Effects on Iron and Cobalt on Methane Production from Dairy Cattle Manure

Fall 1983

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EFFECTS OF IRON AND COBALT
ON METHANE PRODUCTION
FROM DAIRY CATTLE MANURE

BY

MARK ELLIOTT HIMES
B.S., University of Central Florida, 1976

THESIS
Submitted in partial fulfillment of the requirements
for the Master of Science degree in Microbiology
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ABSTRACT

The effects of iron and cobalt on methanogenesis from dairy cattle manure were studied. Four-liter digestors with 3-liter working volumes were charged daily with dairy manure (4.5% W/V volatile solids) to achieve a 3-day retention time. Digestors were incubated at 37°C and pH 7.0 on a rotary shaker. Duplicate digestors were maintained under the following parameters: controls (no ions added), Co⁺⁺ at 4.958 mg/liter, Fe⁺⁺ at 20.64 mg/liter and Fe⁺⁺ and Co⁺⁺ at above stated final digester concentrations.

Significantly higher production of biogas and methane occurred with the addition of iron (p = .05). Iron-amended digestors produced 3.88 ± 0.26 liters/liter/day of biogas and 2.03 ± 0.14 liters/liter/day of methane. Control digestors produced 3.59 ± 0.27 liters/liter/day of biogas and 1.85 ± 0.14.

Cobalt did not stimulate methanogenesis and may have nullified the stimulating effects of iron. Neither the cobalt nor the iron/cobalt-amended digestors demonstrated increases in biogas or methane production. Iron or cobalt did not affect efficiency of fermentation (liters of methane per gm of volatile solids destroyed) or volatile fatty acid conversion.

The total counts of methanogens grown in roll tubes were unaffected by addition of the metals. *Methanobrevibacter smithii* was isolated from all digestors, however, *Methanobacterium formicicum* was isolated only from digestors amended with iron.
ACKNOWLEDGEMENT

I would like to thank Dr. R.N. Gennaro, my major professor, for guidance and assistance through all phases of my studies at UCF. I would also like to thank Dr. J.F. Charba, Dr. W.K. Taylor, and Dr. R.J. Wodzinski for their encouragement and help in this thesis preparation.

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A special thanks is due to Eric Utt, who provided a great deal of assistance and moral support when it was most needed.

To my parents and my wife’s parents, I am extremely grateful for their continual encouragement, understanding, and patience.

Lastly, I express my deepest appreciation to Laura, my wife and
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# TABLE OF CONTENTS

**LIST OF TABLES** .................................................. vii

**LIST OF FIGURES** .................................................. viii

**INTRODUCTION** ..................................................... 1
  Anaerobic digestion ................................................. 1
  Biology of methanogenesis .......................................... 3
  Factors affecting methanogenesis ................................... 11
  Physiological aspects of methanogens ............................. 16
  Taxonomy of methanogens .......................................... 18

**MATERIALS AND METHODS** ........................................... 20
  Digestors ............................................................ 20
  Substrate ............................................................ 20
  Digester start-up and maintenance ................................ 21
  Biogas analysis ..................................................... 22
  Total and volatile solids analysis ................................ 23
  Volatile fatty acid analysis ....................................... 23
  Metal analysis ....................................................... 24
  Cultural procedures ................................................ 24
  Culture media ....................................................... 26
  Statistical analysis ................................................ 26

**RESULTS** ........................................................... 27
  Microscopic examination ........................................... 27
LIST OF TABLES

1. Determinative key to species of the methanogenic bacteria based on simple phenotypic characters ........ 19

2. Biogas and methane produced from fermentation of dairy manure with 4.5% VS concentration at pH 7.0, 37°C, and a 3-day retention time ....................... 31

3. Efficiency of methane fermentation of dairy manure with 4.5% VS concentration at pH 7.0, 37°C, and a 3-day retention time ....................... 33

4. Concentration of volatile fatty acids \(^a\) after methane fermentation of dairy manure with 4.5% VS at pH 7.0, 37°C, and a 3-day retention time ....................... 42

5. Methanogen populations of digestors fermenting dairy manure with 4.5% VS concentration at pH 7.0, 37°C, and a 3-day retention time ....................... 44

6. Methanogens isolated from digestors fermenting dairy manure with 4.5% VS concentration at pH 7.0, 37°C, and a 3-day retention time ....................... 45
LIST OF FIGURES

1. Relation of methanogenic and nonmethanogenic bacteria to the predominant path of carbon and electron flow during anaerobic mineralization of organic matter .......... 4

2. Free energy ($\Delta G_0$) from syntrophic production of hydrogen from ethanol by Methanobacterium bryantii and S species ........................................ 9

3. Fermentation products of pyruvate as the most common intermediate in living cells .................................................. 10

4. Electron transport in Desulfomaculum sp. involving ferredoxin with production of molecular hydrogen ...................... 13

5. Biochemical mechanism for methane formation ......................... 17

6. The effect of addition of iron to dairy manure digestors on biogas production, day 6 through 90 .......... 28

7. The effect of addition of iron and cobalt to dairy manure digestors on biogas production, day 6 through 90 ... 29

8. The effect of addition of cobalt to dairy manure digestors on biogas production, day 6 through 90 .......... 30

9. Methane and biogas production from control digestors, week 1 through 30 .................. 34

10. Methane and biogas production from cobalt-amended digestors (4.958mg/l Co++), week 1 through 15 ........ 35

11. Methane and biogas production from iron-amended digestors (20.64mg/l Fe++), week 1 through 30 ........ 36

12. Methane and biogas production from iron and cobalt-amended digestors (20.64mg/l Fe++; 4.958 mg/l Co++), week 1 through 30 ........ 37

13. Acetate and propionate levels in control digestors, week 1 through 48 .. .......... 38

14. Acetate and propionate levels in cobalt-amended digestors (4.958mg/l Co++), week 1 through 15 . ... 39
15. Acetate and propionate levels in iron-amended digestors (20.64mg/l Fe^{++}), week 1 through 48  

16. Acetate and propionate levels in iron and cobalt-amended digestors (20.64mg/l Fe^{++}; 4.958mg/l Co^{++}), week 1 through 48
INTRODUCTION

Recently, emphasis on the environment and increased use of fossil fuels has stimulated interest in the production of methane via anaerobic digestion. Anaerobic digestion of wastes under certain conditions is an economically feasible solution to environmental pollution and it also provides innocuous and/or useful end products. Credits for end products such as: methane, carbon dioxide, protein feed supplement, and fertilizer can be used to help offset waste disposal and fermentation costs. Conditions that contribute to high-rate anaerobic digestion with maximum yield of useful products can enhance the economic feasibility of methane digestors. The purpose of this study was to investigate the effects of iron and cobalt on anaerobic digestion of dairy cattle wastes.

Anaerobic digestion. Anaerobic digestion has been considered a slow-rate process due to the nature of sludges traditionally treated. The recalcitrant components of sludges are: cellulose, hemicellulose, lignins, lipids, and microbial flocs generated in the aerobic domestic sewage process. Anaerobic digestion destroys approximately 50% of the organic solids in the sludge (60). Thermophilic anaerobic digestion enhances dewaterability of sludge, reduces odor, and destroys pathogenic organisms.

