: DRAW N AT X,Y: NEXT NN: RESTORE
5500    GET N$: N = ASC (N$)
5510    Q = FRE (0)
5515    IF N = 79 THEN GOSUB 6500
5520    IF N = 65 THEN Y = 10: S = 0: I = 0: XX = 1: GOSUB 7000
5525    IF N = 66 THEN GOSUB 5800
5530    IF N = 84 THEN GOSUB 5700
5535    IF N = 80 THEN GOSUB 5900
5540    IF N = 76 THEN GOSUB 8000
5545    IF N = 83 THEN GOSUB 8050
5550    IF N = 70 THEN GOSUB 7700
5555    IF N = 88 THEN GOSUB 5650
5560    IF N = 77 THEN SD = 0: DRAW 28 AT 270,92: DRAW 30 AT 264,92: GOSUB 5850
5565    IF N = 78 THEN N = 0: GOSUB 7500
5570    IF N = 86 THEN GOSUB 7600
5575    IF N = 24 THEN PK = 1: GOSUB 6000
5580    IF N = 16 THEN GOSUB 8500
5585    IF N = 13 THEN PK = PK + 1: GOSUB 6100
5599    GOTO 5500
5650    REM
5655    XX = 0: I = 1: HCOLOR = 0: FOR C = 10 TO 159: HPLOT 20,C TO 276,C:
        HPLOT 9,C TO 15,C: NEXT C: HCOLOR = 3: YY = 0: AB = INT ((BB - AA)/150)
5670    IF AB < 1 THEN AB = 1
5675    F = 37029 - AA: L = 37029 - BB: AX = AA: BX = BB: P = 0: GOTO 100
5700    GET N$: N = ASC (N$): D = A
5711    IF N = 27 THEN X = 1: GOTO 5900
5712    IF N = 66 THEN 5800
5715    IF N = 44 THEN A = A - 1
5716    IF N = 46 THEN A = A + 1
5757    IF N = 8 THEN A = A - 10
5718    IF N = 21 THEN A = A + 10
5719    IF A < 13 THEN A = 13
5720    IF A > 162 THEN A = 162
```

```
5730    XDRAW 13 AT 9,D: DRAW 13 AT 9,A: DRAW 11 AT 9,B: GOTO 5700
5800    GETN$: N = ASC (N$) : D = B
5811    IF N = 27 THEN X = 1: GOTO 5900
5812    IF N = 84 THEN 5700
5815    IF N = 44 THEN B = B - 1
5816    IF N = 46 THEN B = B + 1
5817    IF N = 8 THEN B = B - 10
5818    IF N = 21 THEN B = B + 10
5819    IF B < 13 THEN B = 13
5820    IF B > 162 THEN B = 162
5830    XDRAW 11 AT 9,D: DRAW 11 AT 9,B: DRAW 13 AT 9,A: GOTO 5800
5850    GET N$: N = ASC (N$): DS = SD
5855    IF N = 27 THEN X = 1: GOTO 5900
5865    IF N = 8 THEN S = S + (AB * 10): SD = SD - 1
5870    IF N = 21 THEN S = S - (AB * 10): SD = SD + 1
5875    IF SD > 50 THEN SD = -50
5880    IF SD < -50 THEN SD = 50
5890    XDRAW 30 AT 264,(92 + DS): DRAW 30 AT 264,(92 + SD)
5892    IF BX / (AB * 150) < 0 THEN S = 0
5893    IF AX + (AB * 150) > 22499 THEN S = 0
5895    GOTO 5850
5900    REM PRINT
5903    AA = ((A - 13) * AB) + S: BB = ((B - 12) * AB) + S: S = 0: AA = AA + AX: BB=BB + AX
5906    IF (X + XX) > 0 THEN 5909
5908    AX = AA: BX = BB
5909    HCOLOR = 0: FOR Y = 164 TO 190: HPLOT 30,Y TO 279,Y: NEXT Y
5910    IF BB > 22500 THEN BB = 22500
5912    IF AA < 0 THEN AA = 0
5915    HCOLOR = 3: A$ = STR$ (AA): B$ = STR$ (BB - 1): MM = 30:
        FOR N = 1 TO LEN (A$): N$ = MID$ (A$,N,1): M = 16 + VAL (N$): DRAW M
        AT (MM + (8 * N))),170: NEXT N
5930    FOR N = 1 TO LEN (B$): N$ = MID$ (B$,N,1): M = 16 + VAL( N$): DRAW M
        AT (MM + (8 * N))),180: NEXT N
5935    N$ = "(+)-(-)=" + STR$ (BB - AA): FOR NN = 1 TO LEN (N$): NN$ = MID$
        (N$,NN,1): M = ASC (NN$) - 32:DRAW M AT (145 + (8 * NN)),170: NEXT NN
5940    GOSUB 7260: RETURN
6000    GOSUB 5800: POKE (16350 + PK),B: PK = PK + 1
