THE RESPONSE OF ACETATE UTILIZING METHANOGENS TO TOXICS IN TERMS OF INTERMEDIATE AND PRODUCT GASES

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KAJSA NORGREN

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UNIVERSITY OF MASSACHUSETTS
AT AMHERST

Marston Hall
Amherst, MA 01003
(413) 545-2508

Department of Civil Engineering

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Art:

Enclosed is a copy of Kajsa Norgren's M.S Project. This is for your information only. It will not per se be a technical report. Parts of it will be combined with Eugenio Giraldo's MS Project for a technical report at a later time

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A Master's Project by:

Kajsa Norgren

Department of Civil Engineering

University of Massachusetts

Amherst, Massachusetts

February, 1989

Approved as to style and content:

Michael S. Switzenbaum

Professor Michael S. Switzenbaum,
Project Committee Chairperson

James K. Edzwald

Professor James K. Edzwald,
Project Committee Member

David Reckhow

Professor David A. Reckhow,
Project Committee Member

William H. Highter,
Department Head, Civil Engineering

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ABSTRACT

The trace gases hydrogen and carbon monoxide have shown potential for use as early warning indicators of impending upsets in anaerobic digestion systems. This research was conducted to examine the effects of adding various toxicants on acetate utilizing methanogens in terms of these intermediate gases. The results were then compared to results from a previous study in which a digester fed a particulate substrate (waste activated sludge) was used.

A digester fed sucrose was set up and operated for several months. Conventional monitoring parameters were measured regularly to ensure proper digester operation. Effluent from this reactor was used as inoculum for serum bottle assays. Four heavy metals (Cu, Cd, Ni and Zn) and two organic compounds (bromoethanesulfonic acid (BES) and formaldehyde) were tested. Since the purpose of the assays was to assess the response of the acetate utilizing methanogens, acetate replaced sucrose as the carbon source during these assays.

Based on the results of this study, it was found that hydrogen and carbon monoxide can be used to indicate upsets due to toxicant addition at an early stage in a digester fed a soluble waste . However, in contrast to what was expected, the response for the acetate utilizing methanogens was not faster than the response observed in the sludge digester. The concentrations of toxicant causing similar levels of inhibition in methane production were comparable for both systems. This would indicate that acetate utilizing methanogens are not as sensitive to toxicant addition as is generally thought.

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CHAPTER I

INTRODUCTION

Anaerobic digestion is a biological process in which organic matter is stabilized by conversion to end products, mainly carbon dioxide and methane, in the absence of oxygen. It is a process which occurs naturally in such varied habitats as marshes, rice paddies and cattle. The anaerobic digestion process has been used for over 100 years at wastewater treatment plants to stabilize wastes and reduce the amount of solids generated (McCarty, 1982).

Major advantages of anaerobic digestion over aerobic digestion have been presented by McCarty (1964a) and Speece (1983). A lower amount of sludge is produced since the conversion of substrate to methane yields little energy for the synthesis of new microbial cells. A smaller sludge yield is an important advantage when considering the costs of sludge disposal. Another consideration is that the process is less energy intensive than aerobic digestion since it does not need the addition of oxygen. This is a major benefit now that the cost of energy is high. Since methane, which can be used as fuel, is generated as one of the end products, research has also been directed towards the possibility of methane fuel generation from a variety of agricultural and industrial wastes.

A major reason why anaerobic digestion is not used more widely is that the process has been considered unreliable. Digesters are unable to adjust quickly to changes in hydraulic loading, organic loading, temperature and pH, and especially to the addition of toxic inorganic and organic compounds. Once an upset has occurred, digesters only slowly recover. This inability to respond to sudden shifts in environmental conditions and to toxic loads, is due to the slow growth rate of the methane producing bacteria in the system. It is therefore important to monitor the process closely so that any upsets are detected as early as possible. Remedial measures can then be applied to prevent a complete upset of the digester from occurring.

In recent years, much research has been devoted to gaining a better understanding of the microbiology and biochemistry of the microbial ecosystem involved in anaerobic digestion (Breure and van Andel, 1987, Archer, 1983, Kotze et al., 1969 and Thiel et al., 1968). This knowledge has been used to modify the anaerobic processes (Harper and Pohland, 1986), thereby improving their performance and making them less susceptible to the problems mentioned previously. Also, great efforts have been made to examine the effects which organic overloads and the addition of toxic materials have on anaerobic digestion systems (Rinzema et al., 1988, Ahring and Westermann, 1985, Parkin

and Miller, 1982 and Murray and van den Berg, 1981), and the capability of the systems to recover from these upsets. These advances have contributed to making anaerobic digestion an increasingly more popular alternative when considering options for treating many industrial and municipal wastewaters.

Despite these improvements in the anaerobic digestion process, there is still a need to develop adequate process monitoring techniques and analyses which will provide maximum protection against process upsets and possible failures. The most commonly used methods for monitoring anaerobic processes consist of a combination of gas and liquid phase analyses, which have been well established for many years (McCarty, 1964b). These conventional parameters include monitoring pH, volatile fatty acids (VFA) concentration, and digester efficiency (percent reduction in chemical oxygen demand (COD) or volatile solids (VS)) in the digester liquid, and daily gas production and gas composition in the digester headspace. However, these parameters do not reflect the current status of the microbial population in the digester. They are mostly helpful in detecting slow to develop upsets such as gradual organic overloads.

More recent efforts have focused on developing techniques to estimate the active cell concentrations and

metabolic activities in the system. These include measuring deoxyribonucleic acid (DNA) content, adenosine triphosphate (ATP) activity, dehydrogenase activity and concentration of cofactors such as F_{420} which are unique to methanogens. However, these methods usually require more time consuming analytical procedures.

A common cause of digester upsets is the addition of toxic materials, which frequently occurs as a pulse input. There is therefore a need to develop indicators that can rapidly determine the current status of a digester, so that possible upsets can be detected as early as possible. This will provide more time for remedial actions to be taken.

Hydrogen gas was shown to be an important intermediate in methanogenesis by Bryant et al. (1967). More recent research indicates that trace amounts of carbon monoxide are also evolved in these systems (Nelson and Ferry, 1984 and Conrad and Thauer, 1983). Mosey (1983) and Hickey et al. (1987a & b) have suggested that these intermediate gases may be a more convenient parameter to monitor. They give an indication of the current metabolic status of an anaerobic digester, and are a procedure well suited for real time data acquisition (Hickey, 1987). Only a small sample of the digester headspace gas is needed, and the analysis can be performed quickly and easily compared to the more time consuming liquid phase sample analysis.

This research was undertaken with the following primary objectives:

(1) -To study the response of intermediate (hydrogen and carbon monoxide) and product (methane and carbon dioxide) gases to the addition of various inorganic and organic toxic compounds in an anaerobic digester fed a soluble waste. In this case, glacial acetic acid was used as the carbon source during serum bottle assays, which tested the population of acetate utilizing methanogens.

(2) -To compare the response obtained from this individual population with results from a previous study in which digesters fed waste activatad sludge, a poorly defined, particulate substrate that supports a mixed bacterial population, were used.

This study is part of an ongoing program which has the long range goal of developing an effective monitoring system for anaerobic digestion processes which will detect any upsets as early as possible. This will allow more time for remedial actions to be taken to prevent complete system failures from occurring.

CHAPTER II

BACKGROUND

The objective of anaerobic digestion is to stabilize organic matter by conversion to the gaseous endproducts carbon dioxide and methane. This conversion is accomplished mainly by bacterial populations in the system. The anaerobic process has often been considered unreliable, due to its susceptibility to upsets, mainly from organic overloads and toxic shock loads. Through studies of the microbiological and biochemical principles underlying anaerobic digestion, a better understanding of the process has been achieved. This knowledge has been used to modify process designs, thereby improving process reliability and performance.

However, no wastestream will be completely void of potentially toxic compounds. Therefore, a need exists for developing monitoring techniques that will give sufficiently early warning of impending upsets, so that remedial actions can be taken and complete digester failure may be avoided. Mosey (1983) and Hickey et al. (1987a & b) have suggested that hydrogen and carbon monoxide, which are intermediate gases formed in low concentrations in the digestion process, could be used for this purpose. Analysis of the gas phase would be faster and easier, and lend itself better to real time data acquisition than the gas and liquid phase analysis

techniques presently used to monitor the digestion process.

In the following sections, an overview of the pathways involved in anaerobic digestion will be presented, with an emphasis on the role of hydrogen and carbon monoxide in these systems. In the last section, toxicants in anaerobic digestion systems will be discussed.

2.1 GENERAL MICROBIOLOGY

Over the past 20 years, much research has been conducted to gain a better understanding of the anaerobic digestion process. Recent reviews of the microbiological and biochemical bases of anaerobic digestion were performed by Breure and van Andel (1987), Parkin and Owen (1986), Speece (1983) and Archer (1983).

The breakdown of substrates in anaerobic digestion to produce methane and carbon dioxide as the main endproducts occurs in several stages. Three main groups of bacteria are responsible for these conversions: the acid fermenting bacteria, the hydrogen producing bacteria (acetogens) and the methanogens. A schematic representation of the process is shown in Figure 1.

The preliminary step involves the solubilization of insoluble organic compounds and the reduction in size of large molecules by hydrolytic reactions, which are catalyzed by enzymes released by the bacteria in the system.

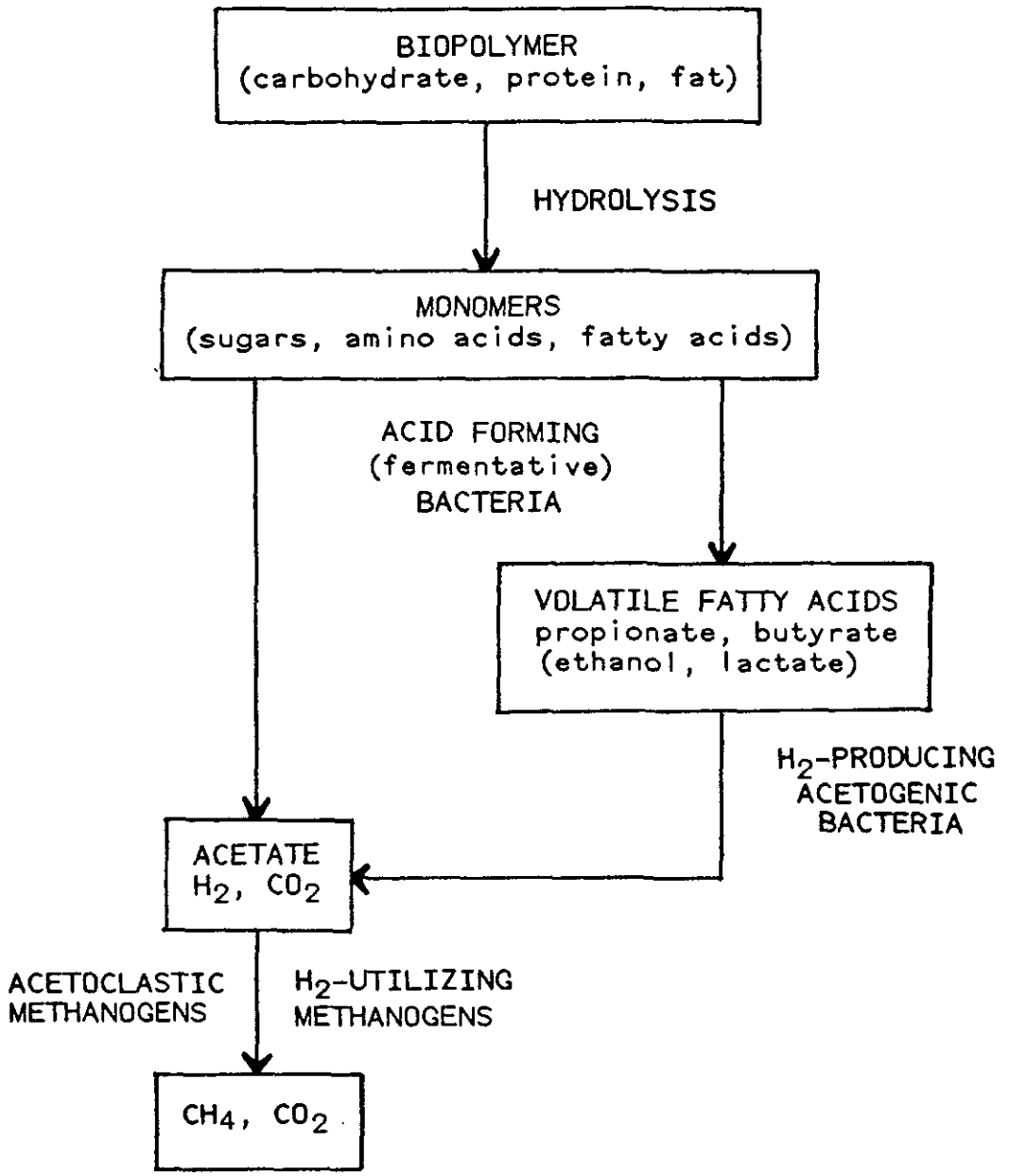

Figure 1: Schematic of steps in anaerobic digestion (adapted from Breure and van Andel, 1987).

Carbohydrates, proteins and fats are thereby converted to sugars, amino acids and fatty acids. This facilitates the transport of the compounds across the cell membranes.

These monomers serve as carbon and energy sources for the fermentative bacteria carrying out the next step in the process. The smaller subunits are fermented into hydrogen, carbon dioxide, volatile fatty acids (such as acetic, propionic and butyric acid), and some other organic compounds such as ethanol and lactic acid. This process is termed acid fermentation or acidogenesis.

The obligate hydrogen producing bacteria (or acetogens) then oxidize some organic acids, ethanol and lactic acid to acetic acid, carbon dioxide and hydrogen. For these reactions to occur, the partial pressure of hydrogen has to be kept extremely low. This is accomplished through a syntrophic relationship with carbon dioxide reducing methanogens which take up hydrogen. Hydrogen can also be removed by sulfate and nitrate reducing bacteria in the system.

The last step is methanogenesis. Here acetate is converted to methane and carbon dioxide by the acetoclastic methane bacteria, while the hydrogen utilizing methanogens form methane and water from carbon dioxide and hydrogen.

Acetate is an important precursor in methanogenesis. McCarty (1964a) reported that about 70 percent of the

methane produced, is formed from acetate even though only few methanogens are capable of utilizing it as a substrate. The remainder is generated through carbon dioxide reduction using hydrogen as the energy source. Smith and Mah (1978) showed that Methanosarcina strain 227 was capable of growing on acetate alone, and Huser et al. (1982) identified Methanothrix soehngenii, which is often present in anaerobic systems, as a non-hydrogen-oxidizing, acetotrophic methane bacterium. Methane is produced by a decarboxylation of acetic acid. The methane is formed exclusively from the methyl group of acetic acid, while the carboxyl group is converted to carbon dioxide (van den Berg et al., 1976 and Zehnder at al., 1980).

The methanogens are a unique group of microorganisms which possess some characteristics not found in any other bacterial groups. Several cofactors have been identified in all methanogens. They include factor F_{420}, an electron tranfer coenzyme, factor F_{430} and coenzyme M (2-mercapto-ethanesulfonate), which takes part in methyl transfer reactions (Balch and Wolfe, 1979).

Methanogenesis is considered the rate limiting step in the digestion of soluble wastes. Especially the conversion of acetate to methane is critical since the acetoclastic methanogens grow extremely slowly (Kaspar and Wuhrmann, 1978b). The maximum specific growth rate of the acetate

utilizing methanogens may be an order of magnitude smaller than that of the hydrogen utilizing methanogens (van den Berg et al., 1976).

2.2 ROLE OF HYDROGEN IN ANAEROBIC DIGESTION

Hydrogen was reported to be an important intermediate in regulating anaerobic digestion by Bryant et al. (1967). Since then, much research has been performed to examine the pathways involved in the formation of methane from various substrates. Especially the degradation of glucose, an easily digested, soluble substrate, has been studied extensively.

Hydrogen acts as a regulator at several points in the Embden-Meyerhof pathway through which glucose is degraded, and in successive steps where pyruvate and volatile fatty acids are converted to acetate (see Figure 2). The nicotine adenine dinucleotide (NAD) which is reduced to NADH must continuously be regenerated for the process to function properly. This occurs through the reduction of protons to form hydrogen gas.

The partial pressure of hydrogen has to be kept low for the degradation reactions to be thermodynamically favorable. Varying limits at which inhibition due to hydrogen occurs have been suggested, depending on the substrate and the microbial population involved. Kaspar and Wuhrmann (1978a) reported that a hydrogen partial pressure of less than 10^{-4}

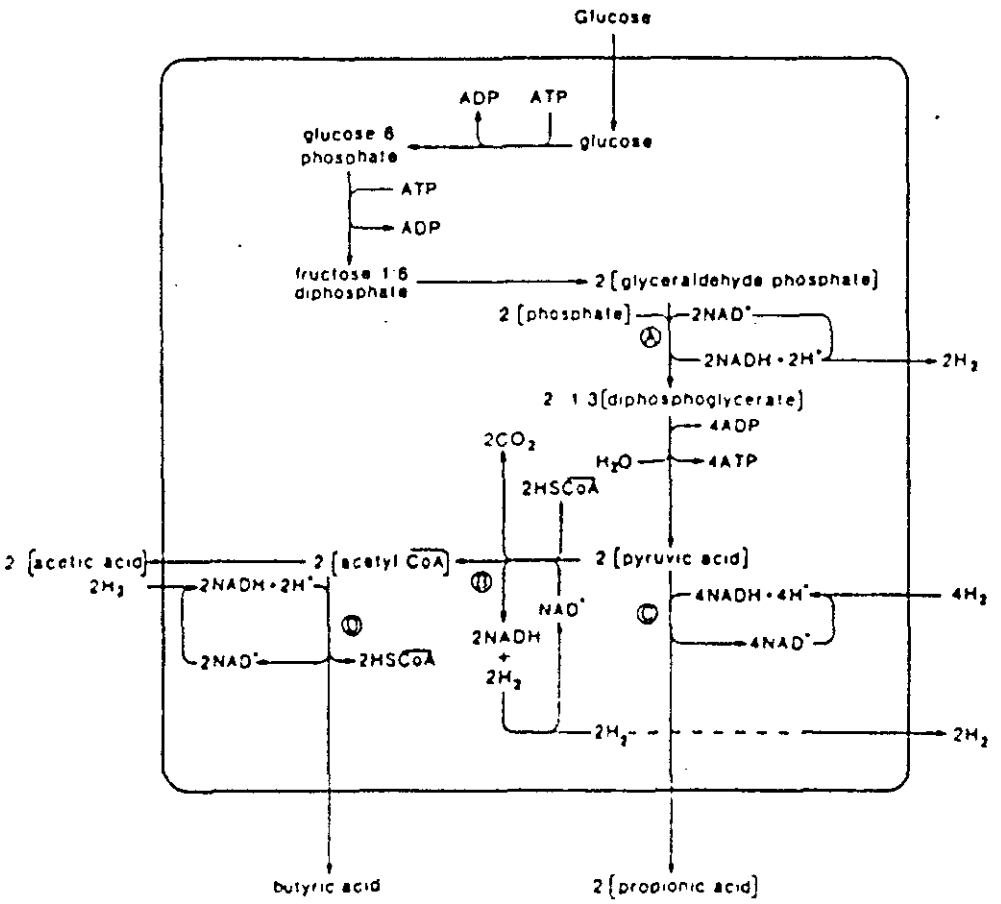

Figure 2: Hydrogen regulated catabolic pathways for glucose degradation (Mosey, 1983).

atm (approximately 10 ppm) was desireable to ensure stable digestion. They found that higher hydrogen concentrations inhibited the degradation of propionate and ethanol. Smith and Mah (1980) showed that propionic acid degradation was inhibited by 0.18 atm hydrogen. Acetate production from ethanol was found to be inhibited by 0.5 atm hydrogen (Bryant et al., 1967). Ferguson and Mah (1983) showed that methanogenesis from acetate was limited by the presence of hydrogen.

A low hydrogen concentration is maintained through hydrogen uptake ("interspecies hydrogen transfer") by hydrogenotrophs, such as the hydrogen-utilizing methanogens. Sulfate and nitrate reducing bacteria also compete for this hydrogen. Existence of these syntrophic relationships between two bacterial populations has been well documented. Some associations which have been studied, include acetogens and methanogens (Henson and Smith, 1985), carbohydrate-fermenters and methanogens (Scheifinger et al., 1975), sulfate-reducers and methanogens (Phelps et al., 1985 and McInerney and Bryant, 1981) and cellulose-degraders and methanogens (Chung, 1976).

Kaspar and Wuhrmann (1978b) showed that hydrogen uptake by methanogens does not occur at a maximum rate. The hydrogen removal system operated at only 1% of the potential rate intrinsic to the sludge ecosystem. The slow growing

methanogens are often considered the rate limiting step in the digestion process. If the system is stressed, hydrogen may accumulate in the digester, thereby inhibiting the hydrogen-producing bacteria. A shift in fermentation products can result, since alternative electron sinks for the regeneration of NAD have to be found. In this case, pyruvate can be fermented to propionate, lactate and ethanol, or butyric acid can be formed from acetyl-CoA (see Figure 2). Methanogens cannot use these substrates directly, leading to an accumulation of hydrogen and volatile acids which can cause a depression of digester pH and a reduction in methane production.

Even though methanogens are considered hydrogen-consumers rather than producers, they have been shown to produce trace levels of hydrogen when grown on carbon monoxide (O'Brien et al. 1984 and Bhatnagar et al., 1987), methanol (Bhatnagar et al., 1987) and acetate (Nelson and Ferry, 1984, Phelps et al., 1985 and Boone et al., 1987). Loveley and Ferry (1985) found that hydrogen accumulated to about 16 to 92 Pa, after which production stopped. The net rates of hydrogen production and consumption were less than one percent of the rate of methane production. Hydrogen levels appeared to interact with electron carriers involved in acetate metabolism. It is unclear, whether the hydrogen is an intermediate in catabolic or anabolic reactions (Boone

et al., 1987 and Krzycki et al., 1987). Boone et al. (1987) suggested that the electron carrier in equilibrium with hydrogen, generates electrons for anabolic reactions.

Hydrogen has been suggested as a potential indicator for monitoring anaerobic digestion (Kaspar and Wuhrmann, 1978b, Heyes and Hall, 1981, Mosey, 1983 and Hickey, 1987). Methods for measuring low concentrations of hydrogen gas in anaerobic digesters were also explored (Robinson et al., 1981, Scott et al., 1983 and Collins and Paskins, 1987). Studies by Hickey et al. (1987a) using laboratory scale digesters have shown the potential of hydrogen to indicate impending reactor upsets. Archer et al. (1986) monitored hydrogen in a pilot scale digester treating brewery effluent. Harper and Pohland (1986) presented a review of hydrogen management options for improving anaerobic biological wastewater treatment.

The role of hydrogen in methanogenesis is difficult to assess. Hydrogen production and accumulation depend on such variable factors as substrate composition, microbial population, temperature, pH, hydraulic retention time, solids retention time and mixing characteristics of the digester, so that monitoring hydrogen alone may not be sufficient to predict impending digester upsets. Hickey et al. (1987b) have indicated that monitoring carbon monoxide and hydrogen simultaneously, may be a better practice.

2.3 ROLE OF CARBON MONOXIDE IN ANAEROBIC DIGESTION

Several genera of anaerobic bacteria, such as Methanobacterium (Daniels et al., 1977) and Acetobacterium (Kerby et al., 1983) have been shown to utilize carbon monoxide as their energy source. O'Brien et al. (1984) showed that Methanosarcina barkeri was capable of growth and production of methane from carbon monoxide as both its carbon and energy source. They also observed net hydrogen formation at a carbon monoxide partial pressure greater than 20 percent, and hydrogen consumption at carbon monoxide concentrations below that value, indicating a possible relationship between hydrogen and carbon monoxide metabolism.

Conrad and Thauer (1983) were the first ones to report carbon monoxide production under strictly anaerobic conditions. They studied Methanobacterium thermo-autotrophicum grown on hydrogen and carbon dioxide as its sole carbon and energy sources, and found between 30 and 90 ppm carbon monoxide in the digester effluent gas. Diekert et al. (1984) found that an acetogenic bacterium produced small levels of carbon monoxide during growth on fructose and glucose. They both proposed that CO dehydrogenase could serve to reduce carbon dioxide to carbon monoxide.

Many methanogens have been shown to possess carbon monoxide dehydrogenase activity (Daniels et al., 1977). Hu

et al. (1982) proposed that CO dehydrogenase takes part in carbonylation of a methyl group during synthesis of acetate from acetyl-coenzyme A in acetogenic bacteria. Kenealy and Zeikus (1982) suggested a similar function in methanogens.

Krzycki et al. (1982) found CO dehydrogenase activity in an acetate-adapted strain of Methanosarcina barkeri to be five times greater than that of a culture growing on methanol or a mixture of hydrogen and carbon dioxide. They hypothesized that CO dehydrogenase functions in the splitting of acetate to methyl and formyl intermediates in the formation of methane and carbon dioxide.

Nelson and Ferry (1984) made similar observations. They found CO dehydrogenase activity to be 40 and 96 times greater in cells grown on acetate, than in cultures grown on hydrogen and carbon dioxide. The conversion of acetate to methane seemed to involve steps similar to a reversal of the pathways through which acetogens and hydrogen/carbon dioxide-utilizing methanogens synthesize acetate. They indicated that a CO-dependent CH_3-S-CoM methylreductase system was active in the conversion of acetate to methane.

Stupperich and Fuchs (1984) showed that the methyl and carboxyl groups of acetic acid originate via two different pathways, with CO dehydrogenase functioning in the formation of the carboxyl group. Diekert et al. (1984) presented

evidence that carbon monoxide in a bound form was the precursor of the carboxyl group in acetate.

Stupperich et al. (1983) and Pezacka and Wood (1984) believed that in the case of hydrogen-utilizing methanogens and acetogens respectively, CO dehydrogenase functions in the anabolic fixation of carbon dioxide into cell carbon.

In contrast to Krzycki et al. (1982), who suggested that formate or carbon monoxide in bound form are intermediates in CO_2 formation from the carboxyl group of methane, Eikmanns and Thauer (1984) showed that neither formate nor CO can be readily converted into the bound forms proposed previously. Based on their research, free formate or free carbon monoxide were also ruled out as possible intermediates. They agreed with results from previous studies suggesting that CO bound tightly to a metal is probably an intermediate in the synthesis of acetyl-CoA from $2CO_2$ in acetogenic bacteria (Hu et al., 1982), and in autotrophically growing methanogens (Stupperich et al., 1983 and Conrad and Thauer, 1983).

Wood et al. (1986) performed an extensive review of autotrophic growth in bacteria using carbon dioxide as their carbon source. Here, two molecules of carbon dioxide are converted to acetyl-CoA, one of which is reduced to the methyl group. CO dehydrogenase was found to be the enzyme playing the major role in this pathway, which they called

the acetyl-CoA pathway. The CO dehydrogenase converts one CO_2 to CO, accepts the methyl and CoA groups and converts them to acetyl-CoA. Fuchs (1986) performed an extensive review of the acetyl-CoA pathway in acetogenic bacteria. Ljungdahl (1986), in his review of autotrophic acetate synthesis, suggested that the pathway be named "the Wood pathway of autotrophic fixation of CO_2".

Smith et al. (1985) studied the inhibition of methanogenesis and carbon metabolism in Methanosarcina barkeri strain 227 by cyanide. Based on their results, they concluded that CO dehydrogenase performs two metabolic functions, one being the oxidation of the methyl group of acetate to form carbon dioxide, the other synthesizing acetyl-CoA for carbon assimilation from methanol and carbon dioxide.

2.4 TOXICANTS IN ANEROBIC DIGESTION

Anaerobic digestion has several advantages over aerobic treatment. However, one of the major drawbacks of the process is its susceptibility to upsets. Most problems are due to either organic or hydraulic overloads, or the addition of toxicants. "Toxicity" is a relative term, and the concentration at which any compound becomes inhibitory varies greatly. The effect of any material depends on such factors as pH, temperature and presence of other compounds,

which may increase or lessen its toxicity. The severity of inhibition also depends on exposure time. Toxicants can appear as one-time shock loads, or be present continuously in the wastestream to be treated (chronic exposure).

The following sections will focus on toxicity in anaerobic digestion systems due to the presence of inorganic and organic compounds.

2.4.1 Inorganic Toxicants

Inorganic compounds which have been the subject of many anaerobic digestion toxicity studies are heavy metals. Heavy metals can be present at high concentrations in municipal wastewater, originating from a variety of sources. Klein et al. (1974) stated that domestic sources contribute large concentrations of heavy metals. Collins and Ridgway (1980) site stormwater runoff as a major source of heavy metals in areas with combined sewers. Industrial applications, such as electroplating can also contribute significant amounts of metals in wastewater.

