Resource Recovery and Epidemiology of Anaerobic Wastewater Treatment Process in a Controlled Ecological Life Support System

FINAL REPORT
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by

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ABSTRACT

Three four-liter packed-bed anaerobic digesters were designed and fabricated to study the resource recovery and epidemiology of a pathogenic bacterial species in a controlled ecological life support system (CELSS). The digesters were operated at 35 ± 2 °C, pH around 7, and hydraulic retention times (HRT) of 20, 10 and 5 days. Polypropylene pall rings with 5/8" size were used as the packing material to immobilize the anaerobic bacteria. Anaerobic seeding from a local municipal wastewater plant was used for acclimation and study. Simulated wastewater was used as the feeding solution. It was prepared following the formulation specified by NASA-JSC (Johnson Space Center) and consisted of shower water, clothwash water, dishwash water, handwash water, and urine flush water. This wastewater had an initial chemical oxygen demand (COD) of 2400 mg/l and total organic carbon (TOC) of 550 mg/l. Under steady-state operation, COD, TOC, pH, total nitrogen (N), total phosphorus (P), and potassium (K) were monitored in the digester input and output solutions. Additionally, the volume and the CH_4/CO_2 mole ratio of the biogas produced from the anaerobic digesters were measured. The results of mass balance indicate about 90% of TOC was converted while only 5 to 8% of N-P-K was consumed in the digesters. This digested solution containing high contents of inorganic nutrients could be used as a good nutrient for plant growth. The maximum organic loading capacity was not reached even at the shortest hydraulic retention time (5 day HRT). This implies that there is a possibility of increasing the organic loading rate to these three anaerobic digesters. To accomplish this, an automatic feeding and sampling system must be used.

A multi-drug resistant strain of *Salmonella choleraesuis* was used as the indicator bacteria in the epidemiology study. This strain is resistant to chloramphenicol, tetracycline, streptomycin, and sulfanilamide. It is not known to be part of the indigenous flora of animals nor is it known to occur in nature. The levels of *Salmonella choleraesuis* in the influent and effluent were determined, biogas productin and pH were measured, and decimal decay rate constants were estimated. The study showed initial rapid declines in viable numbers within 2 to 4 days. During continuous digestion at 10 and 5 d HRT and batch digestion, the period of rapid declines were followed by an equilibrium in which bacteria were maintained at 10 - 10^2 CFU/ml while no detectable residual bacteria population was found at 20 d HRT. *Salmonella choleraesuis* survived at least 15 days from inoculation for 10 and 5 d HRT during continuous and batch digestion, but less than 6 days for 20 d HRT. At 20 d HRT, the indicator bacteria must compete with the anaerobic acetogens and methanogens to use the limited organic nutrient. While at 10 and 5 days HRT, the organic nutrient levels are sufficient for both anaerobic and indicator bacteria. The *Salmonella choleraesuis*, therefore, survived and reached an equilibrium population for at least 15 days.

The decimal decay rate constants, k_d, of *Salmonella choleraesuis* were estimated for batch anaerobic digestion, three single-dose continuous anaerobic digestion, and eight multi-dose continuous anaerobic digestion. Determination of the decimal decay rate constant was based on the following equation,

$$k_d = -\frac{1}{t} \ln\left(\frac{P}{P_o}\right) - \frac{v}{V}$$

where, P is the colony count of indicator bacteria in the withdrawn effluent, P_O is the initial viable count in the digester (CFU/ml), v is the liquid volume of the effluent removed per day (ml/day), V is the liquid volume in the digester (ml), and t is the time interval (day). For a batch digestion system, the flow rate, v, is set to zero. The decimal decay rate (k_d) for the single-dose continuous digestion and batch digestion studies were determined from the slope of a plot of (P/P_0) versus t on a semi-logarithmic scale. Linear regression methods were used to estimate the slope and only those data within 4 days were used. The k_d values were greater at higher initial doses than lower doses for the same HRT, and greater for batch digestion (7.89 day^{-1}) than for continuous digestion (4.28, 3.82 and 3.82 day^{-1} for 20, 10, and 5 day HRT, respectively). No significant difference in k_d values was found among these three HRT.

I. FORWARD

This report documents the results of work accomplished under two different areas:

1. Resource Recovery of an Anaerobic Wastewater Treatment Process , and

2. Epidemiological Study of an Anaerobic Wastewater Treatment Process.

The first part of the work was to set up and test three anaerobic digesters and then run these three digesters with a NASA-simulated wastewater. The second part of the work was to use a multi-drug resistant strain of *Salmonella choleraesuis* as the indicator bacteria for the epidemiological study. Details of these two parts can be found in two master's theses [Cao, 1995; Fu, 1995] and are described in Sections III and IV of this report.

Several important results condensed from these two parts are summarized in the next section (Section II).

II. RESOURCE RECOVERY AND EPIDEMIOLOGY IN AN ANAEROBIC WASTE WATER TREATMENT PROCESS - CONDENSED RESULTS

II.1 INTRODUCTION

Currently, spacecraft life support systems are simple and sufficiently reliable for human space-flight missions of relatively short duration with small crew sizes and limited power availability. However, life support technologies for the coming era of exploration must address longer-duration missions in which humans require substantial amounts of consumable materials to sustain life for long periods of time. If these consumable materials must be provided by re-supply flights from Earth, a substantial logistics infrastructure is required. Consequently, supplying all these consumables from Earth is an extremely expensive proposition. As a result, one of the most important challenges associated with longer-duration manned space flights is the development of a Controlled Ecological Life Support System (CELSS). This includes the technologies of air revitalization, water recovery, waste processing, food production, and food processing, all of which are logistically and economically essential for the resource recovery in a CELSS [Flyn, 1992; Henninger, 1993; Petrie, 1991; Schwartzkopf, 1992].

The major elements in a CELSS are carbon (C), hydrogen (H), oxygen (O), and nitrogen (N); and the minor elements are phosphorus (P), potassium (K), sodium (Na), calcium (Ca), etc. A simplified element flow diagram of a CELSS is presented in Figure II-1. This figure illustrates the fundamental flow of major elements through the system. In this example, crop plants are used to produce food for the crew. In addition to serving as the food production subsystem, the plants take up CO_2 produced by the crew, produce oxygen for the crew to breathe and for oxidation of waste materials, and produce water vapor that can be condensed and collected to supply the crew's drinking and hygiene water. In the food processing subsystem, the foodstuffs produced by the crop plants are converted to a form palatable to the crew. Urine and feces, miscellaneous solid wastes, and waste biomass from the food processing subsystem are treated first in the wastewater treatment subsystem and then supplied to the plant growth chamber. The water out from the plant growth chamber is then further treated by an advanced water treatment subsystem. Any pure water produced from the advanced water treatment subsystem or from the condensate is supplied to the crew chamber.

Technologies for wastewater treatment include physical, chemical and biological methods. In general it is believed that a hybrid treatment system performs better than a single system. A combination process of an anaerobic digester with a plant growth chamber, and an advanced water treatment unit was proposed for resource recovery and epidemiological study in a CELLS [Li and Hunt, 1995]. A conceptual flow diagram of this process is shown in Figure II-2. Advantages for anaerobic bio-process are 1) methane gas is produced, 2) less biomass is generated than in an aerobic process, and 3) the effluent is rich in inorganic nutrients for plant growth. Combining plant-growth with anaerobic bacterial systems provides distinct advantages. For example, the efficiency of removal of ammonium and nitrate nitrogen

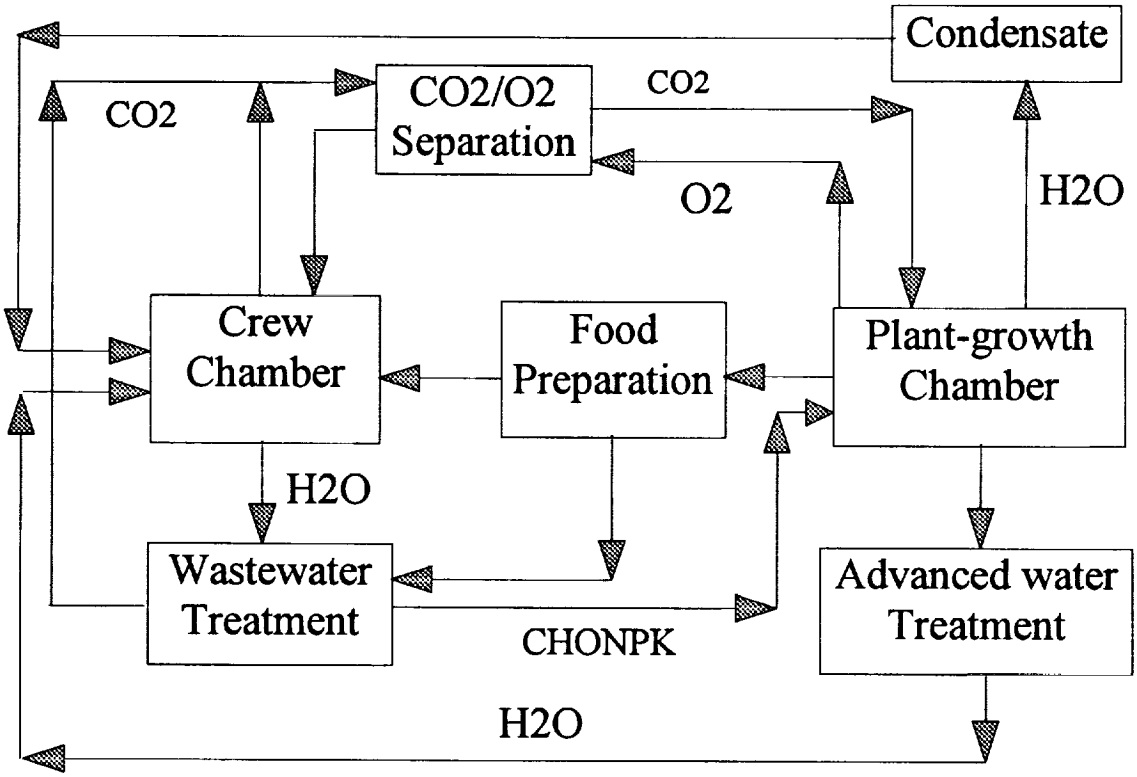

Figure II-1. A simplified element flow-diagram of a CELSS.

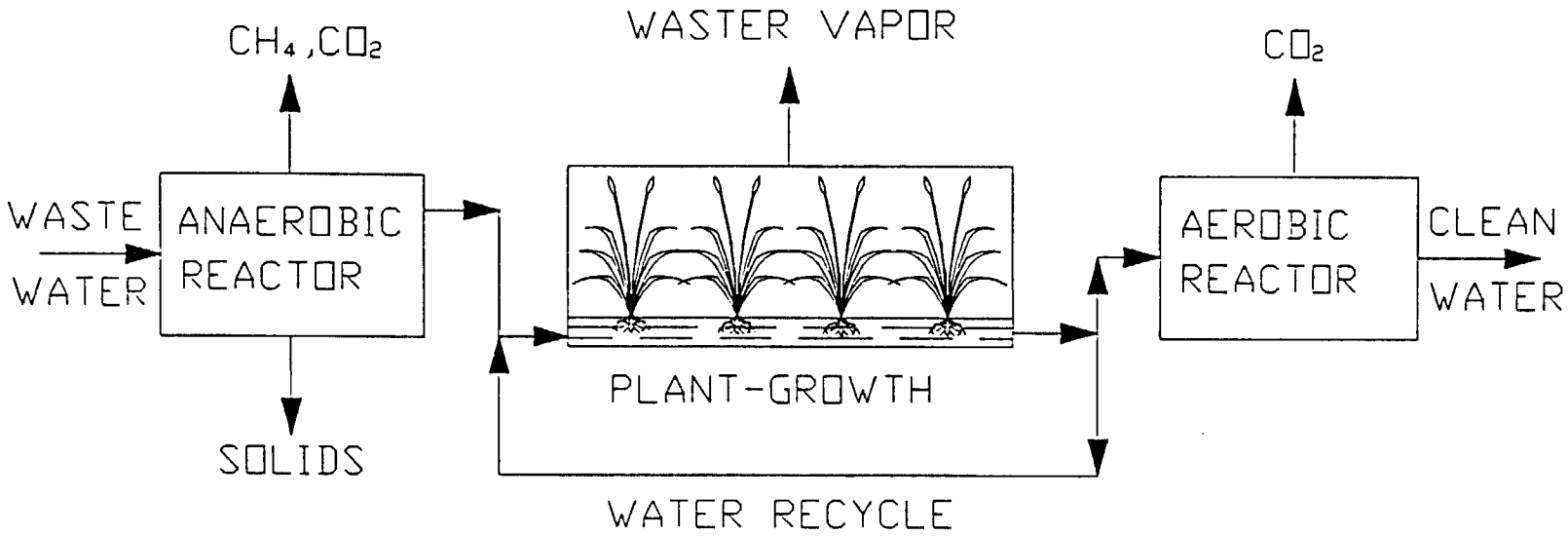

Figure II-2. Flow-diagram of an anaerobic/plant-growth/aerobic process

can be increased during plant growth when compared to bacterial systems without plant growth [Wolverton et al ; 1983].

Wastewater containing human feces can present biological hazards when the intestinal flora consists of pathogenic species of bacteria belonging to genera such as *Salmonella*, *Shigella*, and *Vibrio*. They can cause illness if given the proper environmental conditions that enhance their growth and their transmission. Thus, the risk of transmission of infectious diseases must be a consideration in the treatment of wastewater. In this study, three 4-liter packed-bed anaerobic reactors were build and used to test the resource recovery and epidemiology. Information obtained from this study will be very helpful for the development of a hybrid wastewater treatment system in combining an anaerobic process with a plant growth chamber.

II.2 EXPERIMENT

The experimental setup of each digester is shown in Figure II-3. The digester is a 4-liter glass reaction kettle (ACE Glass 6505) packed with 5/8" size of polypropylene pall rings. Two perforated plexiglass with 1/8" thickness plates were used to hold the packing material inside the reactor. Characteristics of the packing materials are listed in Table II-1.

Table II-1. Characteristics of Pall rings.

material	polypropylene
size	16 x 16 mm
surface area	0.00342 m^2/g
specific area	3.412 cm^{-1}
porosity	0.877

A magnetic drive pump was used to circulate the solution and to keep the liquid phase uniform after the injection of the feed solution. The reactor was placed in a 35°C incubator to obtain a mesophilic condition. Biogas was collected by a gas collection system which also can be seen from Figure II-3. The ratio of CH_4/CO_2 in the biogas was measured by gas chromatography (GOW-MAC 350) with a thermal conductivity detector using a 6 feet Haysep-Q packed column. The total organic carbon (TOC) in the aqueous solution was determined by a TOC analyzer (TOC-5000, Shimadza Scientific Instrument, Inc.). The chemical oxygen demand (COD), total nitrogen (N), total phosphorus (P), and potassium (K) were detected by using a HACH spectrophotometer (Model 2000).

The inorganic nutrients used in this study are listed in Table II-2. This formula supply

the necessary major and micro nutrients, reducing agents to remove the oxygen in the solution, and buffer solution. All of the chemicals used in this study were ordered from Fisher Scientific and Aldrich with ACS grade chemical pure. The simulated wastewater consisted of clothwash water, dishwash water, handwash water, shower water, fresh urine, and urine flush. The formula of the simulated wastewater was specified by NASA-JSC and is shown in Table II-3. Deionized water was used to prepare the simulated wastewater which was prepared freshly prior to each feeding. The soap used in this experiment was provided by NASA-JSC.

Table II. 2. List of inorganic nutrients

chemicals	concentration (mg/ℓ)
$CaCl_2.H_2O$	6.25
$NaPO_3$	0.25
$(NH_4)_2HPO_4$	96.50
L-cystein	2.50
$CoCl_2.6H_2O$	10.00
$FeCl_2.4H_2O$	20.00
$Na_2S.9H_2O$	75.00
$MgCl_2.6H_2O$	266.75
NH_4Cl	369.00
KCl	100.00
KI	.63
$NaHCO_3$	pH adjustment

A multi-drug resistant strain (RS) of *Salmonella choleraesuis*, subspecies: *choleraesuis*, serotype: *typhi*, antigenic formula: 9, 12, Vi:d was obtained from American Type Culture Collection (ATCC No. 19214) and was used as the indicator bacteria in this study. This strain is resistant to chloramphenicol, tetracycline, streptomycin, and sulfanilamide. It is not known to be part of the indigenous flora of animals nor is it known to occur in nature. A wild strain of *Salmonella choleraesuis*, non-resistant to the above antibiotics (NRS), was utilized in the experiment as the control. It was routinely streaked on the antibiotic-

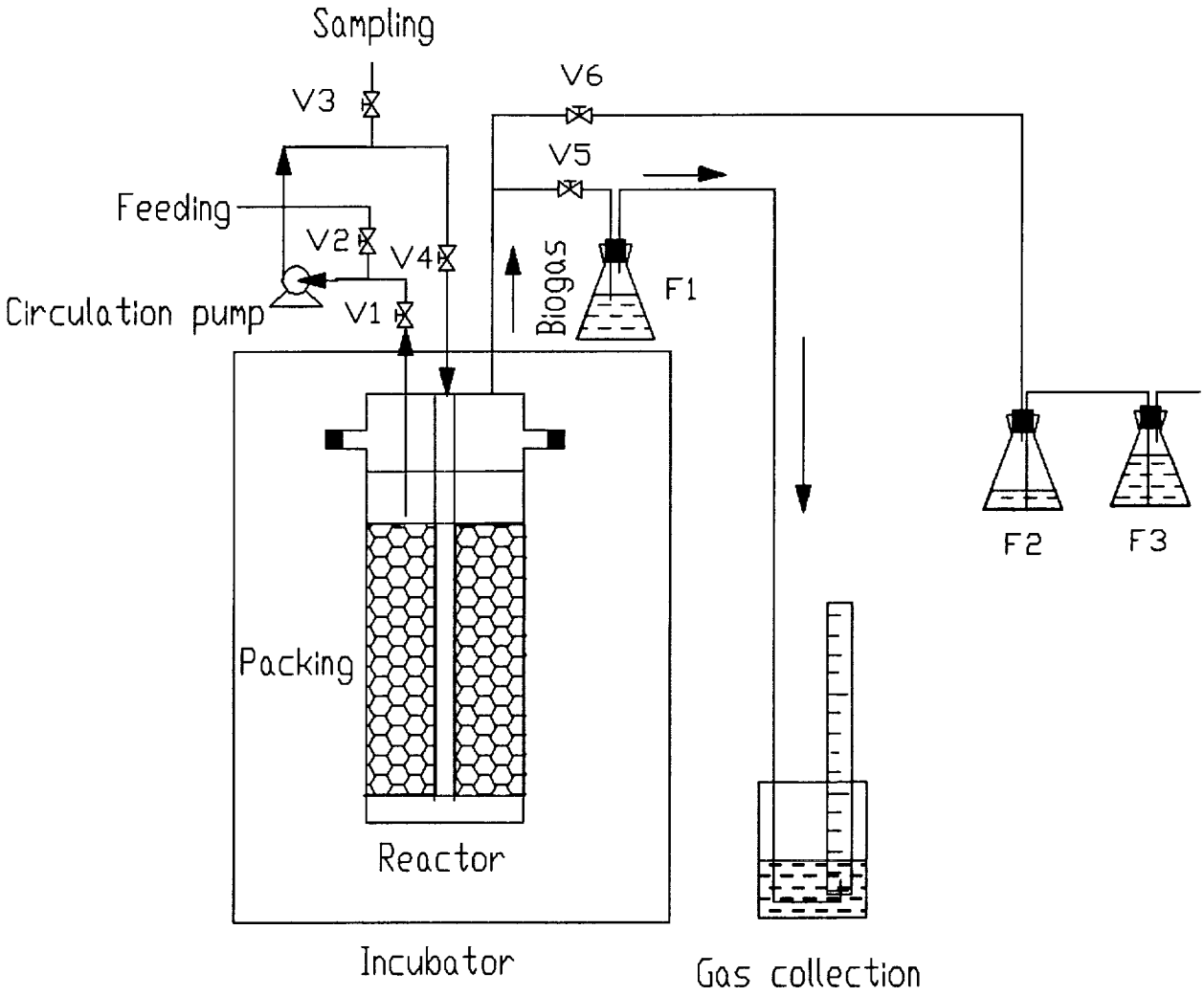

Figure II-3. Experimental setup of an anaerobic digester.

TableII-3. Formulation of NASA simulated wastewater.

Item	ℓ/person/ day
shower water (4 uses /d, 12 g soap/use)	5.32
hand wash (16 uses/d, 2g soap/use)	4.07
clothes wash (30 g soap)	12.44
urine (16 uses/d)	1.51
urine flush	0.49
dish wash	9.07
total	32.90

Nutrient agar and nutrient broth (Fisher, Pittsburgh) were used as growth and storage media for the RS and NRS bacteria. MacConkey agar CS (Difco, Detroit) was used as a bacterial quantification medium. It was supplemented, after sterilization, with chloramphenicol, streptomycin, tetracycline, and sulfanilamide (Sigma, St. Louis) at concentrations that inhibited growth of NRS but not the RS bacteria. This medium was therefore referred to as MacConkey-antibiotic agar.