6010    IF B = 162 THEN PK = 0: RETURN
6020    DRAW 30 AT 18,B: GOTO 6000
6100    B = PEEK (16350 + PK) + 1: A = B - 2
6110    IF B = 163 THEN B = 162: PK = 0: GOTO 8060
6115    X = 1:AX = 0:BX = 0: AB = 150: GOSUB 5900: GOSUB 5650: A = 13: B =162: RETURN
6500    REM
6505    GET N$: N = ASC (N$)
6511    IF N = 27 THEN RETURN
6515    ROT = 32
6520    IF N = 44 THEN XDRAW 62 AT (O+23),0: O = O- 1: DRAW 62 AT (O +23),0
6530    IF N = 46 THEN XDRAW 62 AT (O+23),0: O = O+ : DRAW 62 AT (O + 23),0
6535    ROT = 0: GOTO 6500
7000    REM
```

```
7050    SUM = 0: P = 0: YY = 0: F = 37029 - AA: L = 37029 - BB: Y = A - 3
7200    FOR N = F TO (L-1) STEP - 1
7210    IF N < 24580 THEN P = 8231
7215    IF YY = AB THEN YY = 0: Y = Y + 1
7216    V = (VV * 250 - PEEK (N - P))) + 25
7217    IF V < 21 THEN V = 21
7218    IF V > 275 THEN V = 275
7220    SUM = SUM + (VV * (255 - PEEK (N - P) - O))
7225    IF PEEK (49152) = 155 THEN RETURN
7230    Q = FRE (0)
7235    HPLOT (20 + O),Y TO V,Y: YY = YY + 1: NEXT N
7240    S$ = STR$ (SUM): HCOLOR = 0: FOR Y= 181 TO 190: HPLOT 30,Y TO 140,Y:
        NEXT Y: HCOLOR = 3: MM = 30: FOR N =1 TO LEN (S$): N$ = MID$ (S$,N,1)
7250    M = 16 + VAL (N$): DRAW M AT (MM + (8 * N))),190: NEXT N
7260    N$ = "PEAK#" + STR$ (PK): FOR NN = 1 TO LEN (N$): NN$ = MID$
        (N$,NN,1): M = ASC (NN$) - 32: DRAW M AT (145 + (8 * NN)),180: NEXT NN
7299    RETURN
7500    REM
7510    INPUT N$
7520    HCOLOR = 0: FOR Y = 164 TO 190: HPLOT 141,Y TO 279,Y: NEXT Y: HCOLOR = 3
7525    INPUT N$: N = N + 1
7530    FOR NN = 1 TO LEN (N$): NN$ = MID$ (N$,NN,1): M = ASC (NN$) - 32: DRAW M AT (145 +
      (A * NN)),(160 + (N * 10)): NEXT NN
7540    IF N = 3 THEN RETURN
7550    Q = FRE (0): GOTO 7225
7600    REM
7610    INPUT N$
7620    HCOLOR = 0: FOR Y = 1 TO 8: HPLOT 251,Y TO 272,Y: NEXT Y: HCOLOR = 3:
        INPUT N$: VV = VAL (N$): N$ = "X" + N$
7630    FOR NN = 1 TO LEN (N$): NN$ = MID$ (N$,NN,1): M = ASC (NN$) - 32:
        DRAW M AT (243 + (8 * NN)),A: NEXT NN: RETURN
7700    REM LOAD FILES
7705    INPUT N$
7710    PRINT CHR$ (4);"BLOADTEMPA,A24580": PRINT CHR$ (4);"BLOADTEMPB,A6300"
7720    GOTO 8010
8000    REM
8005    INPUT N$
8010    PRINT CHR$ (4);"BLOADSCREEN,A16350"
8015    A = PEEK (24577): B = PEEK (24578): AB = PEEK(24579): O = PEEK
        (16350): AX = 0: BX = 0: S = 0: RETURN
8050    INPUT N$
8060    POKE 24577,A: POKE 24578,B: POKE 24579,AB: POKE 16350,O:
        POKE -16303,0: POKE -16300,0: INPUT N$: PRINT CHR$ (4);N$:
        POKE -16297,0: POKE -16304,0: POKE -16299,0: POKE  -16302,0: GOTO 8015
8500    INPUT N$: PRINT CHR$ (4);"PR#1": PRINT CHR$ (9);"GDR2": PRINT CHR$
        (4);"PR#0" : RETURN
9000    DATA 54, 85, 8, 47, 110, 8, 44, 135, 8, 52, 160, 8, 51, 185, 8, 11, 2, 10, 8, 8, 0,
        170, 13, 8, 170, 9, 16, 170
9100    DATA 8, 0, 180, 11, 8, 180, 9, 16, 180, 33, 0, 190, 50, 8, 190, 37, 16, 190, 33,
        24, 190, 13, 9, 13, 11, 9, 162
```

SHAPE TABLE ALPHABET FILE (45)

(Builds and saves the shape table alphabet file to the disk)