In conventional wastewater treatment plants, heavy metals were found to be concentrated in both primary and secondary sludges (Nielsen and Hrudey, 1983 and Petrasek and Kugelman, 1983). Overall removal efficiencies for cadmium, chromium and copper were generally high, while nickel and zinc were not removed as efficiently (Nielsen and Hrudey,

1983, and Gould and Genetelli, 1984). These high heavy metals concentrations could then enter the anaerobic digester and possibly cause process upset or failure.

Research into the nutritional requirements of methanogens has revealed a need for several elements, including heavy metals such as zinc and nickel, in low concentrations (Sowers et al., 1984 and Patel et al., 1988). These elements are required for many enzymes and co-enzymes to function properly. Several studies have demonstrated the stimulatory effect of nickel in anaerobic digestion. Murray and van den Berg (1981) showed that anaerobic fixed-film digesters treating food processing waste were stimulated by 100 nM nickel. Canovas-Diaz and Howell (1986) found increased butyric acid conversion by a pilot scale anaerobic downflow fixed-film reactor if nickel were present. Speece et al. (1983) also indicated the positive effect of nickel on an acetate-enriched methanogenic culture.

Vallee and Ulmer (1972) believed toxicity to be caused by the binding of heavy metals to protein molecules or by replacement of naturally occurring metals, thereby disrupting enzyme function and structure. Bacteria can show two types of resistance to inhibition by heavy metals (Sterritt and Lester, 1980), either non-specific resistance due to the particular physiological state of the organism or resistance due to specific inheritable factors.

Studies on heavy metal toxicity have indicated varying concentrations of metals which caused an effect on the digestion process (Ashley et al., 1982 and Ahring and Westermann, 1985). Mosey et al. (1971) indicated the order of decreasing toxicity of heavy metals to anaerobic digestion to be Cu > Pb > Cd > Zn. A recent study by Hickey (1987) using a reactor fed waste activated sludge, also produced the same results. The order of decreasing toxicity was found to be Cu > Cd > Zn. Hayes and Theis (1978) showed similar results, concluding that the order was Ni > Cu > Pb > Cr > Zn. The dosages at which they applied cadmium did not produce any inhibition.

A major difference between heavy metals and other toxic pollutants is that the metals are not biodegradable (Sterritt and Lester, 1980). The toxicity of a heavy metal will not only depend on the concentration applied. The physical environment in the digester also plays a major role. The soluble form of a metal is generally thought to be the toxic species (Kugelman and Chin, 1971). The insoluble forms are of little consequence in terms of toxicity (Hayes and Theis, 1978). Factors such as pH, temperature, chelating agents and presence of other ions will determine how much of the metal is in the soluble form, and therefore available for uptake by the bacteria. Depending on concentrations

available, metals can be stimulatory, inhibitory or toxic (McCarty, 1965c).

In anaerobic environments, heavy metals can form insoluble precipitates with sulfide (except chromium), carbonate and hydroxide (Mosey et al., 1971, Gould and Genetelli, 1978 and Pearson et al., 1980). It should be noted that sulfide in itself can also be toxic. McCarty (1965c) stated that up to 100 mg/l of sulfide added as a shock load, or up to 200 mg/l with acclimation, could be tolerated. The extent to which the metals form precipitates is variable and depends highly on pH (Mosey and Hughes, 1975, Nelson et al., 1981 and Parkin et al., 1983). Jarrell et al. (1987) indicated that methanogenesis in a buffer solution was inhibited by much lower heavy metal concentrations than methanogenesis in sludge, due to formation of metal sulfides and complexation with ligands in the sludge.

The effect of temperature on solubilization was noted by Gould and Genetelli (1978). As temperature was decreased, cadmium, nickel and zinc were solubilized. The change in temperature did not affect solubilization of copper.

The presence of other compounds may also change the toxicity effect of a metal. If the effect of one substance is reduced by the presence of another, it is termed antagonism; if the effect is increased, synergism (Kugelman

and Chin, 1971). Several studies have demonstrated such relationships between different metals (Mosey, 1976, Patterson and Hao, 1980 and Ahring and Westermann, 1985).

Yang and Speece (1985) studied the response of acetate-utilizing methanogens to the addition of cyanide. Cyanide compounds are used widely for industrial applications. The study showed that the severity of inhibition depended on toxicant concentration and exposure time, while recovery was a function of biomass concentration, retention time and temperature.

2.4.2 Organic Toxicants

There are many organic compounds found in wastewaters which can be potentially toxic to anaerobic digestion systems. Swanwick et al. (1969) identified formaldehyde and chlorinated compounds as being the most prevalent ones in sludge digesters. Like metals, these compounds can be concentrated in wastewater treatment sludges (Swanwick and Foulkes, 1971) and cause digester upset or failure. A major difference with heavy metals, is that many of the organic compounds are potentionally biodegradable.

Inhibition of anaerobic digestion by formaldehyde has been well documented. Pearson et al. (1980) observed that a slug dose of 200 mg/l formaldehyde reduced gas production by 50%. In addition, they found that anaerobic degradation

acclimated to the addition of phenol, but not to that of formaldehyde. Parkin and Miller (1983) studied the response of acetate enrichment cultures to the continuous addition of formaldehyde and chloroform. They found that significant acclimation to both toxicants was possible. They hypothesize that acclimation could be due to the compounds being metabolized, adsorbed or volatilized, or that the bacterial enzyme system could adjust to tolerate the toxicant. Bhattacharya and Parkin (1988) showed that acetate and propionate enrichments could tolerate higher concentrations of formaldehyde, added as slug doses and continuously, than reported in previous studies. They concluded that acclimation to continuous addition of formaldehyde was the result mainly of biodegradation. Hickey (1987) reported a rapid accumulation of hydrogen gas as a result of formaldehyde addition.

Of the chlorinated compounds, chloroform, carbon tetrachloride and methylene chloride have been shown to be extremely toxic to anaerobic digestion and rumen systems (Bauchop, 1967, Thiel, 1969 and Bhattacharya and Parkin, 1988). Thiel (1969) observed accumulation of hydrogen at the same time as methane production was inhibited. The same response was observed by Hickey (1987) who examined the effects of chloroform and trichloroacetic acid. In addition, he noted that severe inhibition of methane production was

accompanied by a substantial rise in carbon monoxide concentrations.

Inhibitory or toxic levels of many other compounds which can be found in industial wastestreams were reported in an extensive review by Henze and Harremoes (1983).

A compound not usually found in wastewaters, which has been used in toxicity studies with methanogens, is 2-bromo-ethanesulfonic acid (BES). BES is an analog of coenzyme M (2-mercaptoethanesulfonate), a coenzyme for methyl-transfer reactions in methanogens (Balch and Wolfe, 1979). BES will therefore inhibit methanogens while not affecting other microbial populations in a digester. Methanogenesis by Methanothrix spp. was inhibited by the addition of 0.5 nM BES (Zehnder et al., 1980). Zinder et al. (1984) studied methanogens in a thermophilic digester. They found methanogenesis from acetate to be more sensitive to the addition of BES than CO_2 reduction was. One mM and 50 mM, respectively, were needed to cause complete inhibition. In his studies on a digester fed waste activated sludge, Hickey (1987) found that addition of 5 mM BES was required to cause complete inhibition of methanogenesis after 24 hours.

CHAPTER III

METHODS AND MATERIALS

3.1 SCOPE OF STUDY

This research was conducted to examine the potential of using hydrogen and carbon monoxide as indicators of upsets due to toxic shock loads in an anaerobic digester fed a soluble waste. In addition, the results are compared to the responses obtained from previous research by Hickey (1987) in which a digester fed a particulate substrate (waste activated sludge (WAS)) was used.

Simulating an actual digester's response was accomplished using serum bottle assays. This technique was used by Hickey (1987), and is an adaptation of the method developed by Miller and Wolin (1974) and later modified by Owen et al. (1979). To provide inoculum for the serum bottle assays, a digester was set up and operated for one month before starting any tests. Specific details related to the operation of the reserve digester and the conducting of the serum bottle assays are provided in following sections.

3.2 REACTOR DESIGN

To provide inoculum for the serum bottle assays, a 12 liter (liquid volume) fill and draw reactor was set up. To provide a constant temperature environment, the digester was

kept in a walk-in incubator where a temperature of 35±0.5°C was maintained. The digester was operated in a semi-continuous mode, being fed and wasted once a day. It was operated at a solids retention time (SRT) of 10 days. The digester contents were mixed using a Manostat Varistaltic Pump, Series S connected to a timer device providing a 15 minutes on/15 minutes off cycle.

Gas production was measured continuously with a Wet Test Meter (GCA/Precision Scientific) while a septum in the gas line allowed for gas sampling using a syringe to determine the composition of the headspace gas. The digester configuration is shown in Figure 3.

3.3 FEED SOLUTIONS

The feed for the digester consisted of a carbon source, nutrient salts, vitamins, a buffer solution and well water. In addition, 10 mg/l of cysteine was added. The composition of the feed was based on calcuations from previous research by Pause and Switzenbaum (1983), and modified using data from Owen *et al*. (1979).

Originally, a digester fed glacial acetic acid as the sole carbon source (20000 mg/l as COD) was set up, since the objective of this study was to evaluate the response of the acetate utilizing methanogens to the addition of toxics. However, this digester had to be abandoned due to problems

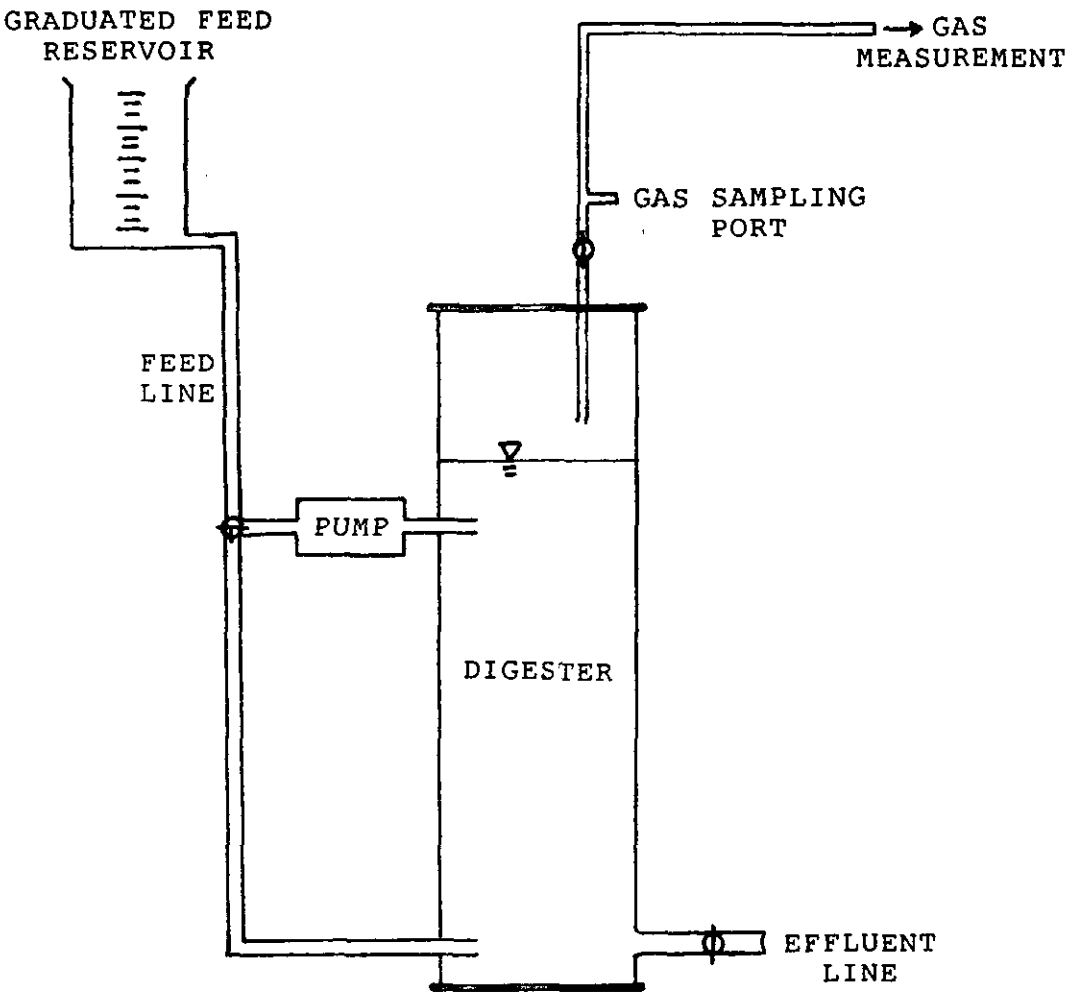

Figure 3: Digester configuration.

with excessive wall growth. Instead, a reactor fed sucrose (5000 mg/l-day as COD), added in the form of common table sugar, was set up. This resulted in a mixed population of fermentative, acetogenic and methanogenic bacteria. During the serum bottle assays, acetate replaced sucrose as the carbon source in the feed.

The formulae for the different feed constituents are given in Table 1, while the composition of the feed is listed in Table 2. Each component of the feed was made up separately and stored at $4^{o}C$. The feed was allowed to reach room temperature before feeding it to the digester.

3.4 DIGESTER OPERATIONAL PARAMETERS

To ensure that the digester was operating at steady state, pH, gas production, gas composition, total alkalinity and volatile fatty acids were determined daily. In addition, chemical oxygen demand (COD), soluble COD, total suspended solids (TSS) and volatile suspended solids (VSS) were measured on a weekly basis. A summary of the digester operational parameters is given in Table 3.

3.5 ANALYTICAL METHODS

3.5.1 pH

pH was measured daily on the digester effluent and at the conclusion of each serum bottle assay. A Fisher Accumet

Table 1: Feed Constituents.

Salt I	Concentration (g/l)
$(NH_4)_2.HPO_4$	11.4
$MgCl_2.6H_2O$	1.118
Salt II	
NH_4Cl	14.0
$MgCl_2.6H_2O$	5.0
$FeCl_2.4H_2O$	3.7
$CaCl_2.4H_2O$	2.2
KCl	2.0
$NiCl_2.6H_2O$	0.6
$CoCl_2.6H_2O$	0.3
Salt III	
H_3BO_3	0.19
$CuCl_2.2H_2O$	0.09
$Na_2MoO_4.2H_2O$	0.09
$ZnCl_2$	0.07

Table 1 (continued)

Buffer	Concentration (g/l)
$NaHCO_3$	50.0
Vitamins	
Pyridoxine hydrochloride	0.1
Biotin	0.02
Folic acid	0.02
Riboflavin	0.05
Thiamine	0.05
Nicotinic acid	0.05
Pantothenic acid	0.05
p-Aminobenzoic acid	0.05
Thioctic acid	0.05
B_{12}	0.001
Cysteine	2.50

Table 2: Feed Composition.

Sucrose	6.8 g
Salt I	68.0 ml
Salt II	68.0 ml
Salt III	20.0 ml
Buffer	235.0 ml
Cysteine	6.0 ml
Vitamins	2.0 ml
Water	1051.0 ml

Table 3: Digester Operational Parameters.

Volume	12 l
Solids Retention Time	10 days
Gas Production	3.0 l/day
pH	7.2
Percent Methane	49
Percent Carbon Dioxide	51
Volatile Suspended Solids	500 mg/l
Carbon Source	Sucrose

Model 630 pH meter with a combination electrode was used. The pH values were measured to the nearest 0.1 pH unit.

3.5.2 Gas Production

Gas production of the digester was measured continuously using a wet test meter (GCA/Precision Scientific). Gas production in the serum bottles was measured by plunger displacement of a water lubricated ground glass syringe inserted into each serum bottle.

3.5.3 Gas Composition

To determine the composition of the headspace gas, 0.5 ml gas samples were collected daily from the digester using a gastight glass syringe. 0.5 ml samples were also taken for gas analysis during the serum bottle assays.

Methane and carbon dioxide (CH_4 and CO_2) concentrations of the digester headspace gas were determined using a GOW-MAC 550 thermal conductivity gas chromatograph. Separation was accomplished with a stainless steel column, 2 m long by 4 mm in diameter, using Porapak Q packing and helium as the carrier gas. An HP-3390A integrator connected to the chromatograph quantified the CH_4 and CO_2 concentrations by area integration. The chromatograph was calibrated daily by injections of CH_4 and CO_2 standards. Operating conditions of the chromatograph are given in Table 4.

Table 4: Operating Conditions for Methane/Carbon Dioxide Gas Chromatograph.

Carrier Gas	Helium
Gas Pressure	30 psig
Gas Flow Rate	30 ml/min
Injection Port Temperature	110^{o}C
Column Temperature	80^{o}C
Detector Temperature	70^{o}C
Bridge Current	6 mA
Sample Volume	0.5 ml

The carbon monoxide and hydrogen (CO and H_2) levels in the gas from the digester and the serum bottles were measured using an RGD2 reduction gas detector with an RGA2 chromatograph module (Trace Analytical) having a dual 0.1 ml sample loop. Separation was achieved with a molecular sieve 5A column using prepurified nitrogen as the carrier gas. Attached to the instrument was a Fisher Recordall Series 5000 strip chart recorder. The instrument was calibrated prior to each use, with H_2 and CO standard gas mixtures (Scott Specialty Gases). Gas concentrations were quantified by peak height analysis. Hydrogen and carbon monoxide responses were linear up to approximately 250 ppm and 20 ppm, respectively. The hydrogen analysis had a measured coefficient of variation of 0.48 percent (Hickey, 1987). Based on 15 injections of 11 ppm standard, CO measurements had a coefficient of variation of 0.38 percent. Operating conditions of the chromatograph are given in Table 5.

3.5.4 Volatile Fatty Acids and Total Alkalinity

Volatile fatty acids (VFA) concentration and total alkalinity of the digester were determined daily, using the method of O'Brien and Donlan (1977). A sample is titrated with sulfuric acid to give total alkalinity. Carbon dioxide is evolved by heating the sample. The sample is backtitrated with sodium hydroxide to determine the VFA concentration.

Table 5: Operating Conditions for Hydrogen/Carbon Monoxide Gas Chromatograph.

Carrier Gas	Nitrogen (prepurified)
Gas Pressure	30 psig
Gas Flow Rate	40 ml/min
Column Temperature	125^{o}C
Detector Temperature	280^{o}C
Sample Loop Volume	0.1 ml
Recorder Setting	0.01 V
Recorder Chart Speed	1 cm/min

3.5.5 Chemical Oxygen Demand

Chemical oxygen demand (COD) and soluble COD were measured once a week on the influent feed and the effluent from the digester. The procedure followed is method 508 C in Standard Methods (1985), which has an accuracy of ± 20 mg/l. All samples were digested for 2 hours at 150°C in a block heater (COD reactor, Hach Chemical Co). After cooling, absorbance at 600 nm was measured on a Spectronic 70 spectrophotometer. A standard curve was prepared using standard solutions of potassium hydrogen phthalate. All samples, blanks and standards were prepared in duplicate.

3.5.6 Suspended Solids Analysis

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured regularly on the digester effluent. The procedures used were methods 209 C and 209 D in Standard Methods (1985). The filters used were Gelman Type A/E glass fiber filters, 47 mm in diameter (Gelman Instrument Co.).

3.6 SERUM BOTTLE ASSAYS

The serum bottle assay technique followed was the procedure used by Hickey (1987). It is an adaptation of the method developed by Miller and Wolin (1974) and later modified by Owen et al. (1979). The assay simulates as

closely as possible the operation of an actual fill and draw anaerobic digester.

3.6.1 Procedure

- Inoculum for the serum bottle assays was provided by a 12 liter reserve digester, operated at a 10 day SRT. The digester was kept in a walk-in incubator maintaining a temperature of 35°C. Once a day, the digester was fed a nutrient solution containing sucrose as the carbon source. During feeding of the digester, effluent was collected in a sealed bottle which had been purged of oxygen by flushing it with a 70/30 mixture of nitrogen/carbon dioxide gas for 15 minutes. The effluent was then used as inoculum for the serum bottle assays.

- Nominal 125 ml serum bottles, having an average total volume of 162 ml, were purged of oxygen using the 70/30 mixture of N_2/CO_2 gas. To each bottle a teflon coated stir bar was added. Generally, four controls were set up, while all toxified samples were prepared in duplicate.

- Feed was added to the digester effluent in a ratio simulating the 10 day SRT of the reserve digester. The feed was the same as for the reserve digester, except that glacial acetic acid (10000 mg/l-day as COD) was added as the carbon source. The acetic acid was used, since the objective of the assays was to examine the response of only the

acetate utilizing methanogens to the addition of toxicants. A few drops of resazurin were added to the inoculum to indicate any oxygen contamination.

- 50 ml aliquots of the inoculum/feed solution were dispensed into the bottles using a repeating pipet. The inoculum/feed solution was stirred continuously with a magnetically driven stirrer. During this step, both the bottles and the inoculum/feed solution were still gassed with the N_2/CO_2 mixture using a gassing manifold (see Figure 4 for a schematic of the set-up).

- The serum bottles were stoppered with butyl rubber septa and crimp sealed with aluminum seals. Then, the serum bottles were incubated for 30 minutes at $35^{o}C$, while being stirred constantly using a gang pulley system.

- After the incubation period, the pressure in the serum bottles was equilibrated to atmospheric pressure by inserting a ground glass syringe into the bottles. The various concentrations of the toxicant to be studied were now injected into the sample bottles in microliter quantities, using a glass syringe with a 24 gauge needle.

- The serum bottles were reincubated and the assay started. Composition of the headspace gas (CH_4, CO_2, H_2 and CO) was determined by gas chromatography at regular intervals, usually at 0, 4, 8, 12 and 24 hours. At these times, gas production was also measured by plunger

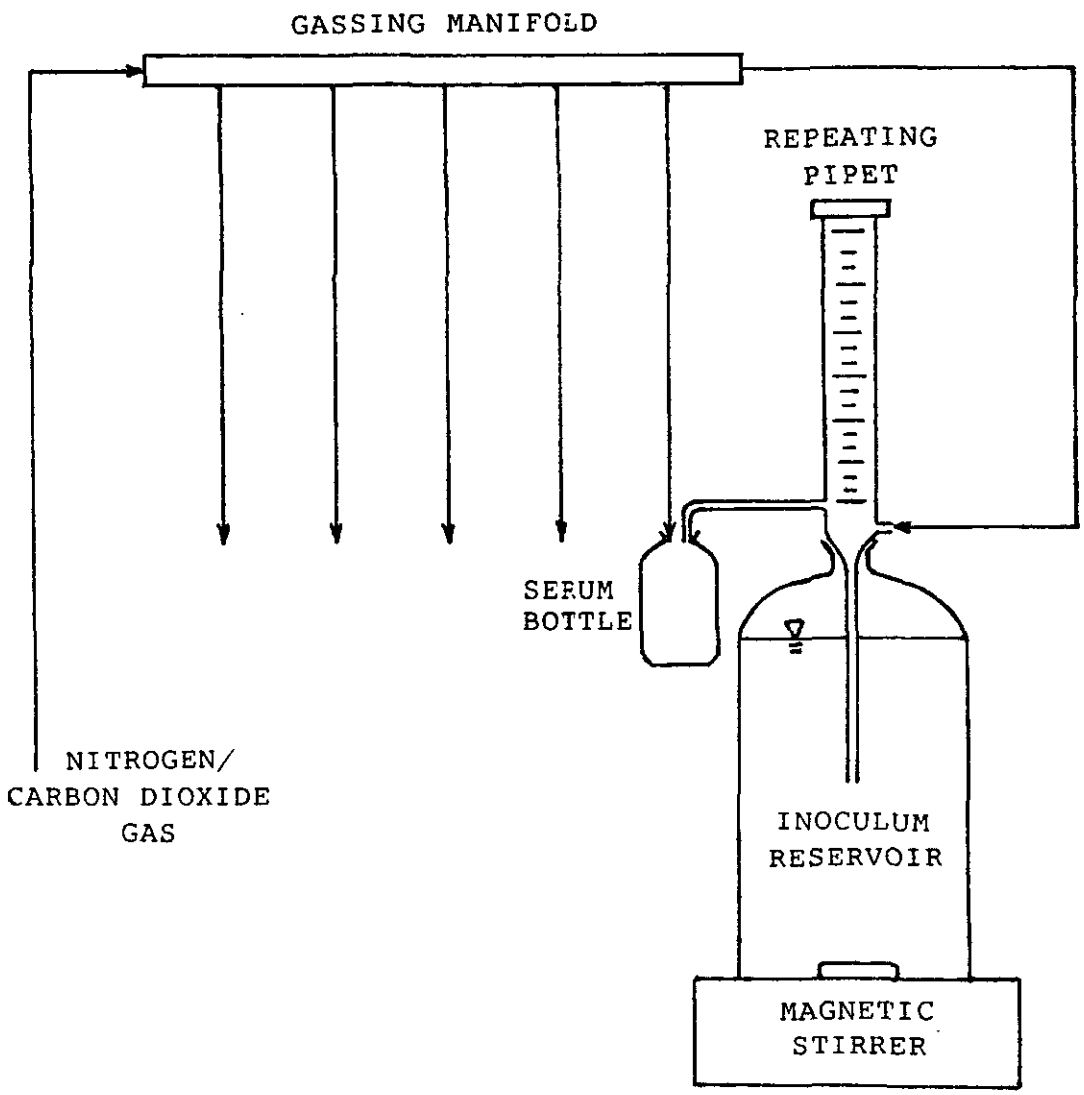

Figure 4: Schematic of serum bottle set-up.

displacement of a water lubricated ground glass syringe. If gas production was low, the gas was reinjected into the serum bottle immediately after measuring without any loss of sample.

3.6.2 Quality Control of Serum Bottle Assays

For each assay, four control samples were set up while toxified samples were run in duplicate. Samples which fell outside the 95% confidence intervals were rejected (Section 801, Standard Methods, 1985).

Based upon analysis of the controls in all toxicant assays, methane production had an average coefficient of variation of 6.8 percent at the conclusion of the assays. At the start of each assay, the coefficient of variation was higher due to the small amounts of gas produced.

Hydrogen analysis generally had a standard deviation of 3 to 4 ppm. At the conclusion of the assays, the average coefficient of variation for the controls was 10.3 percent. The standard deviation of the carbon monoxide measurements was 100 ppb. The average coefficient of variation at the conclusion of the assays was 7.8 percent for the controls.

3.6.3 Toxicants

Several inorganic and organic compounds capable of inhibiting methanogens were selected for the serum bottle

assays. They include cadmium (Cd), copper (Cu), nickel (Ni), zinc (Zn), 2-bromoethanesulfonic acid (BES) and formaldehyde. The four metals were added as chloride salts.

Cd, Cu, Ni and Zn were selected to represent common heavy metals often found in wastewater, and to which previous researchers have shown anaerobic digestion systems to be particularly sensitive (Hayes and Theis, 1978, Ahring and Westermann, 1985 and Hickey, 1987).

Formaldehyde was selected to represent a typical organic toxicant. It is often found in industrial wastewater and has been shown to be inhibitory to anaerobic digestion systems (Parkin and Miller, 1982 and Bhattacharya and Parkin, 1988).

BES, a compound not usually found in wastewater, was chosen since it is an analog of coenzyme M (2-mercapto-ethanesulfonic acid), which is a coenzyme for the methyl transfer reactions in methanogens (Balch and Wolfe, 1979). This makes BES a specific toxin for methanogens, while it is not toxic to the fermentative and acetogenic organisms in an anaerobic digester.

Concentrations of toxicants to be tested were selected based on literature values from previous research, and on results obtained from trial assays.

CHAPTER IV

EXPERIMENTAL RESULTS

The impact of the addition of toxicants on product (methane) and intermediate (hydrogen and carbon monoxide) gases in anaerobic digestion was studied using serum bottle assays. In this chapter, the results from these serum bottle assays will be presented.

The effects of several organic and inorganic toxicants were examined. Copper, cadmium, nickel and zinc were selected to represent common heavy metals found in wastewater. Their inhibitory effect on anaerobic digestion systems has been well documented previously (Hayes and Theis, 1978, Ahring and Westermann, 1985 and Hickey, 1987). Formaldehyde was selected to represent a typical, potentially biodegradable organic compound. It is often found in industrial wastewater, and has been shown to have an inhibitory effect on anaerobic digestion systems (Parkin and Miller, 1982 and Bhattacharya and Parkin, 1988). 2-Bromoethanesulfonic acid (BES) was chosen, since it is a specific inhibitor of methanogens while it does not affect the fermentative organisms in an anaerobic digester.