Because aeration is not necessary in anaerobic digestion, the
necessity of providing adequate oxygen to concentrated organic suspensions is circumvented. The process is suitable for primary treatment of concentrated solid wastes from vegetable or meat-processing factories, slaughterhouses, fermentation industries, and animal wastes. The annual solid waste production by farm animals has been estimated at 2 billion tons (8), with approximately one-half of this waste generated by intensive animal production systems (69). Intensive animal and dairy cattle farming practices have been increasing due to higher yields from feedlots and decreasing land availability. The concentration of livestock on feedlots has created stream pollution, odor, and waste disposal problems (69). These problems are evident in Lake Okeechobee (located in southern Florida), where 33 dairies representing 35,000 cattle are located throughout the water basin. The concentration of animals and the seriousness of the pollution problem provided the motivation for funding by the State of Florida to study the economics of using anaerobic digestors to alleviate pollution.

Many factors must be considered in an economic analysis of anaerobic digestion of dairy wastes. Methane from anaerobic digestion can be sold or utilized on-site to generate electricity, heat water, or cool milk. These credits help to offset the operational and capital costs of the digestor (28,30,69,73,77). Protein from the effluent may be used as a feed supplement for nonruminant animals (30). The cost of processing the digestor residue to provide protein may not be economical (77). Carbon dioxide, produced from
digestors fed with wastes generated by 22,593 head of cattle, has been estimated to provide 25% of the $112,000 net annual profit (77). Stabilized effluent can be recovered and utilized as fertilizer (30,77). Wodzinski and Gennaro (77) analyzed the economics of the system and have determined that it was economically feasible to produce methane from dairy manure if the solids were upgraded to a 6-6-6 fertilizer and sold.

Optimization of the process which increases the amount of end products or reduces capital expenditures will enhance its economic feasibility. Investigators have concentrated on maximizing methane production and volatile solids (VS) destruction. This has been achieved by varying retention times (RT), temperature, pH, loading rates and substrates. An important goal in dairy manure digestion which decreases capital costs, is to use the shortest possible RT that provides adequate VS destruction. This has been accomplished with thermophilic cattle manure digestors fed 8.2% VS and a 3-day RT. The methane production rate was 4.5 liters per liter of digester per day (69).

Biology of methanogenesis. Bioconversion of complex organic matter into methane and carbon dioxide via anaerobic digestion is achieved by diverse microbial populations. Bryant et al. (13) and Zeikus (84) have suggested that there are three trophic based on substrate utilization. (Fig. 1).

A complex group of microorganisms in the first level catabolize polysaccharides, proteins, and lipids to alcohols, fatty acids,
Fig. 1. Relation of methanogenic and nonmethanogenic bacteria to the predominant path of carbon and electron flow during anaerobic mineralization of organic matter. Modified from Zeikus (84).
hydrogen, carbon dioxide and acetate. Genera involved in the first trophic level include Bacteroides, Clostridium, Desulfovibrio, Ruminococcus, Selenomonas, and various facultative anaerobes. The second trophic level catabolizes the products of the first level to acetate, hydrogen, and carbon dioxide which are ultimate substrates for methanogenic bacteria. Bacteria found in the second trophic level include the S species described by Bryant et al. (14); Desulfovibrio sp., Ruminococcus sp., Syntrophobacter wolinii, Acetobacterium woodii, and Selenomonas ruminantium. Factors that limit growth or alter end products of these fermentative bacteria will affect methanogenesis. Nonmethanogens are often referred to as acid-forming or acetogenic bacteria. Methanogens convert end products of the acetogens to methane and carbon dioxide in the third trophic level. The three orders of methanogens involved in this level are Methanobacterales, Methanococcales, and Methanomicrobiales. Methane, the final reduced fermentation product, assures continued organic decomposition because it serves as an electron sink that is vented to the atmosphere.

Methanogenic bacteria are ubiquitous in areas containing large quantities of organic compounds and small amounts of oxygen. Examples of such ecosystems include intestinal tracts (rumen and large intestine), swamps, freshwater and marine sediments, and wetwood of living trees (25,32,47,53,75,86).

Hydrogen, carbon dioxide, and acetate are major energy and carbon sources; however, formate, methanol, methylamines, and carbon
monoxide may also be used. Nutrients for the methanogens are provided by the diverse nonmethanogenic populations. Mineralization of these nutrients results in the removal of carbon and electrons in the form of methane and carbon dioxide. Consequently, methanogenesis is considered the terminal step in the microbial food chain of anaerobic environments. Hydrogen sulfide is a major sulfur source even though it is toxic to methanogens under certain conditions (43,50). Ammonium ion is the major nitrogen source of methanogens.

High levels of oxygen or alternate electron acceptors, such as sulfate and nitrate, must be absent. These electron acceptors when present are either toxic to methanogens or enable other bacteria to compete for nutrients. Redox potentials must be lower than \(-200\, \text{mv}\) (7). Oxygen toxicity to \textit{Methanobacterium formicicium} is due to the formation of superoxide and formate radicals which result in lipid peroxidation and cell damage (5).

Methanogenesis occurs at temperatures between \(20 - 65^\circ\text{C}\), respectively. Thermophilic species are found in thermal springs and self-heated environments of anaerobic soils and composts. Mesophilic species inhabit the large intestine, rumen, and freshwater and marine sediments.

Neutral pH values (5.5 to 8.5) are required for methanogenesis. The optimum pH is between 7.0 and 8.0 (32). Mesophilic dairy manure digestors with 3-day RT showed a marked decrease in biogas and methane production at pH levels less than 6.0 (M.L. Stafford, M.S. thesis, University of Central Florida, Orlando, Florida 1982).
Methanogens regulate methane fermentations by stabilizing the pH and controlling the fermentation products of the acetogenic bacteria (81). Zeikus (84) has suggested that methanogens perform both proton and electron regulation. Proton regulation, accomplished by the catabolism of acetic acid, removes inhibitory protons and maintains an optimal pH for the system. Electron regulation, via oxidation of molecular hydrogen, creates favorable thermodynamic conditions for the metabolism of multicarbon compounds. Electron regulation prevents accumulation of toxic metabolites and also increases the metabolic rates of acetogens.

Interspecies hydrogen transfer is a mechanism by which methanogens regulate methane formation. Hydrogenase activity demonstrated in the periplasmic space of Desulfovibrio gigas provides evidence that hydrogen transfer may occur through somatic interactions (6). Somatic interspecies hydrogen transfer has also been implicated between the anaerobic ciliates of the family Ophyroscoleidae and methanogens (70).

Although large quantities of hydrogen are produced in microbial ecosystems, it is used rapidly by methanogens via interspecies hydrogen transfer (75). Rapid hydrogen catabolism induces fermenters to produce more hydrogen and thereby alter their electron sink products.