```
1       REM             SHAPE TABLE ALPHABET FILE
2       REM             SAVES A SHAPE TABLE ALPHABET ON DISK
10      FOR I = 24576 TO 25364
20      READ X:POKE I, X:NEXT I
50      PRINT CHR$ (4);"BSAVE SHAPE ALPHABET,A24576,L793"
60      END
70      DATA 63,0,128,0,134,0,142,0,157,0: REM ————————
80      DATA 172,0,185,0,200,0,206,0,214,0
90      DATA 222,0,236,0,245,0,249,0,255,0
100     DATA 2,1,9,1,24,1,32,1,44,:     REM   SHAPE TABLE
110     DATA 56,1,67,1,79,1,93,1,102,1
120     DATA 116,1,127,1,133,1,139,1,148,1
130     DATA 156,1,165,1,175,1,190,1,202,1: REM   ADDRESSING
140     DATA 216,1,228,1,240,1,254,1,8,2
150     DATA 19,2,31,2,40,2,48,2,61,2
160     DATA 70,2,82,2,94,2,106,2,116,2:   REM    STRUCTURE
170     DATA 130,2,143,2,155,2,163,2,174,2
180     DATA 185,2,198,2,210,2,219,2,231,2
190     DATA 245,2,253,2,9,3,17,3:      REM ————————
200     DATA 73,4,32,36,36,0: REM !
210     DATA 9,64,24,32,108,54,4,0: REM "
220     DATA 9,36,103,60,5,32,13,246,45,23,30,45,23,38,0: REM #
230     DATA 1,40,53,12,12,28,55,28,28,12,37,22,12,37,0: REM $
240     DATA 73,41,60,223,12,12,12,5,248,35,55,4,0: REM %
250     DATA 73,9,28,28,28,28,100,21,190,26,174,101,8,4,0: REM &
260     DATA 73,64,24,32,36,0: REM '
270     DATA 73,28,28,36,12,12,4,0: REM (
280     DATA 73,12,12,36,28,28,4,0: REM )
290     DATA 73,36,36,36,141,23,31,28,150,98,13,21,4,0: REM *
300     DATA 73,32,60,111,41,31,32,4,0: REM +
310     DATA 9,12,36,0: REM ,
320     DATA 64,24,41,45,37,0: REM -
330     DATA 73,4,0: REM .
340     DATA 1,96,12,12,12,4,0: REM /
350     DATA 9,45,12,36,36,28,63,23,54,54,12,12,12,4,0: REM 0
360     DATA 9,45,28,36,36,188,4,0: REM 1
370     DATA 73,9,63,63,100,12,101,228,63,23,4,0: REM 2
380     DATA 8,21,45,12,228,103,5,32,63,63,4,0: REM 3
390     DATA 73,33,44,31,63,100,12,12,54,38,0: REM 4
400     DATA 8,21,45,12,36,28,63,39,44,45,37,0: REM 5
410     DATA 9,45,12,228,63,55,38,64,3,12,12,45,4,0: REM 6
420     DATA 9,36,12,12,12,60,63,39,0: REM 7
430     DATA 9,45,12,228,63,23,38,64,3,100,45,21,38,0: REM 8