4.1 INORGANIC TOXICANTS

4.1.1 Copper

The effect of copper was studied using dosages ranging from 2 to 20 mg/l as Cu (added as $CuCl_2$). The effect of applied copper dose on methane production rate is shown as a function of time in Figure 5. All samples containing $\geq$ 6 mg/l Cu showed moderate to severe inhibition during the first hours of the assay. After 24 hours, the samples containing up to 6 mg/l Cu showed signs of recovery, while the sample containing the highest dose of copper (20 mg/l) remained severely inhibited (methane production $\leq$ 5% of controls). Figure 6 shows cumulative methane production over 23 hours as a function of applied copper dose. The dose required to produce 50% inhibition of methane production was estimated to be approximately 5 mg/l.

The response of hydrogen as a function of time and applied copper dose is shown in Figure 7. The controls and samples containing up to 10 mg/l copper showed a steady increase in hydrogen concentration throughout the assay. The most toxified sample showed no accumulation of hydrogen.

The effect of applied copper dose on CO levels is presented as a function of time in Figure 8. The samples which exhibited a moderate reduction in methane production, show a continuous increase in CO levels throughout the duration of the assay. The sample containing 20 mg/l copper,

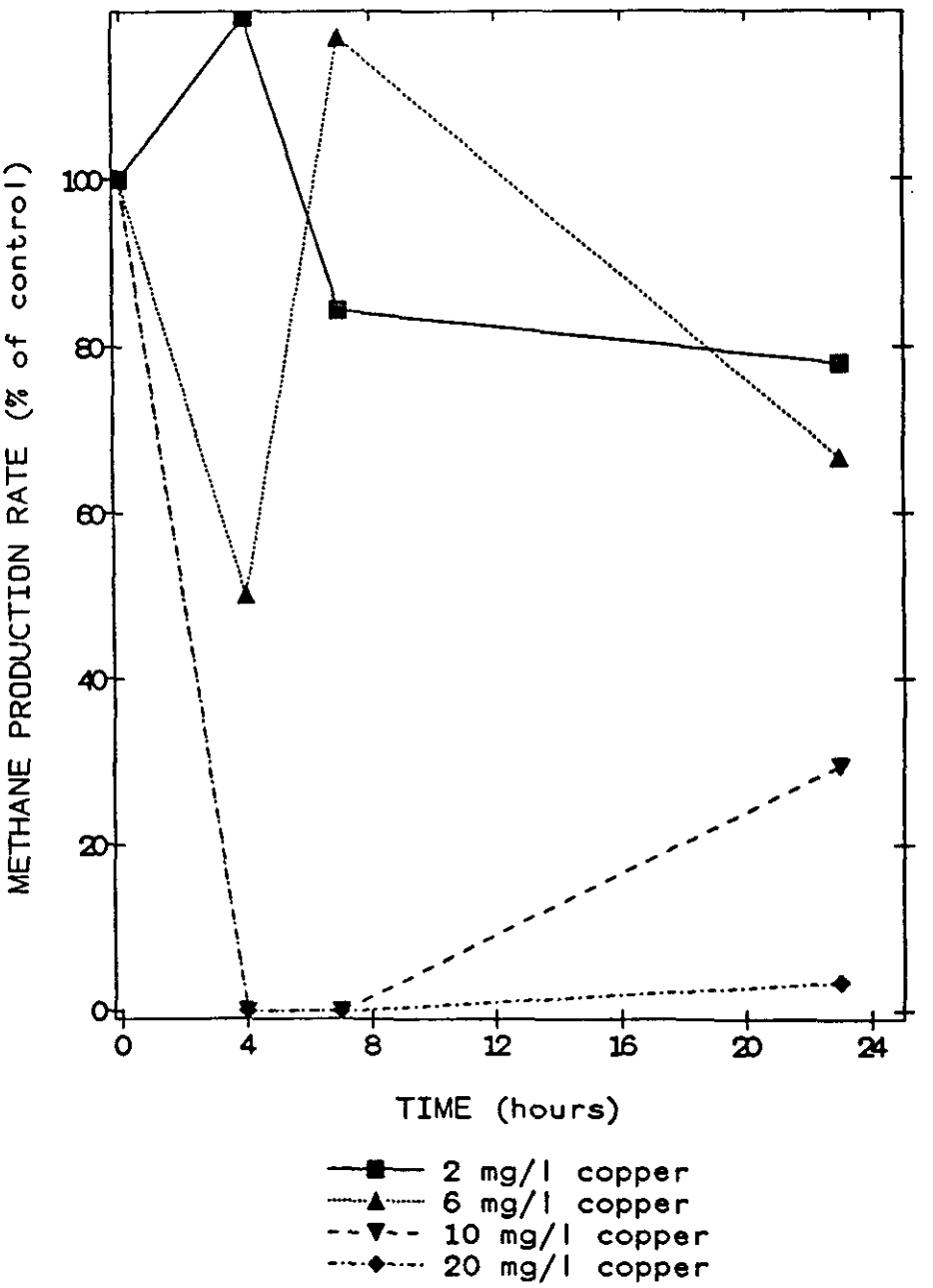

Figure 5: Effect of copper on methane production rate.

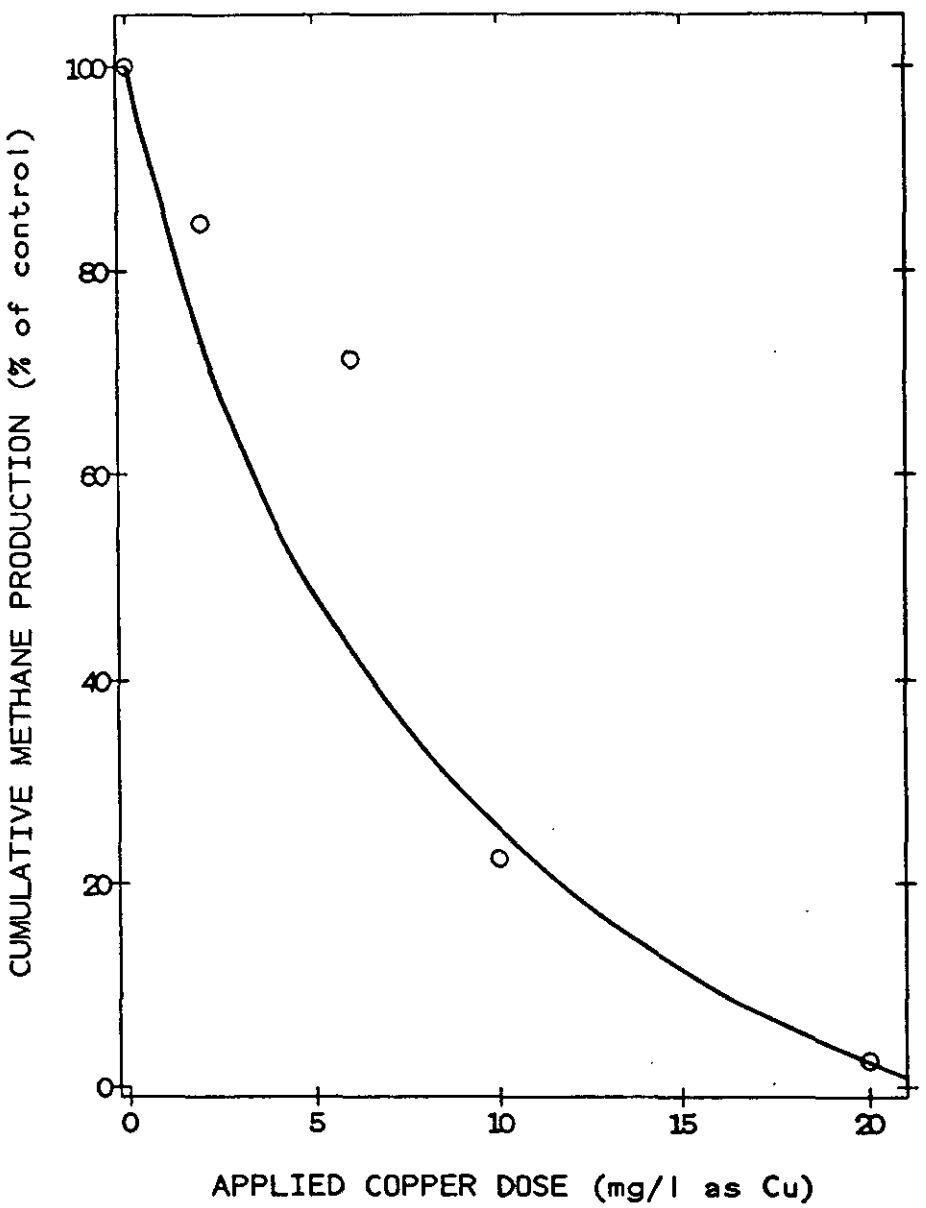

Figure 6: Cumulative 23 hour methane production as a function of copper dose.

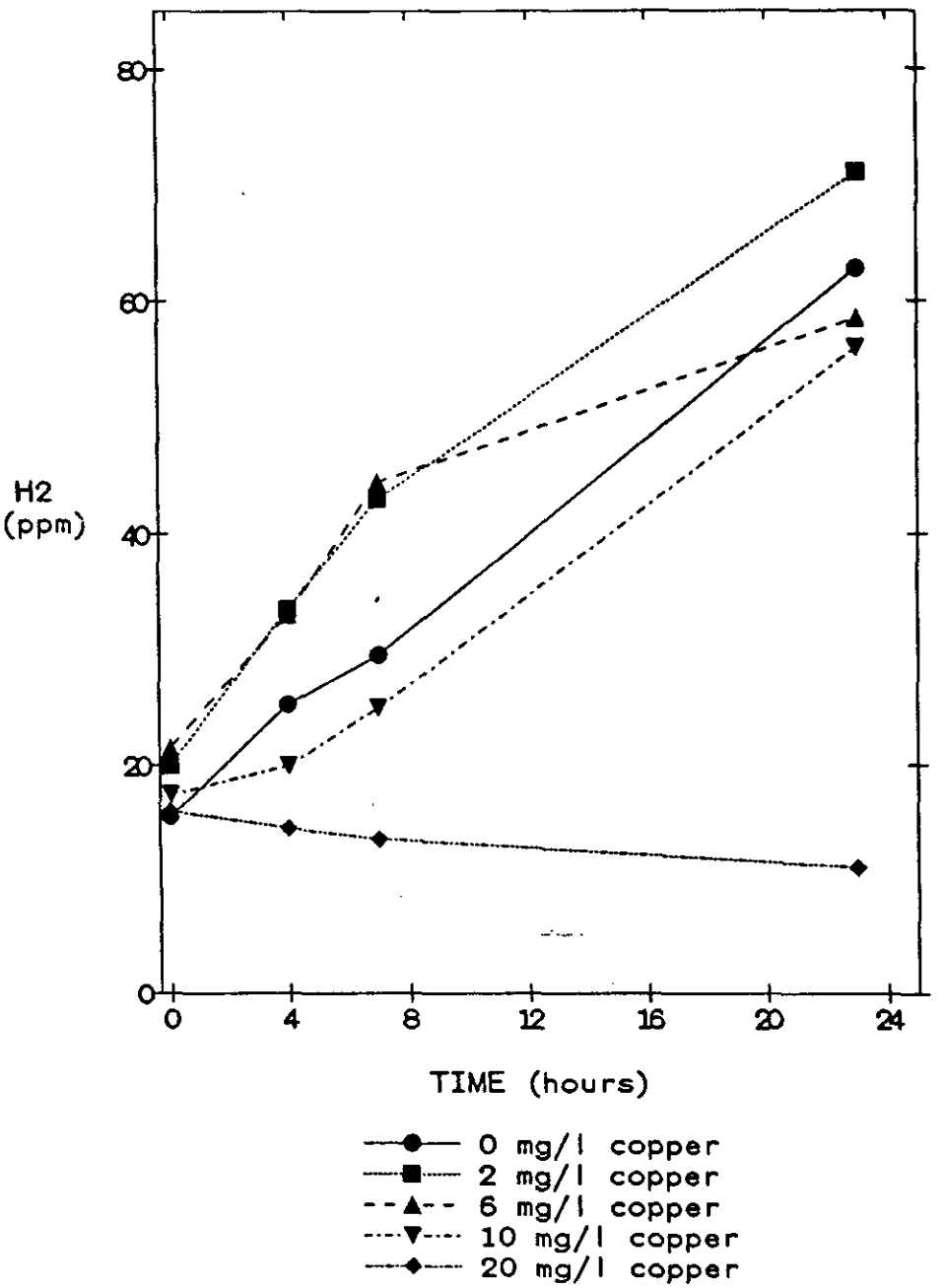

Figure 7: Hydrogen concentration as a function of time and applied copper dose.

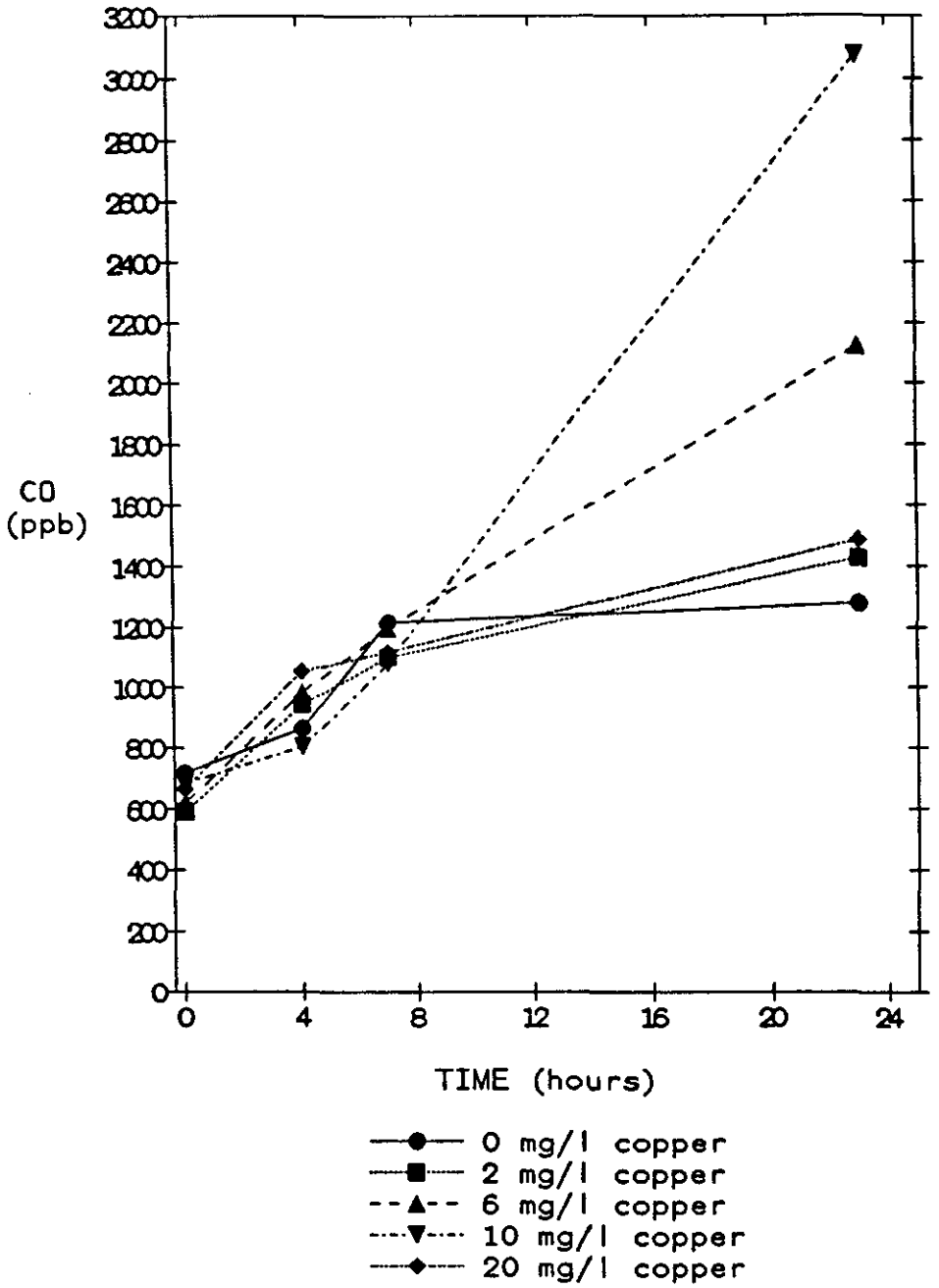

Figure 8: Carbon monoxide concentration as a function of time and applied copper dose.

which showed severe inhibition of methane production, showed a pattern of CO accumulation similar to that of the controls. CO levels rose slightly over the first 8 hours, then leveled off during the last half of the assay.

4.1.2 Cadmium

Cadmium toxicity was tested by applying dosages ranging from 4 to 48 mg/l as Cd (added as $CdCl_2$). All samples showed some inhibition of methane production rate (see Figure 9). Inhibition was severe for samples with more than 16 mg/l of cadmium applied. Using Figure 10, which shows the effect of cadmium dose on cumulative methane production, the cadmium dose required to produce 50% inhibition after 24 hours was determined to be approximately 10 mg/l.

The effect of applied cadmium dose on hydrogen concentration is shown as a function of time in Figure 11. All samples showed a pattern of increasing concentrations with time similar to that of the controls. However, the maximum concentration achieved seemed related to the dose of cadmium applied. Samples with the highest doses of cadmium applied had accumulated the most hydrogen after 24 hours.

The response of CO concentration to the addition of cadmium is shown as a function of time in Figure 12. Carbon monoxide accumulated during the first 12 hours of the assay, then leveled off in both the controls and samples containing

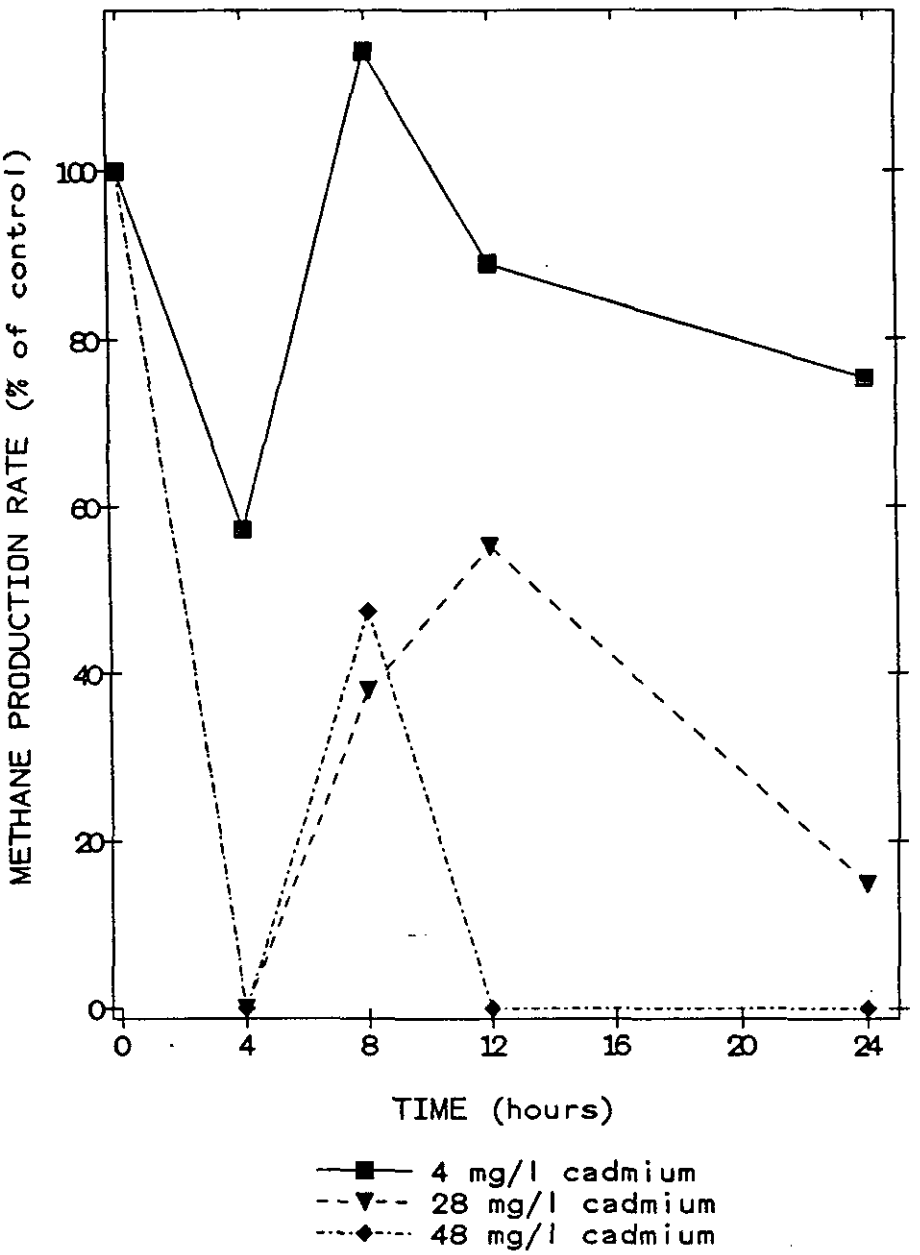

Figure 9: Effect of cadmium on methane production rate.

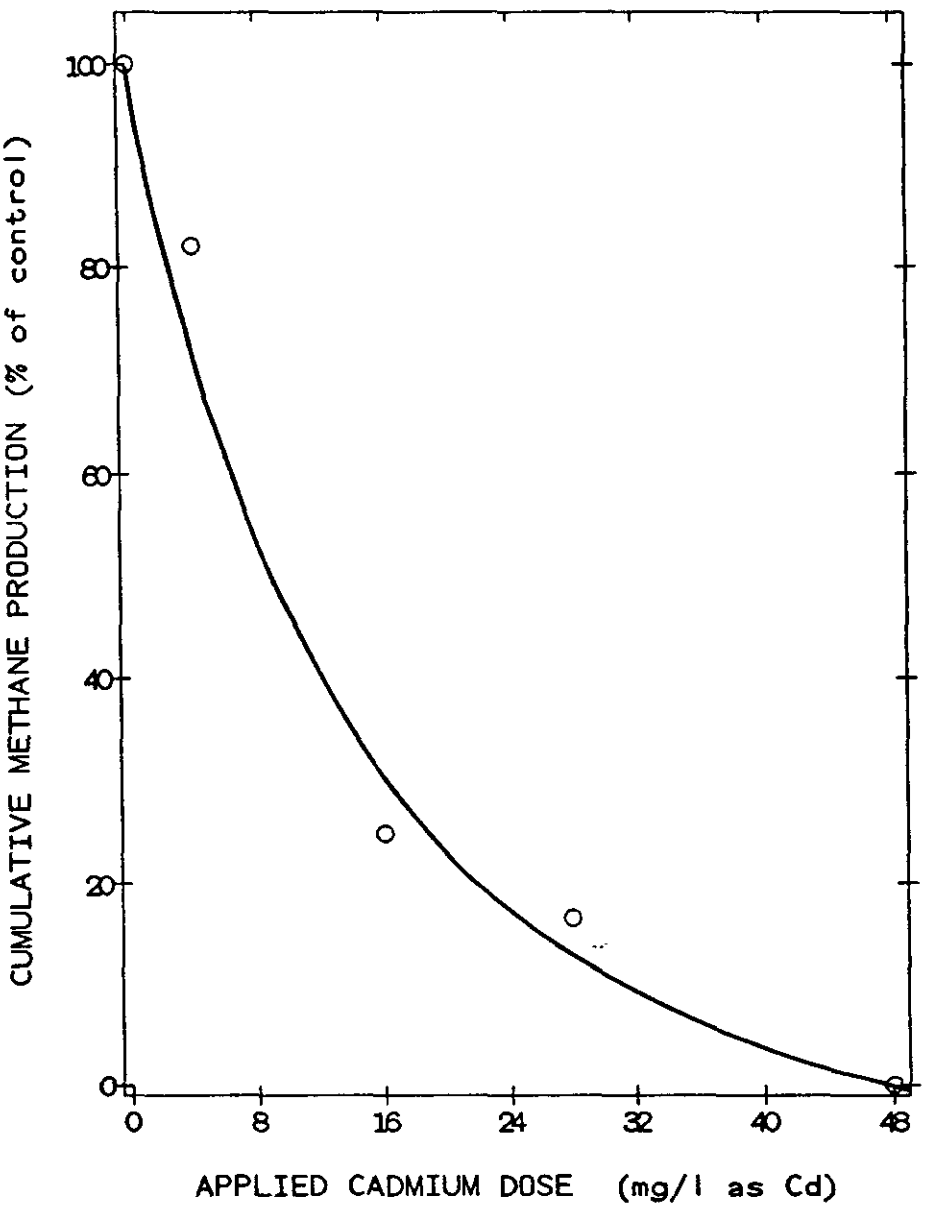

Figure 10: Cumulative 24 hour methane production as a function of cadmium dose.

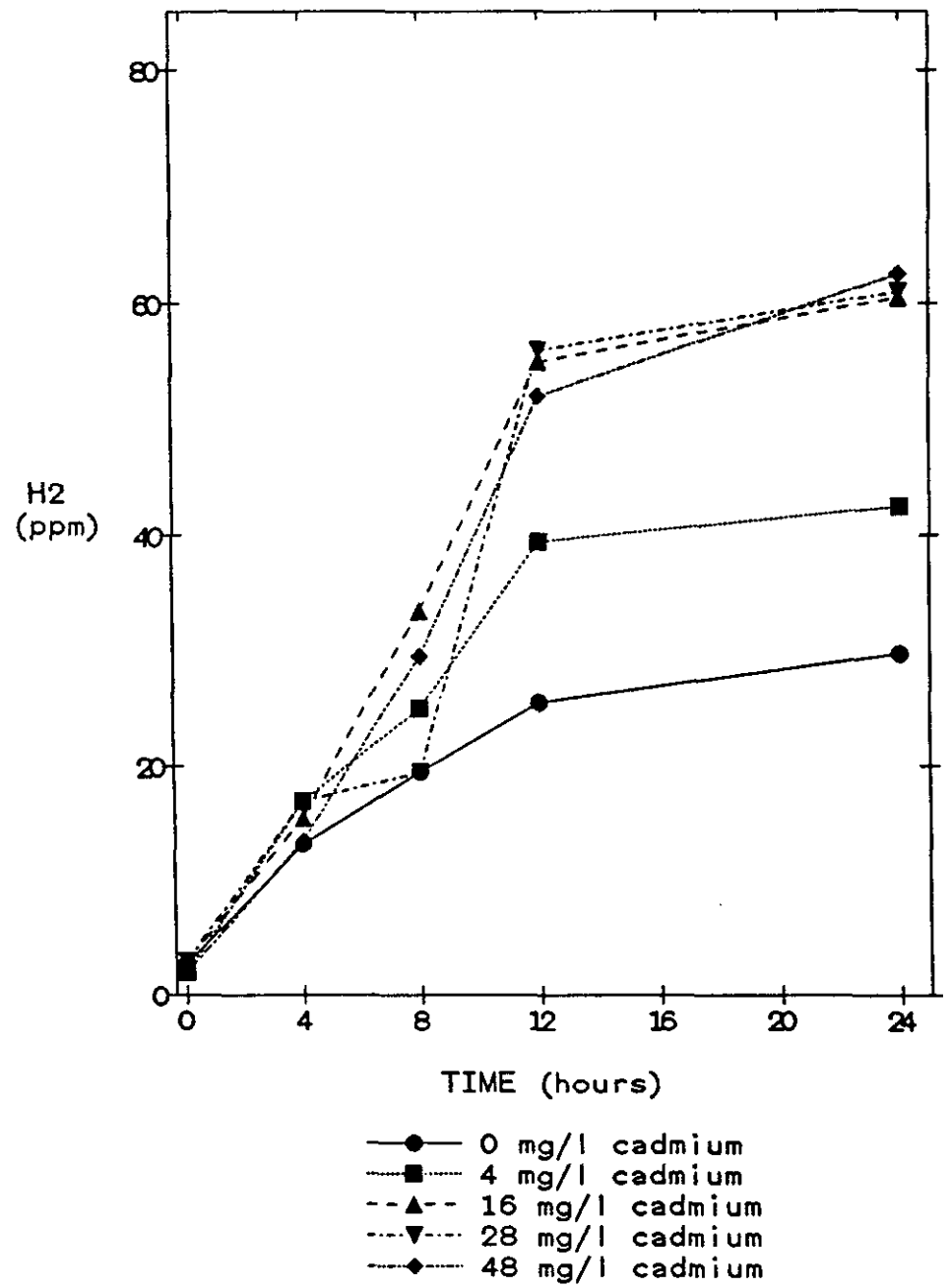

Figure 11: Hydrogen concentration as a function of time and applied cadmium dose.