Interspecies hydrogen transfer can be either syntrophic or non-syntrophic. Some hydrogen-producing organisms are inhibited by the build-up of hydrogen and can only grow syntrophically with methanogens. The first demonstration of syntrophic interspecies hydrogen
transfer occurred with the discovery that *Methanobacillus omelianskii* was actually an association of two distinct organisms (14). *M. omelianskii*, now designated *M. bryantii*, (4) derives its only source of energy from the oxidation of hydrogen. The other species, a non-methanogen known as *S* species, oxidizes ethanol to acetate and hydrogen. Hydrogen accumulation inhibits *S* species by preventing oxidation of ethanol to acetate (Fig. 2). The step most sensitive to the accumulation of hydrogen is the oxidation of NADH to NAD+ and hydrogen. The reverse reaction is favored thermodynamically.

The presence of methanogens alters the end products of *Ruminococcus flavefaciens*, *Selenomonas ruminantium*, and *R. albus* from succinate, propionate, and lactate/ethanol, respectively, to acetate and hydrogen (19,80). These are nonsyntrophic associations. Methanogens maintain a very low partial pressure of hydrogen. It then becomes thermodynamically more favorable to produce acetate from pyruvate than the various electron sink products described above. ATP generated in the substrate level phosphorylation of pyruvate to acetate by coenzyme A and pyrophosphate also enhances the thermodynamics of interspecies hydrogen transfer (81).

Reduced pyridine nucleotides formed during glycolysis oxidize pyruvate to various electron sink products. Pyruvate is considered a key metabolic intermediate of fermentative bacteria (Fig. 3). Most 6-carbon compounds are converted initially to pyruvate from which further catabolic or synthetic reactions proceed. Excess hydrogen in a methane fermentation promotes an accumulation of electron sink products from pyruvate such as acetate, propionate,
\[ \text{Fig. 2. Free energy (} \Delta G'_\text{f} \text{) from syntrophic production of hydrogen from ethanol by } M. \text{ bryantii} \text{ and } S \text{ species (5).} \]

\[
\begin{align*}
\text{C}_2\text{H}_5\text{OH} + \text{NAD}^+ & \rightarrow \text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+ & \Delta G'_\text{f} \text{ (kJ)} \\
\text{NADH} + \text{H}^+ & \rightarrow \text{NAD}^+ + \text{H}_2 & +23.8 \\
\text{CH}_3\text{CHO} + \text{H}_2\text{O} & \rightarrow \text{CH}_3\text{CO}_2^- + \text{H}_2 + \text{H}^+ & +18.0 \\
\text{SUM: } \text{C}_2\text{H}_5\text{CHO} + \text{H}_2\text{O} & \rightarrow \text{CH}_3\text{CO}_2^- + 2\text{H}_2 + \text{H}^+ & -32.2
\end{align*}
\]
Fig. 3. Fermentation products of pyruvate as the most common intermediate in living cells. Dashed lines represent nonfermentative processes (83).
butyrate, and other volatile fatty acids. This accumulation, along with a concomitant decrease in pH, causes methane fermentations to fail.

**Factors affecting methanogenesis.** Chemical factors such as rumen fluids (79), fatty acids (2,79), sulfate (16,54,64,67), sulfide (49,62,64,74), cobalt (9,36,44,51,56,61,62), iron (16, 31,49,54,56,61,62,64,67), and nickel (16,36,39,49,51,56,62) affect methanogenesis. Analysis for these chemical factors in methanogenic substrates has not been investigated thoroughly. Deficiencies of these compounds may be rate-limiting in the methane fermentation.

Iron has been demonstrated to stimulate the conversion of volatile fatty acids to methane (31,61). Both Fe$^{+++}$ (54,61) and Fe$^{++}$ (16,31,56,62,64,67) have been used to stimulate methanogenesis. However, it is not critical which form is used since Fe$^{+++}$ is immediately reduced to Fe$^{++}$ in anaerobic digestors (31). The equilibrium ratio of Fe$^{+++}$ to Fe$^{++}$ at an Eh of -265 mV is 1:3 x $10^{17}$ (49).

The addition of Fe$^{+++}$(20.64 mg/liter) and Co$^{++}$(4.958 mg/liter) to dairy waste digestors fed 8% VS at pH 7.0 and 37°C stimulated methane production. (R.J. Wodzinski, M.E. Himes, and R.G. Gennaro, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, 022, p. 243). Biogas production increased, total volatile acids decreased and acetate decreased up to 24 fold in these studies. Taylor and Pirt (64) and Schonheit et al. (56) determined that iron was rate-limiting for growth of *M. thermoautotrophicum* in conventional media. Iron
is rapidly precipitated by sulfide which further aggravates iron deficiency. Patel et al. (54) demonstrated that hydrogen-oxidizing methanogens required iron levels between 0.3 and 0.9 mM for optimum growth in synthetic media.

Iron is an important micronutrient for many bacteria and is a component of various enzyme systems of anaerobic organism. Ferredoxin and phosphogluconate dehydratase are key iron-containing enzymes of the fermentative bacteria. When iron is growth-limiting, addition of iron stimulate the production of these enzymes and hence certain substrates for methanogenic bacteria.

Ferredoxin is especially important in methanogenesis due to its role in interspecies hydrogen transfer. Ferredoxin is an electron carrier for the nonmethanogens that is reduced by pyruvate and oxidized by hydrogenase (66). Acetyl phosphate, carbon dioxide, and hydrogen are the end products (Fig. 4).

The hydrogenase enzyme is thought to be located in the periplasmic space of acetogenic bacteria (6). This allows a direct transfer of electrons to an acceptor in methanogens. The formation of acetyl phosphate and substrates is proportional to the ferredoxin concentration (1), and ferredoxin synthesis is proportional to iron concentration.

Phosphogluconate dehydratase is active in the Entner-Doudoroff pathway which results in the production of pyruvate and glyceraldehyde 3-phosphate. Both products are further catabolized to carbon dioxide, acetate, and hydrogen.
Fig. 4. Electron transport in Desulfomaculum sp. involving ferredoxin with production of molecular hydrogen (24).
Addition of iron to anaerobic digestors enhances conditions for methanogenesis by precipitating sulfide below toxic levels (49,54,64). The FeS may also enhance methanogenesis by functioning as a reducing agent. FeS reacts more rapidly with oxygen than either Na$_2$S or cysteine (10).

Iron, added to low sulfide media, can adversely affect methanogenesis (87). This low sulfide concentration is growth limiting for M. thermoautotrophicum (64) and its availability further aggravated by precipitation as FeS. The low solubility of FeS [3.7 x 10$^{19}$ (mole/liter)$^2$] effectively removes sulfide from solution (71). Methanogens require sulfide for the synthesis of coenzyme M, amino acids, and Factor 420.

Cobalt stimulation of methanogenesis has been demonstrated with pure cultures in synthetic media and with mixed fermentations (44,51,56,62). Total biogas from fixed-film digestors increased 42% with the addition of 50 mM cobalt (51). Schonheit et al. (56) determined that cobalt at concentrations less than 10 nM were rate limiting for growth of M. thermoautotrophicum in pure culture. Methanosarcina sp. are stimulated by adding cobalt to basal medium (44).