The experiment started by immobilization and acclimation of the bacteria in the digester. Seeding solution (3.5 liter) was obtained from a local municipal wastewater treatment plant and was added to each anaerobic digester. Initially, local municipal wastewater was used as the feed solution. However, after biogas production was observed, the feed solution was changed gradually to NASA-simulated wastewater. No indicator bacteria were isolated on the MacConkey-antibiotic plate from seeding or simulated wastewater. However, *Pseudomonas aeruginosa* from the seeding solution was detected. Experimentation was begun when the digesters achieved a steady-state of gas production and pH.

A volume of 350 ml of the digester solution was withdrawn from the anaerobic digesters after steady-state had been achieved. Afterward, 3.5 ml of bacterial suspension was injected into the digesters through the rubber tube of the input valve. Following injection, the bacterial suspension was flushed with 346.5 ml of simulated wastewater. This withdrawn-and-fed was done at an interval determined by the hydraulic retention time (HRT). The

Salmonella population in the digester was measured from the 350 ml withdrawn solution. For the decay study of *Salmonella* bacteria in a batch digester, five ml of solution was withdrawn from the batch digester after 3, 6, 12, and 24 hours for pH measurements and colony counts. The excess solution was re-injected into the digester after each measurement.

Viable counts of the indicator bacteria were determined by preparing 10-fold serial dilutions of the effluent in 0.1% peptone water. One-tenth ml volumes of the dilutions were spread with sterile glass L-rods over the MacConkey-antibiotic agar plates. Colony counts (CFU/ml) were determined after 24 hours of incubation at 35°C [Greenberg, 1992]. The biofilm on the pall rings was examined at the conclusion of the study for the presence of attached indicator bacteria. One pall ring was selected and was mixed with peptone water with the use of a votex mixer. The mixed suspension was then streaked on a MacConkey-antibiotic plate, and the suspect colonies were identified as indicator bacteria by the Crystal Identification System after 24 hours incubation.

II.3 RESULTS AND DISCUSSION

II.3.1 Resource Recovery

The performance of the anaerobic digester may be seen from Table II-4. At the steady state operating condition, the TOC removal percent (87.13 ± 0.03, 89.01 ± 0.03, and 88.78 ± 0.02 for 20, 10, and 5 d HRT, respectively) seemed independent from the HRT. This result is consistent with the biogas produced from the digester. However, the COD removal percent (81.92 ± 5.25, 74.61 ± 8.67, and 68.18 ± 9.90 for 20, 10, and 5 d HRT, respectively) decreased as the HRT decreased as shown in the table. The standard deviation of COD values was quite high compared with that of TOC values. With this large standard deviation of COD, it is not possible to conclude that the decrease of COD with HRT is significant.

Mass balances of carbon for 10 days HRT are shown in Table II-5. The TOC in the influent has four fates: 1) converted into IC (inorganic carbons) in the effluent, 2) converted into biogas, 3) adsorbed by the microbial cells , and 4) left as the residue TOC in the effluent. The amount of carbon used in the biosynthesis of microbial cells may be too small to be considered in the mass balance. When the amount in the fates 1, 2, and 4 were measured, the amount of carbon adsorbed by the microbial cells could be estimated from the overall carbon mass balance. The results, as shown in Table II-5, indicate that 40% of the influent TOC was converted into IC, 25% into biogas, 25% was adsorbed, and 10% was left as TOC in the effluent. The amount of carbon absorbed by the microbial cells was either converted eventually or desorbed back to the solution. The desorption was observed when the TOC in the influent was reduced from 265.7 to 183.4 and then to 170.8. Under this condition, the mass balance indicates a negative adsorption as shown in Table II-5.

Table II-4. Performance of the anaerobic digester.

HRT, day	20	10	5
TOC inf mg/ℓ	601.1	600.4	465.7
TOC,eff mg/ℓ	77.3	65.98	52.25
TOC load $g/m^3/d$	29	58	106
COD, inf mg/ℓ	2336	2393	2401
COD, eff mg/ℓ	422.3	607.8	764.0
COD load $g/m^3/d$	117	239	480
pH	7.65	6.91	7.11
biogas ml/d	81.2	152.8	299.8

The mass balances of nitrogen, phosphorus, and potassium are shown in Table II-6. It can be seen from the influent and effluent of this table that 95% of the nitrogen and phosphorus and 92% of the potassium were left in the effluent solution. This high N-P-K content solution may serve as a good nutrient for the plant growth.

Table II-5. Mass balance of carbon* for 10 days HRT.

day	1	2	3	4	5	6
TC i	265.6	265.6	267.9	185.9	173.4	192
TOC i	263.4	263.4	265.7	183.4	170.8	189
IC i	2.2	2.2	2.1	2.5	2.6	2.8
TCe	127.2	134.2	130.3	123.1	135.6	102
TOC e	23.9	35.9	17.1	18.3	21.8	22.8
ICe	103.2	98.4	113.6	104.8	113.9	65.1
bio-gas	66.5	74.1	87.4	72.2	61.7	71.2
adsorbed	71.9	57.3	50.2	-9.4	-33.5	28.2

* The unit of the carbon here is mg.
i = influent
e = effluent

TableII-6. Mass balance of N-P-K for 10 days HRT.

element	N	P	K
inf, mg/ℓ	496.5	47.4	142.0
eff, mg/ℓ	470.0	44.5	130.0
consumed	26.5	2.9	12.0

II.3.2 Epidemiology

The declines of viable counts of *Salmonella choleraesuis* during continuous (withdrawn-and-fed) mesophilic anaerobic digestion at different HRT are illustrated in Figure II-4. This figure indicates that the viable counts of *Salmonella choleraesuis* at the three HRT declined rapidly within the first 4 days after inoculation.

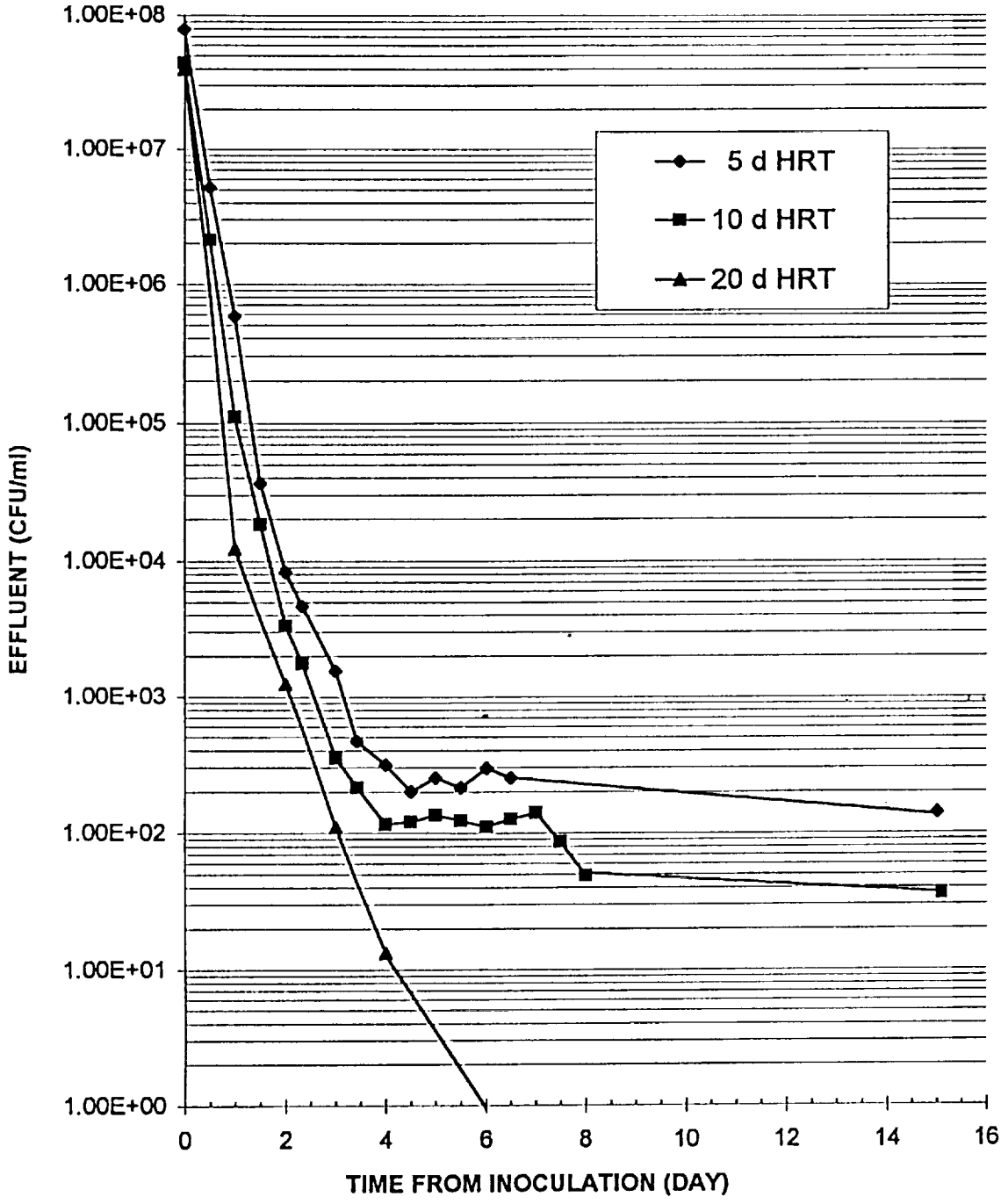

Figure II-4. Survival of *Salmonella choleraesuis* in the single dose continuous anaerobic digestion (pH = 7.12)

The rapid declines of viable counts of *Salmonella choleraesuis* were followed by a period of equilibrium for both 5 and 10 d HRT. However, for the 20 d HRT the viable count droped to a level below the detectable limit. This phenomena could be explained by a lack of sufficient nutrients at the 20 d HRT operation.

The decimal decay rate (k_d) for the continuous digestion studies was calculated using the modified formula by Ginnivan [1980].

$$k_d = -\frac{1}{t} \ln\left(\frac{P}{P_o}\right) - \frac{v}{V}$$

where, P is the colony count of indicator bacteria in the withdrawn effluent, P_o is the initial viable count in the digester (CFU/ml), v is the liquid volume of the effluent removed per day (ml/day), V is the liquid volume in the digester (ml), and t is the time interval (day). For a batch digestion system, the flow rate, v, is set to zero. The decimal decay rate (k_d) for the single-dose continuous digestion and batch digestion studies were determined from the slope of a plot of (P/P_0) versus t on a semi-logarithmic scale as shown in Figure II-5 for 5 d HRT. Linear regression methods were used to estimate the slope.

Differences among the k_d at various HRT during continuous digestion were assessed by calculating upper and lower 95% confidence intervals. The k_d values were significantly different if their 95% confidence intervals did not overlap. The investigation of the effect of the various HRT on the survival time of *Salmonella choleraesuis* showed greater variations. Using an initial dose of approximately 10^7 CFU/ml for each HRT study, at an HRT of 20 days, the indicator bacteria were found to be below the level of detection by the end of the 6th day of the study. However, after a period of rapid decline in viable numbers, at HRT of 10 and 5 days, the indicator bacteria reached equilibrium (the viable numbers were not reduced by one logarithmic unit) by the end of the 15th day at approximately 40 CFU/ml for 10 d HRT and $1.4X10^2$ CFU/ml for 5 d HRT.

The decimal decay rates (k_d) of the *Salmonella choleraesuis* during single-dose continuous mesophilic digestion operated at 20, 10 and 5 d HRT are calculated and listed in Table II-7 with upper and lower 95% confidence intervals [Mosteller et al ; 1983]. The three k_d values were not significantly different because their 95% confidence intervals overlapped. The pH and biogas production during single-dose continuous digestion remained relatively stable throughout the digestion with a mean pH of 7.12 and a mean biogas production of 183 ml/interval which contained about 71% methane.

The effect of bacterial feeding dose on the decimal decay rate (k_d) of *Salmonella choleraesuis* is shown in Figure II-6. The correlation values (r)(0.62 at 20 d HRT; 0.87 at 10 d HRT; 0.90 at 5 d HRT) indicate that there is a strong positive relationship between the bacterial viable count of the initial dose and their k_d values. Biogas production and pH remained relatively stable throughout this digestion, also, with a mean pH of 7.48 and a mean biogas production of 123 ml/interval which contained about 71 % methane.

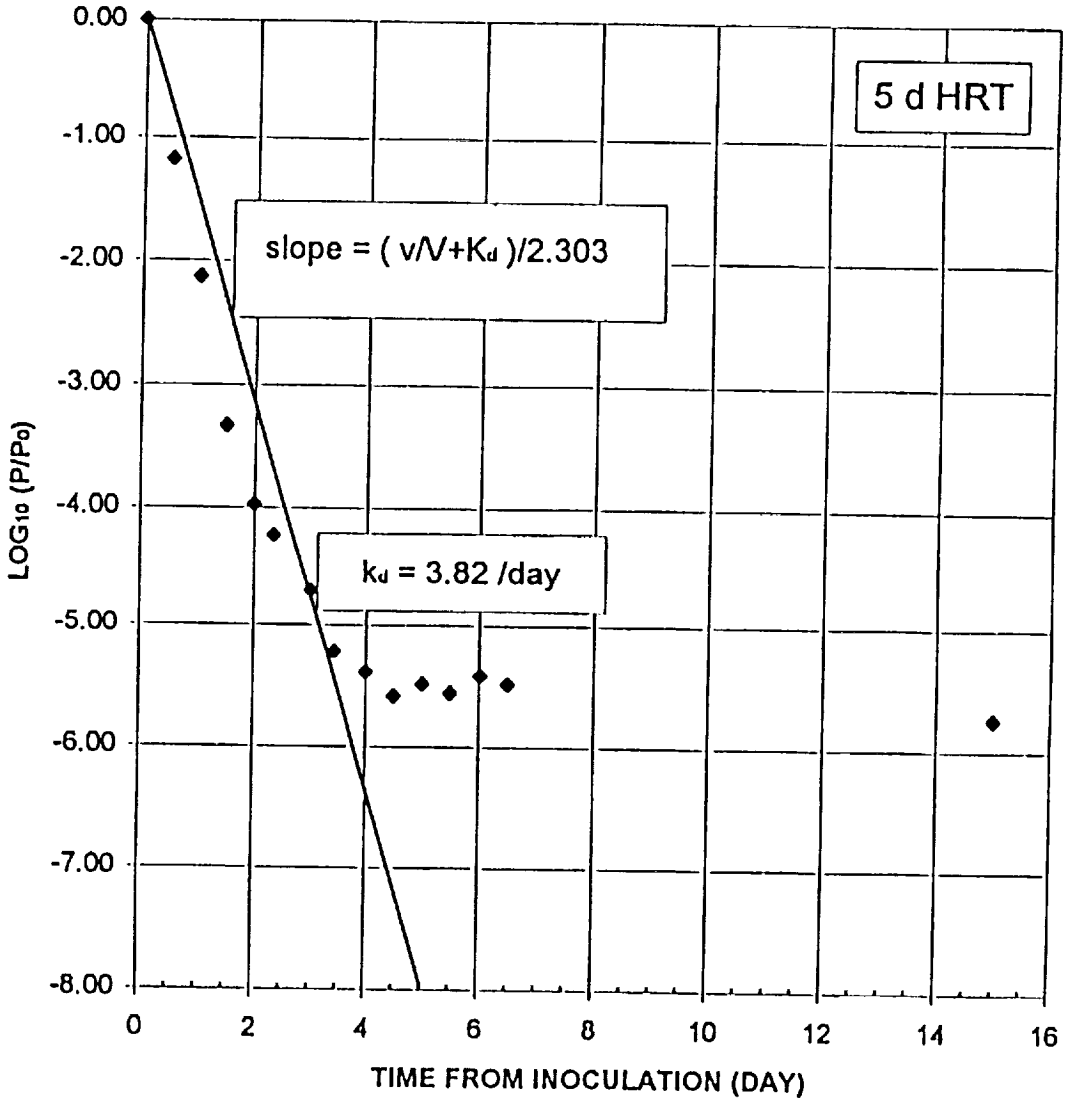

Figure II-5. Determination of k_d of *Salmonella choleraesuis* in the single dose continuous anaerobic digester for 5 d HRT.

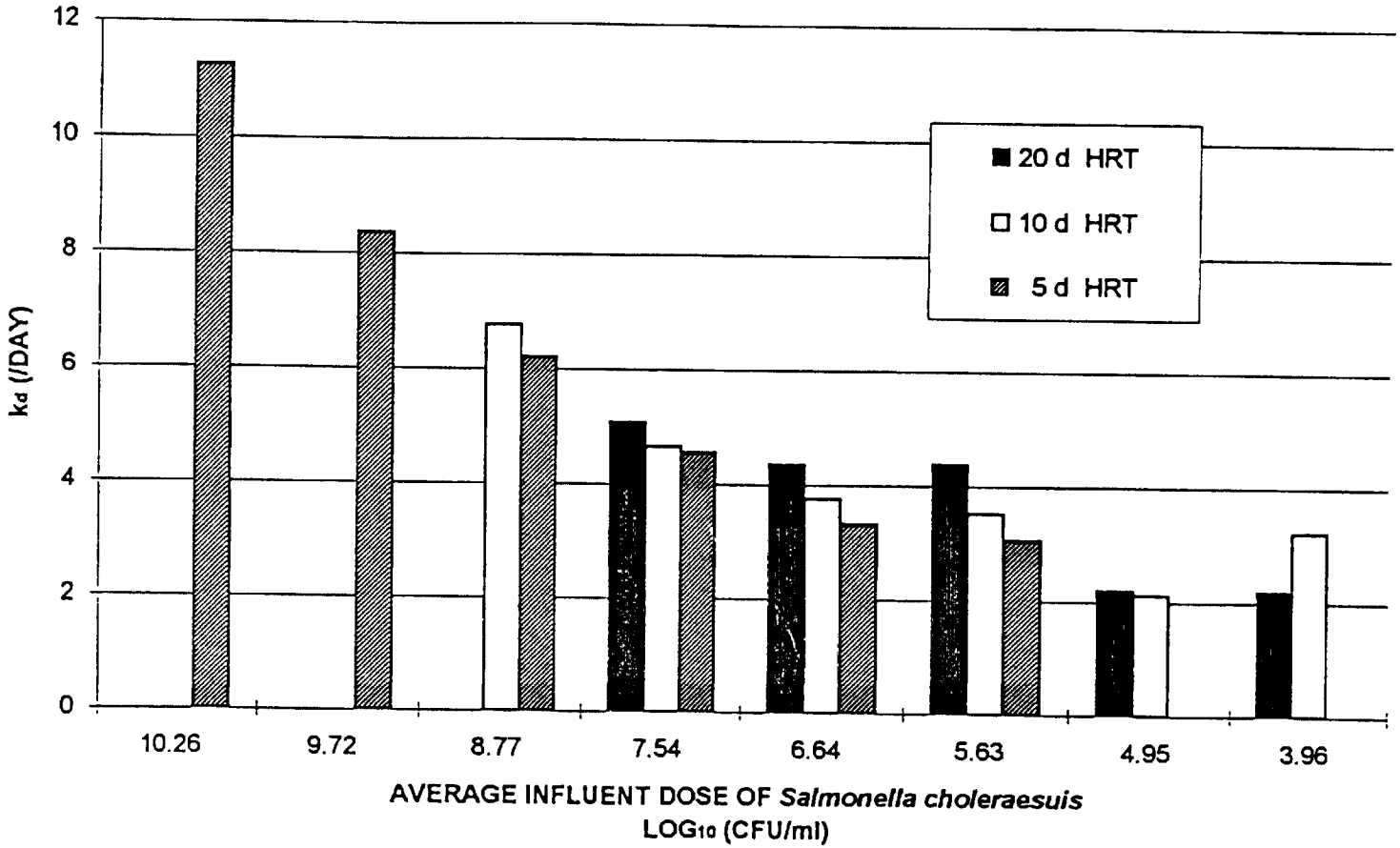

Figure II-6. Influent dose of *Salmonella choleraesuis* and their k_d in the multi-dose study during continuous mesophilic anaerobic digestion under various HRT

Table II-7. Decimal decay rate constant, k_d, of *Salmonella choleraesuis* during single dose continuous anaerobic digestion.

HRT, day	k_d, day^{-1}	lower limit k_d, day^{-1}	upper limit k_d, day^{-1}
20	4.28	3.07	5.48
10	3.82	2.88	4.76
5	3.82	3.36	4.28

A rapid decline in the viable count of *Salmonella choleraesuis* during batch mesophilic anaerobic digestion is shown in Figure II-7. The viable count of *Salmonella choleraesuis* declined rapidly within the first 2-4 days after inoculation. This rapid decline was followed by a period of equilibrium where the indicator bacteria remained at 10^2 CFU/ml until the 15th day from the inoculation. The decimal decay rate (k_d) of the *Salmonella choleraesuis* during batch mesophilic digestion is calculated by the same method used in the single-dose continuous process. The value of k_d calculated was 7.89 day^{-1}.