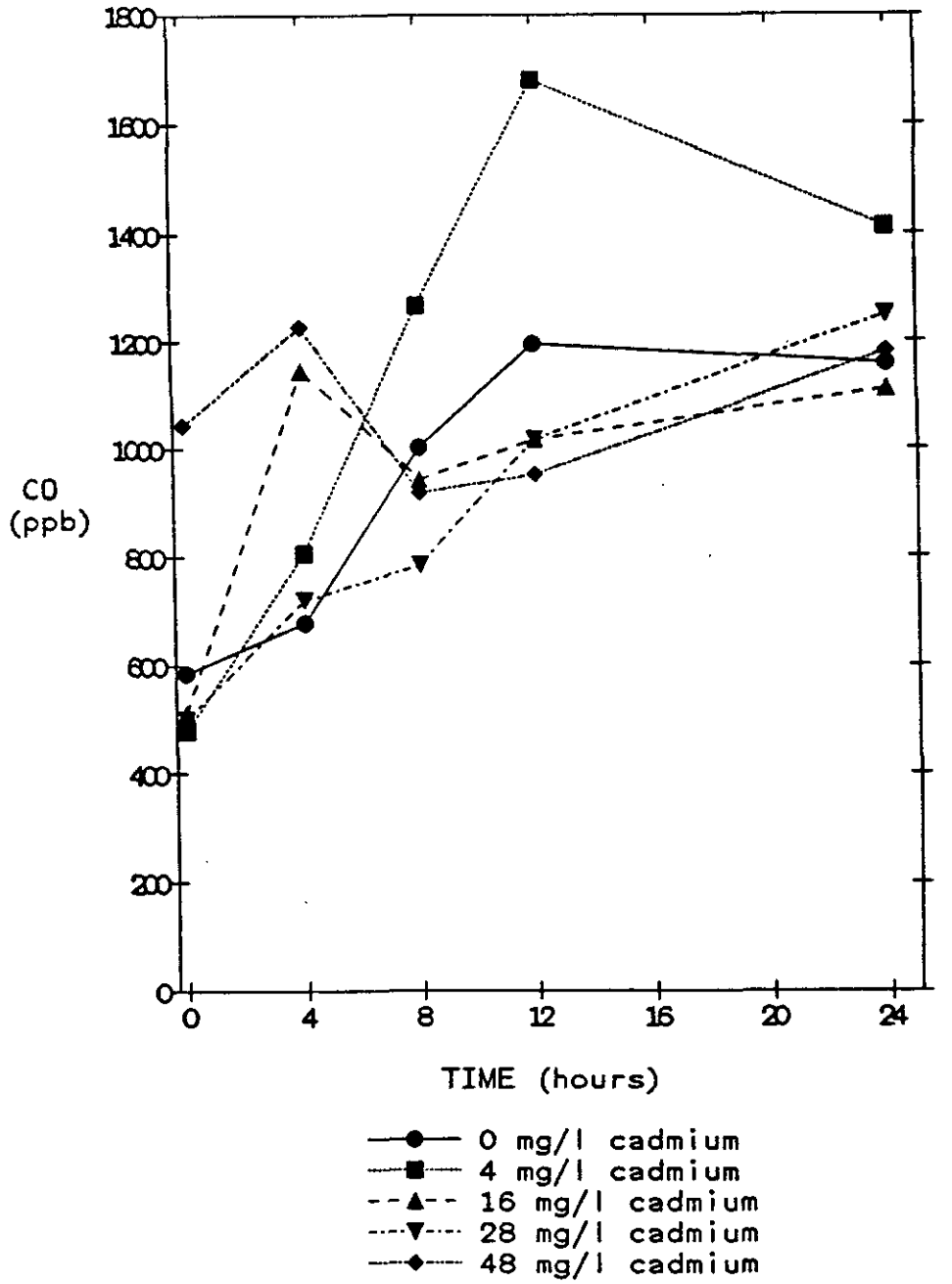

Figure 12: Carbon monoxide concentration as a function of time and applied cadmium dose.

up to 28 mg/l cadmium. The CO concentration in the most toxified sample did not increase. Instead, a small decrease was observed which coincided with the complete inhibition of methane production in that sample.

4.1.3 Nickel

Nickel was added as $NiCl_2$ in concentrations ranging from 30 to 375 mg/l as Ni. All concentrations added caused a reduction in methane production rate compared to the controls. This is shown in Figure 13. Samples containing up to 75 mg/l nickel started to show recovery after 24 hours, while samples containing higher amounts remained severely inhibited. The amount of nickel required to cause 50% inhibition of methane production rate after 24 hours was determined to be approximately 50 mg/l using Figure 14.

The hydrogen accumulation pattern as a function of applied nickel dose and time is shown in Figure 15. All samples and controls accumulated hydrogen as the assay progressed. The rate at which this occurred seemed related to the amount of nickel applied. The samples containing the lowest concentrations of nickel showed rates approximately equal to that of the controls. Samples to which 150 mg/l or more of nickel had been added accumulated hydrogen at a much slower rate.

The response of CO as a function of time and applied

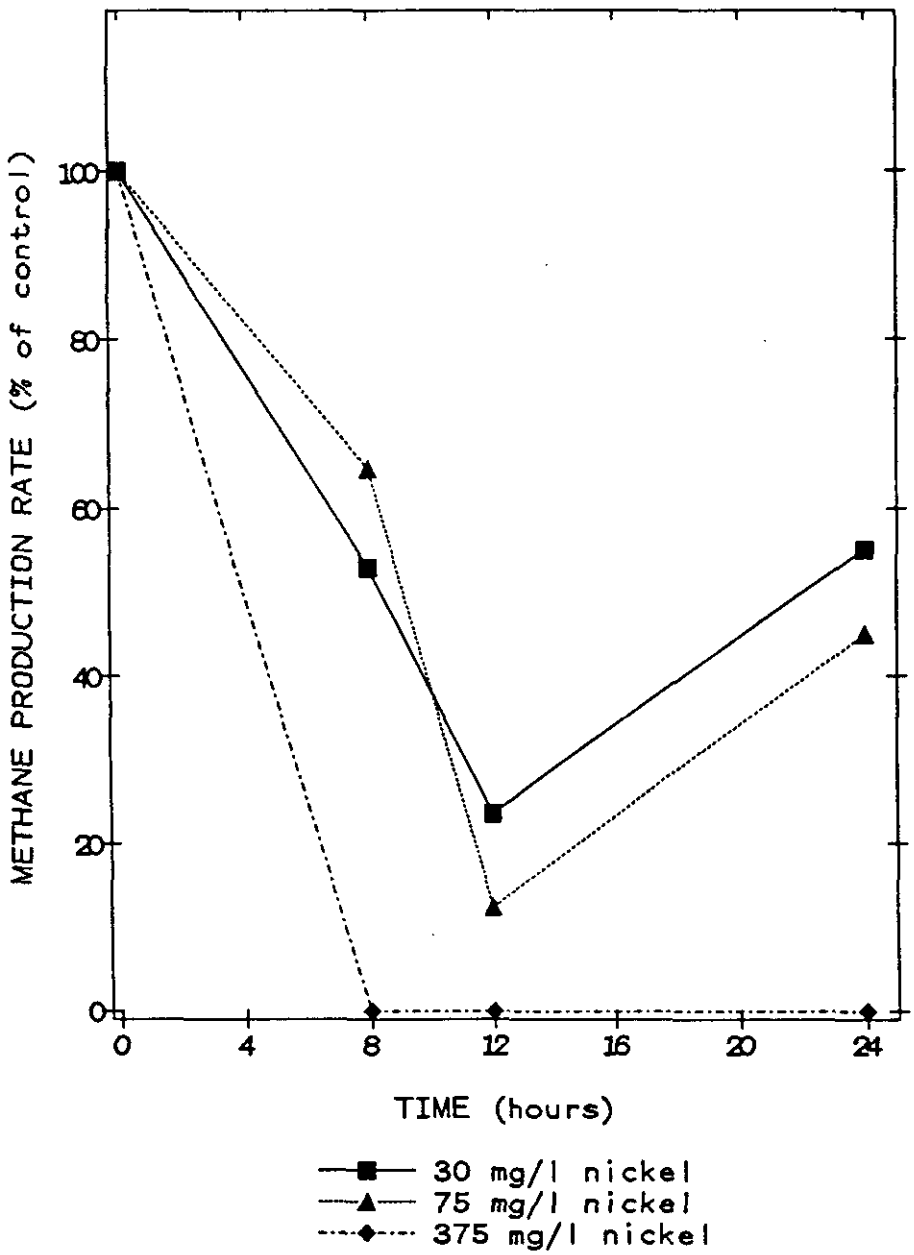

Figure 13: Effect of nickel on methane production rate.

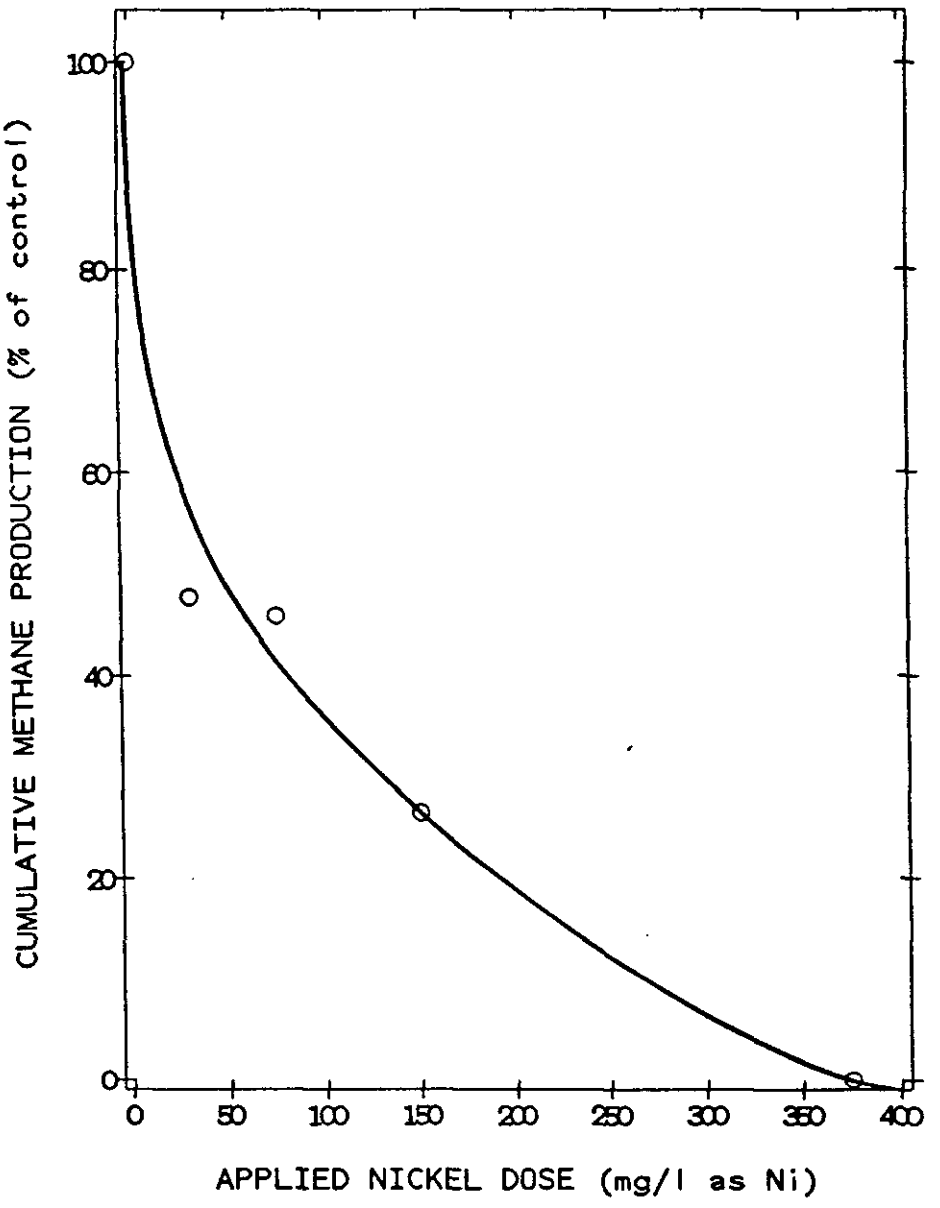

Figure 14: Cumulative 24 hour methane production rate as a function of nickel dose.

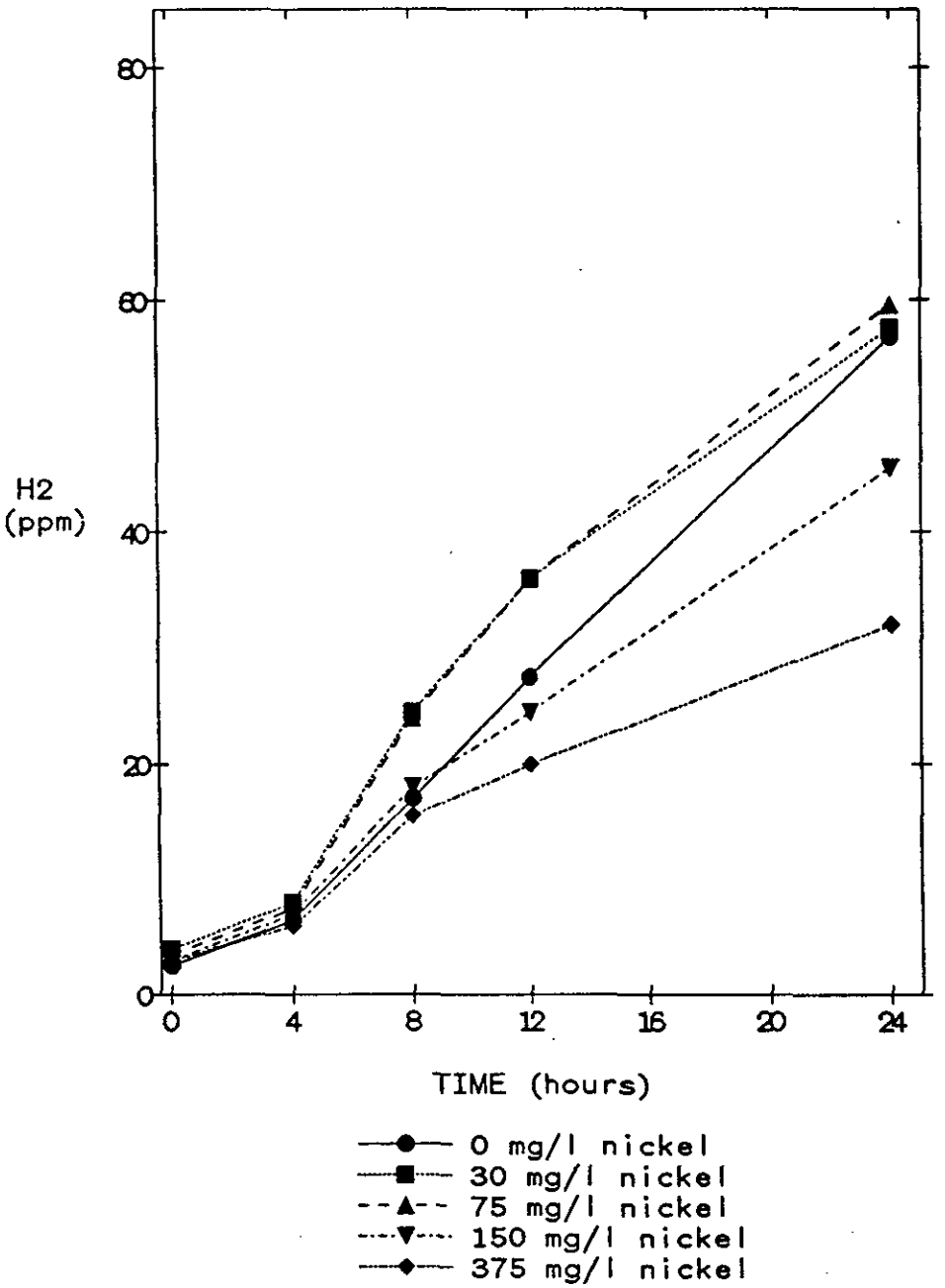

Figure 15: Hydrogen concentration as a function of time and applied nickel dose.

nickel dose is presented in Figure 16. The decreased rate of CO accumulation corresponded with the inhibition of methane production. Samples with up to 150 mg/l nickel added showed CO levels similar to those of the controls after 24 hours. In the sample containing 375 mg/l nickel, which showed no recovery in methane production, the rate of CO accumulation remained low throughout the assay.

4.1.4 Zinc

The effect of zinc was studied using $ZnCl_2$ added in concentrations ranging from 40 to 350 mg/l as Zn. The effect of zinc on the methane production rate as a function of time is shown in Figure 17. A severe decrease in methane production rate was observed in all samples during the first 4 to 8 hours of the assay. A recovery in the samples containing up to 90 mg/l of zinc occurred after 24 hours, while the more toxified samples showed inhibition of $\geq$ 50%. However, at the 48 hour mark, all samples showed a moderate to severe inhibition in the methane production rate.

Figure 18 shows the cumulative 48 hour methane production rate as a function of applied zinc dose. Using this plot, the dose of zinc required to produce a reduction in methane production rate of 50% was estimated to be 175 mg/l. None of the zinc concentrations applied caused inhibition of $\geq$ 50% after 24 hours.

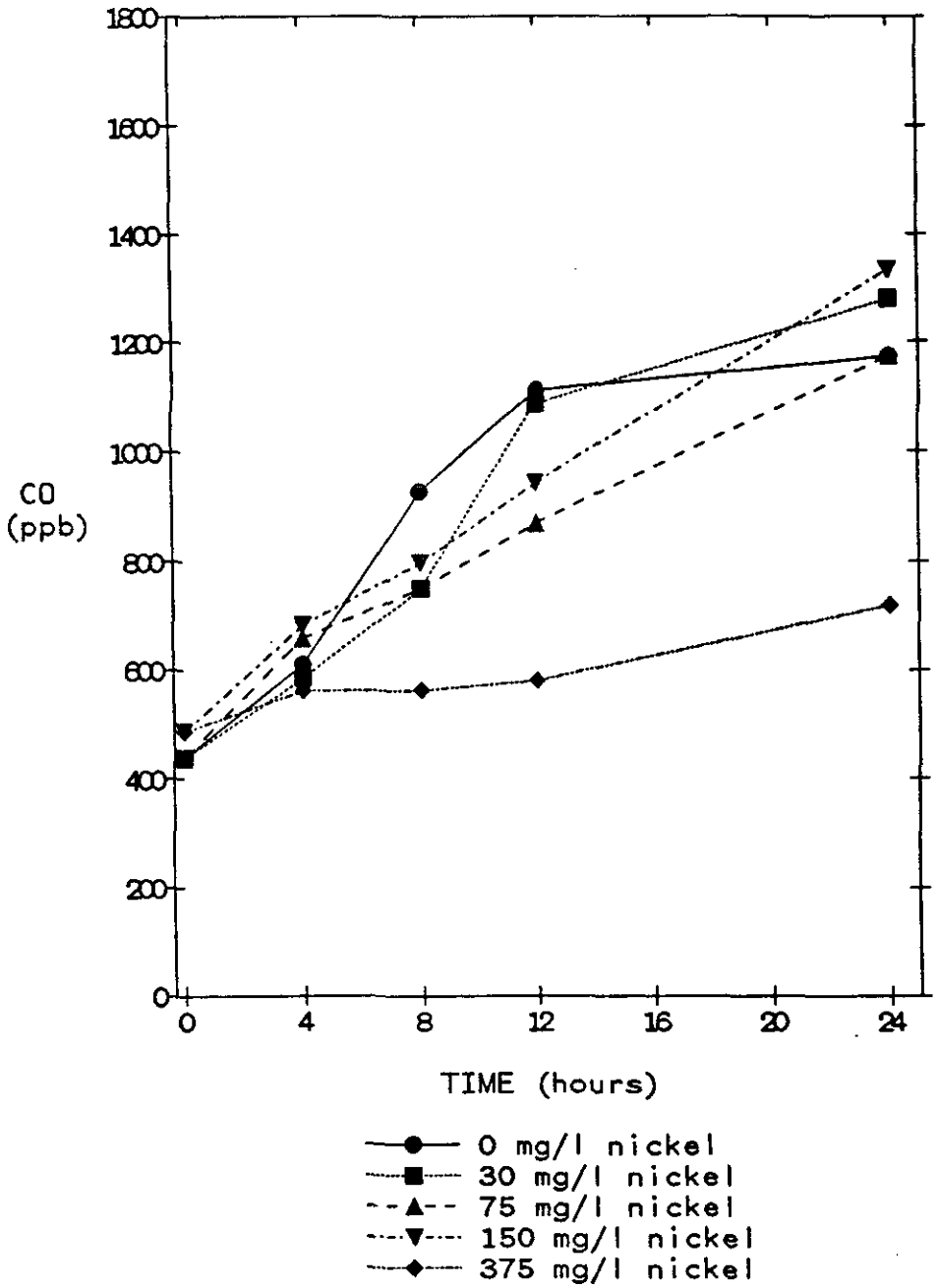

Figure 16: Carbon monoxide concentration as a function of time and applied nickel dose.

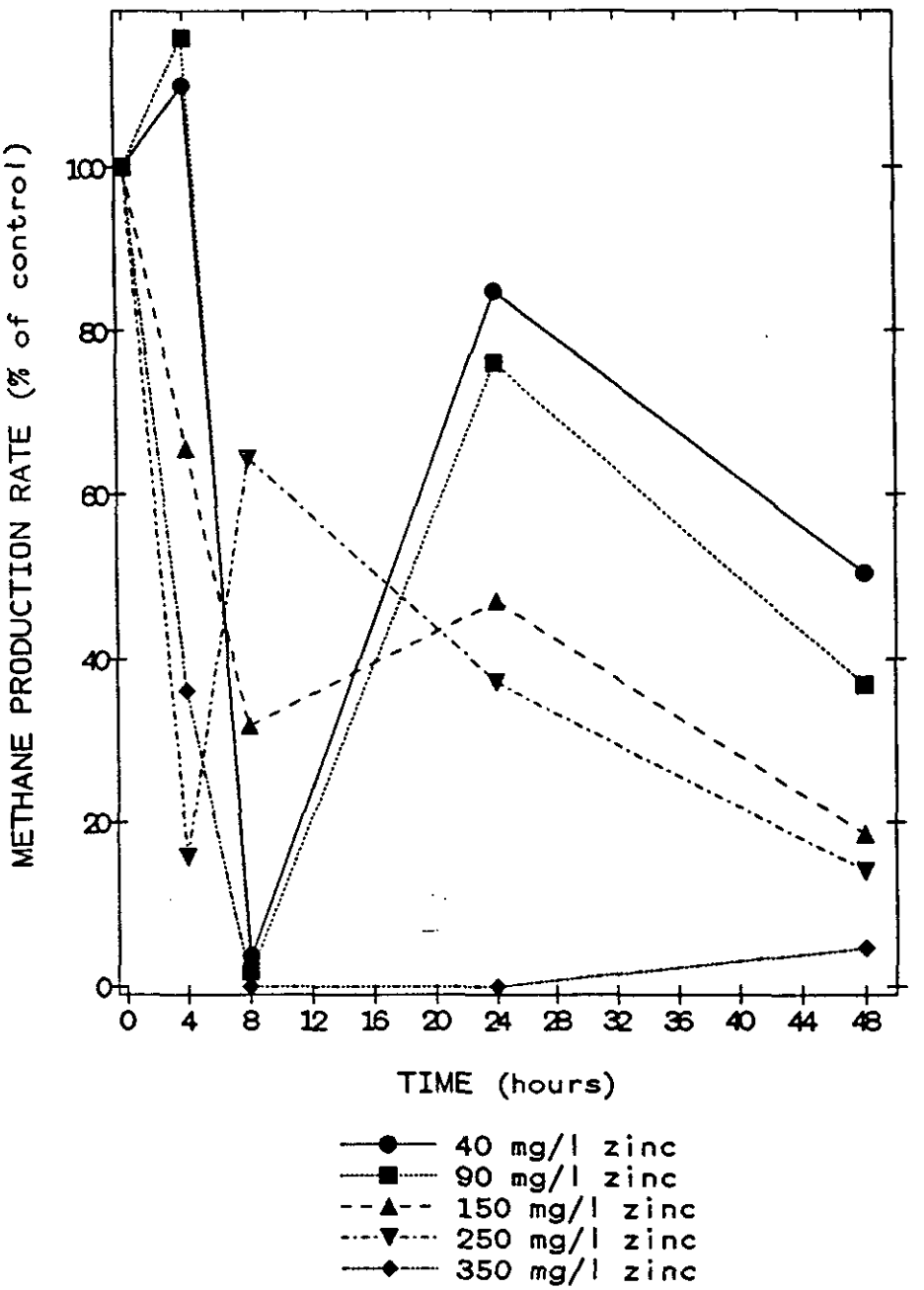

Figure 17: Effect of zinc on methane production rate.

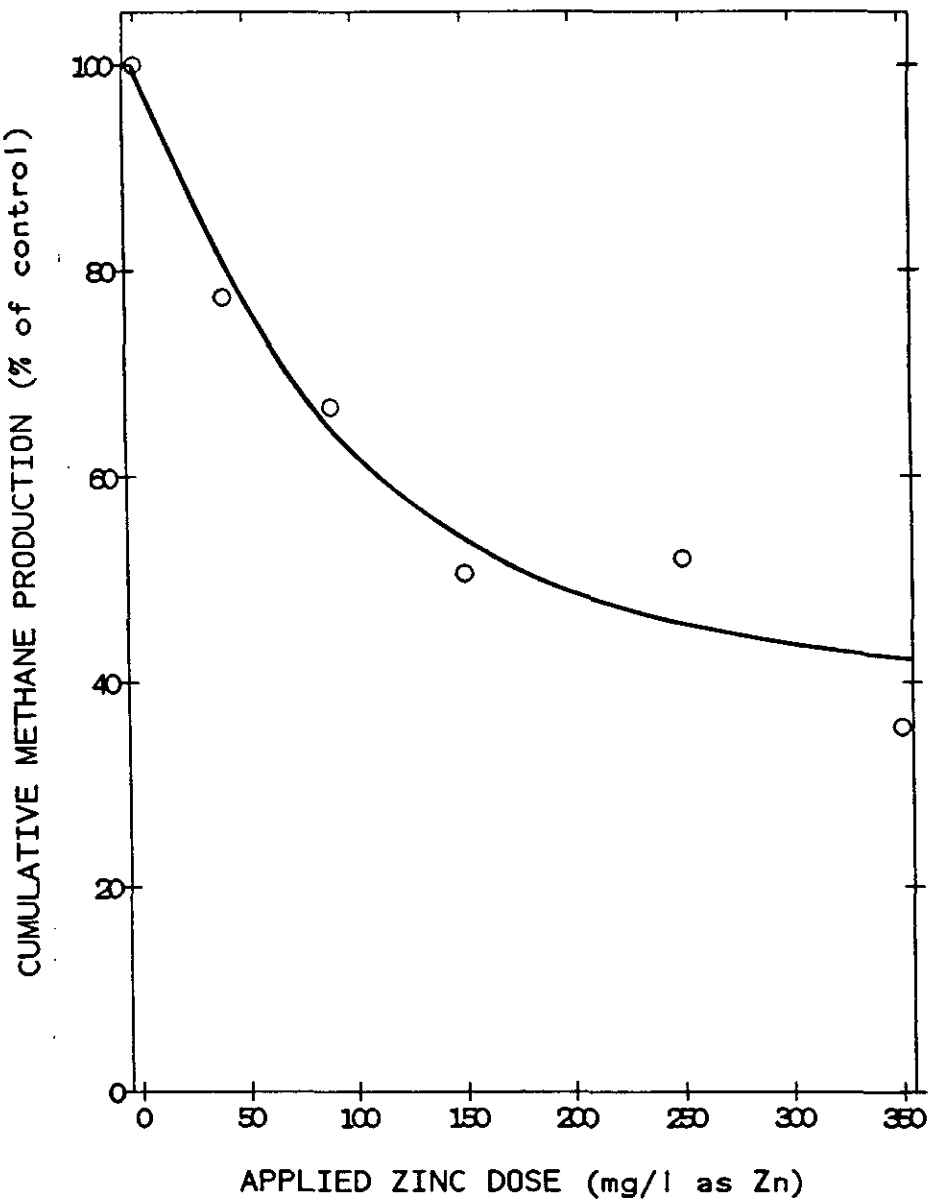

Figure 18: Cumulative 48 hour methane production rate as a function of zinc dose.

The effect of zinc on hydrogen accumulation as a function of time is shown in Figure 19. Samples containing between 40 and 250 mg/l of zinc showed patterns of hydrogen accumulation similar to the controls, at only slightly more elevated levels. Hydrogen levels of the most toxified sample (350 mg/l Zn), which was also the only sample exhibiting complete inhibition of methane production rate, decreased slowly during the entire assay.

The response of the CO concentration to the addition of zinc is shown as a function of time in Figure 20. The degree of CO accumulation seems related to the amount of recovery in methane production rate. At 24 hours, the least toxified samples (40 to 90 mg/l) had reached CO levels similar to the control, whereas more toxified samples showed lower concentrations directly related to the amount of zinc added. The levels of CO in the sample containing 350 mg/l Zn decreased steadily throughout the assay.