Cobalt is an essential micronutrient for bacteria and it is required for the synthesis of corrinoid compounds such as vitamin B$_{12}$ and methylcobalamin (9). Vitamin B$_{12}$ is a coenzyme that functions as a carrier of methyl groups (82). There is also evidence for cobalt inhibition of methanogenesis. Cobalt, at concentrations of 1 mM and greater, inhibits the active transport of nickel across
the membrane of *M. bryantii* (36).

Nickel is an essential nutrient of methanogens and stimulates methanogenesis (16,22,36,39,51,52,56,62). Capone et al. (16) demonstrated stimulation of anaerobic digestors with the addition of 1000 mg/liter of nickel. Nickel concentrations of less than 100 nM are rate limiting for the growth of *M. thermoautotrophicum* when grown on hydrogen and carbon dioxide (56). Nickel concentrations between 250 and 1100 nM are optimum for growth of methanogens in synthetic media (62).

Sulfate stimulation in anaerobic digestors is dependent upon the soluble iron content. Concentrations of greater than 0.5 mM iron are necessary in mixed fermentations for sulfate stimulation to occur (49,67). Optimum sulfate concentrations for methanogens in pure cultures are between 0.16 and 0.52 mM (54).

Sulfate can inhibit methanogenesis at elevated concentrations (54). Methanogenesis in Lake Mendota sediments was inhibited by 150 µg/ml of sulfate (43). Competition of sulfate-reducing bacteria (SRB) with methanogens for substrates has been suggested as the mechanism which may explain the apparent toxicity of sulfate to methanogenesis (54). Anaerobic respiration of SRBs takes precedence over methanogenesis and effectively competes for acetate and hydrogen in anaerobic ecosystems that contain large amounts of sulfate (12,17,41,67,76). Kinetic studies by Lovely et al. (41) suggest that the ability of SRBs to compete with methanogens was related to the SRBs lower half-saturation constant for acetate metabolism even at in situ freshwater sulfate concentrations. Sulfate toxicity
may also be attributed to an increase in Eh (43) and the toxicity of sulfide (50) formed by the reduction of sulfate.

Physiological aspects of methanogens. Taylor and Wolfe (63) have shown that coenzyme M (2-mercaptoethanesulfonic acid) is involved in methyl transfer reactions in methane bacteria. Sulfide is the source of sulfur for the synthesis of coenzyme M. This cofactor is required by methyl coenzyme M reductase, an enzyme present in all methanogens. The enzyme is active in the terminal stages of carbon dioxide reduction to methane (29,63). (Fig. 5).

A low molecular weight enzyme that fluoresces at 420 nM has been detected in all methanogens, but not in other procaryotes or eucaryotes. Coenzyme F420 is a 5′deazaflavin mononucleotide analog (4) and functions as a low redox electron carrier. It is involved in the nicotinamide adenine dinucleotide phosphate-linked hydrogenase and formate dehydrogenase systems in methanogens (27,65). Both coenzyme A-dependent pyruvate and alpha-ketoglutarate dehydrogenases from extracts of M. thermoautotrophicum reduce F420 (85). Ferredoxin normally mediates this reaction in other bacteria (66).

Formate dehydrogenase isolated from M. vaniellii is an iron-sulfur molybdoenzyme that reduces F420 (38). Formate dehydrogenase is the only iron-containing enzyme found in methanogens. The addition of iron to digestors may directly stimulate the synthesis of formate dehydrogenase.

Coenzyme F430 is a low molecular weight, nickel-containing tetrapyrrolo structure (22) that fluoresces at 430 nM. Elefson
Fig. 5. Biochemical mechanism for methane formation (78).
and Wolfe (26) have suggested that F430 may be the prosthetic group of methyl coenzyme M reductase.

Carbon dioxide fixation pathways have not been demonstrated in methanogens. Enzymes necessary for carbon dioxide fixation in the serine, hexulose, or the reductive pentose phosphate pathways have not been demonstrated (72). Only incomplete reductive carboxylic acid cycles have been detected in *M. thermotrophicum* or *M. barkerii* (4). The differences observed between the incomplete carboxylic acid cycles of these two organisms may reflect a phylogenetic divergence.

**Taxonomy of methanogens.** Current taxonomy of the methanogens reflects their phylogenetic divergence as re-evaluated by Balch et al. (4). Phenotypic characteristics and substrate utilization provide the basis for speciation of methanogens (Table 1).

Characteristic blue-green fluorescence at 420 nM is utilized for tentative identification of methanogens. Methanogens are speciated primarily on the basis of their ability to produce methane from various substrates and on their cellular morphology (4). DeMacario et al. (20,21) have developed antisera for rapid identification of most characterized species.
Table 1. Determinative key to species of the methanogenic bacteria based on simple phenotypic characters.

I. Gram-positive to gram-variable rods or lancet-shaped cocci often forming chains and filaments.

<table>
<thead>
<tr>
<th>Order I. Methanobacteriales</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family I. Methanobacteriaceae</td>
</tr>
<tr>
<td>A. Slender, straight to irregularly crooked long rods often occurring in filaments.</td>
</tr>
<tr>
<td>Genus I. Methanobacterium</td>
</tr>
<tr>
<td>1. Mesophilic.</td>
</tr>
<tr>
<td>a. Methane produced from formate. Methanobacterium formicicum</td>
</tr>
<tr>
<td>b. Methane not produced from formate. Methanobacterium bryantii</td>
</tr>
<tr>
<td>2. Thermophilic. Methanobacterium thermoautotrophicum</td>
</tr>
</tbody>
</table>

B. Short rods or lancet-shaped cocci, often in pairs or chains.

<table>
<thead>
<tr>
<th>Order II. Methanobrevibacter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family II. Methanobrevibacteridae</td>
</tr>
<tr>
<td>1. Cells form short, nonmotile rods not utilizing formate. Methanobrevibacter arborophilus</td>
</tr>
<tr>
<td>2. Chain-forming, lancet-shaped cocci that produce methane from formate and require acetate as a carbon source.</td>
</tr>
<tr>
<td>a. Require CoM and D-alpha-methyl butyrate. Methanobrevibacter ruminantium</td>
</tr>
<tr>
<td>b. Do not require CoM or D-alpha-methyl butyrate. Methanobrevibacter smithii</td>
</tr>
</tbody>
</table>

II. Gram negative cells or gram-positive cocci occurring in packets.

A. Gram-negative, regular to slightly irregular cocci often forming pairs.

<table>
<thead>
<tr>
<th>Order II. Methanococcales</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family I. Methanococcaceae</td>
</tr>
<tr>
<td>Genus I. Methanococcus</td>
</tr>
<tr>
<td>1. Cells inhibited by 5% NaCl. Methanococcus vaniellii</td>
</tr>
<tr>
<td>2. Cells not inhibited by 5% NaCl. Methanococcus voltae</td>
</tr>
</tbody>
</table>