The viable counts of effluent correlated with the biogas produced suggesting a strong negative relationship (r = - 0.85). A rapid decline in the viable population of the indicator bacteria was reflected in a large volume of biogas production; as biogas production declined, so did the rate in decline in the viable population of the indicator bacteria in the digester. The pH values remained relatively stable throughout batch mesophilic digestion with a mean value of 6.90. The viable counts and the pH value did not show a relationship (r = - 0.07).

II.4 CONCLUSION

Results indicate that the NASA-simulated wastewater can be treated by anaerobic digestion. Mass balances indicate about 90% of the TOC is converted while only 5 to 8% of N-P-K are consumed in the digester. The maximum organic loading capacity was not reached indicating there is a possibility of increasing the loading rate. *Salmonella choleraesuis* survived at least 15 days from inoculation for 10 and 5 days HRT during continuous and batch digestion, but less than 6 days for 20 days HRT. The k_d values were greater at higher initial doses than lower doses for the same HRT, and greater for batch digestion (7.89 day^{-1}) than for continuous digestion (4.28, 3.82 and 3.82 day^{-1} for 20, 10 and 5 d HRT, respectively).

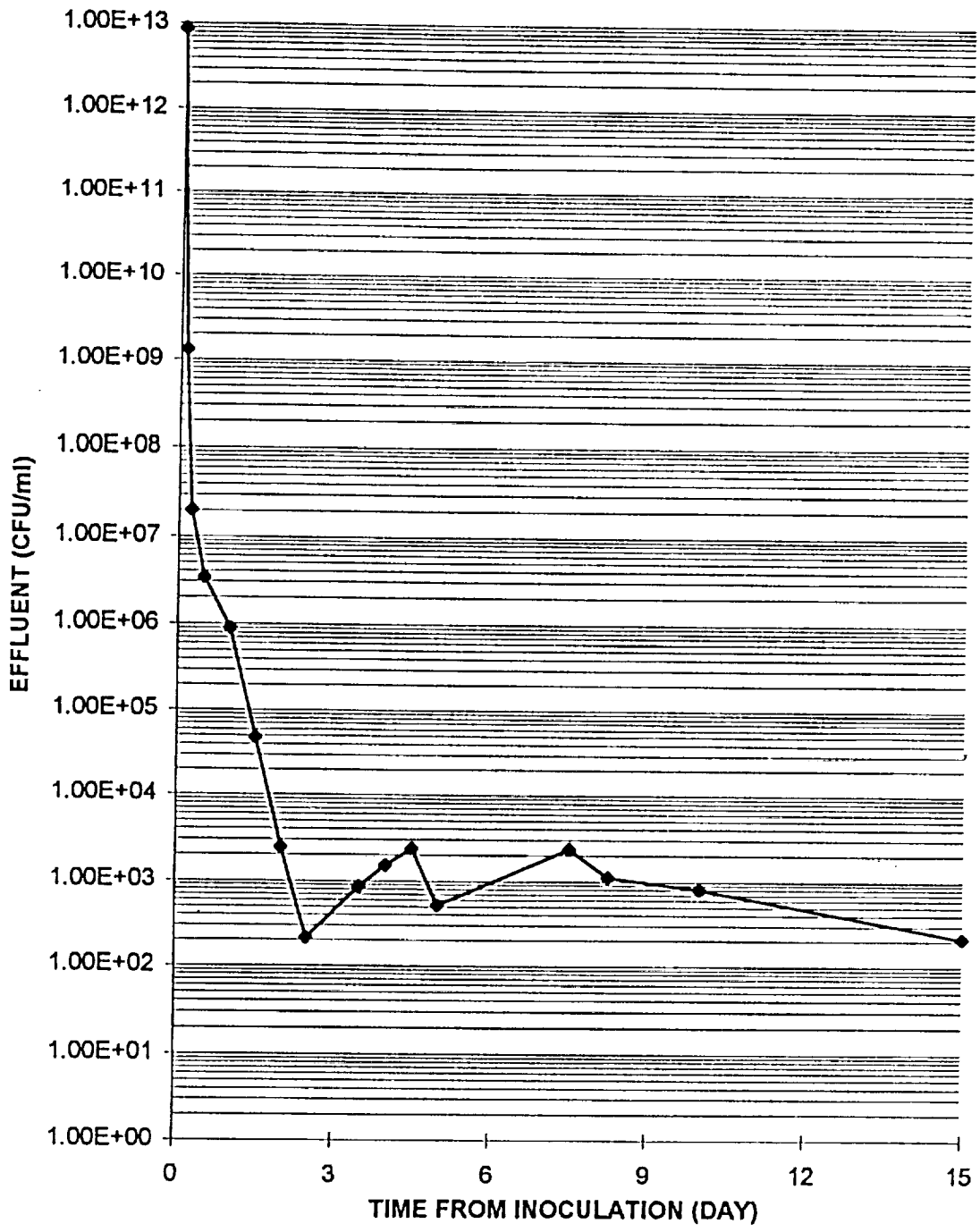

Figure II-7. The inactivation of *Salmonella choleraesuis* during batch mesophilic anaerobic digestion.

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III. RESOURCE RECOVERY OF ANAEROBIC WASTEWATER TREATMENT PROCESS IN A CONTROLLED ECOLOGICAL LIFE SUPPORT SYSTEM

III.1 ABSTRACT

A hybrid, anaerobic/plant-growth/aerobic, wastewater treatment process was proposed to study the resource recovery of carbon, nitrogen, phosphorus, and potassium in a controlled ecological life support system. Three four-liter packed-bed anaerobic reactors (digesters) were fabricated and operated at 35°C, pH around 7, and hydraulic retention times (HRT) of 20, 10 and 5 days. Simulated spacecraft wastewater was used as the feeding solution. It was prepared following the formulation given by NASA-JSC (Johnson Space Center) and consisted of shower water, clothwash water, dishwash water, handwash water, and urine flush water. This wastewater had an initial chemical oxygen demand (COD) of 2400 mg/l and total organic carbon (TOC) of 550 mg/l. Under steady-state operation, COD, TOC, pH, total nitrogen (N), total phosphorus (P), and potassium (K) were monitored in the digester input and output solutions. Additionally, the volume and the CH_4/CO_2 mole ratio of the biogas produced from the anaerobic digesters were measured. The results showed about 90% of TOC was converted while only 5 - 8% of N-P-K was consumed in the anaerobic digesters.

III.2 INTRODUCTION

Currently, spacecraft life support systems rely on open-loop (nonrecycling) technologies. These life support systems are simple and sufficiently reliable for human space-flight missions of relatively short duration, small crew sizes, and limited power availability. Life support technologies for the coming era of exploration, however, must address longer-duration missions in which humans require substantial amounts of consumable materials to sustain life for long periods of time. If these consumable materials must all be provided by resupply flights from Earth, a substantial logistics infrastructure is required. Consequently, supplying these consumables from Earth is an extremely expensive proposition. As a result, one of the most important challenges associated with long-duration manned space flights is in development of closed life support systems (CLSS), including the technologies of air revitalization, water recovery, waste processing, food production and food processing which are logistically and economically essential (Schwartzkopf, 1992).

The two families of technology available to provide these basic functions of human life support are physicochemical and bioregenerative. Although it is conceptually possible to design a life support system based exclusively on either family of technology, analysis indicates that the best design combines the two. By carefully selecting and combining technologies with offsetting advantage and disadvantages, it is possible to develop a hybrid design that offers significant

improvement over purely physicochemical or bioregeneratative system. Such a system combines biological functions such as biotreatment for organic removal, photosynthesis for CO_2 removal and food and oxygen production, with physicochemical function such as gas separation and collection of water vapor on a cooling coil.

In this research, an anaerobic wastewater treatment process was designed and fabricated to treat NASA-formulated wastewater, and at the same time recover the resource of carbon, nitrogen, phosphorus, and potassium. This anaerobic reactor can be used as a component of the hybrid wastewater treatment system which, as shown in Figure III-1, consists of an anaerobic reactor, plant growth chamber, and an advanced water treatment unit (Li and Hunt, 1995).

The objectives of this study were fourfold: (1) design an anaerobic bioreactor, (2) set up the reactors, (3) treat NASA simulated wastewater, and (4) analyze and evaluate the performance of the anaerobic bioreactors under steady state. The TOC was monitored in the inlet and outlet streams as analyzed to determine the removal efficiency of the reactors. The amount and the CH_4/CO_2 mole ratio in the biogas were also monitored.

III.3 LITERATURE REVIEW

III.3.1 Anaerobic Decomposition Process

In order to simplify discussion of the mechanism of anaerobic decomposition, a three-stage process is often used as shown in Figure III-2 (Parkin and Owen, 1986). The stages include (1) hydrolysis, liquefaction, and fermentation, (2) hydrogen and acetic acid formation, and (3) methane formation.

In the first stage, complex or insoluble organics are converted into simple and soluble organics. Thus, these simple and soluble organics can pass into bacteria cells and can be used as their energy sources. Hydrolysis and liquefaction are accomplished by extracellular enzymes released by fermentative bacteria (Group 1 in Figure III-2).

In the second stage, it is believed that hydrogen is produced by the fermentative bacteria and the hydrogen-producing acetogenic bacteria (Groups 1 and 2 in Figure III-2) (McInerney, 1981; Zinder, 1984). Acetate is also produced by these groups in addition to hydrogen-consuming, acetogenic bacteria (McCarty, 1985). If the partial pressure of hydrogen exceeds about 10^{-4} atmosphere, methane production will be inhibited, and the concentration of organic acid such as propionic and butyric acids will be increased (McCarty, 1982). Thus, to maintain efficient anaerobic digestion of wastewater with methane production, the hydrogen level must be maintained below this level.

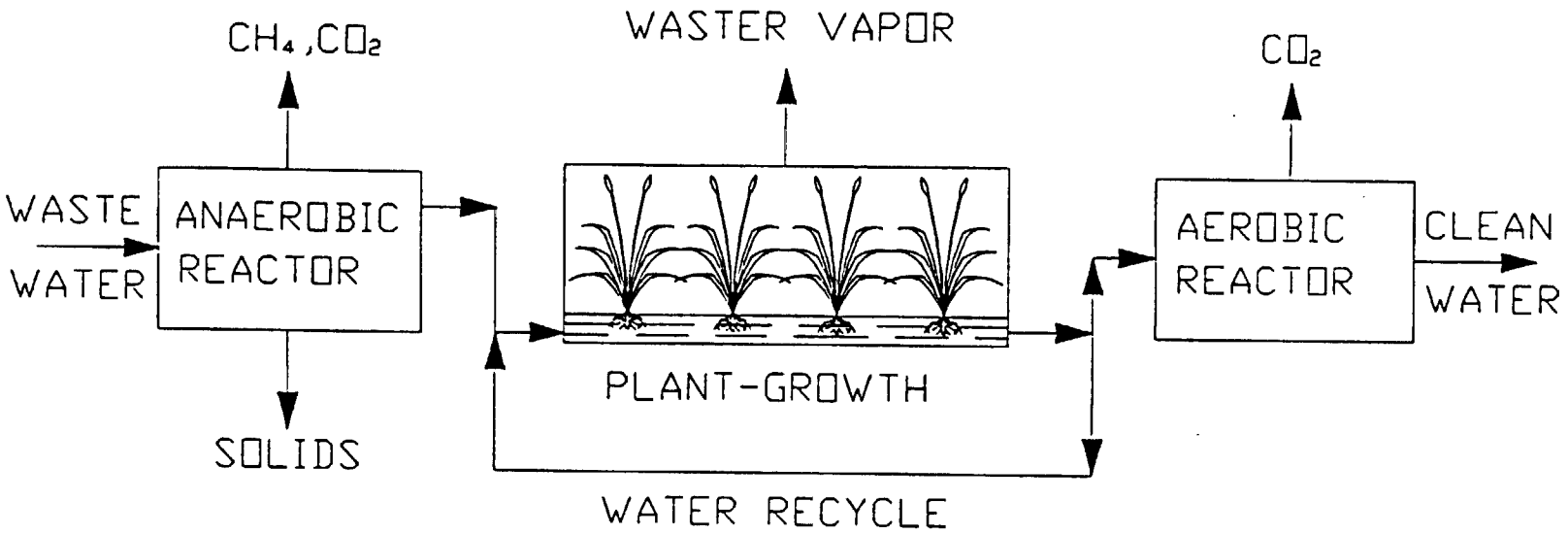

Figure III-1. Flow diagram of the anaerobic/plant-growth/aerobic process

In the third stage, acetic acid is converted to methane by methanogenic bacteria. Carbon dioxide is also produced and either escapes as gas or is converted to bicarbonate (McCarty, 1964). One of the most important characteristics of the methanogenic phase is that only a few substrates can be used as energy sources for the methanogens. It is believed that only formic acid, acetic acid, methanol, and hydrogen can be used as energy sources by the various methanogens (Baresi, et al., 1978). Of these, acetic acid (acetate) and hydrogen serve as the major substrates for methane formation in anaerobic decomposition.

Approximately 72% of the methane formed in anaerobic digestion of wastewater comes from acetate cleavage (Group 5 in Figure III-2.).

$$CH_3COOH \text{ ----------> } CH_4 + CO_2 \qquad (1)$$

The remaining 28% results from reduction of carbon dioxide using hydrogen as the energy source by CO_2-reducing methanogens (Group 4 in Figure III-2.).

$$CO_2 + 4H_2 \text{ ----------> } CH_4 + 2H_2O \qquad (2)$$

The pathways for methane production during anaerobic digestion are shown in Figure III-3.

III.3.2 Operational Condition

Conditions for efficient anaerobic digestion are: sufficient nutrients, optimum pH and temperature, anaerobic condition, and absence of toxic substances (McCarty, 1964). Experimental data indicate that under the operational conditions the methane-forming bacteria are the most sensitive digester organisms.

Nutrients

Nutrients must be present in sufficient quantities to ensure efficient digestion. A commonly used empirical formula of bacteria is $C_5H_7O_2N$ (Mclnerney, et al.,1981), in which nitrogen comprises approximately 12% of bacterial cell mass. The phosphorus requirement for bacteria growth is about 1/7 - 1/5 of the nitrogen requirement (McCarty, 1964).

Domestic sludge usually contain sufficient quantities of nitrogen and phosphorus for efficient digestion (McCarty, 1964). However, treatment of industrial wastes may require addition of supplemental nitrogen and/or phosphorus. Other nutrients may include iron, nickel, cobalt, sulfur, calcium, and some trace organics (Bryant et al., 1971). The complete nutrient requirements for methanogenesis have not been reported (Murray et al., 1981).

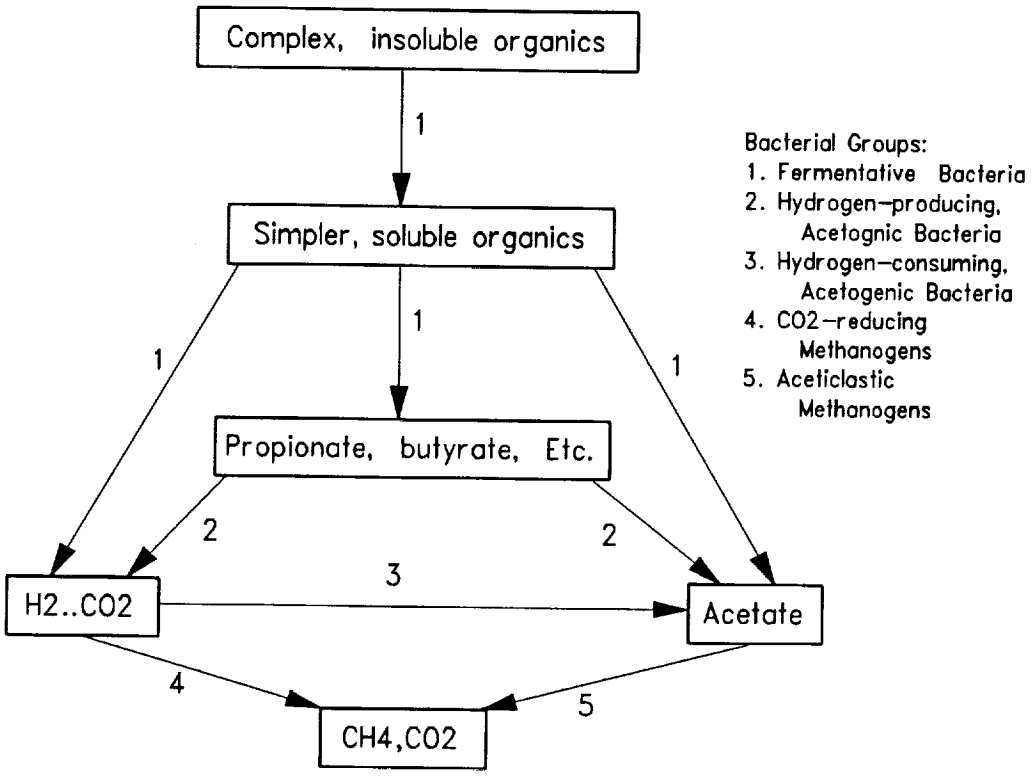

Figure III-2. The mechanism of anaerobic decomposition of organics

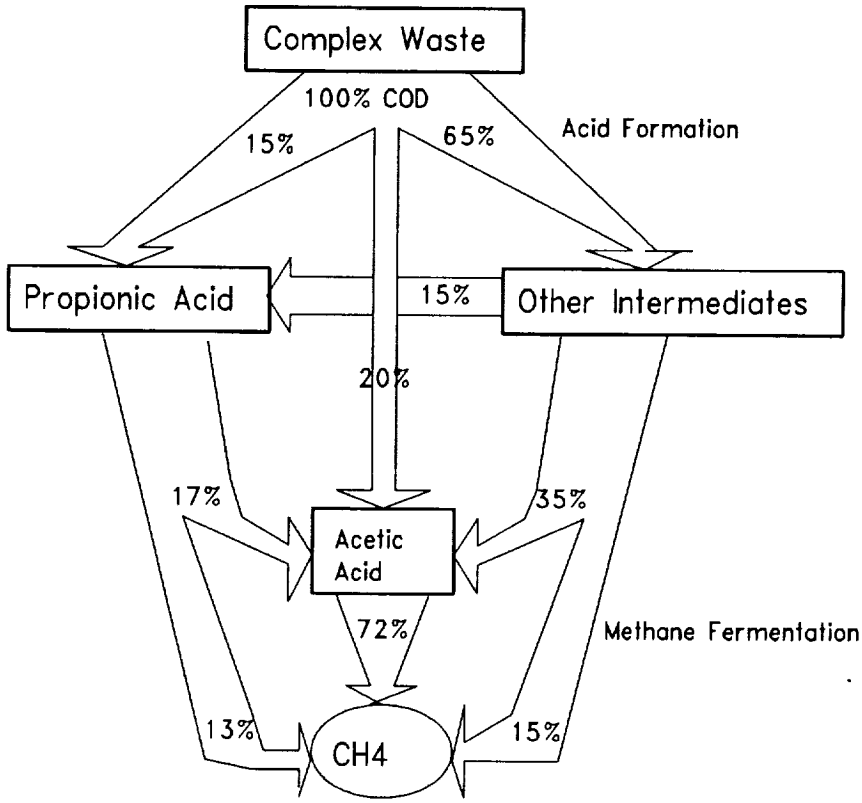

Figure III-3. The pathways for methane production during anaerobic digestion

pH

Maintenance of system pH in the proper range is required for efficient anaerobic digestion. In general, the accepted pH range for optimal process efficiency is 6.5 - 7.6 (McCarty, 1964). In an anaerobic process, the methanogens are the most sensitive to pH changes. During an upset process, volatile acids produced by acetogenic bacteria typically increase at a faster rate than that can be decomposed by the methanogenic bacteria. Under this condition, the pH will drop to unacceptably low levels. At the same time, methane production will be decreased and may eventually cease if the pH drop is too extreme. The common buffering chemical used in anaerobic digestion is bicarbonate.

Temperature

Anaerobic digestion is generally operated in one of two temperature ranges: mesophilic (30-38°C) or thermophilic (50 - 60°C). Most anaerobic digesters are operated in the mesophilic range.

Most reports in the literature confirm that thermophilic digestion results in higher digestion rates, improved sludge dewaterability, and increased pathogen destruction (Buhr et al., 1977). Unfortunately, no controlled research has been conducted to determine the reasons for more rapid stabilization, which could involve differences in microorganisms or physical factors such as improved mixing and contact. So there is no sufficient evidence to demonstrate that thermophilic digestion will yield a significantly greater efficiency of organic matter destruction.