4.2 SUMMARY OF RESULTS FROM INORGANIC TOXICANT ASSAYS

All metals examined (Cu, Cd, Ni and Zn) caused inhibition of methane production to some extent. Samples to which the lowest doses of metals had been added, usually showed recovery within the first hours of the assays. Severe inhibition was observed for the highest dosages of all metals added, and no recovery was evident during the

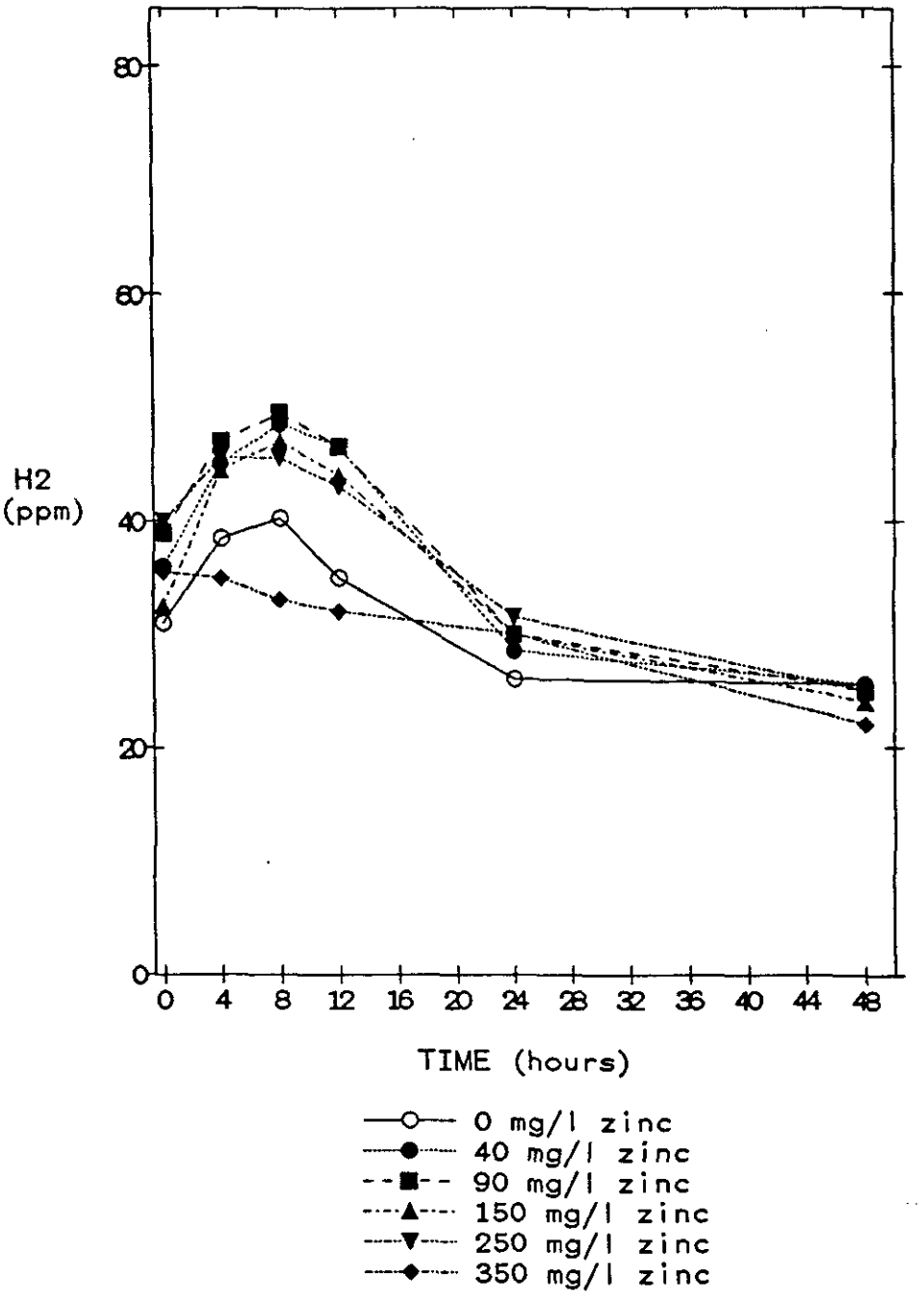

Figure 19: Hydrogen concentration as a function of time and applied zinc dose.

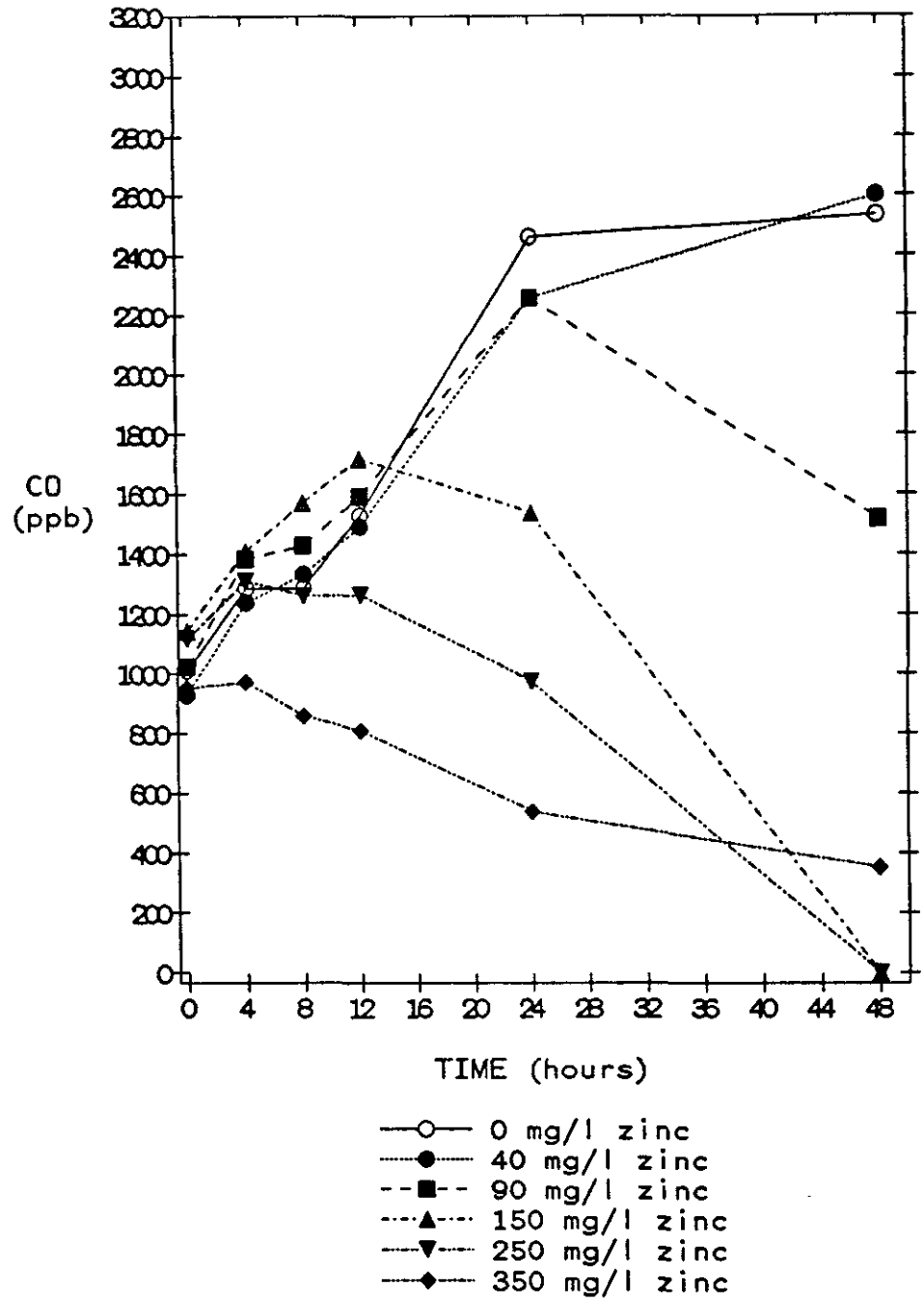

Figure 20: Carbon monoxide concentration as a function of time and applied zinc dose.

duration of the experiments.

The response of hydrogen concentration was similar for the addition of Cu, Ni and Zn. The level of hydrogen in the controls generally increased throughout the assay. The toxified samples showed lower levels of hydrogen accumulation, which seemed dependent on the level of inhibition of methane production in that sample. As toxicity increased, the rate of hydrogen accumulation decreased, and in the most toxified samples hydrogen concentrations never built up. The hydrogen response for the cadmium inhibited samples was completely different. Again, the level of response depended on the amount of toxicant added. However, in this case the rate of hydrogen accumulation increased as samples were more inhibited.

The CO accumulation patterns observed during the assays with Cd, Ni and Zn were similar. A decrease in the rate of accumulation of CO was observed which coincided with the increased inhibition of methane production rate. This was most evident in the assay using zinc. The response pattern for CO was slightly different when copper was tested. Moderately toxified samples accumulated CO at higher levels than the controls. The most inhibited sample exhibited the same pattern of CO accumulation as the controls.

4.3 ORGANIC TOXICANTS

4.3.1 Formaldehyde

Formaldehyde was added in concentrations ranging from 8 to 56 mg/l using a 37% w/w solution. The effect of formaldehyde on methane production rate as a function of time and applied formaldehyde dose is shown in Figure 21. After an initial depression of the methane producton rate, all samples showed some recovery after 12 hours. The amount of recovery seemed related to the dose of formaldehyde added. Using Figure 22, the amount of formaldehyde needed to produce a 50% reduction in the 24 hour methane production rate was determined to be approximately 35 mg/l.

Hydrogen levels in the headspace gas are shown as a function of time and applied formaldehyde dose in Figure 23. The hydrogen response was dose dependent. Hydrogen accumulated rapidly in the sample with the highest dose applied (56 mg/l). Samples containing up to 24 mg/l formaldehyde showed hydrogen levels rising slightly, then dropping to their initial levels after 24 hours. This drop coincided with the recovery in methane production.

The response pattern of the carbon monoxide gas is shown as a function of time and applied formaldehyde dose in Figure 24. All toxified samples show an increased rate of CO accumulation compared to the controls. At 12 hours, there seems to be a change in the rate of CO accumulation which is

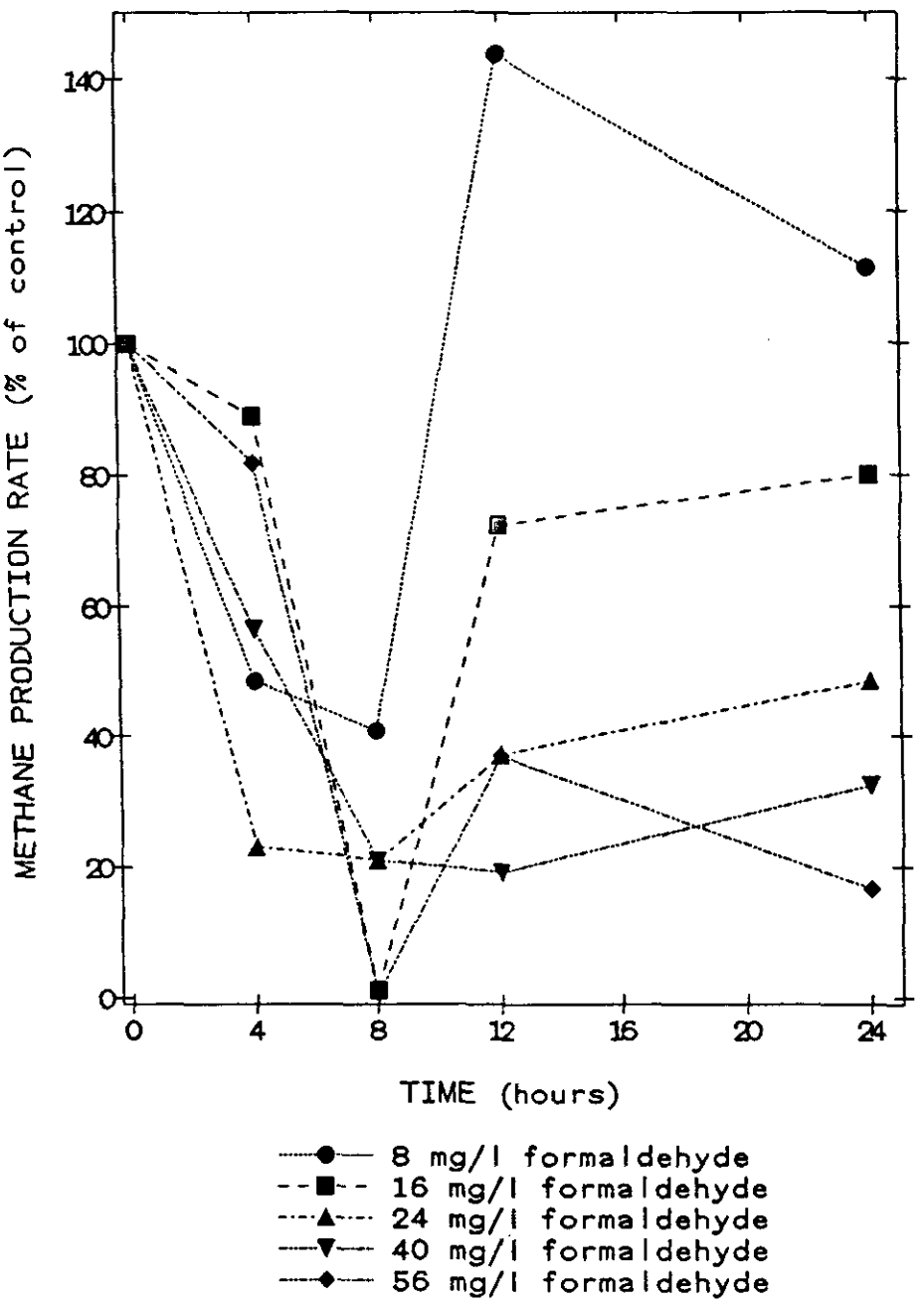

Figure 21: Effect of formaldehyde on methane production rate.

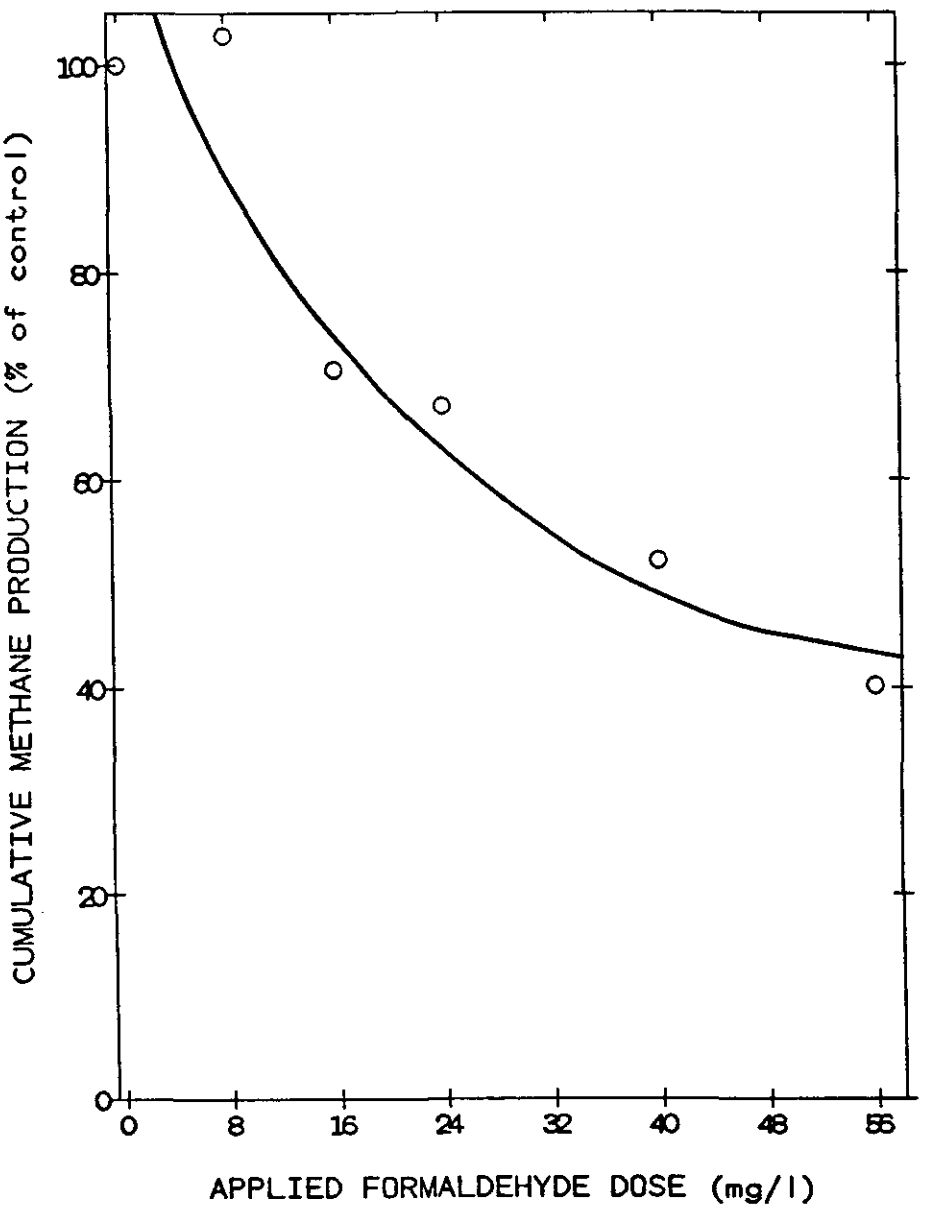

Figure 22: Cumulative 24 hour methane production as a function of formaldehyde dose.

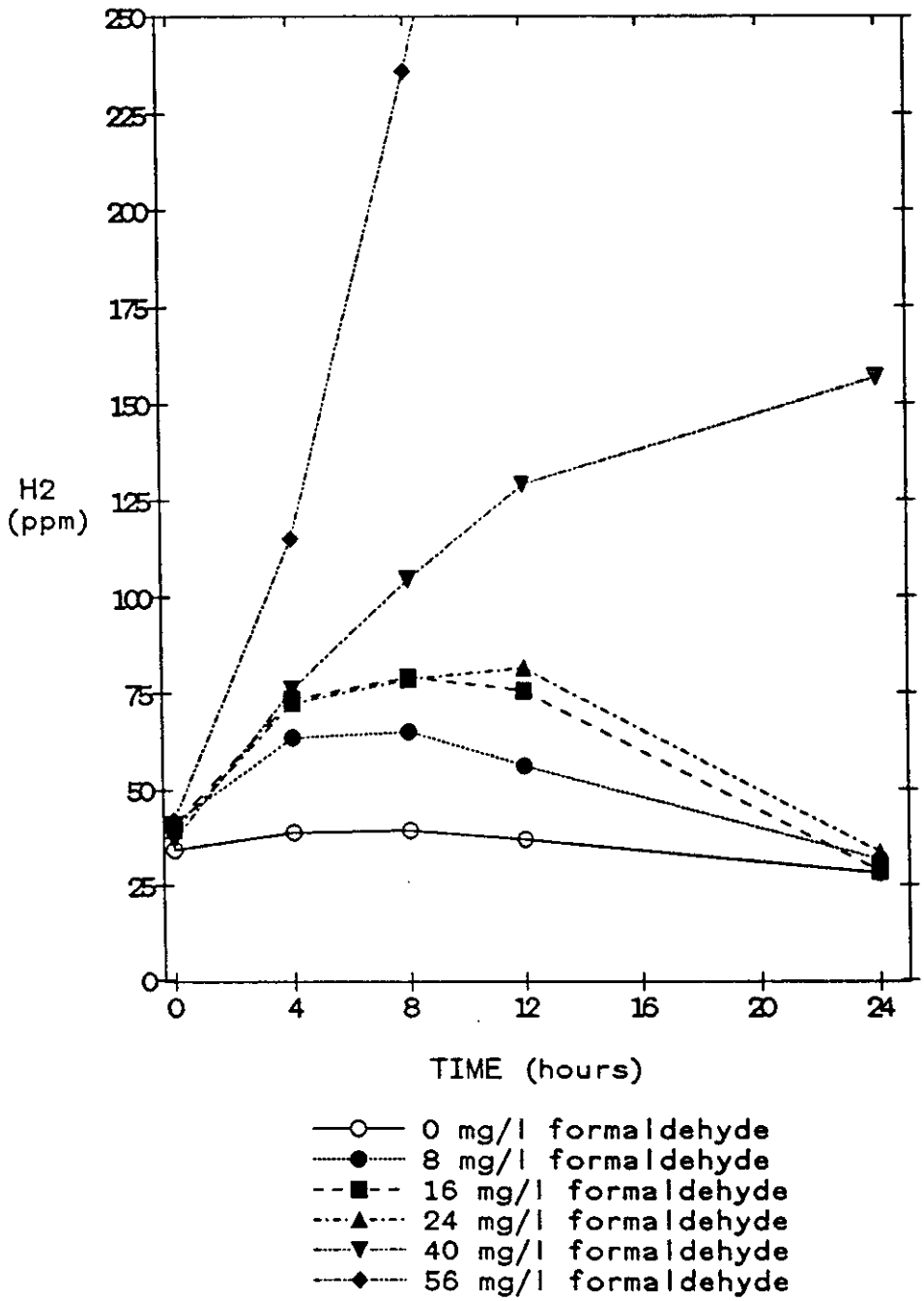

Figure 23: Hydrogen concentration as a function of time and applied formaldehyde dose.

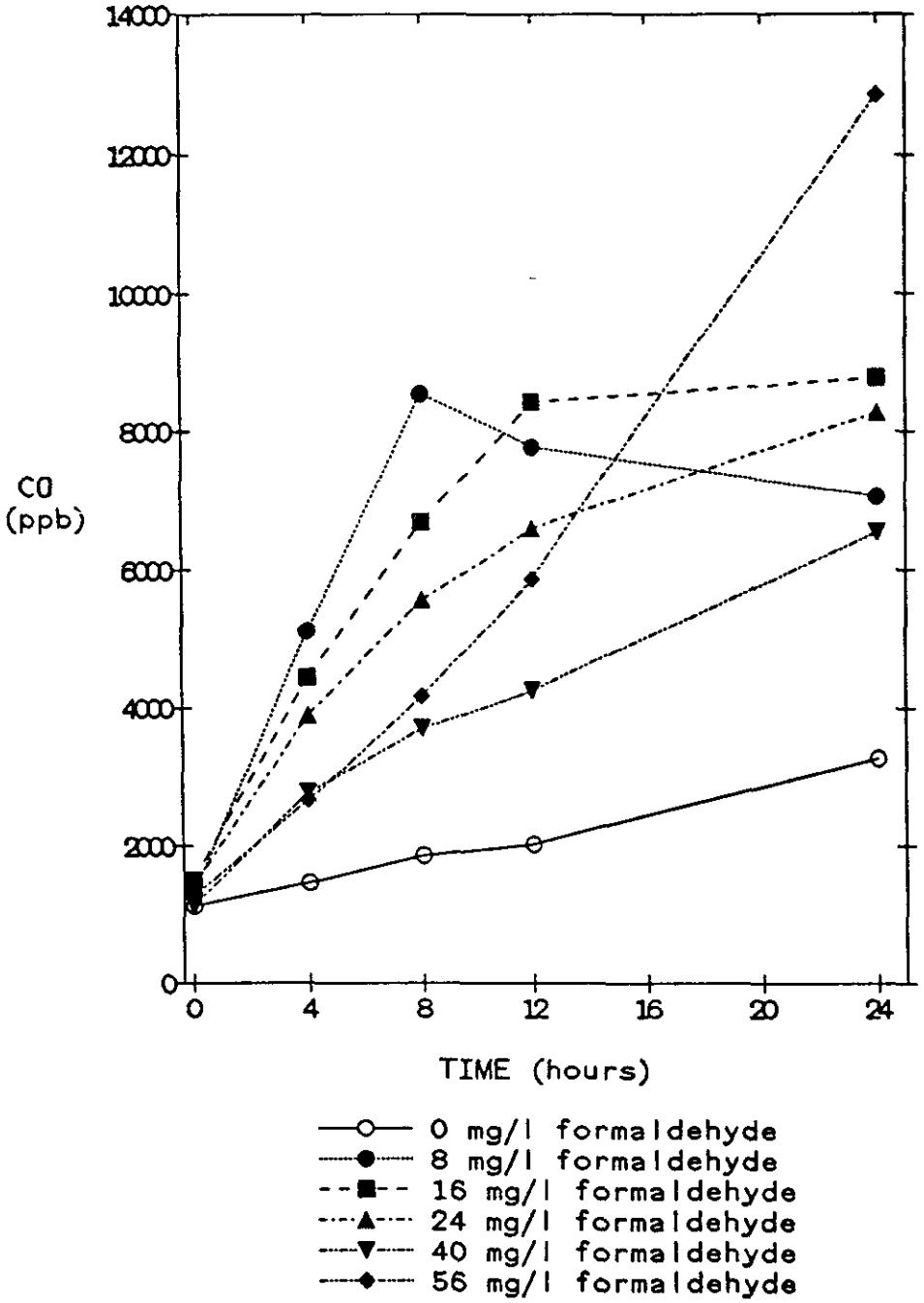

Figure 24: Carbon monoxide concentration as a function of time and applied formaldehyde dose.

related to applied formaldehyde dose. Samples containing between 8 and 24 mg/l formaldehyde show a decrease in their CO accumulation rate, while the more toxified samples continue to show a steady increase.

4.3.2 BES

2-Bromoethanesulfonic acid (BES) toxicity was tested by applying dosages ranging from 211 to 3376 mg/l BES (1 to 16 mM). The effect of this compound on methane production rate is shown as a function of time and applied dose in Figure 25. After an initial increase, the rate dropped dramatically and after 24 hours all samples showed a decrease in methane production rate of more than 65% compared to the controls. The amount of inhibition was directly related to the dose applied. At the conclusion of the assay, all samples showed severe inhibition ($\geq$ 75% compared to the controls). Figure 26 shows cumulative 53 hour methane production as a function of BES dose. The dose required to cause 50% inhibition of the methane production rate was determined to be approximately 400 mg/l. None of the BES doses applied caused $\geq$ 50% inhibition after 24 hours.

The response of hydrogen gas as a function of time and applied BES dose is shown in Figure 27. Here, no accumulation of hydrogen was observed. The response of all toxified samples was similar to that of the controls.

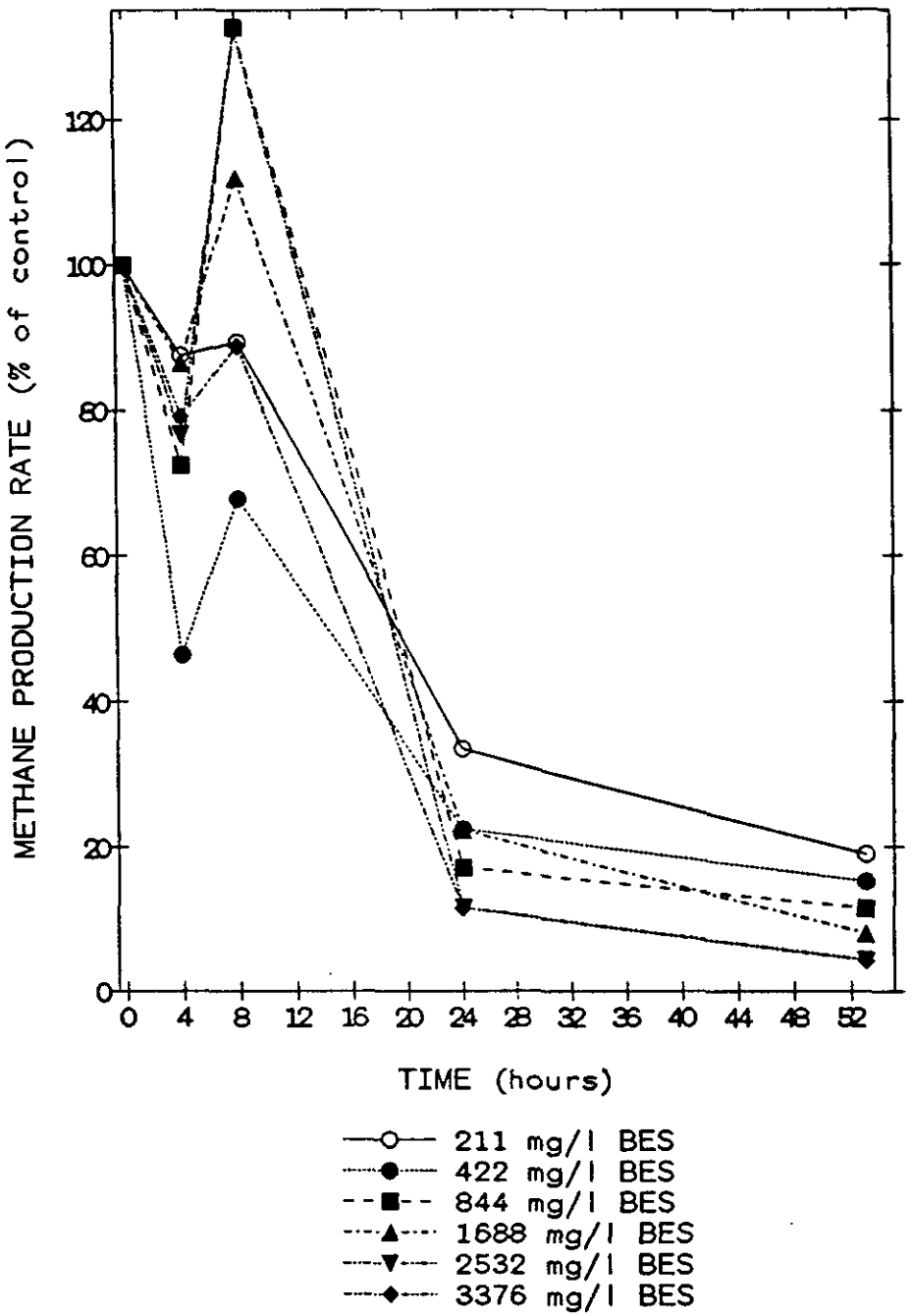

Figure 25: Effect of BES on methane production rate.