B. Gram-negative rods or highly irregular cocci occurring singly.

<table>
<thead>
<tr>
<th>Order III. Methanomicrobiales</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family II. Methanomicrobiaceae</td>
</tr>
<tr>
<td>1. Straight to slightly curved, motile, short rods. Methanomicrobiium mobile</td>
</tr>
<tr>
<td>2. Irregular coccoid cells. Genus II. Methanogenium</td>
</tr>
<tr>
<td>a. Cells require acetate. Methanogenium cariaci</td>
</tr>
<tr>
<td>b. Cells do not require acetate. Methanogenium marisnigri</td>
</tr>
<tr>
<td>3. Regularly curved, slender, motile rods often forming continuous spiral filaments.</td>
</tr>
<tr>
<td>Genus III. Methanospirillum Methanospirillum hungatii</td>
</tr>
</tbody>
</table>

C. Gram-positive cocci, usually in packets and ferment methanol, methylamine and acetate.

<table>
<thead>
<tr>
<th>Family II. Methanosarcinaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus I. Methanosarcina</td>
</tr>
<tr>
<td>Methanosarcina barkeri</td>
</tr>
</tbody>
</table>

Modified from Balch et al. (4)
MATERIALS AND METHODS

Digestors. Four-liter digestors with a working volume of 14 liters were outfitted as described by Varel et al. (69). Biogas production data was assayed for 30 weeks except for reactors amended solely with cobalt which were terminated after 15 weeks. The remaining data are for 48 weeks.

Gas impermeable bags (60 cm x 60 cm) (Pollution Measurement Corporation) equipped with a 200-AS valve, 7 cm from the bottom of the bag, were attached to the digestors for the collection of biogas. The digestors were incubated at 37°C on a rotary shaker (New Brunswick Scientific Company G-10) at 110 rpm with a 5.1 cm displacement.

Substrate. Dairy cow waste was obtained from a commercial dairy. The cattle were fed a ration of 86% Bermuda hay and 14% concentrate. The concentrate contained wheat middlings, soybean meal, peanut meal, cane molasses, ground cornmeal, cotton-seed hulls, and brewer's grain. The concentrations varied with the availability of ingredients. The dairy waste (feces and urine) was collected from concrete feeding slabs.

The waste was placed into 208-liter, plastic-lined, steel barrels and transported to the laboratory at ambient temperature. The waste was kept at 4°C, while total and volatile solids (VS) analyses were performed. After the VS analysis was completed, the waste was diluted with tap water to obtain 4.5% VS. Mixing and
grinding were accomplished with a submersible grinder pump (Peabody Barnes, Model 203) installed in a 208-liter, plastic-lined, steel barrel. The homogenized manure feed was dispensed into 3.76-liter plastic milk jugs and stored at -20°C. The frozen manure was thawed overnight at room temperature and then heated to 37°C in an autoclave (AMSCO Medallion steam sterilizer) at 100°C isothermal for 10 to 15 minutes.

**Digestor start-up and maintenance.** The digestors were innoculated with 300 ml of digestor effluent from active digestors which had been maintained for 10 months at pH 7.0 (M.L. Stafford, M.S. thesis, University of Central Florida, Orlando, 1982). The sample used for innoculation as well as the digestor were gassed with nitrogen during the collection and innoculation procedures. Feed was added to give a final working volume of 3-liters. The digestors were sealed and incubated as described previously.

A 3-day RT was established by sequentially removing one-third of the volume and feeding with 1 liter of thawed manure every 24 hours for 48 weeks. The digestors were adjusted to pH 7 during feeding by titrating an aliquot of wasted digestor effluent with 5N NaOH. The total volume of base necessary to readjust the pH was estimated. The calculated volume of 5N NaOH was added to 1 liter of manure feed and the pH was rechecked with a small aliquot of digestor fluid.

Duplicate digestors were maintained under the following parameters: Controls (no ions added), Co^{++} at 4.958 mg/liter, Fe^{++} ions at 20.60 mg/liter, and Fe^{++} and Co^{++} ions at above stated final
digestor concentrations. The ferrous and cobaltous ions were added daily to the feed substrate to maintain the respective concentrations.

**Biogas analysis.** After achieving steady-state conditions, the gas bags on each digester were changed every 48 hours. The gas volume was measured by fluid volume displacement (68). Gas samples were collected using gas sampling syringes equipped with gas-tight stopcocks (Supelco, Inc.) or sealed 100-ml serum bottles. The sealed gas serum bottles were evacuated and flushed three times with helium, then evacuated to 15 mmHg. The gas sample was taken with a double-draw needle (Becton Dickinson) inserted through the rubber septum located on an in-line T attached to the gas bag. The other end of the double-draw needle was then inserted into the evacuated sample container and allowed to equilibrate. The sample container was flushed three times using a 50-cc syringe equipped with an 18-gauge needles inserted through the serum stopper on the gas sample container. The double-draw needle was removed from the sample container. The contents of the 50-cc syringe from the last evacuation were emptied into the container, resulting in a positive pressure. The serum stopper was sealed with a thin coat of silicone sealant (General Electric Company). The gas samples were stored at 4 C.

A 1.0 ml sample of biogas was obtained from the sample containers using a Hamilton gas-tight syringe equipped with a side-port needle (Supelco, Inc.). Gas analysis was performed using a Shimadzu GC-7A gas chromatograph equipped with a thermal conductivity detector (TCD) and a Spectrophysics SP4100 computing integrator.
A stainless-steel, molecular sieve column of Carbosieve B 120/140, 6.0 m in length by 3.2 mm ID was used to determine the concentration of methane, carbon dioxide, and hydrogen. Optimum resolution was achieved with the following temperature program: 50°C isothermal for 2 minutes, temperature program from 50°C to 130°C at 32°C per minute, 130°C isothermal for 8 minutes. Other instrument parameters were as follows: injector temperature, 60°C; TCD temperature, 170°C; TCD bridge current, 150 milliamps; helium carrier gas with a primary pressure of 4.25 Kg/cm² and a flow rate of 40 ml per minute, integrator chart speed of 0.5 cm per minute, and sample size 1.0 ml.

Standards were obtained commercially from Scott, Inc. Identification of gases present in samples was done by comparison of retention times with those of the standards. The concentrations were determined by peak area integration after correlation of the peak areas with standards (45).

**Total and volatile solids analysis.** Total and volatile solids were determined on duplicate 50-gm samples according to Standard Methods for the Examination of Water and Wastewater (3).

**Volatile fatty acid analysis.** Volatile fatty acids were extracted as outlined in the Virginia Polytechnic Institute Laboratory Manual (33). Volatile fatty acids were determined with a Shimadzu GC-7A gas chromatograph equipped with dual flame ionization detectors (FID). A stainless steel column packed with SP1220, 1% H₃PO₄ on 100/120 Chromosorb W/AW (Supelco, Inc.), 6.0 m in length by 6.4 mm ID was used to resolve fatty acids containing up to seven
carbons. Optimum resolution was obtained using the following temperature program: 135°C isothermal for 5 minutes, temperature program of 130°C to 180°C at 16°C per minute, 180°C isothermal for 8 minutes. Instrument parameters were as follows: injector temperature, 240°C; nitrogen carrier gas at 4.25 Kg/cm² primary pressure with a flow rate of 20 ml per minute; FID air and hydrogen pressure 0.5 Kg/cm²; integrator chart speed, 0.5 cm per minute; and sample size 5 µl. Commercially prepared volatile fatty acid mixtures of 1 meq each, Cl to C7, (Supelco, Inc.) were used to perform a four-point calibration. Identification of volatile fatty acids present in samples was done by comparison of retention times to those of the standards. The concentrations were determined by peak area integration after correlation of the peak areas with standards. Samples containing concentrations above the standards were diluted appropriately and re-assayed.