In mesophilic digesters, there are two optimal temperatures. The acetogenic bacteria have an optimum at 30°C, while methanogenic organisms have their optimum at 35 - 37°C (Mudrack and Kunst, 1981).

Maintenance of a constant and uniform temperature is imperative for consistent and efficient digester operation. Optimal temperature can be accomplished through correct design of heat exchangers and mixers.

Mixing

Anaerobic digesters are mixed to provide efficient utilization of the entire digester volume, prevent stratification and temperature gradients, disperse metabolic and products and any toxic materials contained in the influent sludge, and maintain intimate contact between the bacteria, bacterial enzymes and their substrate. In short, adequate mixing provides a uniform environment for anaerobic bacteria, one of the major factors in obtaining maximum digestion. The effect of inefficient mixing on process kinetics is a decrease in efficient system volume and a decrease in solid retention time.

Insufficient mixing results in failure of an effective system. Studies with full scale digesters have shown that inefficient mixing may reduce the effective volume of a digester by as much as 70%, leaving an actual volume utilization of only 30% (Monteith et al., 1981).

Toxicity

A variety of compounds have been shown to be inhibitory in high concentrations (Zehnder et al., 1977). Ammonia can inhibit methanogenesis. Inhibition in digesters begins at concentration of ammonia near 100 mM if the pH is greater than 7.4. At concentrations greater than 200 mM, the ammonium becomes toxic regardless of the pH (McCarty, 1964). Heavy metal, alkaline and alkaline earth metals, aromatic compounds, and chlorinated hydrocarbons are inhibitory in anaerobic digesters (Peffer, 1980).

III.4 EXPERIMENTAL MATERIALS AND ANALYTICAL METHODS

A flow diagram of the experimental procedures in this research is shown in Figure III-4. In general, the experimental procedures were divided into two phases; operation of anaerobic digesters and sample analysis. The operational phase included preparation of feeding solution, samplings from and feeding to the anaerobic digester. The sample analysis involved measurements of the pH value, biogas volume, TOC, COD, TKN, K, P, and CH_4/CO_2 mole ratio. Each of the above is shown in the flow chart Figure III-4 and is described in the following sections.

Inorganic Nutrients

Inorganic nutrients were added to satisfy the metabolic needs of microorganisms. Inorganic nutrients used in this study are listed in Table III-1 (Liu, 1993). In the nutrient formula, $NaHCO_3$ and NH_4Cl act as buffer solutions to maintain the pH between 6.5 - 7.6. $NaPO_3$ and NH_4CL were used as the sources of major elements, nitrogen and phosphorus. NaS and L-Cysteine served as reducing reagents which maintain anaerobic conditions inside the bioreactors. Other components included trace elements necessary for microbial reproduction.

Simulated Wastewater

The simulated wastewater consists of clothwash water; dishwash water; handwash water; shower water; fresh urine and urine flush. The formula of the simulated wastewater was specified by NASA-JSC. The composition of the simulated wastewater is listed in Table III-2. Deionized water was used to prepare the simulated wastewater. Because more than 80% of the TOC was contributed by urine solutions and the composition of urine changed about 10 % within one hour, it was necessary to use fresh simulated wastewater during the experiment.

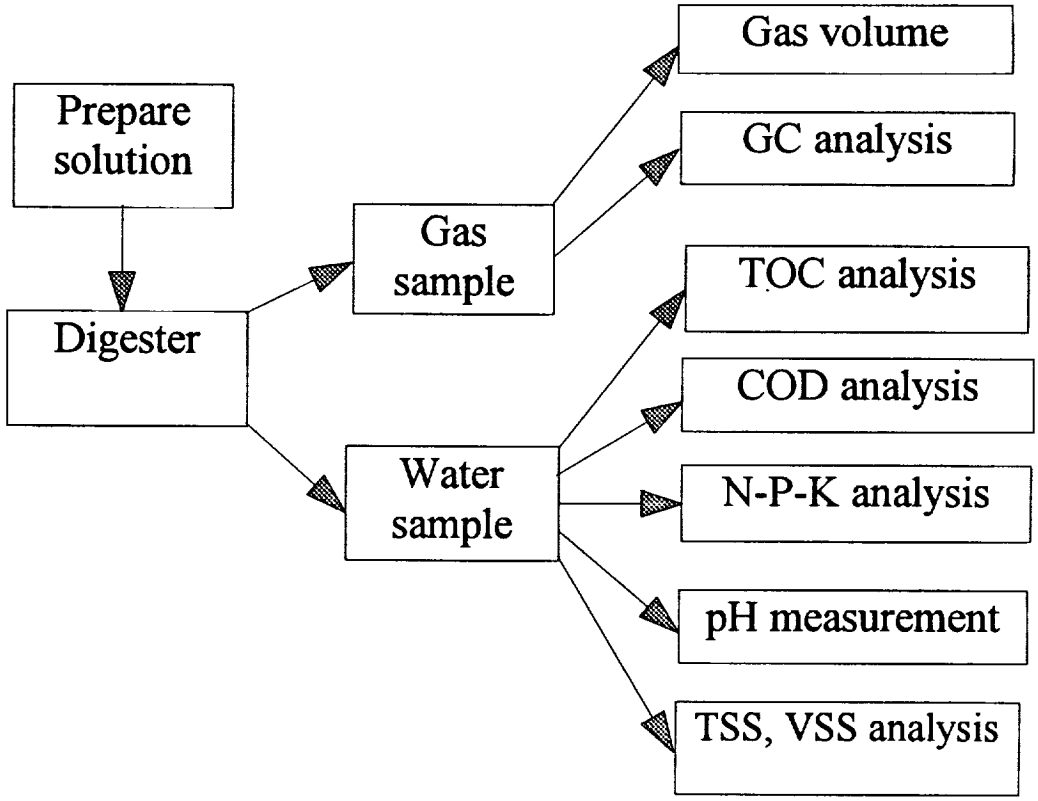

Figure III-4. Flow diagram of experimental procedure

The soap used in this experiment was provided by NASA-JSC.

Bioreactor System

The most important portion is the anaerobic bioreactor system which consists of a reaction kettle, packing section, circulation system, temperature control compartment, and biogas collection system. The schematic diagram of the bioreactor system is shown in Figure III-5. The components of the reactor are described below.

Reaction Kettle

Glass reaction kettle (Ace Glass Model 6505) was chosen as the bioreactor. It consisted of upper and lower portions which were combined to a unit by clamp. The upper portion had four openings on the top. Two of them were used to build a liquid loop, one for releasing biogas as shown in Figure III-5.

Packing Column

Polypropylene pall rings of 5/8" size were used as the packing material. Two perforated plexiglass with 1/8" thickness plates were used to hold the packing material inside the reaction kettle. Characteristics of the packing materials are listed in Table III-3.

Table III-1. Formulation of the inorganic nutrients

Chemicals	Concentration (mg/l)
$CaCL_2.H_2O$	6.25
$NaPO_3$	0.25
$(NH_4)_2HPO_4$	96.50
L-Cystein	2.50
$CoCL_2.6H_2O$	10.00
$FeCL_2.4H_2O$	20.00
$Na_2S.9H_2O$	75.00
$MgCL_2.6H_2O$	266.75
NH_4CL	369.00
KCL	100.00
KI	0.625
$NaHCO_3$	pH adjustment

Table III-2. Composition of NASA Simulated Wastewater

Item	L/Person-day	Four Person Crew
Shower Water (4 uses total per day, 12 g soap per use)	5.32	21.28
Hand wash (16 uses total per day, 2 g soap per use)	4.07	16.28
Clothes wash (30 g soap)	12.44	49.76
Urine (16 uses total per day)	1.51	6.04
Urine flush	0.49	1.96
Dish wash	9.07	36.28
Total	32.9	131.60

Table III-3. Characteristic of the Packing Materials

Media	Pall ring (prolypropylene)
Surface area, m^2/g	0.00342
Area/volume, cm^{-1}	3.412
Porosity	0.877
Size, mm	16x16

Circulation System

A magnetic drive chemical pump was used to circulate the solution and to keep the liquid phase uniform during the assays. The pump was also used to obtain samples from the bioreactors. According to an independent test, it takes 30 minutes to make the whole solution uniform. The test was done by injecting a dye from the feeding port and observe the concentration distribution in the reactor.

Temperature Control Compartment

The bioreactors were placed in 35°C incubators to obtain mesophilic conditions.

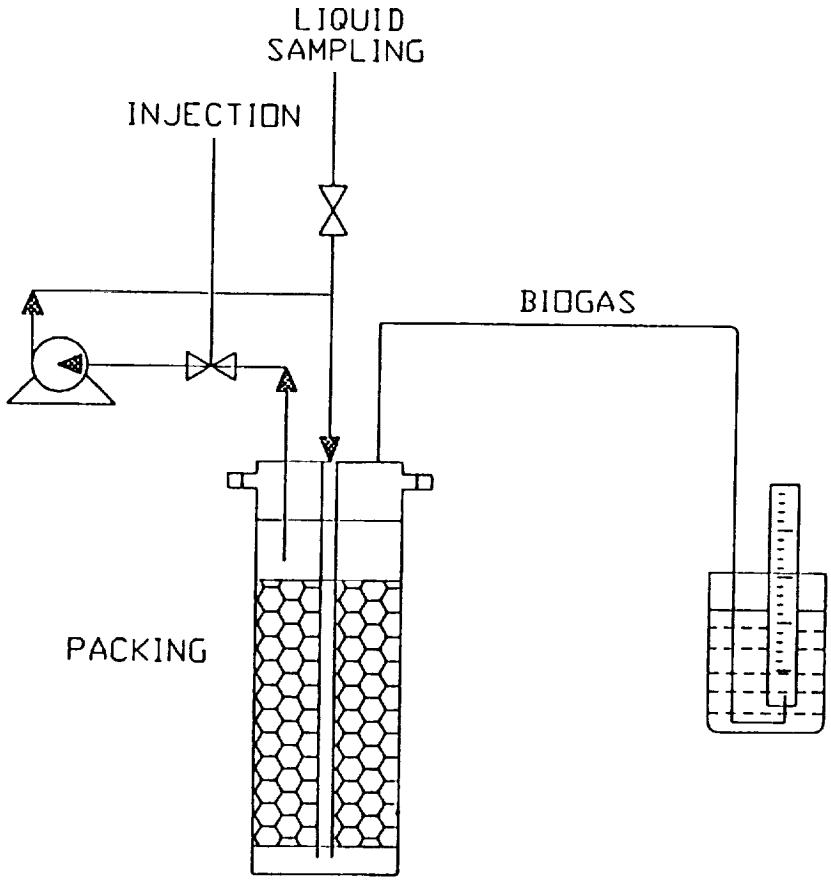

Figure III-5 Flow diagram of the digester

Each of the three bioreactor was placed in an incubator. The tubings ran through an opening located at the top of the incubator. The circulating pump was also placed on the top of each incubator and near the top opening. All of the daily operations of feeding and sampling were performed on the top of the incubator.

Biogas Collection

Biogas was collected by a specially designed gas collection system which is shown in Figure III-6. The gas was collected and measured by a 200 ml graduated cylinder as indicated in Figure III-5. The flask 1 (F1) was used as the water seal. The water in the flask worked as a water seal to prevent air or oxygen from getting into the anaerobic system. The water in this gas collection system was adjusted to acidic by adding HCL solution. Salt or NaCL was also added to the water solution to reduce the solubility and dissolving amount of CO_2. This may be explained from the following reaction equation.

$$CO_2 + H_2O \text{ ----> } H_2CO_3 \text{ -----> } H^+ + HCO_3^- \qquad (3)$$

pH Measurement

The pH was measured using a 720A pH meter. The basic principle of electrometric pH meter is the determination of electromotive force produced in the glass electrode. The electromotive force varies linearly with pH. This linear relationship is described by plotting the measured electromotive force against the pH of different buffers. Sample pH is determined by extrapolation.

TOC Analysis

TOC in the solution was determined by a TOC analyzer (TOC-5000/5050, Shimadzu Scientific Instrument, Inc.). The sample was homogenized and diluted when necessary, and a microportion was injected into a heated reaction chamber packed with an oxidative catalyst, such as cobalt oxide. The water was vaporized, and the organic carbon was oxidized to CO_2 and H_2O. The CO_2 from oxidation of organic and inorganic carbon is transported in the carrier-gas streams and was measured by means of a nondispers infrared analyzer.

Because total carbon was measured, inorganic carbon must be measured seperately, and the TOC obtained by the difference. Measurement of inorganic carbon was performed by injecting the sample into a separate reaction chamber packed with phosphoric acid-coated quartz beads. Under acidic conditions, all inorganic carbon was converted to CO_2, which was measured. Under these conditions organic carbon was not oxidized and only inorganic carbon was measured.

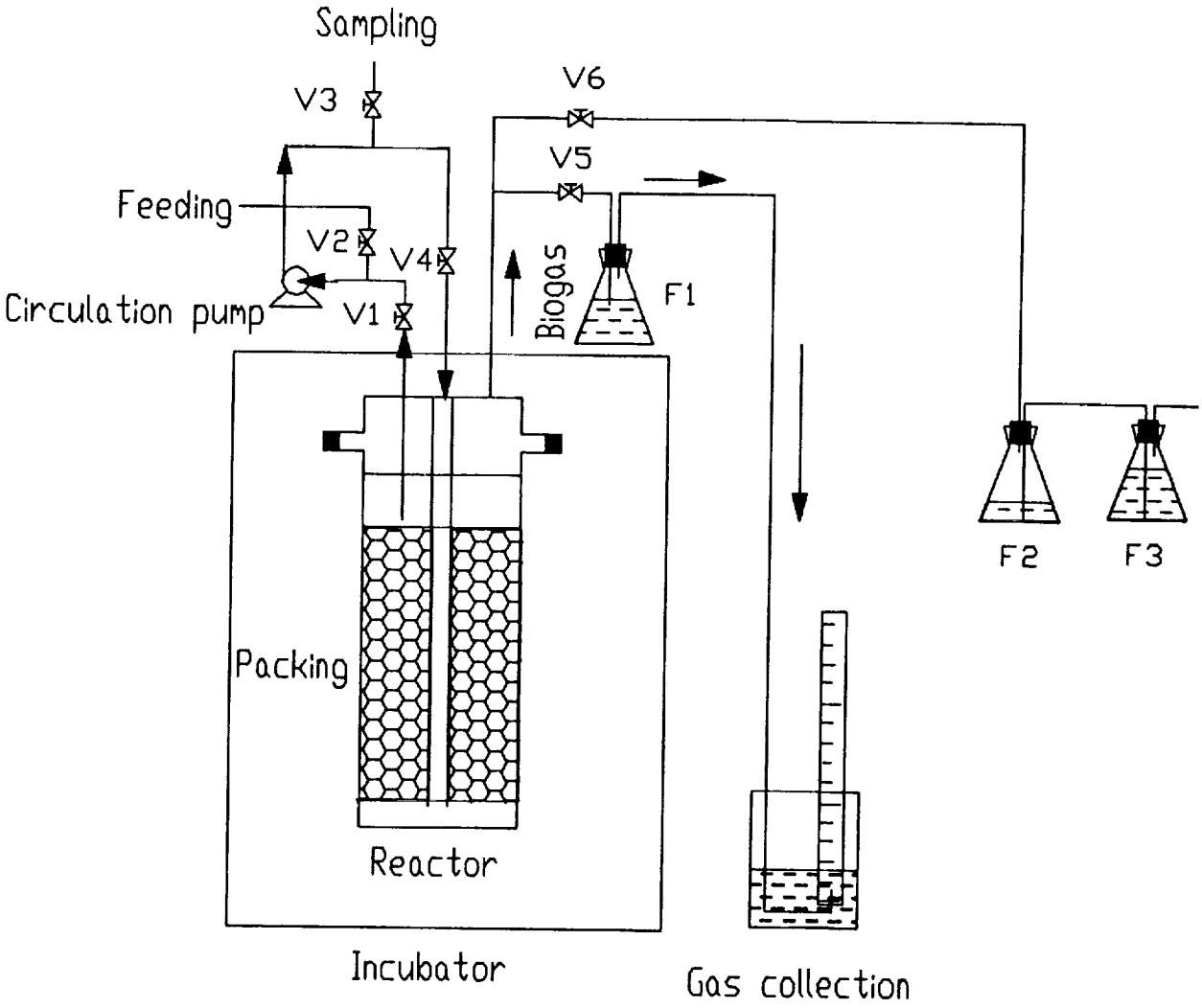

Figure III-6. Schematic diagram of the experimental set-up

The Ratio of Methane and Carbon Dioxide

The ratio of methane and carbon dioxide was measured by a gas chromatograph (GOW-MAC) with a thermal conductivity detector (GC-TCD,Varian 5830A) using a 6 feet x 0.085 inches I.D. Haysep-Q stainless steel column as the separation column (Alltech 80/100 mesh with 275°C maximum temperature). Helium was used as the carrier gas. The operation conditions were

Column temperature = 120°C,
Detector temperature = 140°C,
Injector temperature = 60°C and,
Carrier gas flow rate = 30 ml/min.

The composition of the gas sample was determined from the ratio of the peak areas of CH_4 and CO_2.

COD Concentration

The COD was determined using the HACH spectrophotometer. The reactor digestion method was applied. This method is approved by EPA (Federal Register, April 21, 1980, 45 (78), 26811-26812). The sample was heated for two hours with the strong oxidizing agent, potassium dichromate. Oxidizable organic compounds reacted, reducing the dichromate ion ($Cr_2O_7^{2-}$) to green chromic ion (Cr^{3+}). The amount of Cr^{3+} product was determined using the spectrophotometer set at 435 nm wavelength.

Total Nitrogen Concentration

The total Kjeldahl nitrogen was tested using the HACH spectrophotometer with the Nessler method. The term "Total Kjeldahl Nitrogen" refers to the combination of ammonia and organic nitrogen. However, only the organic nitrogen in the trinegative state are determined in this test. Nitrogen in this form is converted into ammonium salts by the action of sulfuric acid and hydrogen peroxide. The ammonia is then analyzed by a modified Nessler method test set at 460 nm wavelength.

Potassium Concentration

Potassium was detected using the HACH spectrophotometer with the tetraphenylborate method at a wavelength of 650 nm. Potassium in the sample combines with sodium tetraphenylborate to form potassium tetraphenylborate, an insoluble white solid. The amount of turbidity produced is proportional to the potassium concentration.

$$NaB(C_6H_5) + K^+ \text{----->} KB(C_6H_5)_4 + Na^+ \qquad (4)$$

Total Phosphorus Concentration

Total phosphorus was determined using HACH spectrophotometer with the persulfate digestion method which is approved by EPA. Phosphates present in organic and condensed inorganic forms (meta-, pyro- or other polyphosphates) must be

converted to reactive orthophate before analysis. Pretreatment of the sample with acid and persulfates. Organically bound phosphates are thus determined indirectly by subtracting the result of an acid hydrolyzable phosphorus test from the total phosphorus result. The ascorbic acid method is adapted to measure the reactive phosphorus in the sample at a wavelength of 890 nm.

Total Suspended Solid (TSS) and Volatile Suspended Solid (VSS)

A well-mixed sample was evaporated in a weighed dish and dried to a constant weight in an oven at 103 to 105°C for 30 minutes. The increase in weight over that of the empty dish represented the total suspended solids (TSS).

The residue from TSS that was ignited to a constant weight at 550°C for 15 minutes. The remaining solids represent the total suspended solid while the weight lost on ignition was the volatile suspended solid (VSS).

III.5 EXPERIMENT

III.5.1 Reactor Performance

Three four-liter packed-bed reactors were designed to study the resource recovery of carbon, and nitrogen, phosphorus, and potassium (N-P-K) under anaerobic conditions. A schematic diagram of the reactor system is shown in Figure III-6.

The feeding solution was prepared with substrates and necessary inorganic nutrients as shown in Table III-1. A sampling volume of 350 ml was withdrawn from the reactor through valve 3 (V3) shown in Figure 6 with the help of the circulation pump. Next, 350 ml of the feeding solution was injected by a syringe at valve 2 (V2) through a plexiglass tubing into the bottom of reactor. The total liquid volume in the reactor was 3500 ml. The liquid level in the reactor was maintained at 4.5 cm above the packing bed, while 7 cm of head space was allowed for gas-liquid separation. Biogas exited from the top of the reactor and was collected by a graduated cylinder.

In order to maintain anaerobic operating conditions, the following procedures were followed (see Figure III-6).

1. Open valve 5 (V5) to collect gas production. Close valve 5 when sampling and feeding.
2 Open valve 6 (V6) during sampling and feeding.
3. Fill flask 2 (F2) and flask 3 (F3) with H_2O, and fill nitrogen into the head space of flask 2.
4. Seal valve 2 and 3 with parafilm. Remove parafilm when sampling or during

feeding operation.

5. Flush bioreactors with nitrogen when necessary.

Flask 1 (F1) was used as a water seal apparatus so that air cannot enter the bioreactor system.

Although the anaerobic filter was operated as an axial-flow reactor, mixing action in the reactors was produced by circulation pump. As a result, the bioreactors acted as a well-stirred reactor.