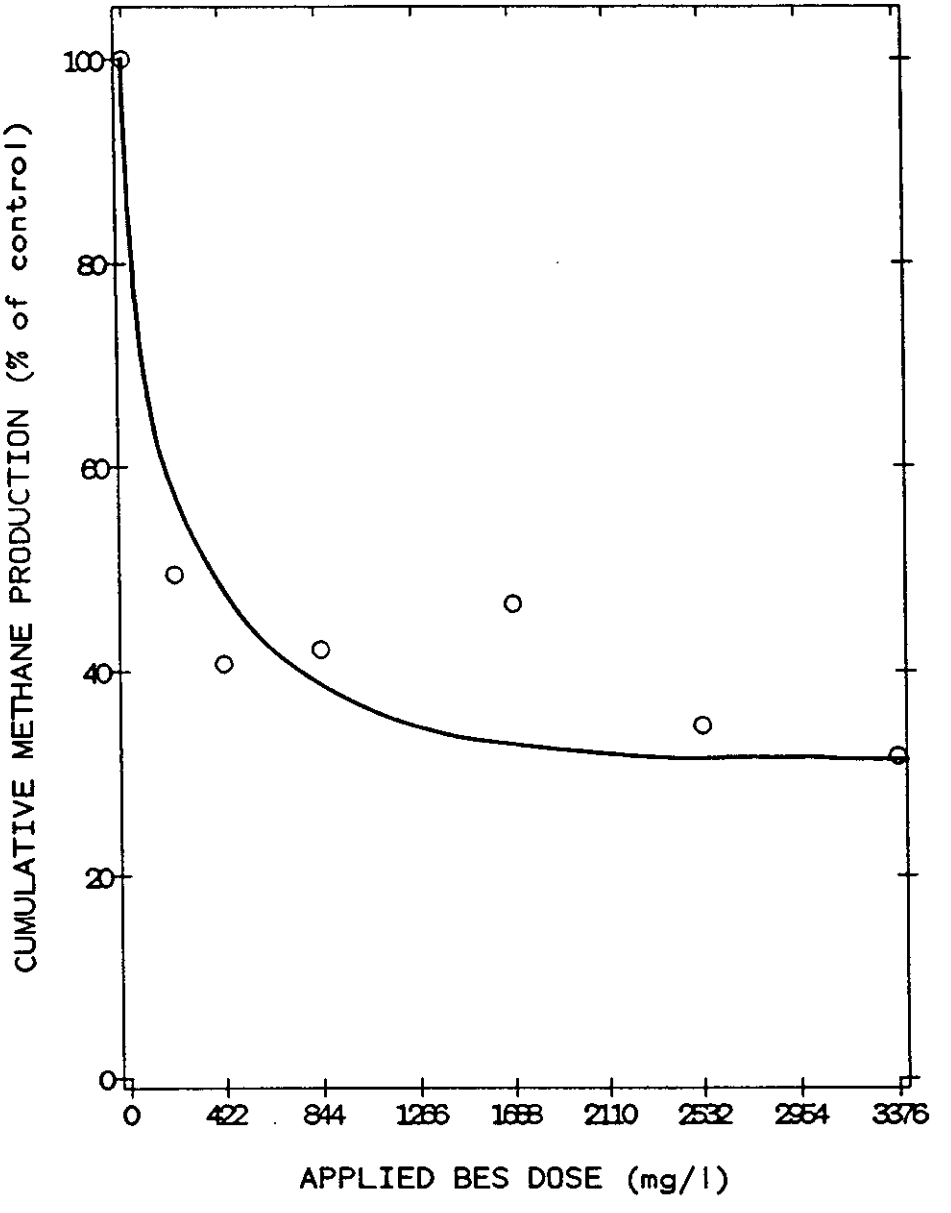

Figure 26: Cumulative 53 hour methane production as a function of BES dose.

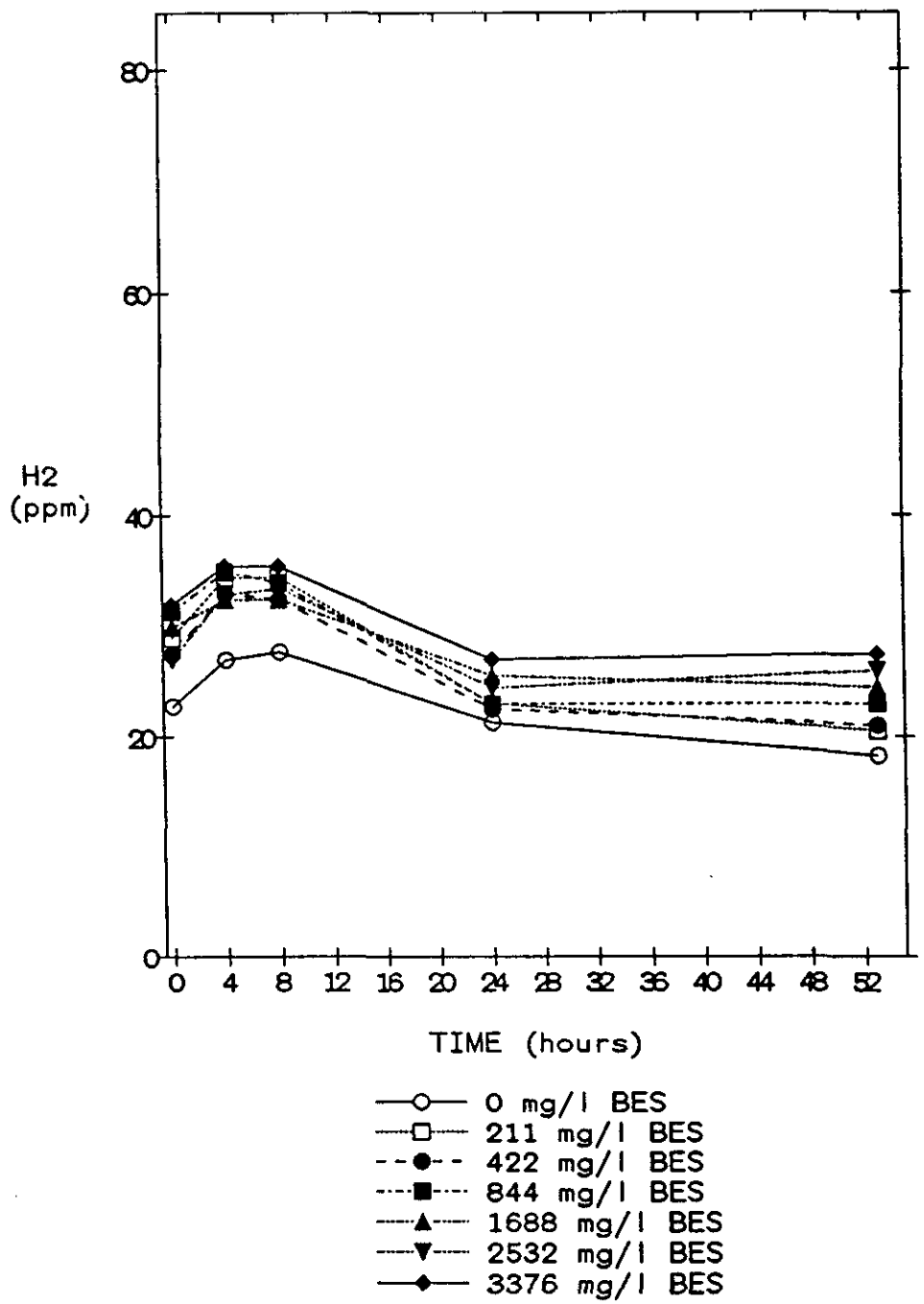

Figure 27: Hydrogen concentration as a function of time and applied BES dose.

Initially, hydrogen levels rose slightly during the first hours of the assay, while decreasing to slightly below their original value by the 24 hour mark.

The CO response pattern is shown as a function of time and applied BES concentration in Figure 28. The controls exhibit a pattern similar to that for formaldehyde. CO is accumulated during the first 12 hours, whereafter a decrease is observed. The toxified samples, on the other hand, do not show a significant accumulation of CO. At 24 hours, their CO levels are at or below their initial concentrations. This decrease in concentration continues for the remainder of the assay. This response is observed for all concentrations of BES tested (211 to 3376 mg/l).

4.4 SUMMARY OF RESULTS FROM ORGANIC TOXICANT ASSAYS

Both organic toxicants tested (formaldehyde and BES) caused a reduction in the methane production rate. All samples tested were severely inhibited by the BES doses used, while the range of concentrations of formaldehyde selected only caused moderate inhibition.

The response of the hydrogen gas was different in both cases. When formaldehyde was used, more hydrogen accumulated as higher doses of the toxicant were added. This response is similar to the one observed with cadmium. When BES was added, all toxified samples showed a hydrogen accumulation

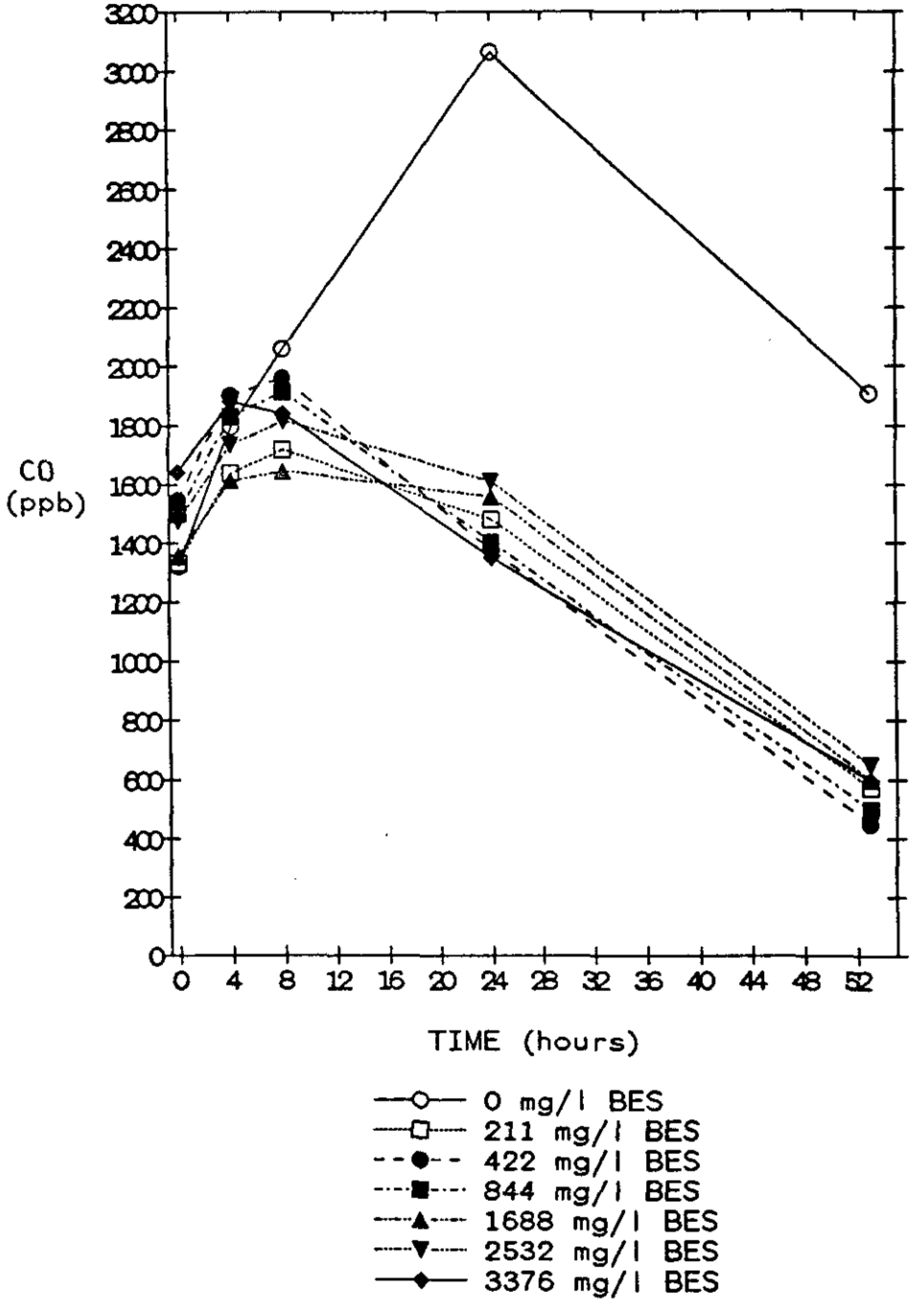

Figure 28: Carbon monoxide concentration as a function of time and applied BES dose.

pattern similar to that of the controls at only a slightly more elevated level.

The CO accumulation pattern was also different for both toxicants. In the samples to which BES was added, CO levels had dropped well below their initial concentrations by the end of the assay. When formaldehyde was used, levels of CO increased as methane production decreased in the samples.

CHAPTER V

DISCUSSION OF RESULTS

The objectives of this research were to study the response of acetate utilizing methanogens to the addition of selected toxicants in terms of hydrogen and carbon monoxide concentrations, and to compare these responses to results obtained in similar studies where waste activated sludge and sucrose, which support mixed bacterial populations, were used as the feed. Hydrogen and carbon monoxide could possibly be used as early warning indicators of impending reactor upsets. To be a good indicator, the response must be fast and distinct. A fast response allows more time for remedial actions to be taken to prevent complete digester failures from occurring. The indicator response must also be significant enough, so that it can be easily distinguished from normal daily fluctuations.

The four heavy metals tested (Cu, Cd, Ni and Zn), caused different responses in the rate of hydrogen accumulation, indicating different toxic actions by the metals.

Copper and nickel showed similar patterns of hydrogen accumulation (see Figures 7 and 15) during the serum bottle assays. Hydrogen levels increased with time in the samples

containing low concentrations of metals. For the highest concentrations applied, hydrogen did not accumulate or did so at a greatly reduced rate. Figure 29 was created to investigate whether the level of hydrogen accumulation could be correlated to methane production. Both hydrogen and methane production are expressed as a percentage of the controls. For both copper and nickel, hydrogen levels rose up to 50% above the controls when inhibition of methane production was moderate. This response was noted early during the assays, approximately 8 hours after the toxicant had been added. Severe inhibition of methane production coincided with a reduction in hydrogen concentration of 50% or more after 24 hours. This would indicate that at low copper and nickel dosages only the methane producing bacteria are moderately inhibited. However, at high toxicant doses, other groups of bacteria are at least as severely affected as the methanogens.

When cadmium was used as the toxicant, a different response was seen for hydrogen accumulation. All toxified samples accumulated hydrogen at a higher rate than the controls (see Figure 11). Figure 30 shows the normalized hydrogen concentrations as a function of methane production. Already after 8 hours, some samples produced 50% less methane than the controls. The corresponding hydrogen levels increased more than 50% over controls during that time

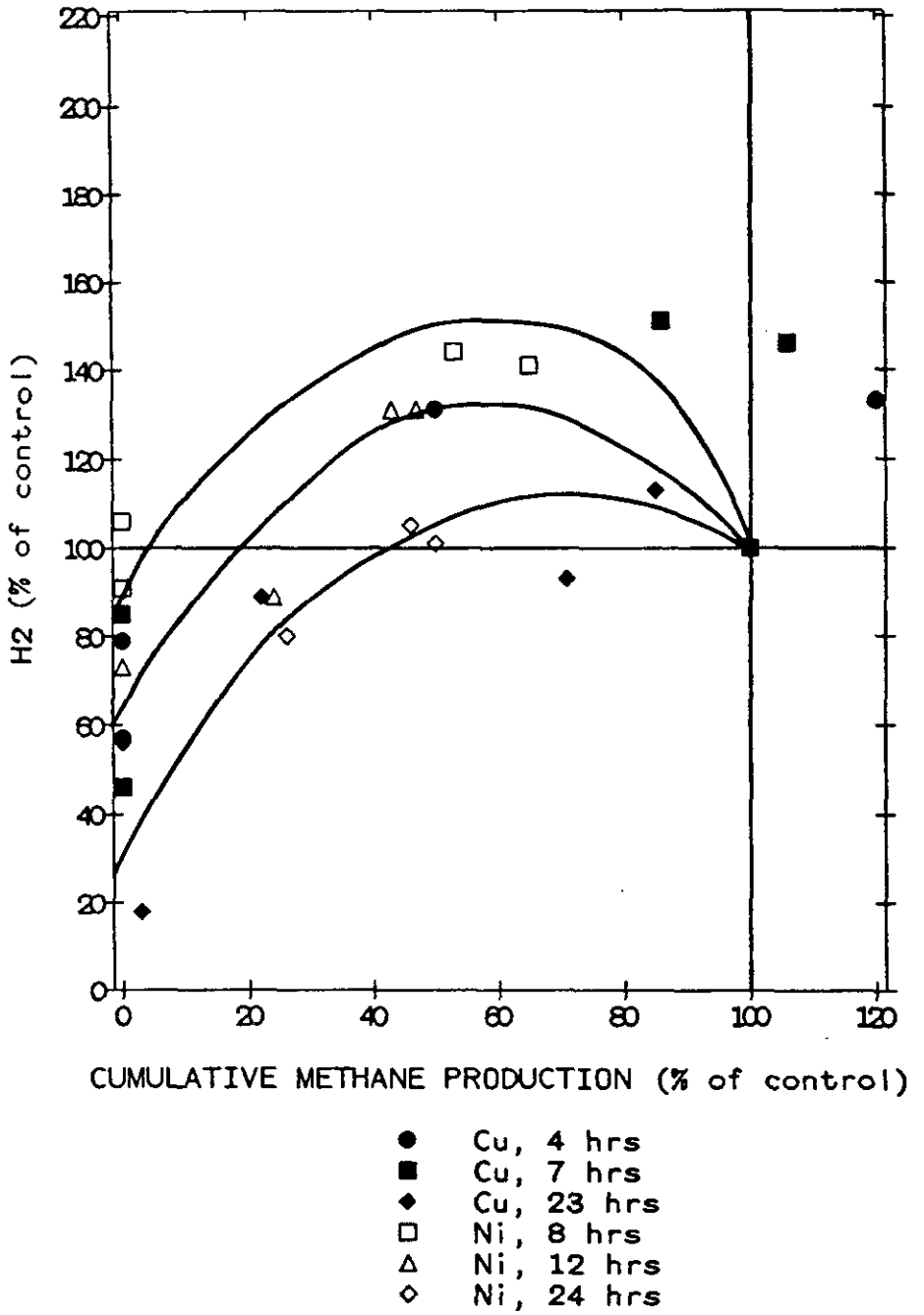

Figure 29: Correlation between hydrogen concentration and level of methane production in copper and nickel inhibited samples.

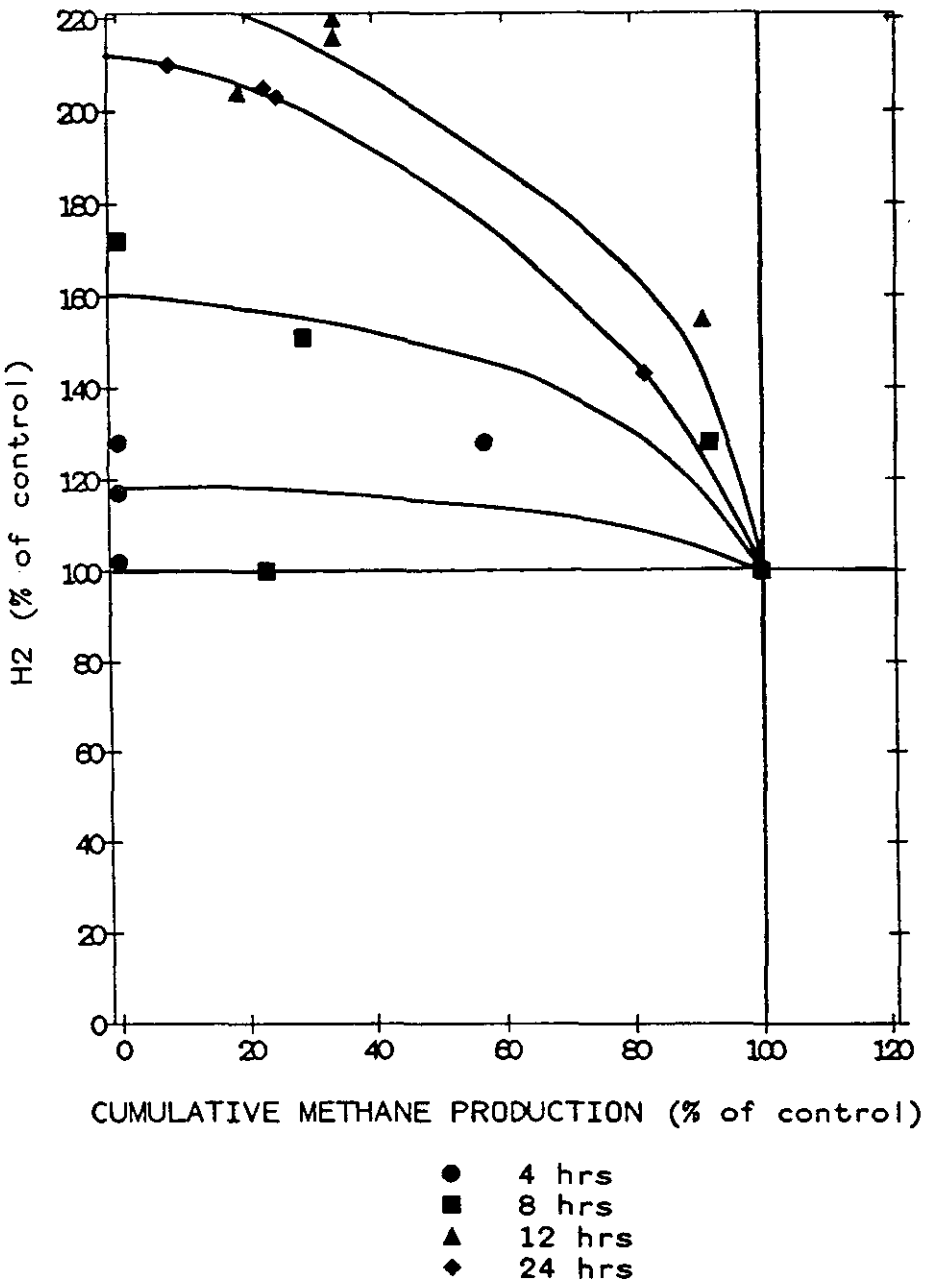

Figure 30: Correlation between hydrogen concentration and level of methane production in cadmium inhibited samples.

period. As the assay progressed, severely inhibited samples accumulated hydrogen up to 100% above levels found in the controls. This would indicate that the methanogens are the bacterial group which is most inhibited by the addition of cadmium.

Hydrogen concentrations remained close to those of the controls throughout the assay with zinc (see Figure 19). When the normalized hydrogen concentrations were plotted as a function of methane production, no distinctive trends were observed (Figure 31). Hydrogen fluctuated between 30% above and 20% below control levels for all samples. It should be noted that none of the zinc dosages applied caused complete inhibition of methane production.

During the heavy metal assays, there was no definite pattern observed in the rate of carbon monoxide accumulation except when zinc was added. Carbon monoxide levels remained close to control levels throughout the assays with copper, cadmium and nickel (see Figures 8, 12 and 16). There was no correlation found between normalized carbon monoxide concentrations and inhibition of methane production. Carbon monoxide levels fluctuated, but for most samples they remained within 20% of the concentration in the controls, even when methane production was severely inhibited (Figures 32, 33 and 34). Only a few exceptions were observed. Carbon monoxide levels increased more than 100% over control levels

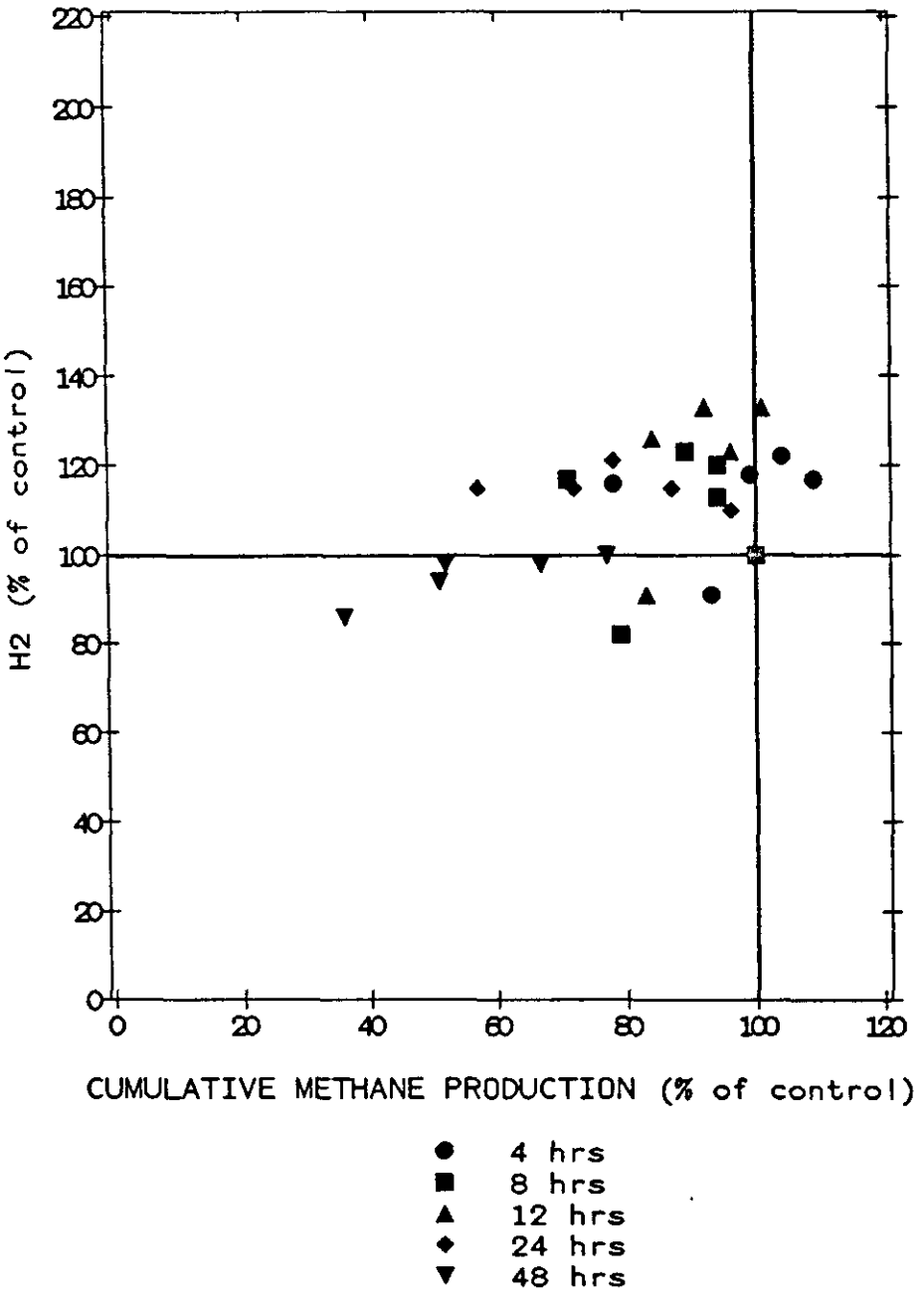

Figure 31: Correlation between hydrogen concentration and level of methane production in zinc inhibited samples.