Metal analysis. Fifty-gram samples were prepared for metal analyses by using a dry-ashing and acid-digestion procedure (Perkin-Elmer, Procedure AY-4, March, 1971). Analyses for iron and cobalt were performed on an atomic-absorption spectrophotometer (Perkin-Elmer model 306) equipped with a 20-µl autosampler. An acetylene air, flame-ionization attachment (Perkin-elmer) was utilized for cobalt determinations. A heated-graphite atomizer (Perkin-Elmer HGA 2200) was utilized for iron determinations.

Culture procedures. Anaerobic culture techniques included serum bottle and roll tube methods (42) modified by using syringes
to dispense media (11,46). All gasses used for anaerobic manipulations were mixed to proper ratios with gas proportioning meters (Air Products, Inc.). The gasses were scrubbed free of oxygen by flowing 400 to 500 ml/min through a 2" OD x 36" column of copper filings heated to 350°C. The copper column was equipped with a 1250-watt heating rod, thermistor, and temperature controller (Thermal Corp.). A gassing manifold described by Balch and Wolfe (4) and a V.P.I. anaerobic culture system (Bellco, Inc.) were utilized to manipulate cultures. Flow rates through the canullas and needles were controlled by valves on the gassing manifold.

Samples were obtained from individual digestors and were serially diluted in 30-ml sealed serum bottles containing pre-reduced media. Syringes flushed three times with oxygen-free nitrogen were used for all transfers and inoculations. The diluted cultures were inoculated into 118 x 150-mm serum tubes containing melted, prerduced, sterile media and maintained in a 44.5°C waterbath. Reducing solution and an antibiotics solution were added after inoculation. The tubes were then placed in a tube spinner (Bellco, Inc.) and allowed to solidify while spinning. The resultant pour-roll tubes were gassed with 2.0 atm of 80% H₂/20% CO₂ using the gassing manifold. All cultures were incubated at 37°C.

Methanogenic colonies were tentatively identified and enumerated by observing the characteristic blue-green fluorescence from irradiation with long-wave, UV light (23,48). Fluorescing colonies
were picked off and innoculated into enrichment and differential media for further identification. Head space from pure cultures was analyzed for methane as previously described to confirm the presence of methanogens.

**Culture media.** The media of Balch et al. (4) was modified by using the trace vitamin solution of deMacario et al. (21) for growth of methanogens. Dilution and enumeration media incorporated penicillin G and D-cycloserine (37,88) to inhibit the growth of non-methanogens.

**Statistical analysis.** One-way analysis of variance (p = 0.05) was used to test significance of data. All statistical calculations were performed on an Apple II+ microcomputer equipped with Interstat software (Serendipity Systems, Inc.).
RESULTS

Microscopic examinations. Direct microscopic examination of the various digester effluents, employing ultraviolet, epi-fluorescence and 400X magnification, revealed a predominance of small, fluorescent nonmotile rods. Low numbers of Methanosarcina-like clumps were observed in all digestors. Small, motile, fluorescent rods and spiral rods were observed in one of the control digestors.

Iron and cobalt concentrations. The concentration of iron in the substrate ranged from 90.4 to 162.5 mg per liter. The concentration of cobalt in the substrate ranged from 2.7 to 6.4 mg per liter.

Effect of cobalt and iron on biogas and methane production. During the first 90 days, digestors supplemented with Fe$^{++}$ (20.64 mg/liter) produced significantly more biogas and methane than the digestors that were not supplemented with Fe$^{++}$ ($p = 0.05$) (Figs. 6,7). There were no significant differences ($p = 0.05$) in the amount of biogas produced in digestors supplemented solely with Co$^{++}$ (4.958 mg/liter) and digestors which were not supplemented with Co$^{++}$ during the first 90 days (Fig. 8).

After the first 90 days, the highest levels of biogas and methane were produced in the digestor supplemented only with Fe$^{++}$ (Table 2). Compared to the controls, digestors supplemented with Co$^{++}$ or with both Fe$^{++}$ and Co$^{++}$ did not vary significantly ($p = 0.05$)
Fig. 6. The effect of addition of iron to dairy manure digestors on biogas production, day 6 through 90. Symbols: (---) no iron added
(○○) 20.64mg/l Fe<sup>++</sup> added.
Fig. 7. The effect of addition of iron and cobalt to dairy manure digestors on biogas production, day 6 through 90.
Symbols: (●●) no iron or cobalt added
(■■) 20.64mg/l Fe^{++} and 4.958mg/l Co^{++} added.
Fig. 8. The effect of addition of cobalt to dairy manure digestors on biogas production, day 6 through 90.
Symbols: (••) no cobalt added
(○○) 4.958mg/l Co^{++} added.
TABLE 2. Biogas and methane produced from fermentation of dairy manure with 4.5% VS concentration at pH 7.0, 37°C, and a 3-day retention time.

<table>
<thead>
<tr>
<th>Digestor</th>
<th>Biogas liter/liter of digestor per day</th>
<th>Biogas 95% C.I. for X</th>
<th>Methane liter/liter of digestor per day</th>
<th>Methane 95% C.I. for X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.59 ± 0.27a</td>
<td>(3.51 - 3.66)</td>
<td>1.85 ± 0.17d</td>
<td>(1.81 - 1.90)</td>
</tr>
<tr>
<td>Cobalt (4.958mg/l)</td>
<td>3.58 ± 0.32b</td>
<td>(3.43 - 3.73)</td>
<td>1.80 ± 0.19b</td>
<td>(1.73 - 1.87)</td>
</tr>
<tr>
<td>Iron (20.64mg/l)</td>
<td>*3.88 ± 0.26a</td>
<td>(3.81 - 3.96)</td>
<td>*2.03 ± 0.14d</td>
<td>(1.99 - 2.08)</td>
</tr>
<tr>
<td>Iron/Cobalt (20.64mg/l; 4.958mg/l)</td>
<td>3.74 ± 0.41c</td>
<td>(3.66 - 3.81)</td>
<td>1.94 ± 0.15e</td>
<td>(1.90 - 1.99)</td>
</tr>
</tbody>
</table>

*a* The data represent the average of 65 paired samples.

*b* The data represent the average of 18 paired samples.

*c* The data represent the average of 63 paired samples.

*d* The data represent the average of 46 paired samples.

*e* The data represent the average of 48 paired samples.

*Significant at p = 0.05.*
in biogas or methane production (Table 2). The control digestors produced 3.59 ± 0.26 liters/liter/day of biogas and 1.85 ± 0.17 liters/liter/day of methane. The highest yields of biogas and methane (3.88 ± 0.26 and 2.03 ± 0.14 liters/liter/day, respectively) were produced in digestors supplemented only with Fe++. There were no significant differences in percent methane in the biogas of any supplemented reactor when compared to the controls (Table 3). All digestors stabilized after 7 weeks as evidenced by the leveling of biogas, methane production (Figs. 9-12), and acetate concentrations (Figs. 13-16).