440     DATA 41,101,12,36,228,63,23,174,45,4,0: REM 9
450     DATA 73,64,3,4,32,0: REM :
460     DATA 9,5,32,4,32,0: REM ;
```

```
470     DATA 73,225,28,28,12,12,12,4,0: REM <
480     DATA 64,45,45,4,56,63,39,0: REM =
490     DATA 9,12,12,12,28,28,28,4,0: REM >
500     DATA 73,4,32,12,12,28,63,23,4,0: REM ?
510     DATA 9,45,37,64,3,36,28,63,23,54,54,76,229,36,0: REM @
520     DATA 33,36,100,12,14,14,54,63,111,17,38,0: REM A
530     DATA 33,36,36,44,45,21,190,31,109,50,23,63,4,0: REM B
540     DATA 73,9,184,63,28,36,36,12,45,21,4,0: REM C
550     DATA 33,36,36,44,45,21,54,54,23,63,4,0: REM D
560     DATA 41,45,37,192,63,55,38,64,3,36,45,45,4,0: REM E
570     DATA 33,36,36,44,45,181,26,63,4,0: REM F
580     DATA 9,45,37,60,223,34,36,100,45,37,0: REM G
590     DATA 33,36,36,180,10,45,37,36,150,50,38,0: REM H
600     DATA 9,45,28,36,36,60,13,4,0: REM I
610     DATA 1,168,45,12,36,36,36,0: REM J
620     DATA 33,36,36,108,9,23,23,23,21,21,21,4,0: REM K
630     DATA 73,9,63,63,36,36,36,4,0: REM L
640     DATA 33,36,36,172,21,102,96,54,54,54,4,0: REM M
650     DATA 33,36,36,172,170,21,149,36,36,36,4,0: REM N
660     DATA 9,45,12,36,36,28,63,23,54,54,4,0: REM O
670     DATA 33,36,36,44,45,21,190,63,4,0: REM P
680     DATA 9,37,168,21,4,32,36,28,63,23,54,54,4,0: REM Q
690     DATA 33,36,36,44,45,21,190,63,21,21,21,4,0: REM R
700     DATA 8,21,45,12,60,56,231,100,45,21,4,0: REM S
710     DATA 73,36,36,36,63,77,37,0: REM T
720     DATA 9,45,12,36,36,252,27,54,54,38,0: REM U
730     DATA 73,12,12,36,36,223,51,54,174,4,0: REM V
740     DATA 33,36,36,108,9,54,54,54,28,28,180,35,0: REM W
750     DATA 33,12,12,28,28,108,9,190,22,21,38,0: REM X
760     DATA 73,36,228,28,108,9,246,4,0: REM Y
770     DATA 73,9,63,63,100,12,12,12,60,63,39,0: REM Z
780     DATA 73,9,63,63,44,60,44,60,44,28,45,45,4,0: REM LEFT BRACKET
790     DATA 72,73,28,28,28,28,4,0: REM BACKSLASH
800     DATA 41,45,37,39,37,39,37,39,253,63,4,0: REM RIGHT BRACKET
810     DATA 64,24,97,12,21,21,4,0: REM ^
820     DATA 41,45,37,0: REM UNDERLINE
```