III.5.2 Acclimation

The bioreactors were set up with a microbial seed and necessary inorganic nutrients. The seed came from a local municipal wastewater facility. Initially, municipal wastewater was used to acclimate the microbes. Afterwards, ethyl acetate was used as a supplemented carbon source to stimulate methanogenesis. Finally, NASA-simulated wastewater was used in place of the municipal wastewater containing ethyl acetate. During acclimation, inorganic nutrients were added as micronutrients (Table III-1).

In order to immobilize the bacterial on the surface of the packings, the circulation pump was stopped for at least four hours after running for one to three hours. At which time, the activity of the bacteria was slowed down and thus the bacteria attached to the surface of the packing materials. A detailed operation is as follows.

1. Fill each reactor with 3.5 L of the sludge seeding and inorganic nutrients.
2. Flush reactors with nitrogen for one hour to remove oxygen in the reactor.
3. Remove a sample of 350 ml. Then, feed with 350 ml of sludge containing inorganic nutrients.
4. Change the feeding solution to 350 ml of municipal wastewater containing inorganic nutrients and ethyl acetate (1000 mg/l). Run reactor for one month.
5. Change the feeding solution to contain 50% simulated wastewater. Operate every other day for three times.
6. Use 100% simulated wastewater containing inorganic nutrients and ethyl acetate (500 mg/l) as the feeding solution. Operate every other day for two times.
7. Feed reactor with 350 ml of simulated wastewater containing inorganic nutrients. Operate every other day for three times.

Acclimation was achieved after these operations.

III.5.3 Experimental Procedure

Experiments were performed to test the biodegradation of NASA-simulated wastewater under anaerobic conditions. After bacterial acclimation, three reactors were run under different HRT: 20 days, 10 days, and 5 days .

HRT of 20 days

1. Sample then feed simulated wastewater supplemented with inorganic nutrients every other day.
2. Operate three reactors under the same condition.
3. Determine the pH, COD, TOC for the three reactors using a liquid sample.
4. Obtain the volume of biogas produced for each of the three reactors.
5. Stop the circulation pump for four hours after running one to three hours. Turn the pump off at night.

HRT of 10 and 5 days

1. Sample and feed every day for 10 days HRT and twice a day for 5 days HRT.
2. Adjust the pH value when necessary. One milliliter of concentrated sulfuric acid in one liter feeding solution was used to maintain the pH within the required range. Sodium bicarbonate and ammonium chloride were not used under these condition.
3. Run the circulation pump at all times.
4. Measure the pH and gas produced for each reactor.
5. Determine the CH_4/CO_2 mole ratio using a GC-TCD.
6. Monitor TOC, COD, N-P-K in one reactor.
7. Measure the VSS and TSS in one reactor.

The following conditions were kept for the above operations:

Total liquid volume = 3500 ml,
Reaction temperature = 35°C, and
Volume of sampling and feeding = 350 ml.

Simulated wastewater was prepared and fed into the digesters as soon as possible or within one hour of preparation.

III.6 RESULTS AND DISCUSSIONS

III.6.1 Acclimation

There were three major stages of the acclimation in this experiment. First, municipal wastewater was used as the substrate to cultivate anaerobic bacteria. Findings from this study demonstrate that municipal wastewater could not be used as a

sole carbon source for anaerobic bacteria. Then ethyl acetate was added as a supplemented carbon source; an increased bacteria growth was observed. Finally simulated wastewater was gradually used to replace municipal wastewater, acetogens and methanogens acclimated to this substrate. The COD and TOC concentrations of five samples of the municipal wastewater containing micronutrients are shown in Table III-4. The average values of the COD and TOC were about 280 mg/l and 40 mg/l, respectively.

Table III-4 TOC and COD Concentrations in Municipal Wastewater

Sample	1	2	3	4	5	Average
TOC (mg/l)	53.25	48.22	44.69	30.34	32.18	41.74
COD (mg/l)	240	350	359	160	278	277.4

According to McCarty (1964), 2000 mg/l COD could possibly serve as an operational low limit for good substrate of anaerobic bacteria. Insufficient carbon source in municipal wastewater may be the reason for the low biogas production (from 30-70 ml. to none).

In order to supply sufficient a carbon source, ethyl acetate (1000 mg/l) was selected. This modified feeding solution consisted of municipal wastewater, ethyl acetate and inorganic nutrients. Five samples of the modified feeding solution were taken to determine COD and TOC. The results are shown in Table III-5. The average values of COD and TOC were about 2300 and 720 mg/l, respectively.

Table III-5 TOC and COD Concentrations in Modified Substrate with Inorganic Nutrients and Ethyl Acetate (1000 mg/l)

Sample	1	2	3	4	5	Average
TOC (mg/l)	728.2	701.6	740.6	649.6	769.6	717.92
COD (mg/l)	2270	2480	2310	2170	2225	2291

Using the modified feeding solution as a carbon source, anaerobic bacteria began growing on the packing material. The color of the packing bed became darker and darker. At the same time, the biogas production increased gradually. Finally, the maximum volume of biogas was reached at 550 ml per feeding interval. Figure III-7 shows biogas production during acclimation in two of the reactors. The COD and TOC in the effluent were 440 mg/l and 155.3 mg/l, respectively. Under these conditions, the bacteria were ready to become acclimated to NASA-simulated wastewater.

During the last stage of acclimation, NASA-simulated wastewater was gradually added to completely replace the municipal wastewater. In all of the stages of acclimation, inorganic nutrients were added as micronutrients, while $NaHCO_3$ and NH_4CL was used as pH buffer solutions. The pH was maintained between 6.4 and 7.4. The circulation pump was operated in the run-stop model (pump stopped 3-5 hours after running 2-3 hours). At the end of the acclimation period, the feeding solution was added every other day at 20 days HRT. One and a half month was required to the acclimate acetogenic and methanogenic bacterial in the reactors.

III.6.2 Digester Stability

Three digesters were operated under the same feeding and operating conditions. The main operating conditions were:

Total liquid volume = 3500 ml,
Operating temperature =35°C,
Feeding volume = 350 ml, and
Operating pH = 6.4 - 8.0.

The stability of the digesters were determined using three parameters: (1) biogas production, (2) COD removal efficiency, and (3) TOC removal efficiency (At the beginning of the study, three reactors were setup. Because reactor 2 did not reach steady state at 20 days HRT operation, data from digester 2 were not used in Figure III-7).

Biogas production was monitored to estimate the performance of bioreaction during the experiments. Figure III-7 shows the relationship of biogas production and feeding times between two reactors during the acclimation phase. Relatively stable feeding conditions were maintained as shown in Table III-6.

As observed, biogas production by the two bioreactors increased with increased feeding times (Figure III-7). This phenomenon demonstrated that the bioreactors were stable in biogas production with the same feeding composition and operational conditions. Although similar results were obtained for the three digesters, the data

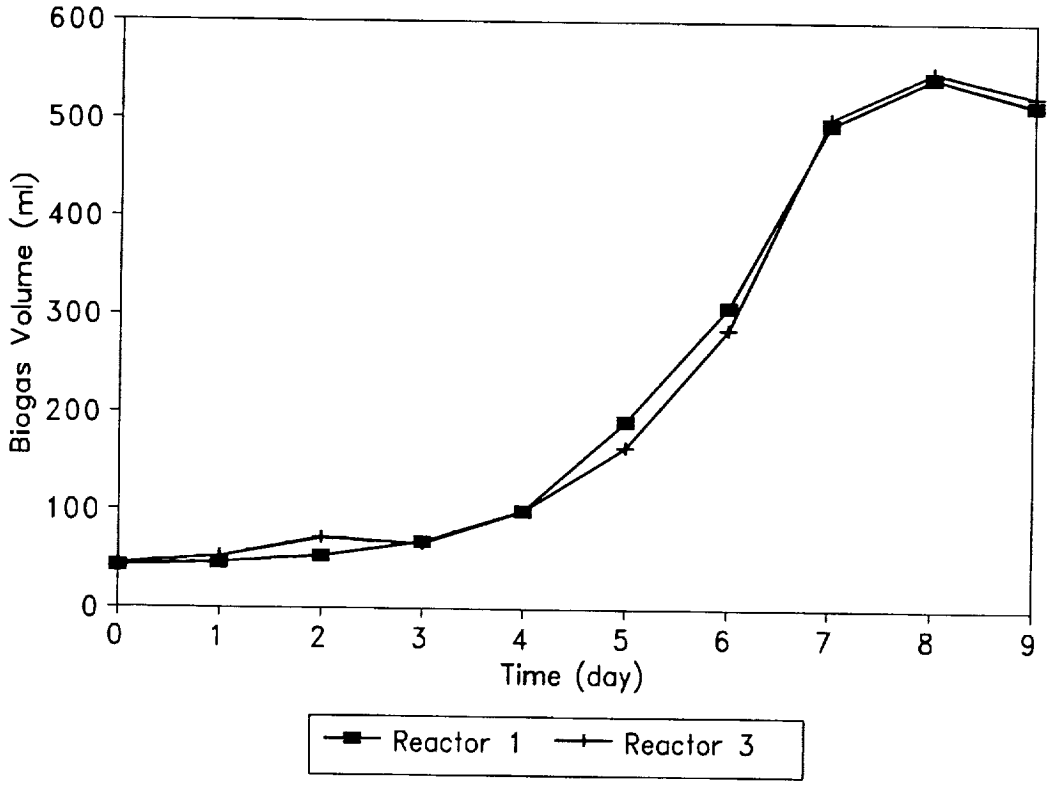

Figure III-7. Biogas production during acclimation

from digester 2 are not as complete as those from digesters 1 and 3. Therefore, only the performances from digesters 1 and 3 will be discussed.

The COD removal efficiency was used to examine the stability of the digesters. Figure III-8 shows the COD removal efficiency of digesters 1 and 3. Both digesters were operated at 35°C and 20 days HRT. During this experiment, the NASA-simulated wastewater was fed as the substrate. The COD removal efficiency varied from 68% to 90% as shown in Figure III-8. However, both digesters had nearly the same of COD removal efficiency with respect to operation time. Therefore, the performance of the digesters was stable in COD removal efficiency.

The TOC removal efficiency was also evaluated to study the stability of the digesters. Figure III-9 shows the TOC removal efficiency of digesters 1 and 3 with respect to feeding time at 20 days HRT. The operational condition and feeding composition were the same as those used in Figure III-8 (see section III.5 experimental procedure, 20 days HRT). The TOC removal efficiency varied from 66% to 90%, as shown in Figure III-9, but was consistent for both digesters.

Table III-6. Feeding conditions for digesters during acclimation

Time	Feeding Solution		
(days)	pH	TOC (mg/l)	COD (mg/l)
0	8.72	763.2	2530
1	8.46	728.2	2250
2	8.46	701.6	2170
3	8.29	740.6	1970
4	7.90	649.6	1730
5	7.75	826.0	2150
6	8.00	769.6	2210
7	7.87	698.7	1860
8	7.81	699.2	1960
9	8.37	484.4	1490

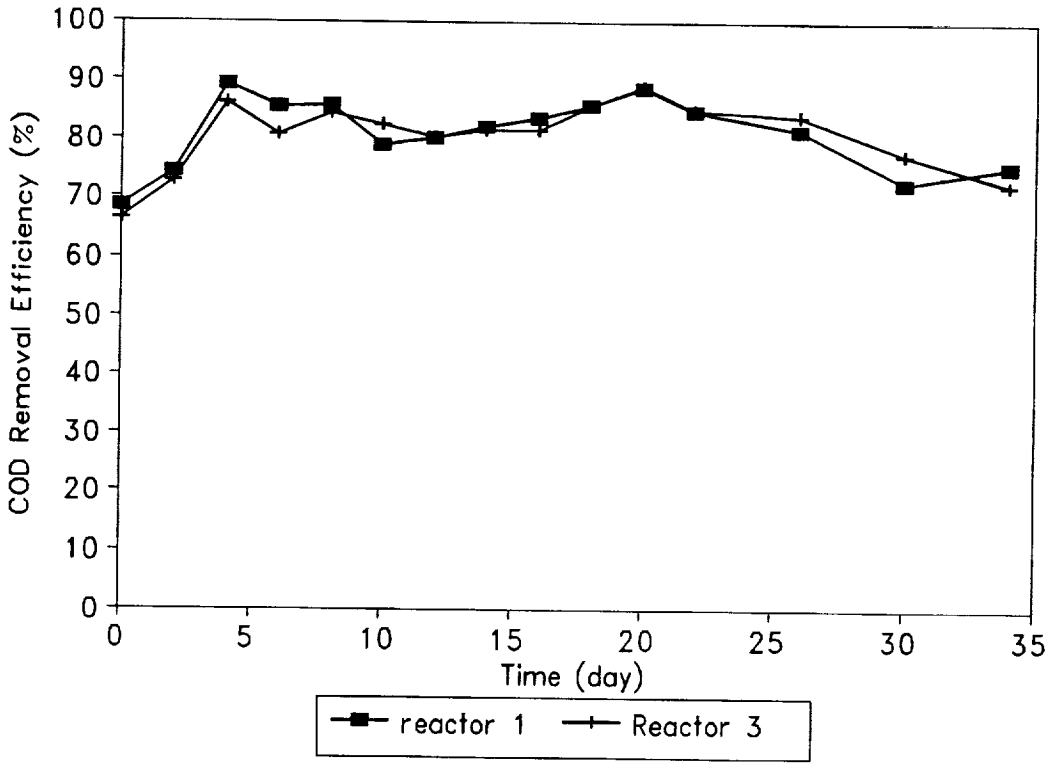

Figure III-8. The COD removal efficiency at 20 days HRT

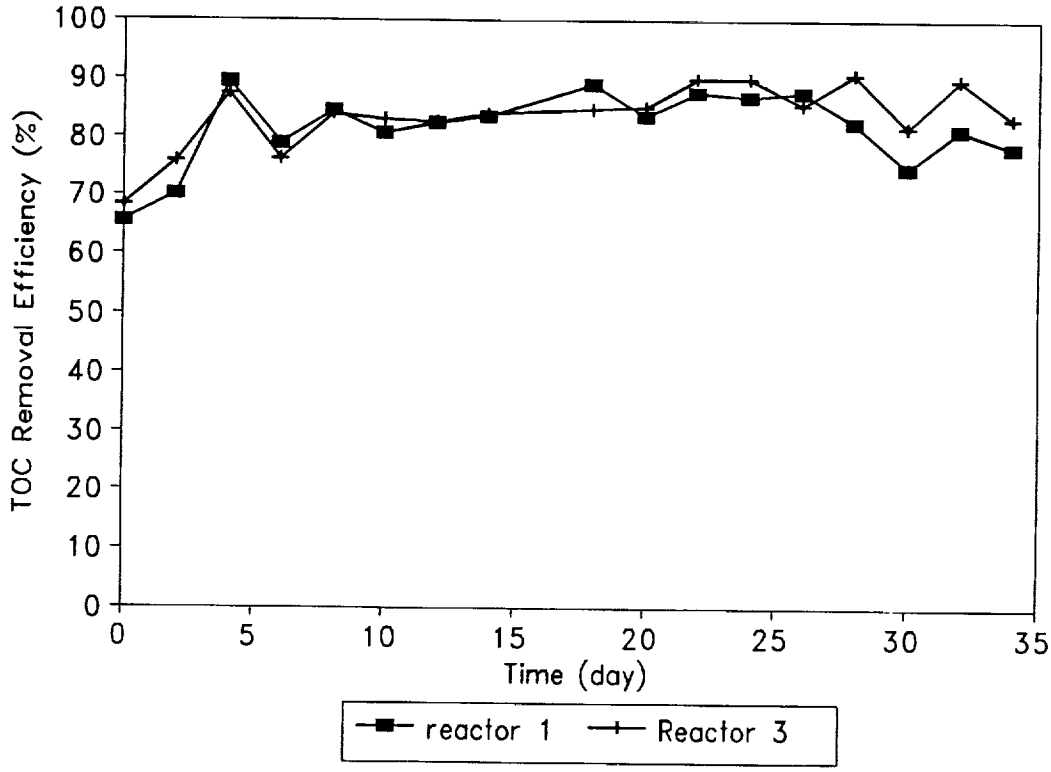

Figure III-9. The TOC removal efficiency at 20 days HRT

Similar performance for different digesters in biogas production, COD removal efficiency, and TOC removal efficiency were observed during the experiments. Based on the measured parameters (i.e. biogas production, COD and TOC removal efficiency), the stability of bioreactors was ensured in this bioreaction system.

Stability of bioreactors is necessary to evaluate the performance of a reaction system. It is also required for providing a steady environment for other biological experiments which were performed in this study.

III.6.3 Organic Loading

The loading limits in anaerobic treatment and removal efficiency of TOC and COD for simulated wastewater are very important to the design an effective biotreatment system. In this experiment, organic loading was changed by decreasing HRT from 20 days to 5 days. At the same time, removal efficiencies of TOC and COD were monitored. Under steady state, the performance of the bioreactors using different organic loading is shown in Table III-7.

Three different organic loadings were used to evaluate the performance of the digesters. TOC in effluent was 77.36 mg/l, 65.98 mg/l, and 52.25 mg/l under 20 days, 10 days, and 5 days HRT, respectively. Because the bioreaction system is very complicated, the feeding composition and pH adjustment could not be controlled to exactly the same levels during the three different HRT. These factors may have contributed to slightly different TOC in the effluent at different HRT.

The COD in the effluent indicated a chang with respect to organic loading as shown in Table III-7. But the change was not significant in comparing with the error of the COD measurement. The range of COD removal efficiency under different HRT is shown in Figure III-10. The average values of COD removal efficiencies at 20 days, 10 days and, 5 days HRT (Figure III-10) were 81.92 $\pm$ 5.25%, 74.61 $\pm$ 8.67% and 68.18 $\pm$ 9.9%, respectively. These data indicated that COD removal efficiency increased with HRT, but the standard deviation decreased with HRT. This means that long HRT were helpful in reducing COD as well as offsetting changes in operational conditions (i.e. loading rate, feeding composition).

According to Young (1989), the usual operating loading for a packed-bed anaerobic biofilm reactor is about 12 kg COD/m^3.d which is 25 times higher than the maximum COD loading (0.48 kg COD/m^3.d) in this study. Because of the limitation of operation for further decreasing HRT and the low COD and TOC values in the simulated wastewater (COD: 2000-3300 mg/l; TOC: 450-750 mg/l), the maximum organic loading rate could not be determined in this study. It is possible to obtain the

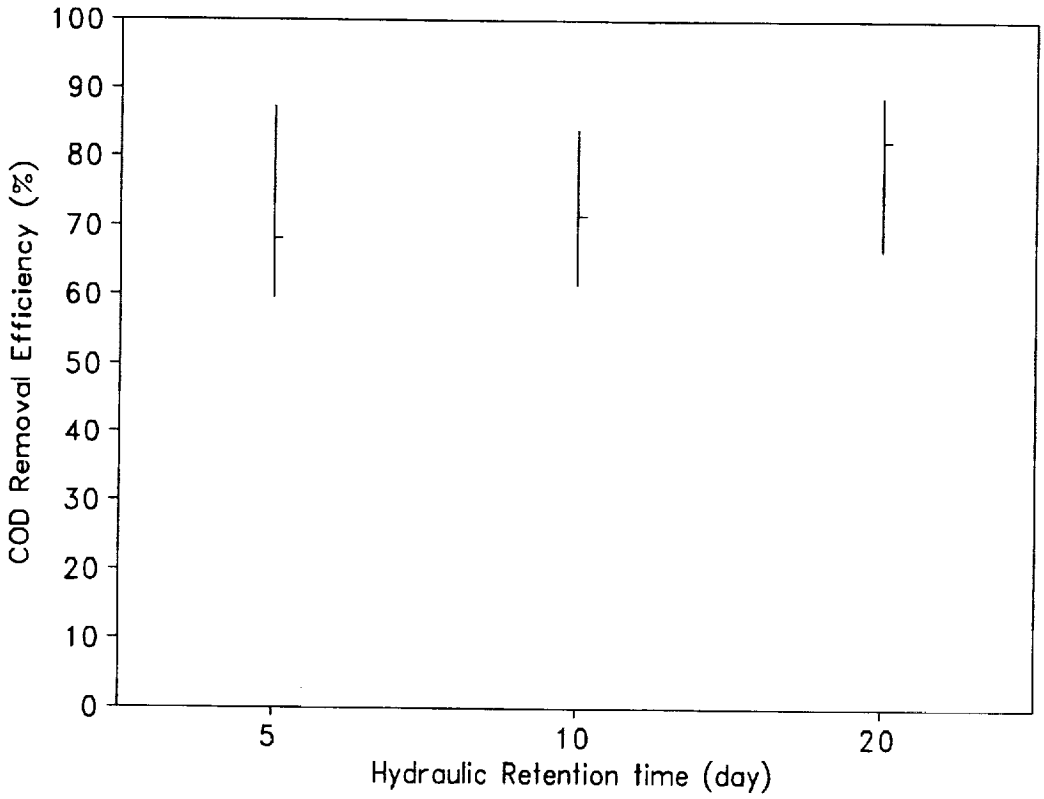

Figure III-10. Effects of organic loading on COD removal efficiency

Table III-7 The Performance of Bioreactors in Different Organic Loading under Steady State

HRT (day)	20	10	5
pH	7.65	6.91	7.11
TOC loading (Kg TOC/m^3.d)	0.029	0.058	0.106
TOC in influent (mg/l)	601.1	600.4	465.7
TOC in effluent (mg/l)	77.36	65.98	52.25
TOC removal rate (%)	87.13	89.01	88.78
COD loading (Kg COD/m^3.d)	0.117	0.239	0.480
COD in influent (mg/l)	2336	2393	2401
COD in effluent (mg/l)	422.3	607.8	764.0
COD removal rate (%)	81.92	74.61	68.18
Biogas (ml/day)	81.2	152.8	299.8

maximum organic loading by increasing the feeding rate. Therefore, a continuous feeding mode may be needed to replace the feeding mode used in this study.