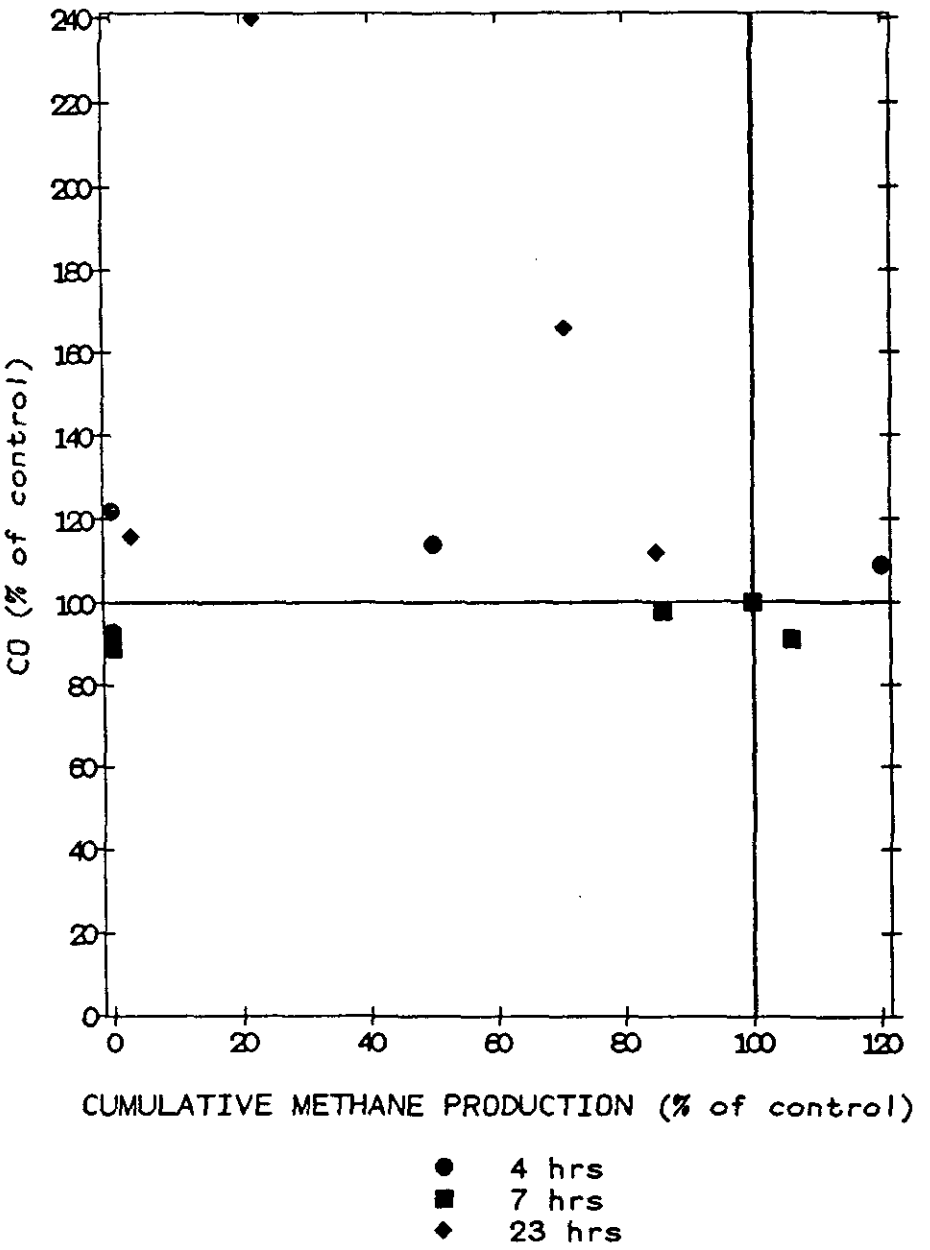

Figure 32: Correlation between carbon monoxide concentration and level of methane production in copper inhibited samples.

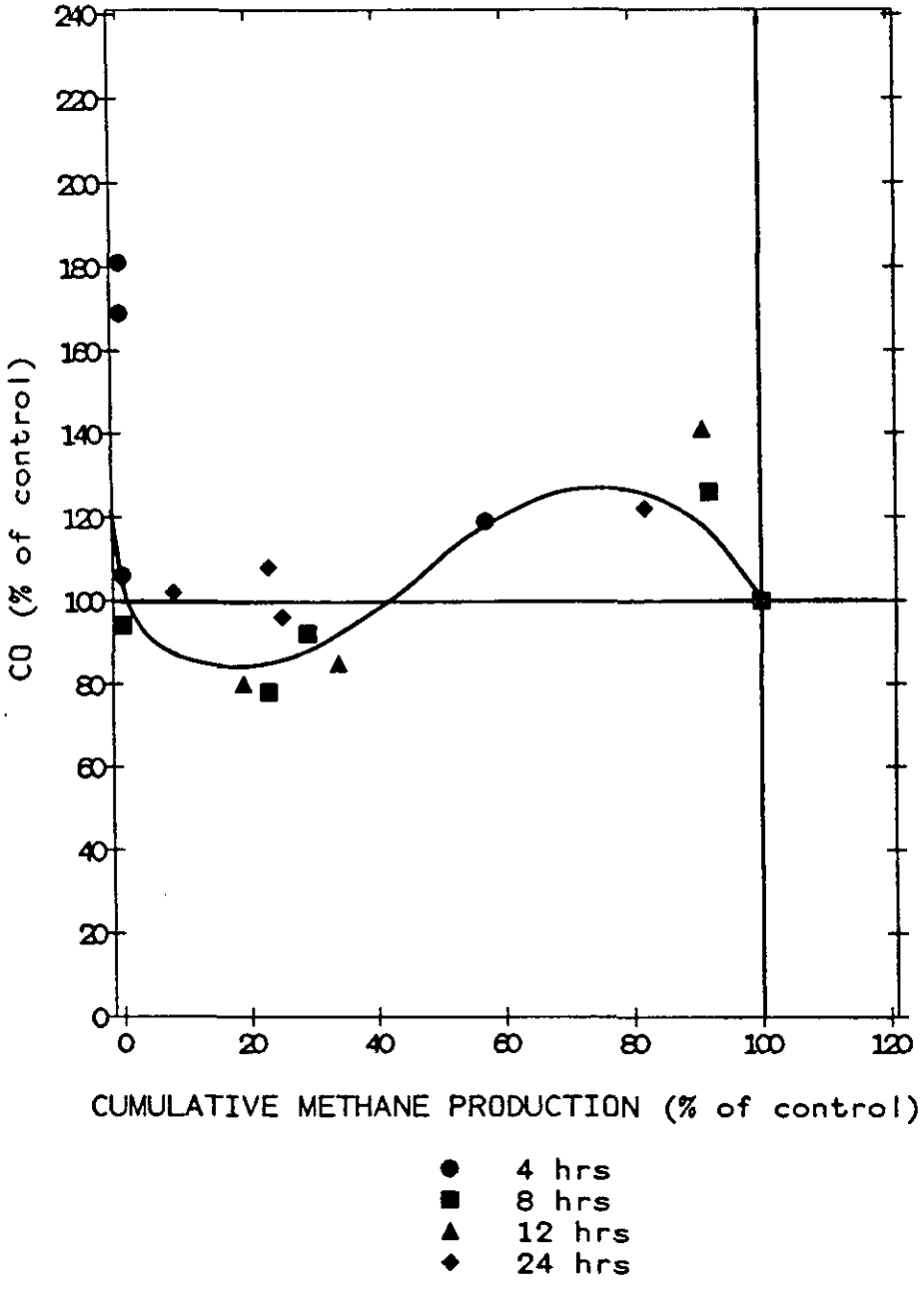

Figure 33: Correlation between carbon monoxide concentration and level of methane production in cadmium inhibited samples.

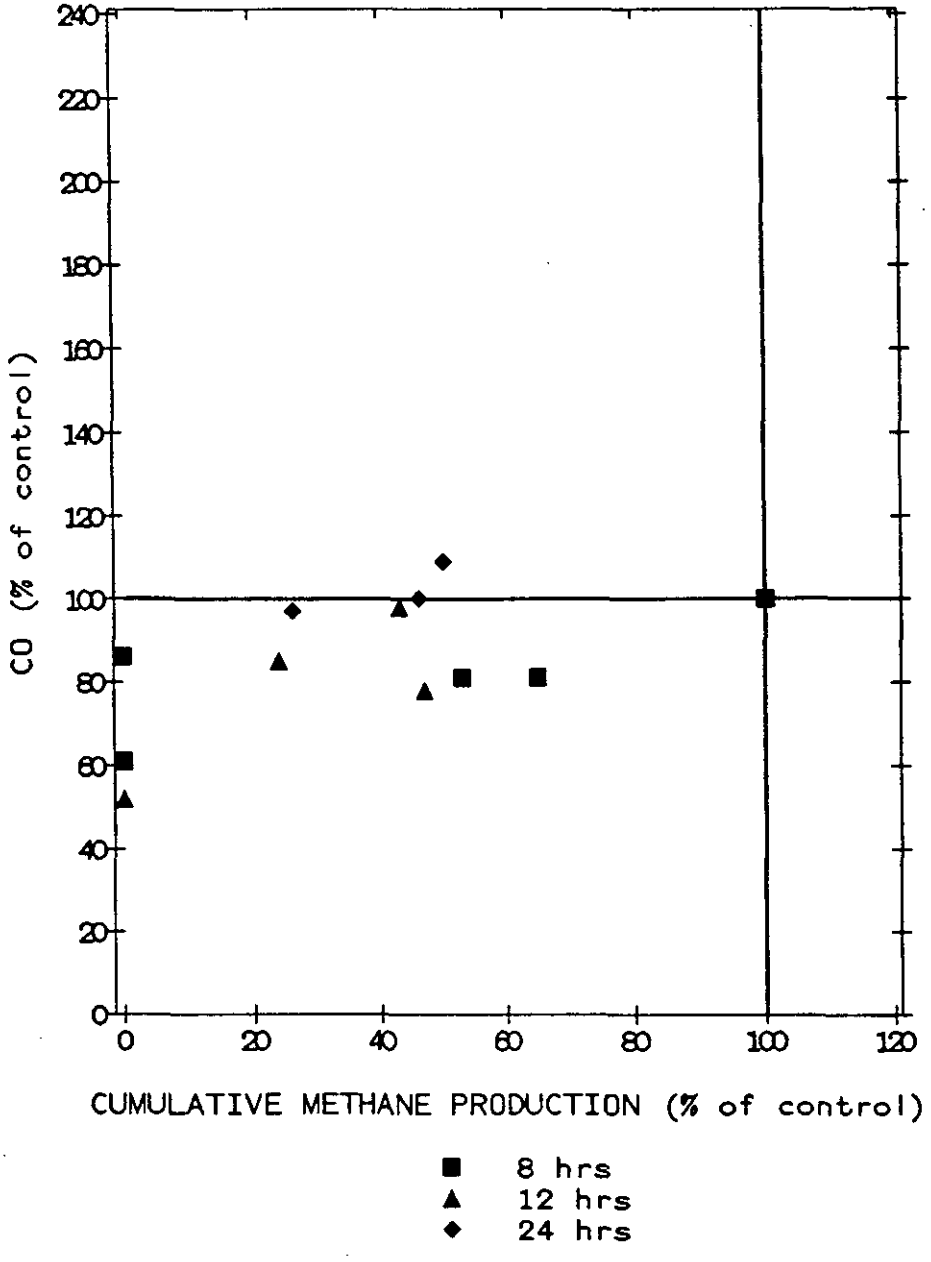

Figure 34: Correlation between carbon monoxide concentration and level of methane production in nickel inhibited samples.

after 24 hours, in the sample containing copper which was most inhibited in terms of methane production. In the sample most inhibited by nickel, the carbon monoxide concentration was reduced by 50% after 8 hours compared to the controls.

Zinc was the only heavy metal to cause a significant change in carbon monoxide concentrations in the toxified samples (see Figure 20). In Figure 35, the normalized carbon monoxide concentrations are shown as a function of methane production. Samples in which methane production was moderately inhibited, had carbon monoxide levels that were reduced by 50% compared to the controls after 12 hours exposure to the metal. Carbon monoxide levels were reduced to only 20% of the control values, when methane production was inhibited more than 50%.

Hydrogen accumulated at higher rates compared to the controls in all toxified samples when BES and formaldehyde were added (see Figures 23 and 27). Addition of BES caused an increase of 30% in hydrogen concentration after only 4 hours, while methane production was not affected (Figure 36). Hydrogen concentrations remained at this level until the 53 hour mark, when a 50% increase was observed, which coincided with a reduction in methane production of about 70%.

Hydrogen levels increased almost immediately after

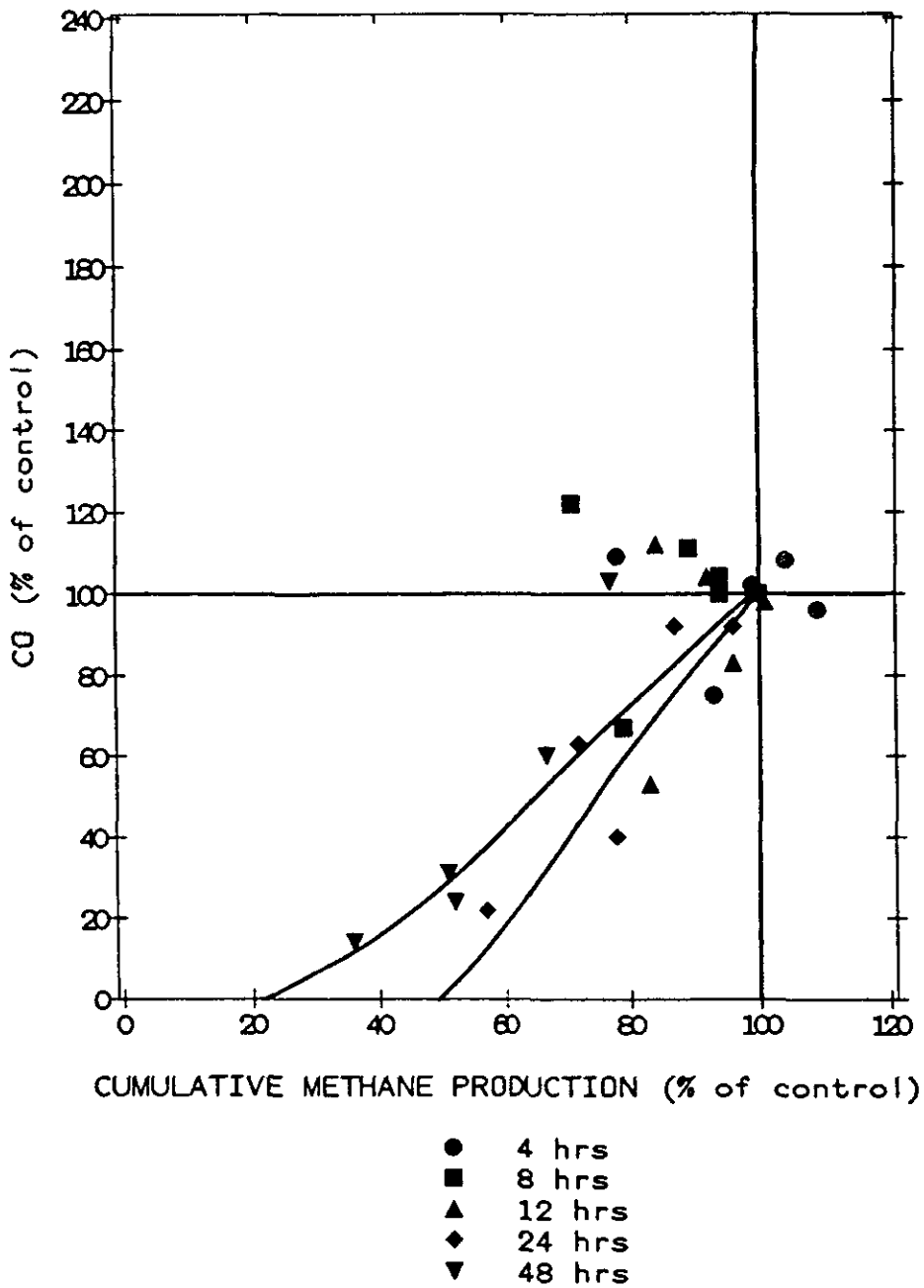

Figure 35: Correlation between carbon monoxide concentration and level of methane production in zinc inhibited samples.

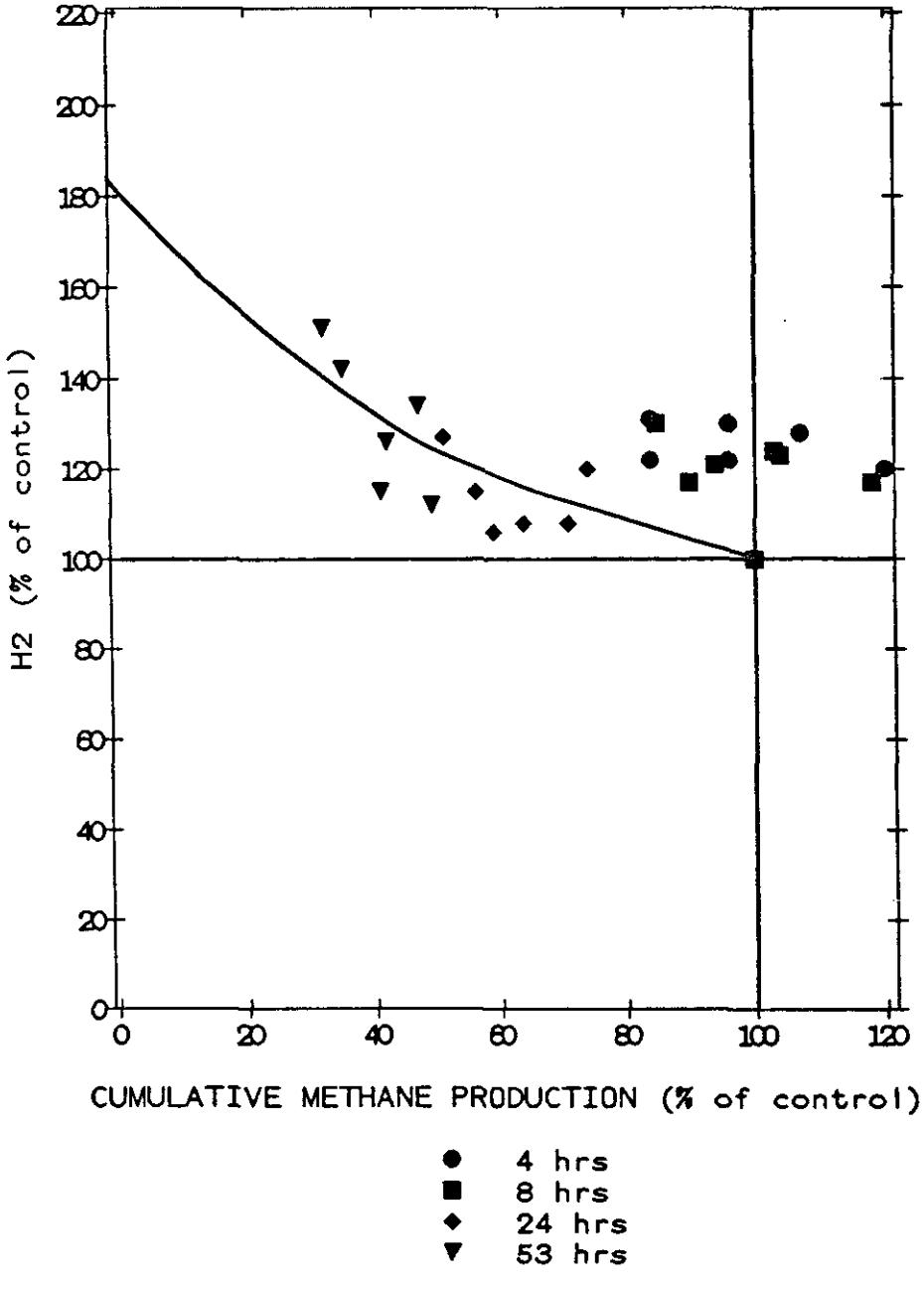

Figure 36: Correlation between hydrogen concentration and level of methane production in BES inhibited samples.

formaldehyde had been added, with the highest amounts of formaldehyde causing the greatest increases in hydrogen levels (see Figure 23). Even though hydrogen increased several hundred percent above control levels, methane production was only reduced by 40% after 24 hours (Figure 37). Escalante-Semerena et al. (1984) found that formaldehyde reacted with formaldehyde activating factor (FAF) in the hydrogen to methane cycle to form (CH_2)=FAF in Methanobacterium thermoautotrophicum. Hydrogen was liberated when (CH_2)=FAF was reduced to (CHO)-FAF. This could also account for the large increase in hydrogen observed in this study.

Different response patterns were observed when comparing the effect of adding BES and formaldehyde on carbon monoxide concentrations. CO levels decreased continuously as methane production was more inhibited by the addition of BES (Figure 38). After 53 hours, CO levels had dropped to 30% of the control values, while a 60% reduction of methane production occurred.

Formaldehyde addition, on the other hand, caused CO levels to rise rapidly after only 4 hours of contact time (see Figure 24). However no definite correlation was found between increase in CO concentrations and inhibition of methane production (Figure 39).

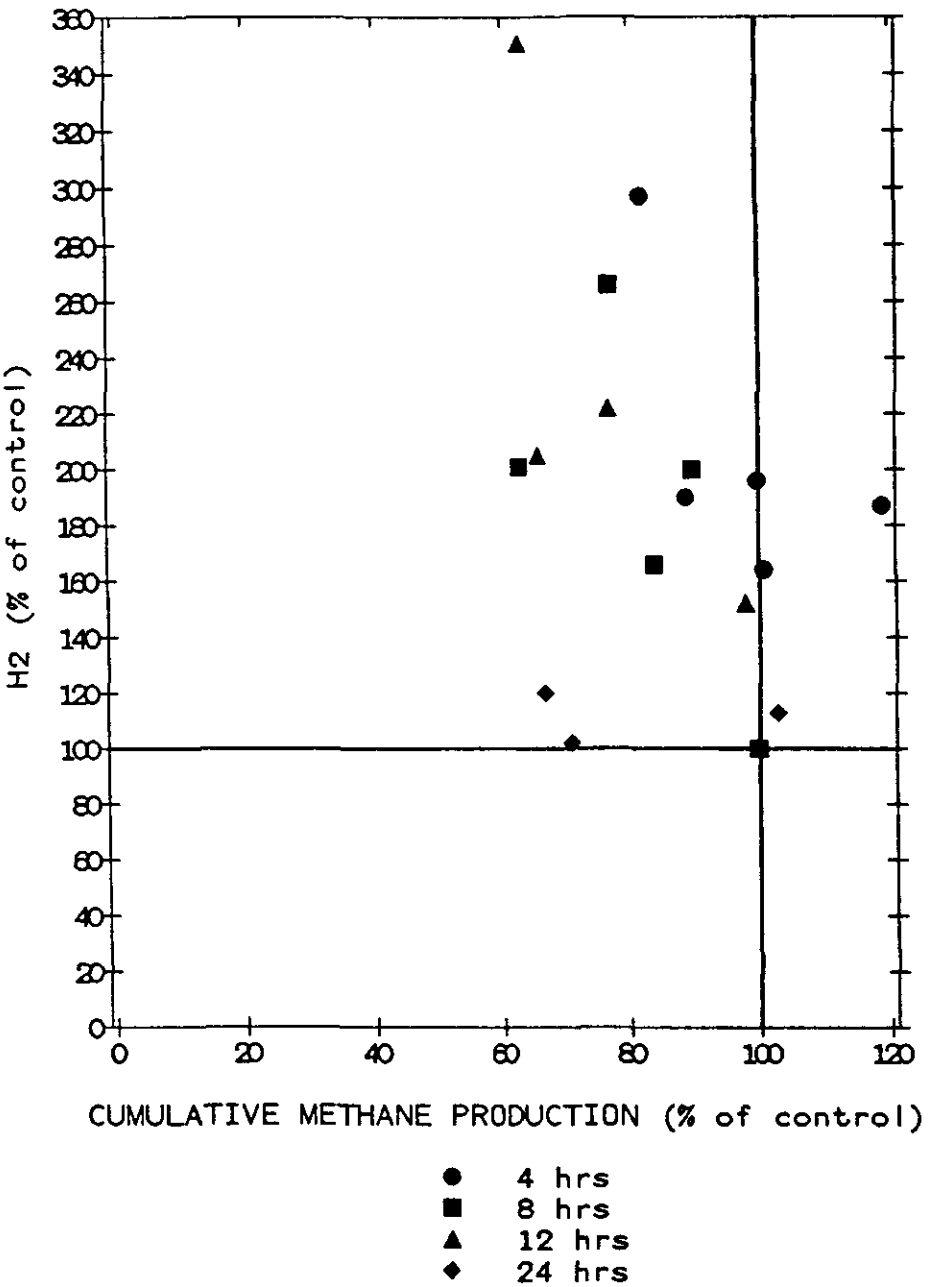

Figure 37: Correlation between hydrogen concentration and level of methane production in formaldehyde inhibited samples.

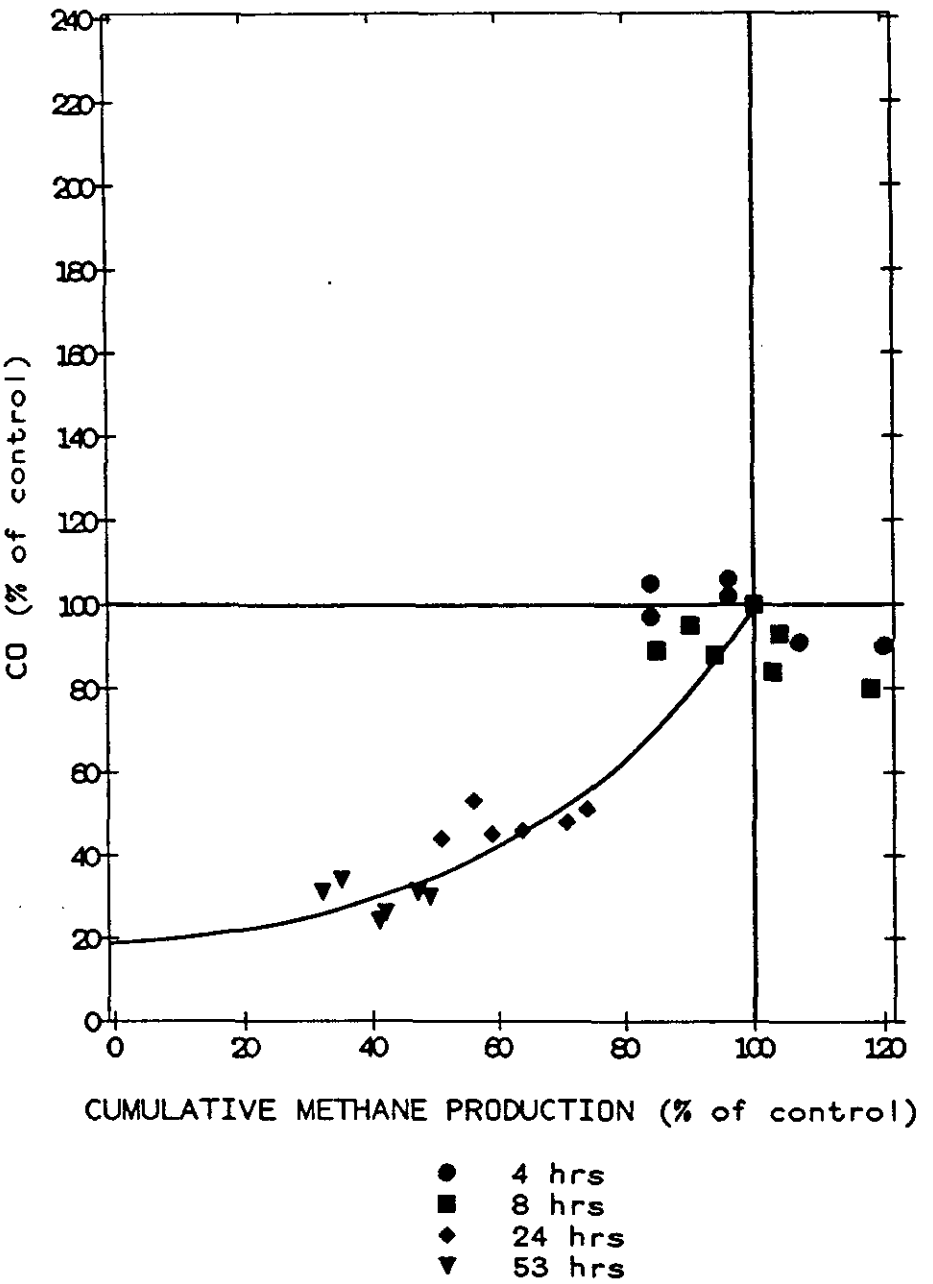

Figure 38: Correlation between carbon monoxide concentration and level of methane production in BES inhibited samples.