Effect of cobalt and iron on volatile fatty acid concentrations. Neither the acetic nor propionic acid concentrations of the Fe++, Co++, and Fe++/Co++ supplemented digestors varied significantly (p = 0.05) from the control digestors (Table 4). Higher levels of acetate, propionate, isobutyrate, isovalerate, and valerate were present in all digestors (Table 4) when compared with dairy waste digestors maintained at thermophilic conditions and 3-day RT (69). The levels of propionate (51 to 62 mM) were high throughout the study (Figs. 13-16).

Efficiency of fermentation. The VS destruction did not vary significantly (p = 0.05) between any of the digestors (Table 3). The control, Fe++, and Fe++/Co++ supplemented digestors had 26.5, 26.7, and 27.2% VS destruction, respectively. A lower VS destruction of 22.9% VS was observed in the Co++ supplemented digestors. Percentages of VS destruction are lower than in the 40 to 60% VS
TABLE 3. Efficiency of methane fermentation of dairy manure with 4.5% VS concentration at pH 7.0, 37°C, and a 3-day retention time.

<table>
<thead>
<tr>
<th>Digestor</th>
<th>%TS W/V</th>
<th>%VS W/V</th>
<th>%VS Destroyed</th>
<th>liters CH₄/g VS Destroyed</th>
<th>%CH₄ in Gas Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>5.60 ± 0.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.49 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Control</td>
<td>4.32 ± 0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.30 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.5 ± 1.3</td>
<td>.177 ± .051&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.0 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cobalt (4.958mg/l)</td>
<td>4.73 ± 0.55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.46 ± 0.42&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22.9 ± 1.8</td>
<td>.163 ± .037&lt;sup&gt;e&lt;/sup&gt;</td>
<td>48.2 ± 4.5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iron (20.64mg/l)</td>
<td>4.31 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.29 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.7 ± 1.4</td>
<td>.193 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.2 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iron/Cobalt (20.64mg/l; 4.958mg/l)</td>
<td>4.28 ± 0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.27 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.2 ± 1.2</td>
<td>.194 ± 0.60&lt;sup&gt;f&lt;/sup&gt;</td>
<td>51.8 ± 2.1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>The data represent the average of 50 samples ± standard error.
<sup>b</sup>The data represent the average of 32 samples ± standard error.
<sup>c</sup>The data represent the average of 36 samples ± standard error.
<sup>d</sup>The data represent the average of 22 samples ± standard error.
<sup>e</sup>The data represent the average of 16 samples ± standard error.
<sup>f</sup>The data represent the average of 42 samples ± standard error.
Fig. 9. Methane and biogas production from control digestors, week 1 through 30. Symbols: (●●) biogas; (○○) methane.
Fig. 10. Methane and biogas production from cobalt-amended digestors (4.958 mg/l Co^{++}), week 1 through 15. Symbols: (●) biogas; (○) methane.
Fig. 11. Methane and biogas production from iron-amended digestors (20.64mg/l Fe^{++}), week 1 through 30. Symbols: (●) biogas; (○) methane.
Fig. 12. Methane and biogas production from iron and cobalt-amended digestors (20.64mg/l Fe\(^{++}\); 4.958mg/l Co\(^{++}\)), week 1 through 30.

Symbols: (●●) biogas; (○○) methane.
Fig. 13. Acetate and propionate levels in control digestors, week 1 through 48.
Symbols: (●–●) acetate; (○–○) propionate.
Fig. 14. Acetate and propionate levels in cobalt-amended digestors (4.958mg/1 Co^{++}), week 1 through 15.
Symbols: (●●) acetate; (○○) propionate.
Fig. 15. Acetate and propionate levels in iron-amended digestors (20.64mg/l Fe++, week 1 through 48.
Symbols: (---) acetate; (-----) propionate.
Fig. 16. Acetate and propionate levels in iron and cobalt-amended digestors (20.64mg/l Fe++; 4.958mg/l Co++), week 1 through 48.
Symbols: (•••) acetate; (○○○) propionate.
TABLE 4. Concentration of volatile fatty acids\(^a\) after methane fermentation of dairy manure with 4.5% VS at pH 7.0, 37°C, and a 3-day retention time.

<table>
<thead>
<tr>
<th>Digestor</th>
<th>Acetic mM</th>
<th>Propionic mM</th>
<th>Isobutyric mM</th>
<th>Butyric mM</th>
<th>Isovaleric mM</th>
<th>Valeric mM</th>
<th>Total Fatty Acid (Acetate) mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^b)</td>
<td>6.7 ± 4.9</td>
<td>59.9 ± 25.5</td>
<td>1.9 ± 1.1</td>
<td>2.0 ± 3.0</td>
<td>2.3 ± 1.0</td>
<td>1.5 ± 0.9</td>
<td>4458</td>
</tr>
<tr>
<td>Cobalt(^c) (4.958mg/l)</td>
<td>9.1 ± 4.5</td>
<td>54.7 ± 10.8</td>
<td>2.4 ± 0.9</td>
<td>3.2 ± 2.0</td>
<td>2.4 ± 0.9</td>
<td>2.0 ± 0.6</td>
<td>4428</td>
</tr>
<tr>
<td>Iron(^b) (20.64mg/l)</td>
<td>4.8 ± 3.2</td>
<td>51.0 ± 29.2</td>
<td>1.6 ± 1.2</td>
<td>1</td>
<td>2.2 ± 1.5</td>
<td>1.4 ± 0.8</td>
<td>3690</td>
</tr>
<tr>
<td>Iron/Cobalt(^d) (20.64mg/l; 4.958mg/l)</td>
<td>9.7 ± 5.8</td>
<td>61.5 ± 27.0</td>
<td>2.1 ± 1.2</td>
<td>2.1 ± 1.2</td>
<td>2.3 ± 1.4</td>
<td>1.7 ± 0.8</td>
<td>4758</td>
</tr>
<tr>
<td>Substrate(^e)</td>
<td>54.4 ± 11.3</td>
<td>11.5 ± 3.3</td>
<td>1</td>
<td>4.7 ± 1.3</td>
<td>1</td>
<td>1</td>
<td>4296</td>
</tr>
</tbody>
</table>

\(^a\) Isocaproic and caproic acids were not detected with a sensitivity of 1 mM.

\(^b\) The data represent the average of 30 samples ± standard error.

\(^c\) The data represent the average of 8 samples ± standard error.

\(^d\) The data represent the average of 28 samples ± standard error.

\(^e\) The data represent the average of 26 samples ± standard error.
destruction reported by Varel et al. (68,69).

The efficiency of methane fermentation, expressed as liters of methane per gm VS destroyed, did not vary significantly (p = 0.05) between any of the digestors (Table 3). The efficiencies are lower than those reported when compared to thermophilic dairy waste fermentations with 3-day RT (69). The percentages of methane (48 to 52) in the biogas were comparable with the 52 to 57% demonstrated in 3-day RT thermophilic dairy waste fermentations (69).