Biogas production was observed to increase with respect to organic loading as shown in Table III-7. Good performance of the digesters was observed during 5 days HRT under steady state conditions. The HRT could have been reduced further if the digesters had been operated in a continue mode. Under different organic loadings, most of the COD and TOC in the effluent (in Table III-7) was observed to be at nearly the same levels.

III.6.4 Biogas Production

Biogas consists of methane and carbon dioxide. Methane and carbon dioxide are the end products of the methanogenic anaerobic process. Consequently, the amount of biogas production for a specific anaerobic process can be an important criterion in assessing the performance of this process. Additionally, the mole ratio of CH_4/CO_2 can be an indication of the balance of two steps in an anaerobic process.

The mole ratio of CH_4/CO_2 was very stable during the entire experiment. Several gas samples were obtained to determine the mole ratio of CH_4/CO_2 by GC with a TCD (Thermal Conductivity Detector). The values obtained were 3.34 ± 0.85. This indicated that a mature bioreactor system had been established.

In an anaerobic process, biogas production, the TOC removal rate and the COD removal rate are often used to demonstrate the degree of biodegradation of an organic waste. In general, high TOC removal rates correspond to high biogas production. Figure III-11 illustrates the TOC removal rate increased with increasing biogas production.

III.6.5 pH Effect

The pH is one of the important parameters in anaerobic digestion. It is recommended to maintain pH within 6.5 - 7.6 in anaerobic process. Several methods were used to adjust the pH value. $NaHCO_3$ and NH_4Cl were used as the buffer solutions during acclimation and 20 days HRT operation. The pH in the bioreactors were maintained about 7.4 - 8.0, which was slightly higher than the recommended and normal range of 6.5 - 7.6. Under this operational condition, biogas production was slow with 80% of total biogas being produced 24-48 hours after feeding. In other words, bioreaction was inhibited in a certain range.

Acetic acid was chosen to adjust the pH at 10 days HRT. The pH in the bioreactors was maintained between 6.44 and 6.85. But acetic acid is also a carbon source for anaerobic bacteria, therefore, carbon balance was required in this study. When the biogas produced at 20 days HRT was compared to that at 10 days HRT, excess biogas was observed due to the extra carbon source from acetic acid. As a result, acetic acid would not be a good chemical to adjust the pH.

Subsequently, concentrated sulfuric acid was used to adjust the pH value. One milliliter of concentrated sulfuric acid was added to 1 liter of simulated wastewater, thus, the pH in the bioreactor were controlled within 6.4-7.2. The rate of biogas production increased with 80 % of the biogas being produced within the first 8 hours after feeding. Figure III-12 illustrates the effects of pH of the feeding solution on

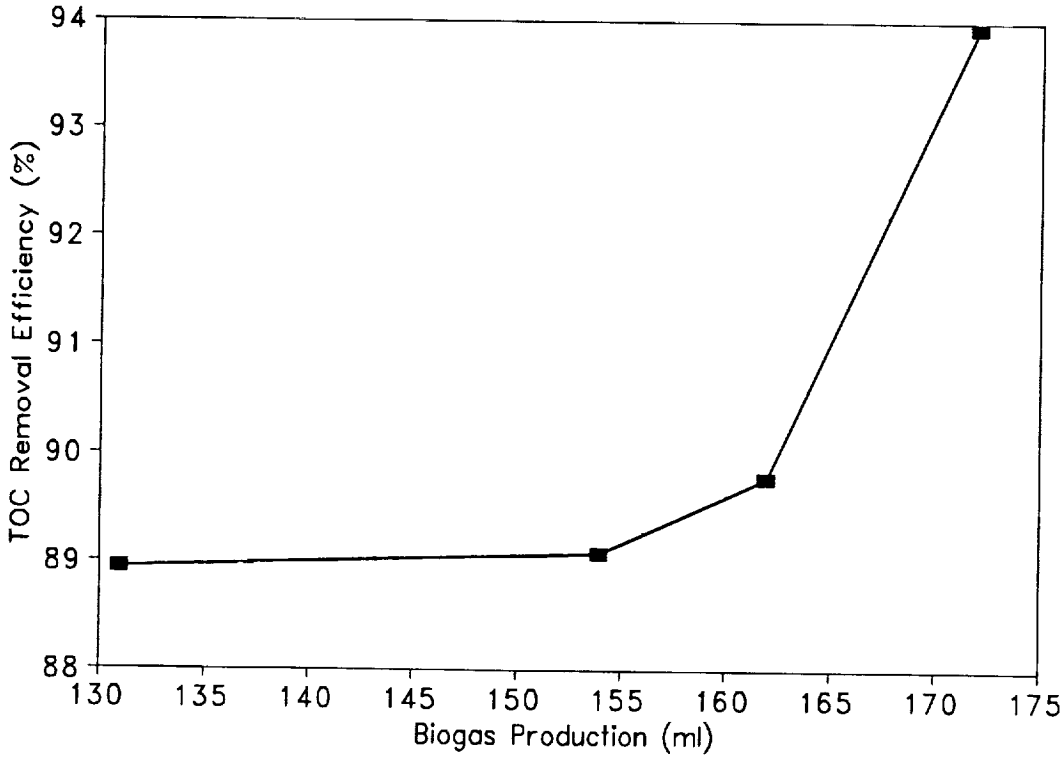

Figure III-11. TOC removal efficiency vs biogas production at 5 days HRT

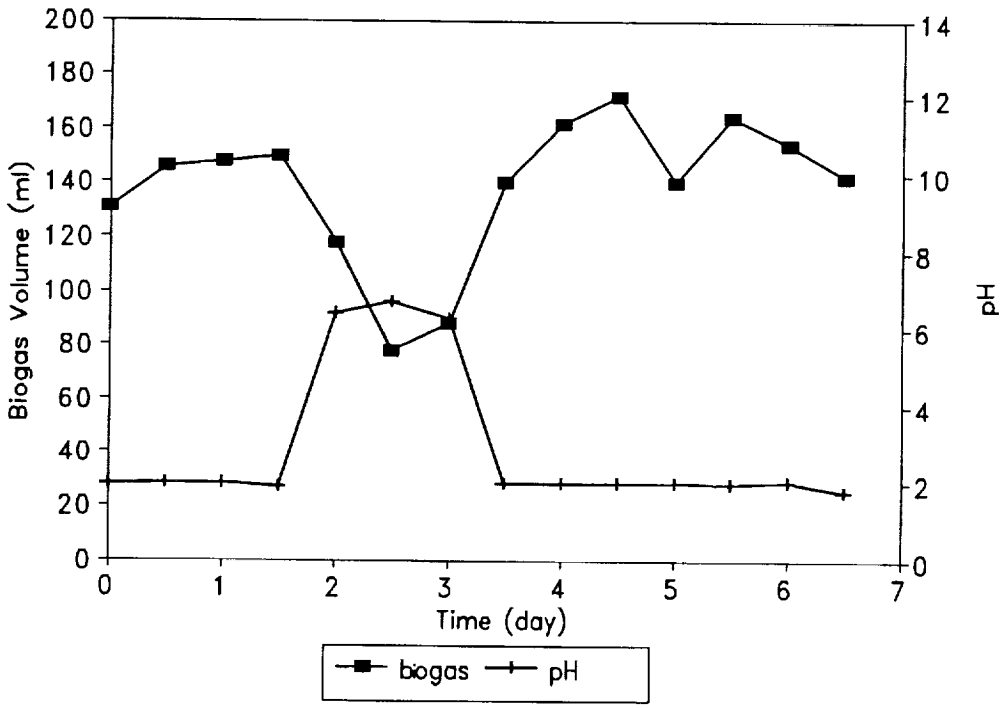

Figure III-12. The response of biogas to the pH in the influent solution (at 5 days HRT)

biogas production. As noted, biogas production was decreased when concentrated sulfuric acid was not added.

III.6.6 Mass Balance of Nitrogen, Phosphorus and Potassium

There are four possible sources/sinks for nitrogen(N), phosphorus(P), and potassium(K) in the bioreactors: 1) The seed and sludge during acclimation; 2) The nutrient broth containing multi-antibiotic resistant bacteria used in the epidemiologic study; 3) biotransferred by microbes; 4) simulated wastewater containing added micronutrients.

Mass balance of N,P, and K was studied at operations of 10 days and 5 days HRT. The results are listed in Table III-8.

Nitrogen

The concentration of nitrogen was 496.5 mg/l. After 24 hours bioreaction, the concentration of effluent was 470 mg/l; 26.5 mg/l of nitrogen was considered as the removal portion. Three possible pathways can be used to explain removal of nitrogen: 1) biotransferred by microbes; 2) adsorbed by microbial cell; and 3) stripped by biogas. In Table III-8, similar results are listed for the nitrogen mass balance after 12 hour of reaction.

Phosphorus

The mass balance of phosphorus for the two bioreaction times are listed in Table III-8. For 24 hours bioreaction, the concentration of the total phosphorus in influent was 47.4 mg/l while the concentration of the total phosphorus in the effluent was 44.25 mg/l. Thus, 2.9 mg/l of phosphorus was removed. Similar results were found for the 12 hour bioreaction. The results are shown in Table III-8. Unlike nitrogen, only two possible pathways are involved for phosphorus removal. 1) biodegraded by microbes; or 2) adsorbed by the microbial cell.

Potassium

The mass balance for potassium is listed in Table III-8. For 24 hours bioreaction, the concentration of potassium in the influent was 142 mg/l and 130 mg/l in the effluent; 12 mg/l of potassium was removed. Similar results were obtained for the 12 hour bioreaction time and are shown in Table III-8. As with phosphorus, only two possible pathways are involved for potassium removal. 1) biodegraded by microbes; and 2) adsorbed by microbial cell.

As indicated in Table III-8, the removal of N,P, and K is related to the bioreaction time. The longer the reaction time, the greater the amount removed.

Table III-8 Material Balance Table for Nitrogen, Phosphorus and Potassium

Element	Reaction Time (hour)	Influent (mg/l)	Effluent (mg/l)	Accu. in Digester (mg/l)
N	24	496.5	470.0	26.5
	12	540.0	520.0	20.0
P	24	47.4	44.5	2.9
	12	54.5	52.5	2.0
K	24	142.0	130.0	12.0
	12	163.0	150.0	13.0

Note. Accu. = Accumulation

Conversely, small amounts of N, P, and K are required when using simulated wastewater: 26.5 mg/l of nitrogen, 2.9 mg/l of phosphorus and 12 mg/l of potassium were actually consumed by the bioreaction process. Therefore, it may be concluded that it is not necessary to add N, P, K to simulated wastewater.

Based on our finding, digester effluent can be used as a good growth medium in plant-growth because of its high concentration of N, P, and K. Further study in this area is suggested.

III.6.7 Mass Balance of Carbon

As shown in Table III-9, the total carbon fed to the bioreactors may have four fates: 1) withdrawn from the reactor in the effluent, most of which is the inorganic form; 2) converted to biogas; 3) biotransferred to cell material; and 4) adsorbed by microbial cells in the digester.

In Table III-9, it is shown that 63-75% of the TOC in the influent was destroyed or transferred into inorganic carbon in the liquid or into CH_4 and CO_2 in biogas. Organic carbon in the simulated wastewater can be converted into soluble inorganic carbon or into CH_4 and CO_2 in the biogas by biological reactions. The amount of carbon adsorbed, shown in the last column of the Table III-9, was calculated from an overall mass balance of carbon. A negative value of adsorption may indicate a desorption of carbon from the microbial cells. This occurred when the total carbon in the feeding solution was low. This adsorption/desorption process acts like a reservoir for the mass balance of carbon.

Table III-9 Mass Balance of Total Carbon at 10 days HRT

Date	9/24	9/24*	9/26	9/27	9/28	9/29
TC in inf. (mg)	265.6	265.6	267.9	185.9	173.4	191.5
TOC in inf. (mg)	263.4	263.4	265.7	183.4	170.8	188.7
IC in inf. (mg)	2.2	2.2	2.1	2.5	2.6	2.8
TC in eff. (mg)	127.2	134.2	130.3	123.1	135.6	101.6
TOC in eff. (mg)	23.9	35.9	17.1	18.3	21.8	22.8
IC in eff. (mg)	103.2	98.4	113.6	104.8	113.9	65.1
C in Biogas (mg)	66.5	74.1	87.4	72.2	61.7	71.2
Adsorbed (mg)	71.9	57.3	50.2	-9.4	-33.5	28.2

Note. * = this data set was from digester 2. The others were from digester 3.
inf. = influent
eff. = effluent
TC = Total carbon
TOC = Total organic carbon
IC = inorganic carbon

III.7 CONCLUSION

The following conclusions may be summarized from this study.

1. NASA-simulated wastewater can be biodegraded easily by an anaerobic packed bed digester. The removal efficiency was nearly 90% for TOC and 80% for COD.

2. The maximum organic loading rate was not reached indicating there is a possibility of increasing the loading rate or decreasing the HRT. Further research is required in order to obtain the organic loading capacity of the digester.

3. Small amounts of N-P-K (5-8%) were consumed in the anaerobic process. The digested effluent could be used as a good nutrient source for plant growth.

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IV. EPIDEMIOLOGICAL STUDY OF AN ANAEROBIC WASTEWATER TREATMENT PROCESS

IV.1 ABSTRACT

The inactivation of a multi-drug resistant strain of *Salmonella choleraesuis* during continuous and batch mesophilic anaerobic digestion of NASA-simulated waste water was investigated at 35°C, pH 7, and hydraulic retention times (HRT) of 20, 10 and 5 days. The levels of *Salmonella choleraesuis* in the influent and effluent were determined, biogas production and pH were measured, and decimal decay rates (k_d) were estimated. This study showed initial rapid declines in viable numbers within 2 to 4 days. During continuous digestion at 10 and 5 d HRT and batch digestion, the period of rapid declines were followed by an equilibrium in which bacteria were maintained at 10 - 10^2 CFU/ml while no detectable residual bacteria population was found at 20 d HRT. *Salmonella choleraesuis* survived at least 15 days from inoculation for 10 and 5 d HRT during continuous and batch digestion, but less than 6 days for 20 d HRT. The k_d values were greater at higher initial doses than lower doses for the same HRT, and greater for batch digestion (7.89/d) than for continuous digestion (4.28, 3.82 and 3.82/d for 20, 10 and 5 d HRT, respectively). No significant difference in k_d values was found among these three HRT.

IV.2 INTRODUCTION

IV.2.1 Controlled Ecological Life Support System (CELSS)

Currently, spacecraft life support systems are simple and sufficiently reliable for human space-flight missions of relatively short duration with small crew sizes and limited power availability. However, life support technologies for the coming era of exploration must address longer-duration missions in which humans require substantial amounts of consumable materials to sustain life for long periods of time. If these consumable materials must be provided by re-supply flights from Earth, a substantial logistics infrastructure is required. Consequently, supplying all these consumables from Earth is an extremely expensive proposition. As a result, one of the most important challenges associated with longer-duration manned space flights is the development of a Controlled Ecological Life Support System (CELSS). This includes the technologies of air revitalization, water recovery, waste processing, food production, and food processing, all of which are logistically and economically essential (Pertrie, 1991; Schwartzkopf, 1992a; Flyn, 1992; Henninger, 1993).

A diagram of a CELSS is presented in Figure IV-1. This figure illustrates the fundamental flow of life support materials through the system. In this example, crop plants are used to produce food for the crew. In addition to serving as the food production subsystem, the plants take up CO_2 produced by the crew, produce oxygen

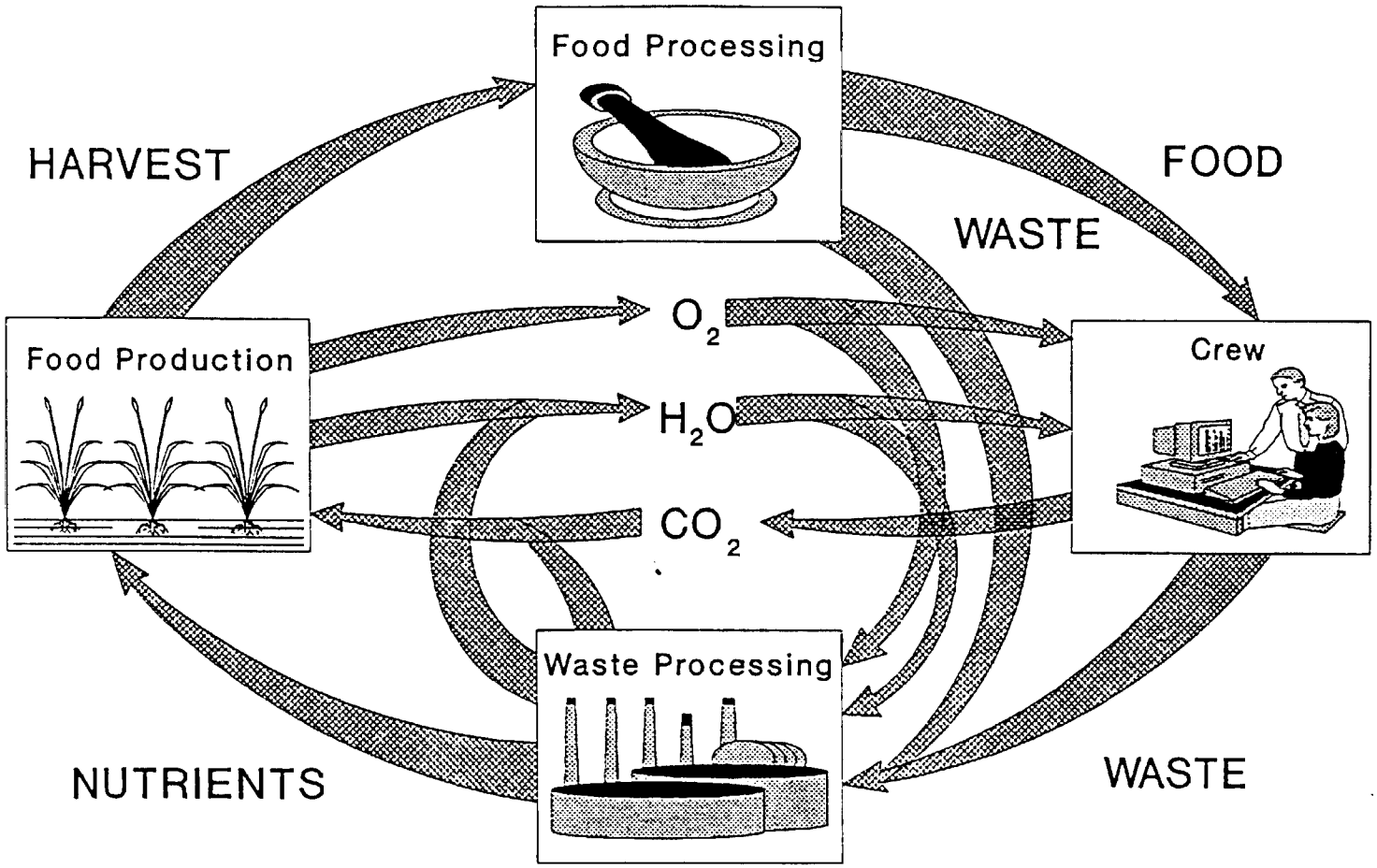

Figure IV-1. Diagram of a CELSS illustrating the fundamental flow of life support materials through the system

for the crew to breathe and for oxidation of waste materials, and produce water vapor that can be condensed and collected to supply the crew's drinking and hygiene water. In the food processing subsystem, the foodstuffs produced by the crop plants are converted to a form palatable to the crew. Urine and feces, miscellaneous solid wastes, and waste biomass from the food processing subsystem are oxidized in the waste processing subsystem to supply the crop plants with inorganic nutrients and CO_2. Any pure water produced as a by-product of the waste processor is supplied to the crew or recirculated through the waste processing subsystem.

IV.2.2 Waste processing subsystem in CELSS

Technologies for waste processing include bacterial reactors and plant-bacteria combination systems.