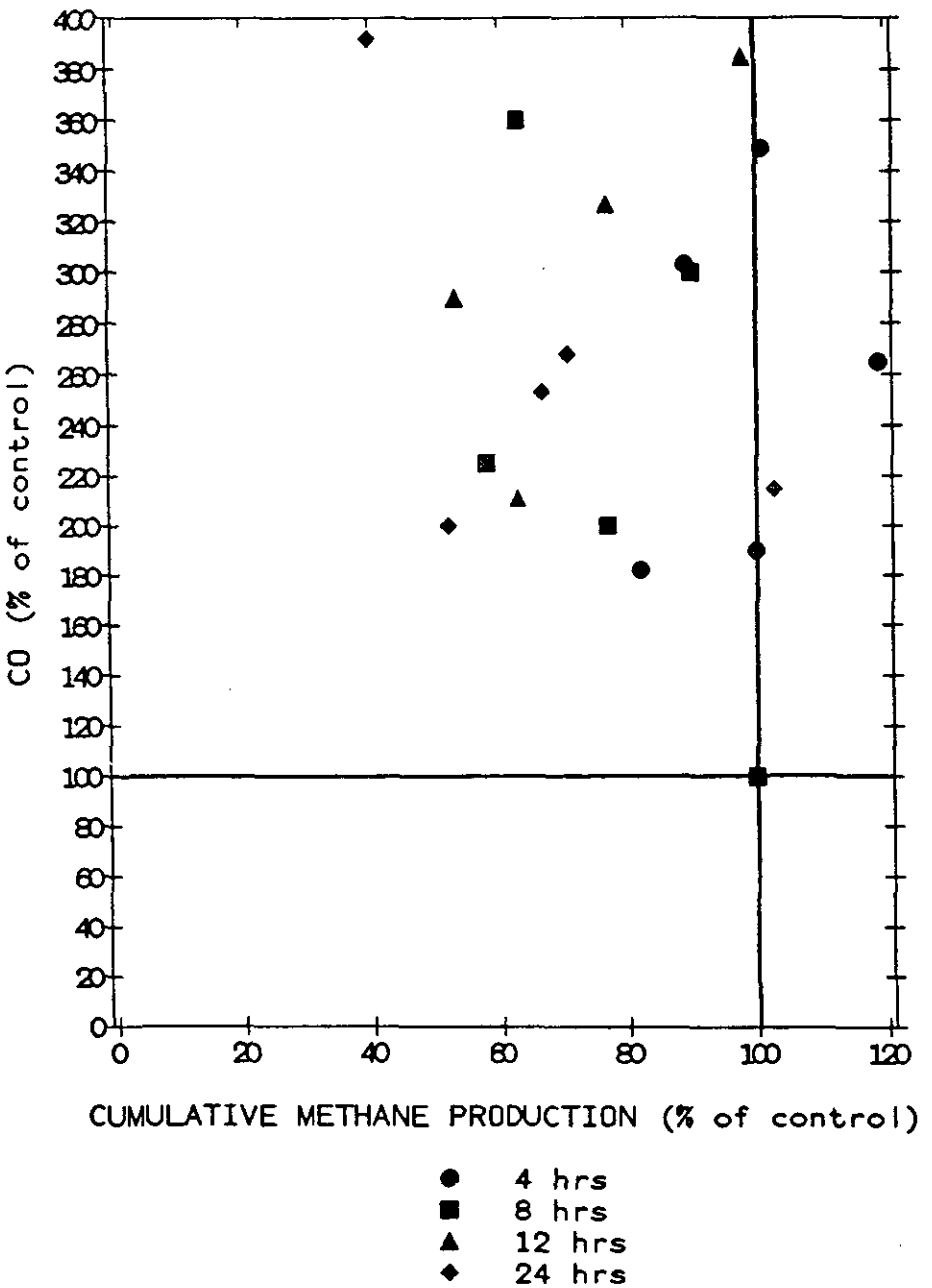

Figure 39: Correlation between carbon monoxide concentration and level of methane production in formaldehyde inhibited samples.

In Tables 6 and 7, the toxicant doses which caused a significant change in hydrogen and carbon monoxide levels, respectively, are summarized. A significant change was defined as at least a 50% change in hydrogen or carbon monoxide concentration, as compared to the control levels. The 50% value was selected to ensure that the change would be significantly greater that any normal daily fluctuations in the gas levels.

It can be seen that hydrogen and carbon monoxide, especially if monitored concurrently, have the potential to indicate possible upsets at an early stage after toxicants have been added. All toxicants, except BES, caused significant changes in hydrogen and/or carbon monoxide concentrations after 4-8 hours of exposure to the toxicants. BES only showed significant changes in hydrogen and carbon monoxide levels after 24 and 53 hours, respectively.

These results were then compared to the ones obtained in the study by Hickey (1987) which used waste activated sludge, a particulate substrate. It was expected that the responses would occur faster in the system fed acetate (a soluble substrate), since the first step (hydrolysis) in the breakdown of substrate is eliminated (see Figure 2). However, this trend was not observed. All the toxicants tested in that study (formaldehyde, BES, Cd, Cu and Zn) also caused significant changes in hydrogen and carbon monoxide

Table 6: Toxicant Doses Causing Significant Changes in Hydrogen Concentrations[1].

TIME EXPOSED TO TOXICANT	H_2 RESPONSE		
	INCREASE	NO CHANGE	DECREASE
4 hrs	20 mg/l Cu 8-56 mg/l HCHO	4-48 mg/l Cd 2-10 mg/l Cu 30-375 mg/l Ni 40-350 mg/l Zn 211-3376 mg/l BES	
8 hrs[2]	16-48 mg/l Cd 2-6 mg/l Cu 30-75 mg/l Ni 8-56 mg/l HCHO	4-16 mg/l Cd 10 mg/l Cu 150-375 mg/l Ni 40-350 mg/l Zn 211-3376 mg/l BES	20 mg/l Cu
12 hrs[3]	4-48 mg/l Cd 8-56 mg/l HCHO	4 mg/l Cd 30-375 mg/l Ni 40-350 mg/l Zn	
24 hrs[4]	16-48 mg/l Cd 40-56 mg/l HCHO	4-16 mg/l Cd 2-10 mg/l Cu 30-150 mg/l Ni 40-350 mg/l Zn 211-3376 mg/l BES 8-24 mg/l HCHO	20 mg/l Cu 375 mg/l Ni

1 A significant change was defined as an increase or decrease of at least 50% as compared to control values.
2 Cu analyzed @ 7 hrs.
3 Cu and BES not analyzed @ 12 hrs.
4 Cu analyzed @ 23 hrs.

Table 7: Toxicant Doses Causing Significant Changes in Carbon Monoxide Concentrations[1].

TIME EXPOSED TO TOXICANT	CO RESPONSE		
	INCREASE	NO CHANGE	DECREASE
4 hrs	16-48 mg/l Cd 8-56 mg/l HCHO	4 mg/l Cd 2-20 mg/l Cu 30-375 mg/l Ni 40-350 mg/l Zn 211-3376 mg/l BES	
8 hrs[2]	8-56 mg/l HCHO	4-48 mg/l Cd 2-20 mg/l Cu 30-150 mg/l Ni 40-350 mg/l Zn 211-3376 mg/l BES	375 mg/l Ni
12 hrs[3]	8-56 mg/l HCHO	4-48 mg/l Cd 30-150 mg/l Ni 40-250 mg/l Zn	375 mg/l Ni 350 mg/l Zn
24 hrs[4]	6-20 mg/l Cu 8-56 mg/l HCHO	4-48 mg/l Cd 2 mg/l Cu 30-150 mg/l Ni 40-90 mg/l Zn	375 mg/l Ni 150-350 mg/l Zn 211-3376 mg/l BES

1 A significant change was defined as an increase or decrease of at least 50% as compared to control values.
2 Cu analyzed @ 7 hrs.
3 Cu and BES not analyzed @ 12 hrs.
4 Cu analyzed @ 23 hrs.

levels after only 4 to 8 hours. Additionally, the levels of response of hydrogen and carbon monoxide in the system fed waste activated sludge were higher. It should be noted that due to the protocol used in testing the acetate utilizing methanogens, any early changes in hydrogen and carbon monoxide concentrations (i.e. occurring before the gas analysis at the four-hour mark) may have gone undetected.

When comparing the effect of the different heavy metals on acetate utilizing methanogens, it was found that the order of decreasing toxicity was Cu > Cd > Ni > Zn. This agrees with results obtained by previous researchers (Mosey et al., 1971 and Hayes and Theis, 1978). In Table 8, the toxicant doses required to cause 50% inhibition of methane production after 24 hours are listed. Results from similar studies using a sucrose enrichment (Giraldo, in progress) and a digester fed waste activated sludge (Hickey, 1987) are also shown. The toxicant dosages are expressed as mg toxicant per g volatile solids (unless otherwise stated), since this has been found to minimize differences between digesters with different solids retention times and solids concentrations (Mosey, 1976, Yang and Speece, 1985 and Hickey, 1987). When waste activated sludge was used, the toxicity of the metals was found to be Cu > Cd > Zn, which is identical to the results obtained with acetate. When using the sucrose enrichment, the order of decreasing

Table 8: Comparison of Toxicant Doses Causing 50% Inhibition of Methane Production After 24 Hours.

DIGESTER SYSTEM	TOXICANT DOSE (mg/g VS)			
	Cu	Cd	Ni	Zn
ACETATE	10[1]	20	100	350[2]
SUCROSE	*	9[2]	380	150
WASTE ACTIVATED SLUDGE	15	28	-	70

DIGESTER SYSTEM	TOXICANT DOSE (mg/l)	
	BES	HCHO
ACETATE	400[3]	35
SUCROSE	200[2]	30
WASTE ACTIVATED SLUDGE	400	40

* Did not reach 50% inhibition during assay
1 Time = 23 hrs
2 Time = 48 hrs
3 Time = 53 hrs

toxicity was Cd > Zn > Ni.

The dosages causing 50% inhibition were found to be only slightly lower for the acetate culture than when waste acetate sludge was used as the feed, when cadmium and copper were added. However, zinc could be tolerated in 2 to 5 times higher concentrations. This could be due to a higher degree of complexation of the zinc. The acetate culture was less resistant to nickel than the sucrose culture. Nickel was not tested on the reactor fed waste activated sludge.

The organic toxicant doses causing 50% inhibition were not reported on a per gram solids basis, so that a direct comparison between the three digester systems is more difficult to make. The results seem to indicate that the acetate system could tolerate higher doses of both formaldehyde and BES, while the digester fed waste activated sludge was the most sensitive to the addition of these toxicants.

These results would be in contrast to most theories which consider the acetate utilizing methanogens to be the most sensitive organisms in an anaerobic digestion system.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Based on the results of this research, the following can be concluded:

1. Hydrogen gas levels in the anaerobic digester system were affected by the addition of heavy metals and organic compounds.
2. The addition of zinc, formaldehyde and BES caused significant changes in carbon monoxide concentrations.
3. Therefore, monitoring of H_2 and CO concurrently will allow more rapid detection of inhibition by heavy metals and organics in an anaerobic digester fed a soluble waste than with analyses presently in use.
4. A decrease in hydrogen levels during some of the heavy metal assays indicates that other bacterial groups in the system were at least as severely inhibited as the methanogens.
5. The trends of the responses in H_2 and CO levels due to toxic inhibition are different for the acetate utilizing methanogens than for the waste activated sludge system. In addition, the level of response of the two indicators is higher in the sludge digester.

6. The responses of H_2 and CO in the system fed a soluble waste (acetate) did not occur more rapidly than in the digester fed a particulate substrate (waste activated sludge).
7. The toxicant doses required to cause the same level of inhibition are comparable for both systems. This would indicate that the acetate utilizing methanogens are not as sensitive to toxicant addition as is generally thought.

6.2 RECOMMENDATIONS

1. The response of trace gases, especially carbon monoxide, should be studied using pure cultures or defined co-cultures, to gain a better understanding of the behavior of these individual bacterial groups to toxicant addition.
2. Experimental work using soluble substrates should be performed with fixed-film digesters.
3. In fixed-film systems, the effect which mass transfer of substances into and out of the biofilm has on the trace gas concentrations should be studied.
4. The effect which metal speciation within the digester has on toxicity should be examined.

5. Hydrogen and carbon monoxide gas monitoring should be used in field work.

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APPENDIX

Data from Serum Bottle Assays

COPPER
H2 (ppm) vs TIME

SAMPLE	Cu DOSE (mg/l)	PPM H2 t=0 hrs	PPM H2 t=4 hrs	PPM H2 t=7 hrs	PPM H2 t=23 hrs
A	0	15	27	31	53
B	0	16	23	27	71
C	0	9	21	25	60
D	0	22	30	35	67
1A	2	18	28	35	69
1B	2	22	39	51	73
2A	6	18	32	47	48
2B	6	25	34	42	69
3A	10	16	18	24	54
3B	10	19	22	26	58
4A	20	19	18	16	14
4B	20	13	11	11	8

COPPER
CO (ppb) vs TIME

SAMPLE	Cu DOSE (mg/l)	PPB CO t=0 hrs	PPB CO t=4 hrs	PPB CO t=7 hrs	PPB CO t=23 hrs
A	0	667	1004	2124	1508
B	0	593	803	1004	1230
C	0	1037	843	888	1151
D	0	574	803	849	1230
1A	2	593	924	1081	1429
1B	2	593	964	1120	1429
2A	6	630	1004	1197	2302
2B	6	611	964	1197	1944
3A	10	685	863	1081	2897
3B	10	685	743	1081	3254
4A	20	648	1024	1158	1627
4B	20	685	1084	1081	1349

COPPER
CUMULATIVE CH4 PRODUCTION (ml) vs TIME

SAMPLE	Cu DOSE (mg/l)	ML CH4 t=0 hrs	ML CH4 t=4 hrs	ML CH4 t=7 hrs	ML CH4 t=23 hrs
A	0	0.00	0.00	0.90	4.10
B	0	0.00	0.65	8.92	10.38
C	0	0.00	0.75	1.09	4.43
D	0	0.00	0.75	1.09	4.31
1A	2	0.00	0.65	1.09	3.50
1B	2	0.00	0.65	1.09	3.75
2A	6	0.00	0.54	1.09	3.38
2B	6	0.00	0.00	0.67	2.71
3A	10	0.00	0.00	0.00	0.90
3B	10	0.00	0.00	0.00	1.02
4A	20	0.00	0.00	0.00	0.00
4B	20	0.00	0.00	0.00	0.23

CADMIUM
H2 (ppm) vs TIME

SAMPLE	Cd DOSE (mg/l)	PPM H2 t=0 hrs	PPM H2 t=4 hrs	PPM H2 t=8 hrs	PPM H2 t=12 hrs	PPM H2 t=24 hrs
A	0	3	13	20	25	20
B	0	3	13	19	22	27
C	0	3	14	21	28	37
D	0	2	13	18	27	35
1A	4	3	13	20	22	33
1B	4	1	21	30	57	52
2A	16	3	18	30	53	58
2B	16	3	13	37	57	63
3A	28	3	16	33	56	63
3B	28	3	18	6	56	59
4A	48	3	16	30	56	64
4B	48	1	11	29	48	61

CADMIUM
CO (ppb) vs TIME

SAMPLE	Cd DOSE (mg/l)	PPB CO t=0 hrs	PPB CO t=4 hrs	PPB CO t=8 hrs	PPB CO t=12 hrs	PPB CO t=24 hrs
A	0	600	678	1063	1106	1204
B	0	652	678	966	1195	1250
C	0	607	636	918	1372	1111
D	0	478	720	1063	1106	1065
1A	4	435	636	966	1681	1065
1B	4	522	975	1570	1681	1759
2A	16	543	636	966	1062	1065
2B	16	478	1653	918	973	1157
3A	28	522	783	918	1062	1204
3B	28	478	678	652	973	1296
4A	48	522	720	1063	929	1204
4B	48	1565	1737	773	973	1157

CADMIUM
CUMULATIVE CH4 PRODUCTION (ml) vs TIME

SAMPLE	Cd DOSE (mg/l)	ML CH4 t=0 hrs	ML CH4 t=4 hrs	ML CH4 t=8 hrs	ML CH4 t=12 hrs	ML CH4 t=24 hrs
A	0	0.00	0.00	0.90	1.34	3.19
B	0	0.00	0.55	1.00	1.45	3.40
C	0	0.00	0.44	1.00	1.56	3.29
D	0	0.00	0.55	1.00	1.56	3.62
1A	4	0.00	0.44	1.00	1.56	3.17
1B	4	0.00	0.00	0.78	1.12	2.37
2A	16	0.00	0.00	0.00	0.56	0.89
2B	16	0.00	0.00	0.00	0.45	0.78
3A	28	0.00	0.00	0.00	0.56	0.56
3B	28	0.00	0.00	0.45	0.45	1.01
4A	48	0.00	0.00	0.33	0.33	0.33
4B	48	0.00	0.00	0.22	0.22	0.22

NICKEL
H2 (ppm) vs TIME

SAMPLE	Ni DOSE (mg/l)	PPM H2 t=0 hrs	PPM H2 t=4 hrs	PPM H2 t=8 hrs	PPM H2 t=12 hrs	PPM H2 t=24 hrs
A	0	2	6	18	22	52
B	0	3	6	16	26	55
C	0	2	6	9	28	61
D	0	3	8	25	34	59
1A	30	4	8	27	42	56
1B	30	4	8	22	30	59
2A	75	3	7	25	39	62
2B	75	4	8	23	33	57
3A	150	4	8	21	28	56
3B	150	2	6	15	21	35
4A	375	3	7	18	23	36
4B	375	3	5	13	17	28

NICKEL
CO (ppb) vs TIME

SAMPLE	Ni DOSE (mg/l)	PPB CO t=0 hrs	PPB CO t=4 hrs	PPB CO t=8 hrs	PPB CO t=12 hrs	PPB CO t=24 hrs
A	0	485	634	704	1932	1173
B	0	437	634	657	918	1120
C	0	388	585	1643	773	1173
D	0	437	585	704	821	1227
1A	30	437	585	798	1014	1387
1B	30	437	585	704	1159	1173
2A	75	485	634	751	870	1227
2B	75	388	683	751	870	1120
3A	150	485	585	751	821	1120
3B	150	485	780	845	1063	1547
4A	375	485	585	563	580	747
4B	375	485	537	563	580	693

NICKEL
CUMULATIVE CH4 PRODUCTION (ml) vs TIME

SAMPLE	Ni DOSE (mg/l)	ML CH4 t=0 hrs	ML CH4 t=4 hrs	ML CH4 t=8 hrs	ML CH4 t=12 hrs	ML CH4 t=24 hrs
A	0	0.00	0.00	0.90	1.36	2.95
B	0	0.00	0.00	1.01	1.48	3.28
C	0	0.00	0.00	1.02	1.59	3.51
D	0	0.00	0.00	0.90	1.36	2.94
1A	30	0.00	0.00	0.34	0.34	1.57
1B	30	0.00	0.00	0.68	0.91	1.57
2A	75	0.00	0.00	0.45	0.57	1.46
2B	75	0.00	0.00	0.79	0.80	1.46
3A	150	0.00	0.00	0.00	0.68	1.23
3B	150	0.00	0.00	0.00	0.00	0.44
4A	375	0.00	0.00	0.00	0.00	0.00
4B	375	0.00	0.00	0.00	0.00	0.00

ZINC
H2 (ppm) vs TIME

SAMPLE	Zn DOSE (mg/l)	PPM H2 t=0 hrs	PPM H2 t=4 hrs	PPM H2 t=8 hrs	PPM H2 t=12 hrs	PPM H2 t=24 hrs	PPM H2 t=48 hrs
A	0	31	35	34	26	22	23
B	0	35	42	44	38	29	26
C	0	28	37	41	38	26	27
D	0	30	40	42	38	27	26
1A	40	34	43	47	47	27	25
1B	40	38	47	50	46	30	26
2A	90	38	45	49	47	30	25
2B	90	40	49	50	46	30	25
3A	150	26	40	45	45	29	24
3B	150	39	49	49	43	31	24
4A	250	42	47	46	45	30	25
4B	250	38	44	45	41	33	25
5A	350	35	34	32	33	29	22
5B	350	36	36	34	31	31	22

ZINC
C0 (ppb) vs TIME

SAMPLE	Zn DOSE (mg/l)	PPB C0 t=0 hrs	PPB C0 t=4 hrs	PPB C0 t=8 hrs	PPB C0 t=12 hrs	PPB C0 t=24 hrs	PPB C0 t=48 hrs
A	0	1333	1553	1095	1566	2513	2222
B	0	905	1214	1238	1465	2667	2873
C	0	952	1214	1476	1667	2308	2385
D	0	857	1165	1333	1414	2359	2656
1A	40	810	1068	1190	1364	2205	2710
1B	40	1048	1408	1476	1616	2308	2493
2A	90	1048	1456	1429	1667	2000	1572
2B	90	1000	1311	1429	1515	2513	1463
3A	150	1095	1408	1619	1869	1744	867
3B	150	1190	1408	1524	1566	1333	705
4A	250	1286	1408	1333	1364	1028	650
4B	250	952	1214	1190	1162	923	542
5A	350	857	874	857	808	564	325
5B	350	1048	1088	857	808	513	379

ZINC

CUMULATIVE CH4 PRODUCTION (ml) vs TIME

SAMPLE	Zn DOSE (mg/l)	ML CH4 t=0 hrs	ML CH4 t=4 hrs	ML CH4 t=8 hrs	ML CH4 t=12 hrs	ML CH4 t=24 hrs	ML CH4 t=48 hrs
A	0	0.73	1.00	1.23	1.24	1.81	2.86
B	0	0.73	1.10	1.34	1.34	2.02	3.42
C	0	0.73	1.00	1.12	1.24	1.81	3.20
D	0	0.73	1.00	1.12	1.35	1.92	3.20
1A	40	0.85	1.12	1.13	1.24	1.81	2.51
1B	40	0.73	1.12	1.12	1.35	1.80	2.39
2A	90	0.73	1.02	1.02	1.13	1.59	1.94
2B	90	0.72	1.13	1.13	1.24	1.70	2.28
3A	150	0.48	0.58	0.69	0.91	1.25	1.49
3B	150	0.73	1.03	1.03	1.25	1.48	1.71
4A	250	0.97	0.97	1.20	1.32	1.54	1.66
4B	250	0.96	1.06	1.06	1.17	1.39	1.63
5A	350	0.73	0.94	0.94	1.05	1.05	1.17
5B	350	0.97	0.97	0.97	1.08	1.08	1.09

BES
H2 (ppm) vs TIME

SAMPLE	BES DOSE (mg/l)	PPM H2 t=0 hrs	PPM H2 t=4 hrs	PPM H2 t=8 hrs	PPM H2 t=24 hrs	PPM H2 t=53 hrs
A	0	20	26	25	21	15
B	0	23	29	29	21	20
C	0	18	20	24	22	19
D	0	30	33	33	21	19
1A	211	27	32	33	22	20
1B	211	31	37	36	24	21
2A	422	25	32	32	24	22
2B	422	30	34	33	21	20
3A	844	32	35	33	23	22
3B	844	31	35	35	23	24
4A	1688	28	30	31	27	25
4B	1688	32	35	34	24	24
5A	2532	34	37	36	25	27
5B	2532	20	29	31	24	25
6A	3376	32	36	36	26	27
6B	3376	32	35	35	28	28

BES
CO (ppb) vs TIME

SAMPLE	BES DOSE (mg/l)	PPB CO t=0 hrs	PPB CO t=4 hrs	PPB CO t=8 hrs	PPB CO t=24 hrs	PPB CO t=53 hrs
A	0	1381	1976	2082	3013	2943
B	0	1095	1542	1695	3065	1796
C	0	1286	1831	2421	3221	1247
D	0	1524	1831	2034	2961	1646
1A	211	1190	1446	1598	1558	698
1B	211	1476	1831	1840	1403	449
2A	422	1381	1831	1937	1506	499
2B	422	1714	1976	1985	1247	399
3A	844	1524	1831	1937	1403	449
3B	844	1476	1831	1889	1403	549
4A	1688	1190	1542	1598	1558	599
4B	1688	1524	1687	1695	1558	599
5A	2532	1571	1735	1743	1455	599
5B	2532	1381	1735	1889	1766	698
6A	3376	1571	1783	1792	1351	549
6B	3376	1714	1976	1889	1351	648

BES
CUMULATIVE CH4 PRODUCTION (ml) vs TIME

SAMPLE	BES DOSE (mg/l)	ML CH4 t=0 hrs	ML CH4 t=4 hrs	ML CH4 t=8 hrs	ML CH4 t=24 hrs	ML CH4 t=53 hrs
A	0	0.61	0.98	1.21	2.24	4.55
B	0	0.73	0.89	1.23	2.15	3.06
C	0	0.36	0.76	0.99	1.91	2.83
D	0	1.09	1.09	1.32	2.57	4.68
1A	211	0.61	0.78	1.12	1.47	1.82
1B	211	0.98	1.22	1.34	1.68	1.92
2A	422	0.85	0.90	1.02	1.36	1.49
2B	422	0.72	0.89	1.13	1.24	1.59
3A	844	0.60	0.78	1.12	1.35	1.48
3B	844	0.85	1.01	1.35	1.47	1.71
4A	1688	0.98	1.22	1.46	1.80	1.92
4B	1688	0.84	1.01	1.35	1.47	1.60
5A	2532	0.72	0.89	1.24	1.36	1.37
5B	2532	0.48	0.67	1.01	1.13	1.25
6A	3376	0.72	0.89	1.13	1.24	1.37
6B	3376	0.47	0.67	0.90	1.02	1.03

FORMALDEHYDE
H2 (ppm) vs TIME

SAMPLE	HCHO DOSE (mg/l)	PPM H2 t=0 hrs	PPM H2 t=4 hrs	PPM H2 t=8 hrs	PPM H2 t=12 hrs	PPM H2 t=24 hrs
A	0	36	41	37	33	23
B	0	34	38	41	39	31
C	0	30	35	34	35	28
D	0	37	41	45	40	30
1A	8	37	62	61	56	30
1B	8	46	65	69	56	33
2A	16	37	76	76	77	28
2B	16	45	71	82	74	29
3A	24	36	72	74	80	32
3B	24	43	73	83	83	35
4A	40	31	73	98	126	138
4B	40	43	79	111	132	175
5A	56	41	111	208	337	885
5B	56	43	119	264	398	730

FORMALDEHYDE
CO (ppb) vs TIME

SAMPLE	HCHO DOSE (mg/l)	PPB CO t=0 hrs	PPB CO t=4 hrs	PPB CO t=8 hrs	PPB CO t=12 hrs	PPB CO t=24 hrs
A	0	1250	1570	1796	2037	3323
B	0	1080	1337	1796	1852	3448
C	0	1023	1570	2043	2346	3197
D	0	1136	1395	1796	1852	3135
1A	8	1193	4767	7554	7593	6458
1B	8	1420	5465	9536	7963	7649
2A	16	1534	4709	6873	8951	9028
2B	16	1477	4186	6502	7901	8527
3A	24	1250	4070	5697	7284	8276
3B	24	1591	3721	5449	5926	8276
4A	40	1136	2791	3591	4321	6708
4B	40	1136	2791	3839	4198	6395
5A	56	1250	2849	4149	5864	11724
5B	56	1307	2500	4211	5864	13981

FORMALDEHYDE
CUMULATIVE CH4 PRODUCTION (ml) vs TIME

SAMPLE	HCHO DOSE (mg/l)	ML CH4 t=0 hrs	ML CH4 t=4 hrs	ML CH4 t=8 hrs	ML CH4 t=12 hrs	ML CH4 t=24 hrs
A	0	0.00	0.73	0.95	1.19	1.87
B	0	0.00	0.73	1.07	1.31	2.22
C	0	0.00	0.63	0.97	1.31	1.89
D	0	0.00	0.73	0.96	1.42	2.11
1A	8	0.00	0.63	0.75	1.20	2.01
1B	8	0.74	0.79	0.90	1.36	2.16
2A	16	0.00	0.62	0.62	0.86	1.43
2B	16	0.00	0.63	0.63	0.86	1.43
3A	24	0.73	0.89	1.01	1.13	1.59
3B	24	0.61	0.78	0.78	0.90	1.13
4A	40	0.61	0.78	0.90	0.90	1.24
4B	40	0.00	0.63	0.63	0.75	0.87
5A	56	0.00	0.52	0.53	0.65	0.76
5B	56	0.00	0.63	0.63	0.74	0.86