Effect of cobalt and iron on methanogen populations. The numbers of methanogens did not vary significantly (p = 0.05) between any of the digestors (Table 5). The concentration of methanogens ranged from $1.9 \times 10^7$ to $8.0 \times 10^8$ CFUs per ml. The numbers of methanogens are comparable to the levels reported in the literature (25,34,35,58,59).

Methanogens isolated from the Fe$^{++}$ and Fe$^{++}$/Co$^{++}$ supplemented digestors were Methanobrevibacter smithii and M. formicicum. M. smithii was the only methanogen isolated from the control digester. Direct microscopic observation revealed other fluorescent, morphological types. However, these types were not isolated.

M. smithii was the predominant species present in all digestors (Table 6). The numbers of M. smithii were $2.95 \times 10^8$ CFUs per ml in the control digestor, $2.1 \times 10^8$ CFUs in the Fe$^{++}$ reactor, and $1.5 \times 10^8$ CFUs per ml in the Fe$^{++}$/Co$^{++}$ supplemented digestors. M. formicicum was not isolated from the control digestor.
**TABLE 5. Methanogen populations of digestors fermenting dairy manure with 4.5% VS concentration at pH 7.0, 37°C, and a 3-day retention time.**

<table>
<thead>
<tr>
<th>Digestor</th>
<th>Control</th>
<th>Iron (20.64mg/l)</th>
<th>Iron/Cobalt (20.64mg/l; 4.958mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Date</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/15(^b)</td>
<td>1/17(^b)</td>
<td>2/02(^b)</td>
</tr>
<tr>
<td>Control</td>
<td>30.0</td>
<td>18.5</td>
<td>12.0</td>
</tr>
<tr>
<td>Iron</td>
<td>80.0</td>
<td>31.0</td>
<td>18.5</td>
</tr>
<tr>
<td>Iron/Cobalt</td>
<td>12.0</td>
<td>20.5</td>
<td>9.5</td>
</tr>
</tbody>
</table>

\(^a\)Blue-green fluorescence from irradiation with UV light at 420nm.

\(^b\)The data represent the average of duplicate samples.

\(^c\)The data represent the average of triplicate samples.

\(^d\)The data represent the average of quadruplicate samples.
TABLE 6. Methanogens isolated from digestors fermenting dairy manure with 4.5% VS concentration at pH 7.0, 37°C, and a 3-day retention time.

<table>
<thead>
<tr>
<th>Digester</th>
<th>Methanobrevibacter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Methanobacterium&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>smithii</td>
<td>formicicum</td>
</tr>
<tr>
<td>Control</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>Iron (20.64mg/l)</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Iron/Cobalt (20.64mg/l; 4.958mg/l)</td>
<td>32</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>The data represent the numbers of methanogens isolated from seven separate sample dates (1/15, 1/17, 2/02, 2/14, 2/19, 2/29, 3/07).
DISCUSSION

This study indicates that iron (20.64 mg/liter) stimulates biogas and methane production when added to 37°C dairy waste digestors with 3-day RT. Iron stimulation has been reported previously (16,30,31, 38,44,49,51,61,64). Iron has been determined to affect methanogenesis by stimulating the conversion of volatile acids to methane (61).

Significant decreases in volatile acids with concurrent increases in methane from iron-amended digestors were not observed. Iron-amended digestors produced more methane, but did not convert greater amounts of VS when compared to the control. The low VS content of the substrate made it difficult to detect significant VS conversion.

Variation in the efficiency of fermentation, due to substrate inconsistency, is magnified when the VS concentration is lowered. The substrate, containing dairy cattle manure, urine, straw and sand, was ground and diluted to 4.5% VS in 208-liter drums. The diluted substrate tended to rapidly form gradients due to settling of sand and floating plant matter. The settling out was partially compensated for by stirring before feeding and sampling for gravimetric determinations. However, inconsistencies in the samples were still evident.

Lower percent VS destruction rates are observed at low influent VS concentrations, at shorter RTs (30,68,69), and at mesophilic
temperatures (68). This study was conducted under all three conditions. The low percent VS destruction resulted in low biogas production. Variability of biogas and methane production increases with use of low-percent VS feedstock.

All digesters required long start-up periods of 7 weeks (14 Rts) to stabilize which indicates stressed conditions. Stabilization of nonstressed digestors should occur within 3 RTs (69). The pH was maintained during start-up by the addition of excessive amounts (10 to 30 mls) of 5N NaOH daily. Indications of stressed digestors were still present after steady-state conditions were established. VFA concentrations were above the 2000 mg/liter that is considered inhibitory to methanogenesis (15,55,57). Acetic and propionic acid concentrations were especially high as well as percent carbon dioxide in the biogas. The VS destruction rates were low. Digester failure did not occur as a result of these stressed conditions.

Cobalt (4.958 mg/liter) was not found to be stimulatory to methanogenesis and may have been inhibitory. The cobalt-only amended digestors demonstrated 22.9 ± 1.8% VS destruction, whereas 26.5 ± 1.3% of the VS were destroyed in the control digestors. Significantly higher (p = 0.05) amounts of biogas and methane were produced in digestors amended only with iron. Stimulation was not evident in the iron/cobalt-amended digestors. This suggests that cobalt may interfere with the effect of iron on methanogenesis. Pilot studies performed in this laboratory and results reported elsewhere contradict this finding (44,51,56,62).
Jarrell and Sprott (36) have reported that cobalt inhibits the active transport of nickel across the membrane of *M. bryantii*. Nickel is an essential nutrient of methanogens (16,22,36,39,51,52,56,62). It is possible that the cobalt levels (amount present in the substrate plus the supplemental cobalt) may have reached concentrations that prevent transport of nickel in methanogens.

The addition of nickel to anaerobic digestors has been associated with the predominance of *Methanosarcina* sp. (62). These studies indicate addition of iron may also affect the methanogenic flora (Table 5). *M. formicicum* was isolated from all iron-amended digestors but not from the control digestors. Addition of iron to anaerobic digestors has been determined to enhance methanogenesis by precipitating sulfides (49,56,64), stimulating substrate production (31,61), and decreasing the O/R potential as FeS (49). Sulfide concentrations that inhibit methanogenesis in mixed fermentations range from 0.003 to 12.5 mM (40,50,76). Pure cultures of *Methanococcus* sp. vary greatly in their sensitivity to sulfide (39). It is suggested that precipitation of sulfide to low levels by iron may favor growth of sulfide-sensitive methanogens.

Additional studies are required to determine optimal effects of metals on methane fermentation of dairy manure. The effects of iron and cobalt are dependent on a wide variety of environmental factors such as pH, VS concentration, VFA levels, RT, and feed ration. Concentrations of sulfate, sulfide, carbonate, phosphate, other trace metals, and the consortium of microorganisms are also interrelated.
These variables and the physiology of the methanogens involved need to be investigated in greater detail before optimal conditions for methane fermentations can be predicted.
LITERATURE CITED