Bacterial reactors.

Bacterial reactors, both aerobic and anaerobic systems, have an extensive history of application in domestic sewage treatment plants (Farrah, 1983; Larsen & Munch, 1983). Aerobic systems typically require higher energy inputs to maintain oxygenation. Anaerobic systems require little energy, but they have slow process rates, and anaerobic bacteria are more susceptible to changes in environmental conditions. However, anaerobic digestion, when compared to aerobic digestion, has the advantage of high efficiency removal of organic matter by converting it to biogases such as methane and carbon dioxide. Methane can be used as a heating source, and carbon dioxide can be used in photosynthetic reactions. More importantly, anaerobic digestion produces approximately one tenth the biomass of that produced by aerobic digestion (El-Abagy & El-Zanfaly, 1984).

Anaerobic digestion, a naturally occurring biological process, as illustrated in Figure IV-2, includes three phases: The first phase consists of the conversion of complex organic molecules such as fats, carbohydrates, and proteins to organic acids and hydrogen. The bacteria involved in this phase are hydrolytic and acidogenic bacteria. The second phase of the process consists of the conversion of organic acids to simpler forms such as acetic acid and the formation of CO_2 by the acetogenic bacteria. Both acidogenic and acetogenic bacteria are not very sensitive to changes in their environment and reproduce rapidly. The third phase of the process consists of the transformation of the acids to biogas. Bacteria which accomplish this phase are methanogenic bacteria and are very sensitive to changes in their environment and do not reproduce rapidly (Schwartzkopf, 1992b; Archer & Kirsop, 1991).

Plant-anaerobic bacterial systems.

Combining plants with anaerobic bacterial systems provides several distinct advantages. One is to recover methane and basic nutrients (in the water after anaerobic treatment) by using an anaerobic wastewater treatment process. The methane gas, after purification, can be used as an energy source, and the nutrients (in

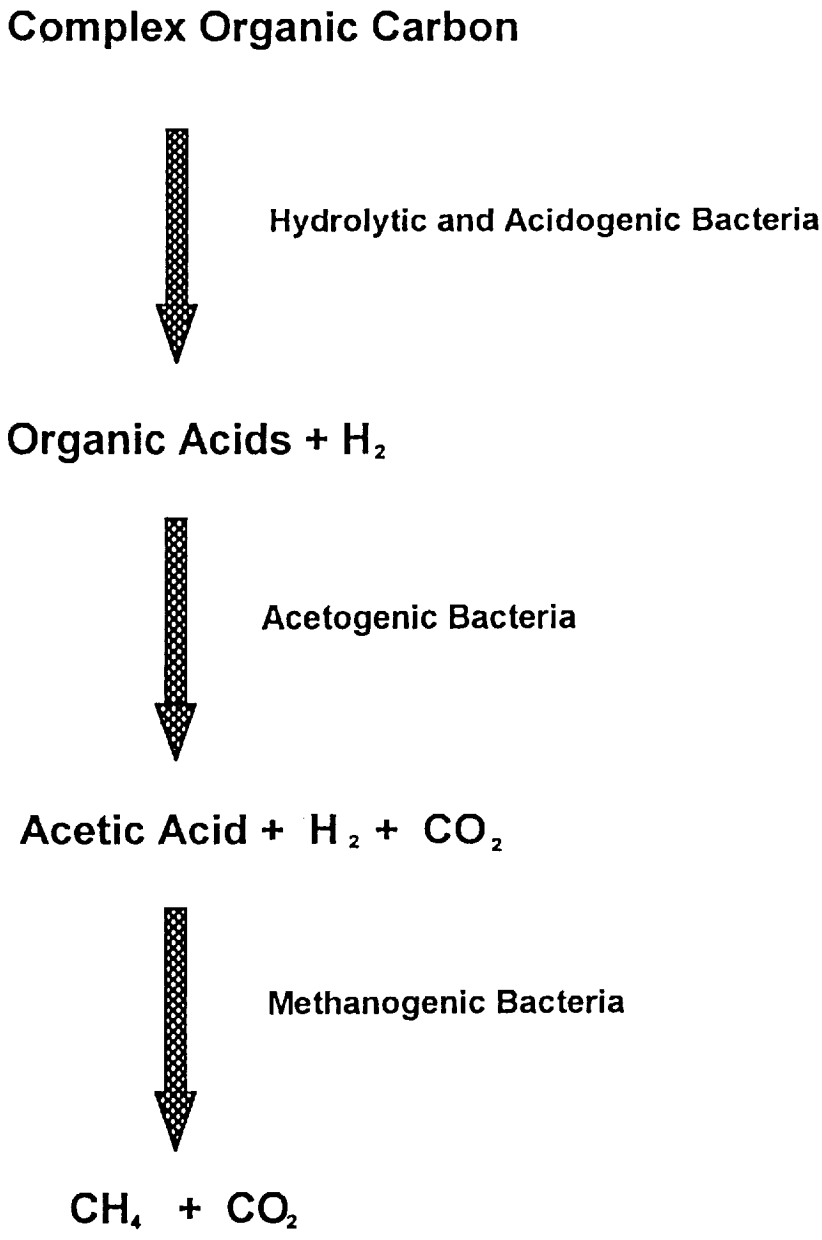

Figure IV-2. Anaerobic digestion process

the water) can be used for plant growth. More significantly, the efficiency of removal of NH_3^- and NO_3^- nitrogen can be increased during plant growth when compared to bacterial systems without plants (Wolverton et al., 1983).

Thus, an ideal wastewater treatment process in a CELSS is a combination of anaerobic bio-process, plant growth, and aerobic bio-process. A conceptual flow diagram of this process is shown in Figure IV-3. The wastewater together with solid waste are discharged into an anaerobic reactor which is kept at 35°C. In the anaerobic reactor, most of the organic material is converted into methane and carbon dioxide. After the anaerobic digestion, the effluent is used in the plant growth as a nutrient-rich solution. During growth of plants, water is transpired into the air as water vapor. This water vapor can be condensed and reused. Water from the plant-growth chamber can be treated by an aerobic process.

IV.2.3 Biohazards in wastewater

Wastewater containing human feces can present biological hazards when the intestinal flora consists of pathogenic species of bacteria belonging to genera such as *Salmonella*, *Shigella*, and *Vibrio*. They can cause illness if given the proper environmental conditions that enhance their growth and their transmission. Thus, the risk of transmission of infectious diseases must be a consideration in the treatment of wastewater.

IV.2.4 Inactivation of pathogens by anaerobic digestion

Previous studies

Inactivation of pathogenic bacteria in sewage sludge using anaerobic digestion has been successful in reducing certain pathogens to a level where the risk of transmission of these disease agents to man is low (Carrington et al., 1982; Turner et al., 1983; Gadre et al., 1986; Olsen & Larsen, 1987; Olsen, 1988; Kearney et al., 1993). From these studies, the degree of inactivation of pathogens in anaerobic digestion seems dependent upon the bacterial species, the design of the digester, as well as a variety of operational parameters (Mergaert & Verstraete, 1987; Sorlini et al., 1987). Operational parameters considered to be of major importance in determining the rate of gas production and the survival of pathogenic bacteria during anaerobic digestion are as follows: temperature (Jones, 1976); total solids (Summers, 1980; Hirn et al., 1983; Forshell, 1983); hydraulic retention time (HRT) (van Veslen, 1977); volatile fatty acids (VFA) (Geopfert & Hicks, 1969; Henry et al., 1983); and pH (van Velsen, 1980; Henry et al., 1983; George, 1988).

Studies of anaerobic digestion have been conducted using different species of pathogenic indicator bacteria under various operational parameters during different types of digestion. In each situation, optimal inactivation required specific conditions.

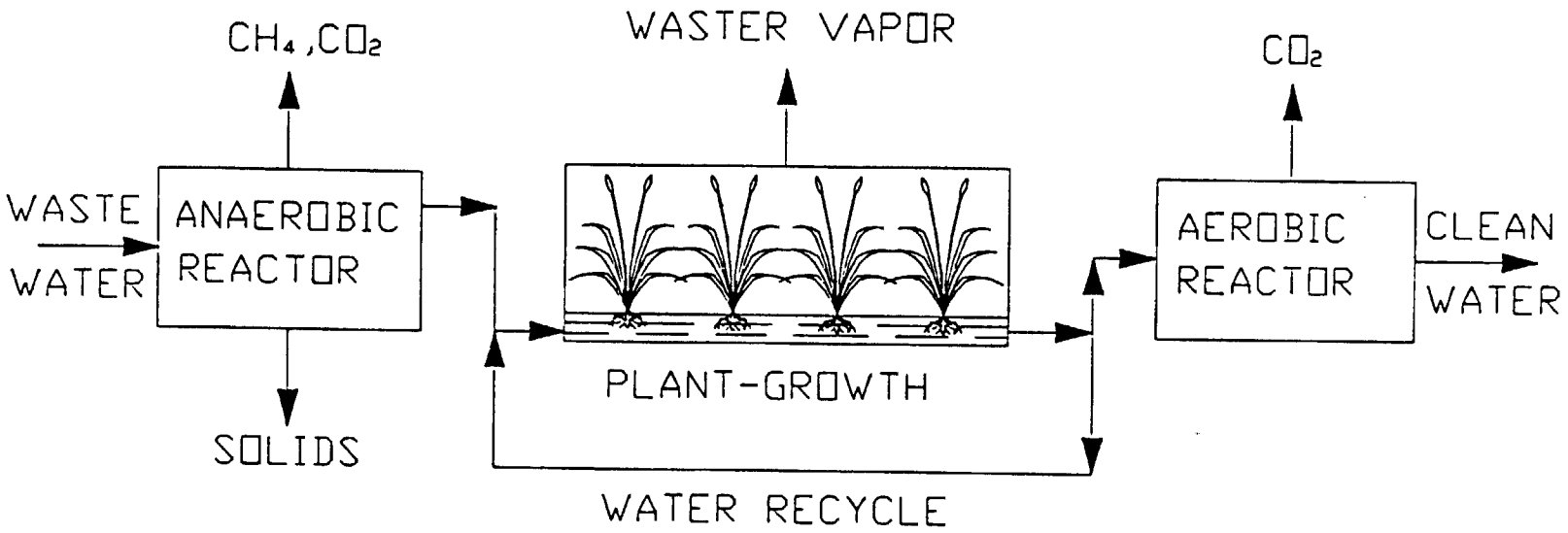

Figure IV-3. Flow diagram of the anaerobic/plant-growth/aerobic hybride process

For example, Carrington et al. (1982) found the decimal decay rate of *Salmonella duesseldorf* in sewage sludge during continuous anaerobic digestion to be greater at 48°C than at 35°C at the same HRT; the decimal decay rates were also found to be greater for HRT of 16 days than for 10 days, but showed no difference when the HRT was over 16 days; and gas production was gradually lost when the mean retention period was reduced to 6-7 days. Jones (1976) found that survival of salmonellae in cattle slurry was longest in slurries with a solids content of 5% or more with temperatures below 10°C. Kearney et al. (1993) reported a decline in viable numbers of pathogenic bacterial species in slurry storage under mesophilic anaerobic digestion conditions and proposed that this decline was temperature-dependent because the bacteria declined more rapidly at 17°C than at 4°C. Gadre et al. (1986) found that an antibiotic-resistant strain of *Salmonella typhimurium* was totally eliminated in 9 days in a 37°C anaerobic digester. Goepfert and Hicks (1969) reported that the maximal death rate was associated with the concentration of VFA and pH.

The importance of present study

Due to crew space limitation in a CELSS, one very important consideration is the volume of the digester. The digester should be as small as possible. This means that the HRT has to be set as short as possible without allowing washout of the digesting bacteria which are immobilized in the digester. This, along with pH and temperature, is important in setting the operational parameters of anaerobic digestion. Inasmuch as these parameters may create optimal conditions for treatment of the waste water, they may not create optimal conditions for inactivation of pathogenic bacteria present in the wastewater. Thus, there remains the possibility of the presence of viable pathogenic bacteria in the treated wastewater and its recycling through the CELSS. If the reduction of pathogens to a safe level cannot be achieved, entry into the environment of the CELSS can create a substantial risk to its occupants (Dudley et al., 1980). Rodgers (1986) reported that enteric bacteria were frequently recovered from past NASA missions. In another study, Roman (1992) monitored the microbial distribution in the environment of a CELSS and found the pathogenic species *Shigella sonnei*.

Defining the survivability of pathogenic bacteria during the wastewater treatment process in an anaerobic bioreactor to be used in a CELSS is of extreme importance. If the specific decay rates of a pathogen in the wastewater treatment facility operating under specified conditions are known, it should be possible to predict the remaining number of pathogens in the effluent of the digester. Thus, it should be possible to control the water quality relative to the presence of pathogens in the water recycling system.

Salmonellosis

Salmonellosis is regarded as the most important disease that is spread by slurry and is thought to provide a suitable model for the dissemination of other bacterial diseases in the environment (Jones & Matthews, 1975). Most species of *Salmonella*

cause infection in a wide range of hosts and can cause different problems depending on the species. For example, *S. typhi* can cause enteric fever (typhoid fever) with an infective dose of less than 10^5 cells. Moreover, *S. choleraesuis* and *S. enteritidis* can cause acute enterocolitis if the organisms invade the small intestine and the colon. They can also cause bacteremia if the organisms invade the intestinal mucosa and blood (Baron et al., 1994). Additionally, a secondary infection may result anywhere in the body. Modes of transmission for these diseases are food, water, dirt, and fecal-oral spread (McCoy, 1962).

Salmonella species are facultatively anaerobic Gram-negative bacilli belonging to the family Enterobacteriaceae. The infective dose of *Salmonella* species is 10^5-10^6 cells, or fewer if ingested with a meal or with a high gastric pH (Krieg et al., 1984). Salmonellae give positive test results with methyl red, citrate and motility. Negative test results are obtained with indole, lactose and urease. Serological tests are necessary in confirming their identification.

IV.2.5 Objectives

The objectives of this study were threefold: (1) to measure the decimal decay rates and extent of inactivation of a multi-drug resistant strain of *Salmonella choleraesuis* during mesophilic anaerobic digestion of NASA simulated wastewater within laboratory model digesters, (2) to study the effects of HRT and type of digestion on inactivation, and (3) to investigate the size of inoculum on inactivation.

IV.3 MATERIALS AND METHODS

IV.3.1 Bacterial strains

A multi-drug resistant strain (RS) of *Salmonella choleraesuis*, subspecies: *choleraesuis*, serotype: *typhi*, antigenic formula: 9, 12, Vi:d was obtained from American Type Culture Collection (ATCC No. 19214) and was used as the indicator bacteria in this study. This strain is resistant to chloramphenicol, tetracycline, streptomycin, and sulfanilamide. It is not known to be part of the indigenous flora of animals nor is it known to occur in nature.

A wild strain of *Salmonella choleraesuis*, non-resistant to the above antibiotics, was utilized in the experiment as the control (NRS). It was routinely streaked on the antibiotic-containing agar plates to check the inhibitory action of the medium.

IV.3.2 Media

Nutrient agar and nutrient broth (Fisher, Pittsburgh) were used as growth and storage media for the RS and NRS bacteria.

MacConkey agar CS (Difco, Detroit) was used as a bacterial quantification medium. It was supplemented, after sterilization, with chloramphenicol, streptomycin, tetracycline, and sulfanilamide (Sigma, St. Louis) at concentrations that inhibited growth of NRS but not the RS bacteria. This medium was therefore referred to as MacConkey-antibiotic agar.

IV.3.3 Substrates

The laboratory anaerobic digesters were inoculated with a wastewater seeding (3.5 l) obtained from a local municipal wastewater treatment plant.

A nutrient feed stock solution (NFSS) was used as a nutrient source to feed the digesters. This solution was prepared from wastewater generated in our NASA Simulated Waste Water Station housed at Lamar University - Beaumont, Texas, in the Department of Biology. Its composition was based on NASA wastewater composition guidelines listed in Table IV-1. When necessary, the waste water was supplemented with micronutrients listed in Table IV-2. NFSS was prepared freshly prior to each feeding. In order to reduce its total volume but still keep the correct proportion of ingredients, one use of shower water (5.32 l), one use of clothes wash water (12.44 l), and one use of dish wash water (9.07 l)) were mixed together in a container. One-fourth of this suspension (6.71 l) was mixed with one use of urine (0.37 l), one use of urine flush water (0.123 l), and one use of hand wash water (1.01 l). Three-hundred and fifty milliliters of the final suspension were used to feed each anaerobic digester.

Table IV-1. Composition of NASA-simulated waste water

Item	l/person-day	Four person crew
Shower Water (4 uses total per day,12g soap per use)	5.32	21.28
Hand Wash (16 uses total per day, 2g soap per use)	4.07	16.28
Clothes Wash (30g soap)	12.44	49.76
Urine Flush	0.49	1.96
Urine (16 uses total per day)	1.51	6.04
Dish Wash	9.07	36.28
TOTALS	32.90	131.60

Table IV-2. Micronutrients supplemented to the NFSS

Chemicals	Concentration (mg/l)
$CaCl_2 \cdot H_2O$	6.25
$NaPO_3$	0.25
$(NH_4)_2HPO_4$	96.5
$C_3H_7NO_2S \cdot HCl \cdot H_2O$ (L-cysteine)	2.5
$CoCl_2 \cdot 6H_2O$	10.0
$FeCl_2 \cdot 4H_2O$	20.0
$Na_2S \cdot 9H_2O$	75.0
$MgCl_2 \cdot 6H_2O$	266.75
NH_4Cl	369.0
KCl	100.0
$NaHCO_3$	1500.0
KI	0.625
$CH_3COOC_2H_5$ (Ethyl acetate)	1.25 (ml/l)

IV.3.4 Anaerobic digesters

Anaerobic wastewater digestion was carried out in three 4-liter laboratory model digesters constructed with ports for the withdrawal of the fermenting mass, feeding with NFSS, and withdrawal of the biogas samples (Figure IV-4). The digesters were filled with polypropylene pall rings for immobilization of bacteria. The digesters were maintained at 35°C in incubators. Performance of the anaerobic digesters was monitored by the pH, biogas production, and percentage of methane present. The pH of the influent and effluent was measured using an Orion, 720A pH meter. Biogas generation was monitored, and the amount produced between each feeding was recorded. The percentage of methane present was measured using gas

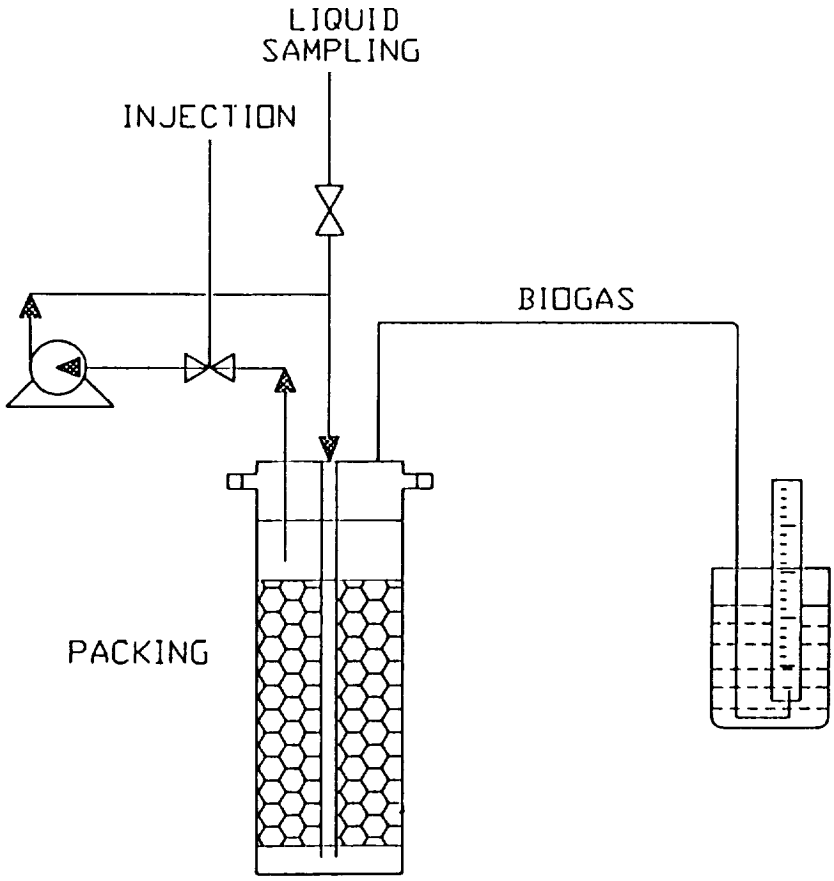

Figure IV-4. Schematic diagram of experimental set-up

chromatography. Continuous cycling of the contents of the digesters was accomplished by pumping for mixing.

IV.3.5 Experimental procedure (see details in Appendix B)

Bacterial quality assurance

The stock indicator bacteria growing on nutrient agar was inoculated into nutrient broth. After 18 hours of incubation at 35°C, one loopful of the broth culture was streaked to a MacConkey-antibiotic agar plate. After another 18 hours of incubation, three to five colonies from the MacConkey-antibiotic agar plate were identified by the Crystal Identification Systems (Becton Dickinson, Maryland) and confirmed serologically using commercially available antisera (Sigma, St. Louis).

The RS and NRS were assayed to determine the concentration of the four antibiotics required to inhibit growth of the NRS but not the RS. The final concentration of each of the four antibiotics supplemented in MacConkey media was based on these findings.

Examination of wastewater seed and NFSS

One milliliter of the seed solution and one milliliter of the NFSS were transferred to two separate nutrient broths. After 18 hours of incubation at 35°C, one loopful of each broth culture was streaked to MacConkey-antibiotic agar plates. After another 18 hours of incubation at 35°C, colonies on these plates were identified by the Crystal Identification Systems as described above. Indicator bacteria were not expected to be isolated from the wastewater seed nor the NFSS.

Establishment of steady-state anaerobic digestion

Seeding solution (3.5 l) containing digesting bacteria was added to each anaerobic digester and subsequently fed with NFSS (350 ml) after withdrawing 350 ml of the fermenting mass from the digester. Initially, local municipal wastewater was used as the feed solution. However, after biogas production was observed, the feed solution was changed to NASA formulated NFSS. Experimentation was begun when the digesters achieved a steady-state of gas production and pH.

After a steady-state was established in each digester, the experiments to determine the survival curve were conducted in single-dose continuous, multi-dose continuous and batch digesters. The single-dose digester was inoculated with indicator bacteria only once with its first feeding; the multi-dose continuous digester received indicator bacteria with each feeding. The batch digester received indicator bacteria in its first and only feeding, and hence no fresh nutrients were supplied to the digester flora.

Establishment of bacterial growth curve

The absorbance and colony counts over a period of 36 hours were obtained to establish a bacterial growth curve of the indicator bacteria. This growth curve was used in preparing indicator bacterial inocula concentrations. Indicator bacteria from a nutrient agar slant were inoculated into nutrient broth. After 18 hours of incubation at 35°C, 100 µl of the broth were inoculated into 200 ml of nutrient broth. This 200 ml nutrient broth culture was incubated at 35°C. During incubation, absorbance readings at 420 nm using a spectrophotometer (HACH, Loveland) were taken and recorded every 30 minutes; one ml of the broth was withdrawn simultaneously for 10-fold serial dilution in 0.1% peptone water for quantification of the bacteria on MacConkey-antibiotic agar plates (Greenberg, 1992).

Preparation of bacterial culture

Indicator bacteria from a nutrient agar slant were inoculated into nutrient broth. After 18 hours incubation at 35°C, 100 µl of this broth culture were inoculated into 200 ml of nutrient broth and incubated at 35°C. During incubation, spectrophotometric absorbance readings were taken until an absorbance reading corresponding to that of a desired concentration, as determined by the growth curve, was obtained.

Injection of the indicator bacteria

A volume of 350 ml of the fermenting mass was withdrawn from the anaerobic digesters after steady-state had been achieved. Afterward, 3.5 ml of bacterial suspension were injected into the digesters through the rubber tube of the input valve. Following injection, the bacterial suspension was flushed with 346.5 ml of NFSS. At this point, the digesters contained a total volume of 3500 ml. A volume of 350 ml of fermenting mass was withdrawn and 350 ml of NFSS was added to the digesters at an interval as determined by the HRT.

At the conclusion of the studies described above, inactivation of salmonellae was investigated during batch anaerobic digestion. Unlike the continuous digestion studies, the batch anaerobic digester was not fed with fresh NFSS. Five ml of fermenting mass was withdrawn from the batch digester after 3, 6, 12, and 24 hours for pH measurements and colony counts. The excess fermenting mass was re-injected into the reactor after each measurement.

Enumeration of the indicator bacteria

Viable counts of the indicator bacteria were determined by preparing 10-fold serial dilutions of the fermenting mass in 0.1% peptone water. One-tenth ml volumes of the dilutions were spread with sterile glass L-rods over the MacConkey-antibiotic agar plates. Colony counts (CFU/ml) were determined after 24 hours of incubation at 35°C (Greenberg, 1992).

The biofilm on the pall rings was examined at the conclusion of the study for the presence of attached indicator bacteria. One pall ring was selected and was mixed

with peptone water with the use of a votex mixer. The mixed suspension was then streaked on MacConkey-antibiotic plate, and the suspect colonies were identified by the Crystal Identification System after 24 hours incubation.

IV.3.6 Statistical analysis

The decimal decay rate (k_d) for the multi-dose continuous digestion studies was calculated using the modified formula by Ginnivan (1980).

$$k_d = -\frac{1}{t} \ln\left(\frac{P}{P_o}\right) - \frac{v}{V}$$

where, P is the colony count of indicator bacteria in the withdrawn effluent, P_O is the viable count in the influent (CFU/ml), v is the liquid volume of the effluent removed per day (ml/day), V is the liquid volume in the digester (ml), and t is the time interval (day).

The decimal decay rate (k_d) for the single-dose continuous digestion and batch digestion studies were determined during the rapid decline of the population and were calculated by the slope of the linear regression of the nature log (ln) fraction surviving against time from inoculation. The period of rapid decline was fitted with the line of best fit by linear regression.

Pearson's correlation coefficients (r) were calculated to determine the relationship between the viable count and time from incubation; viable count and biogas production; viable count and pH; initial feeding dose and decimal decay rate.

Differences among the k_d at various HRT during continuous digestion were assessed by calculating upper and lower 95% confidence intervals. The k_d values were significantly different if their 95% confidence intervals did not overlap.

IV.4 RESULTS

IV.4.1 Assay for the determination of the concentration of antibiotics supplemented to media

Concentrations of the antibiotics required in the media that allow growth of the resistant strain (RS) but not the non-resistant strain (NRS) are shown in Table IV-3. The resistant strain grew on the medium containing the four antibiotics with concentrations up to 15 μg/ml while growth of the non-resistant strain was inhibited at concentrations of 1 μg/ml and higher for each antibiotic.

Table IV-3. Growth of the *Salmonella choleraesuis* resistant strain (RS) and non-resistant strain (NRS) on MacConkey agar plates containing four antibiotics (choramphenicol, streptomycin, tetracycline, and sulfanilamide) in different concentrations

Concentration of each antibiotic (μg/ml)						
Bacteria	20	15	10	5	1	0
Resistant strain	-	+	+	+	+	+
Non-resistant strain	-	-	-	-	-	+

+, growth of bacteria
-, no growth of the bacteria

IV.4.2 Examination of seeding and NFSS

No indicator bacteria were isolated on the MacConkey-antibiotic plate from seeding and NFSS. However, *Pseudomonas aeruginosa* from the seeding solution was detected.

IV.4.3 Growth curve of the indicator bacteria

The growth curve of the indicator bacteria in nutrient broth incubated aerobically at 35°C is shown in Figure IV-5. The incubation time, absorbance reading, and viable numbers of *Salmonella choleraesuis* are listed in Table IV-4. It can be seen that under optimal conditions, a typical growth curve occurred with indications of a lag, log, stationary, and death phase.

IV.4.4 The single-dose study of inactivation of *Salmonella choleraesuis* during continuous mesophilic anaerobic digestion

The declines of viable counts of *Salmonella choleraesuis* during continuous mesophilic anaerobic digestion at different HRT are illustrated in Figure IV-6 and Table IV-5. Figure IV-6 indicates that the viable count of *Salmonella choleraesuis* at the three HRT declined rapidly within the first 4 days after inoculation. The correlation values ($r = -0.71$ at 20 d HRT; -0.71 at 10 d HRT; -0.55 at 5 d HRT) indicate that there is a strong negative and linear relationship between viable counts and time from inoculation during the period of rapid decline in the three HRT.

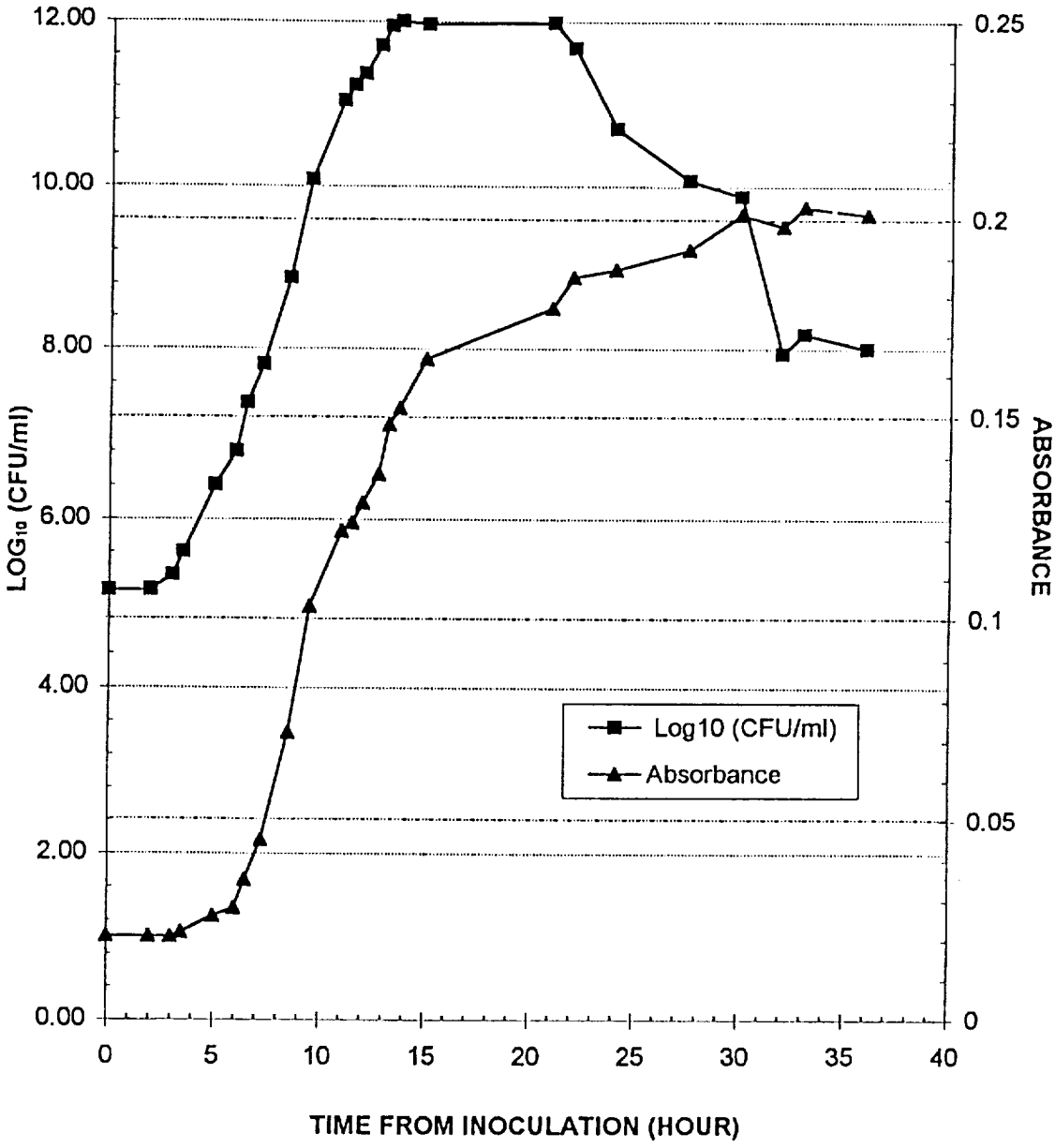

Figure IV-5. The growth curve of *Salmonella choleraesuis*

Table IV-4. The incubation time, viable count, and absorbance reading of *Salmonella choleraesuis* in nutrient broth at 35°C under aerobic condition

Time (hour)	CFU/ml	Log_{10}(CFU/ml)	Absorbance
0.00	1.36E+05	5.13	0.021
2.00	1.38E+05	5.14	0.021
3.00	2.10E+05	5.32	0.021
3.50	4.00E+05	5.60	0.022
5.00	2.51E+06	6.40	0.026
6.00	6.20E+06	6.79	0.028
6.50	2.26E+07	7.35	0.035
7.25	6.42E+07	7.81	0.045
8.50	7.33E+08	8.87	0.072
9.50	1.20E+10	10.08	0.103
11.00	1.09E+11	11.04	0.122
11.50	1.67E+11	11.22	0.124
12.00	2.30E+11	11.36	0.129
12.75	5.10E+11	11.71	0.136
13.25	8.80E+11	11.94	0.148
13.75	9.95E+11	12.00	0.152
15.00	9.25E+11	11.97	0.164
21.00	9.63E+11	11.98	0.177
22.00	4.79E+11	11.68	0.185
24.00	5.11E+10	10.71	0.187
27.50	1.19E+10	10.08	0.192
30.00	7.41E+09	9.87	0.201
32.00	8.79E+07	7.94	0.198
33.00	1.51E+08	8.18	0.203
36.00	1.01E+08	8.00	0.201

Table IV-5. Viable counts of *Salmonella choleraesuis* in the single-dose study during the continuous mesophilic anaerobic digestion under various HRT (Mean pH = 7.12; Mean biogas = 183 ml/interval)

Time Day	5 HRT		10 HRT		20 HRT	
	CFU/ml	$Log_{10}(P/P_0)$	CFU/ml	$Log_{10}(P/P_0)$	CFU/ml	$Log_{10}(P/P_0)$
0.0	7.80E+07	0.00	4.40E+07	0.00	3.90E+07	0.00
0.5	5.16E+06	-1.18	N/A	N/A	N/A	N/A
1.0	5.75E+05	-2.13	1.10E+05	-2.60	1.21E+04	-3.51
1.5	3.61E+04	-3.34	N/A	N/A	N/A	N/A
2.0	8.26E+03	-3.98	3.30E+03	-4.12	1.23E+03	-4.50
2.5	4.61E+03	-4.23	N/A	N/A	N/A	N/A
3.0	1.55E+03	-4.70	3.50E+02	-5.10	1.09E+02	-5.55
3.5	4.68E+02	-5.22	N/A	N/A	N/A	N/A
4.0	3.13E+02	-5.40	1.16E+02	-5.58	13	-6.47
4.5	2.00E+02	-5.59	N/A	N/A	N/A	N/A
5.0	2.51E+02	-5.49	1.35E+02	-5.51	N/A	N/A
5.5	2.14E+02	-5.56	N/A	N/A	N/A	N/A
6.0	2.95E+02	-5.42	1.10E+02	-5.60	UD	N/A
6.5	2.51E+02	-5.49	N/A	N/A	N/A	N/A
7.0	N/A	N/A	1.39E+02	-5.50	N/A	N/A
7.5	N/A	N/A	N/A	N/A	N/A	N/A
8.0	N/A	N/A	4.90E+01	-5.95	N/A	N/A
15.0	1.40E+02	-5.75	4.00E+01	-6.04	N/A	N/A

UD: Undetectable Level; N/A: Not Available

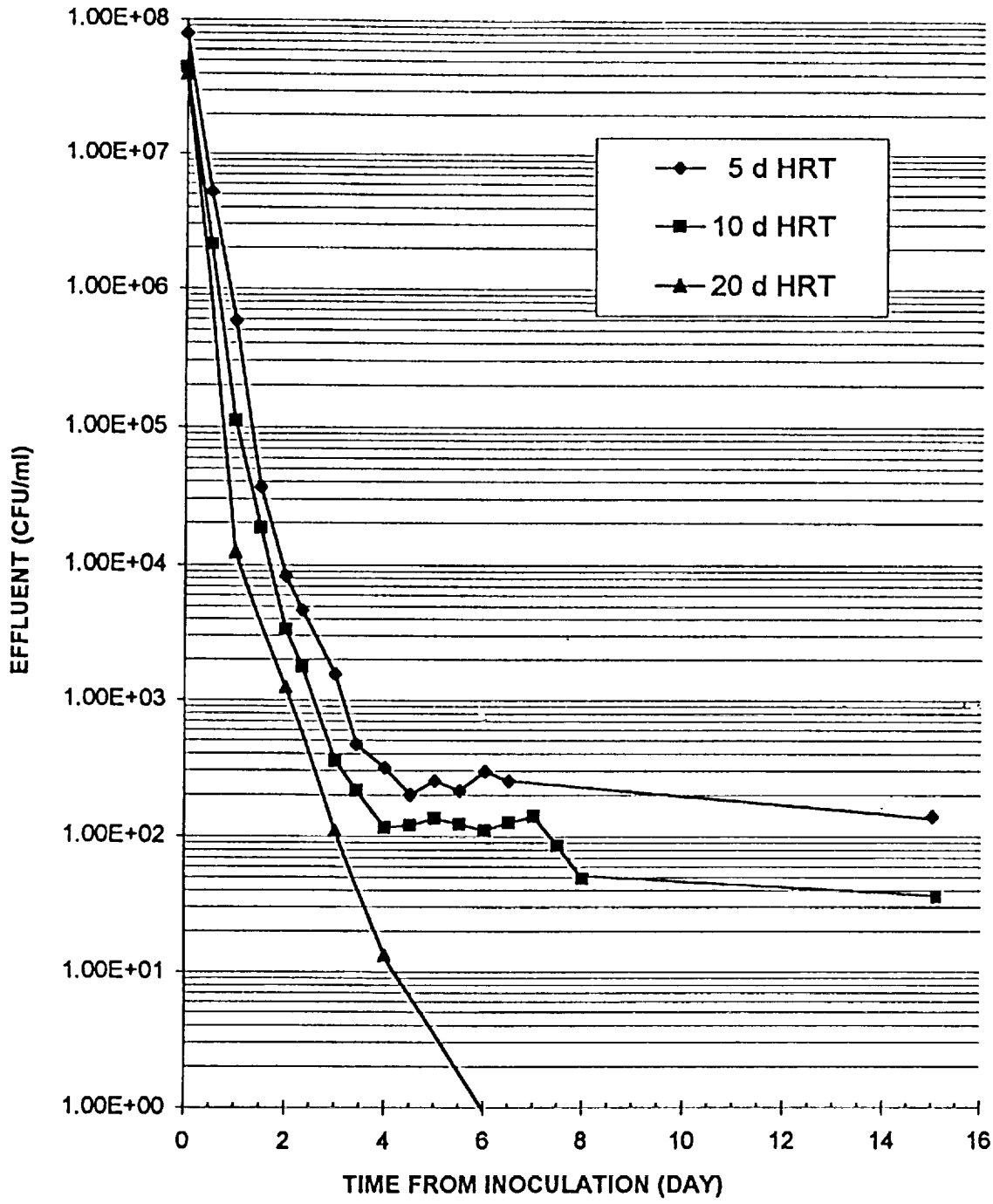

Figure IV-6. Hydraulic retention time (HRT) and survival of *Salmonella choleraesuis* in the single-dose study during continuous mesophilic anaerobic digestion (Mean pH = 7.12; Mean biogas = 183 ml/interval)

The investigation of the effect of the various HRT on the survival time of *Salmonella choleraesuis* showed greater variations. Using an initial dose of approximately 10^7 CFU/ml for each HRT study, at an HRT of 20 days, the indicator bacteria were found to be below the level of detection by the end of the 6th day of the study. However, after a period of rapid decline in viable numbers, at HRT of 10 and 5 days, the indicator bacteria reached equilibrium (the viable numbers were not reduced by one logarithmic unit) by the end of the 15th day at approximately 40 CFU/ml for 10 d HRT and $1.4X10^2$ CFU/ml for 5 d HRT.

The decimal decay rates (k_d) of the *Salmonella choleraesuis* during single-dose continuous mesophilic digestion operated at 20, 10 and 5 d HRT are shown in Figures 7, 8, and 9, respectively. Differences among the k_d values, at various HRT were assessed by calculating upper and lower 95% confidence intervals (Table IV-6) (Mosteller et al., 1983). The three k_d values were not significantly different because their 95% confidence intervals overlapped.

Table IV-6. Decimal decay rate constants (k_d) of Salmonella choleraesuis during songle-dose continuous mesophilic anaerobic digestion

HRT (day)	k_d (day^{-1})	Lower Limits (day^{-1})	Upper Limits (day^{-1})
20	4.28	3.07	5.48
10	3.82	2.88	4.76
5	3.82	3.36	4.28

The pH and biogas production during single-dose continuous digestion remained relatively stable throughout the digestion with a mean pH of 7.12 and mean biogas production of 183ml/interval which contained a mean of 71% methane.

IV.4.5 The multi-dose study of inactivation of *Salmonella choleraesuis* during continuous mesophilic anaerobic digestion

The effect of bacterial feeding dose on the decimal decay rate (k_d) of *Salmonella choleraesuis* is shown in Figure IV-10 and Table IV-7. The correlation values (r)(0.62 at 20 d HRT; 0.87 at 10 d HRT; 0.90 at 5 d HRT) indicate that there is a strong positive relationship between the bacterial viable count of the initial dose and their k_d values.

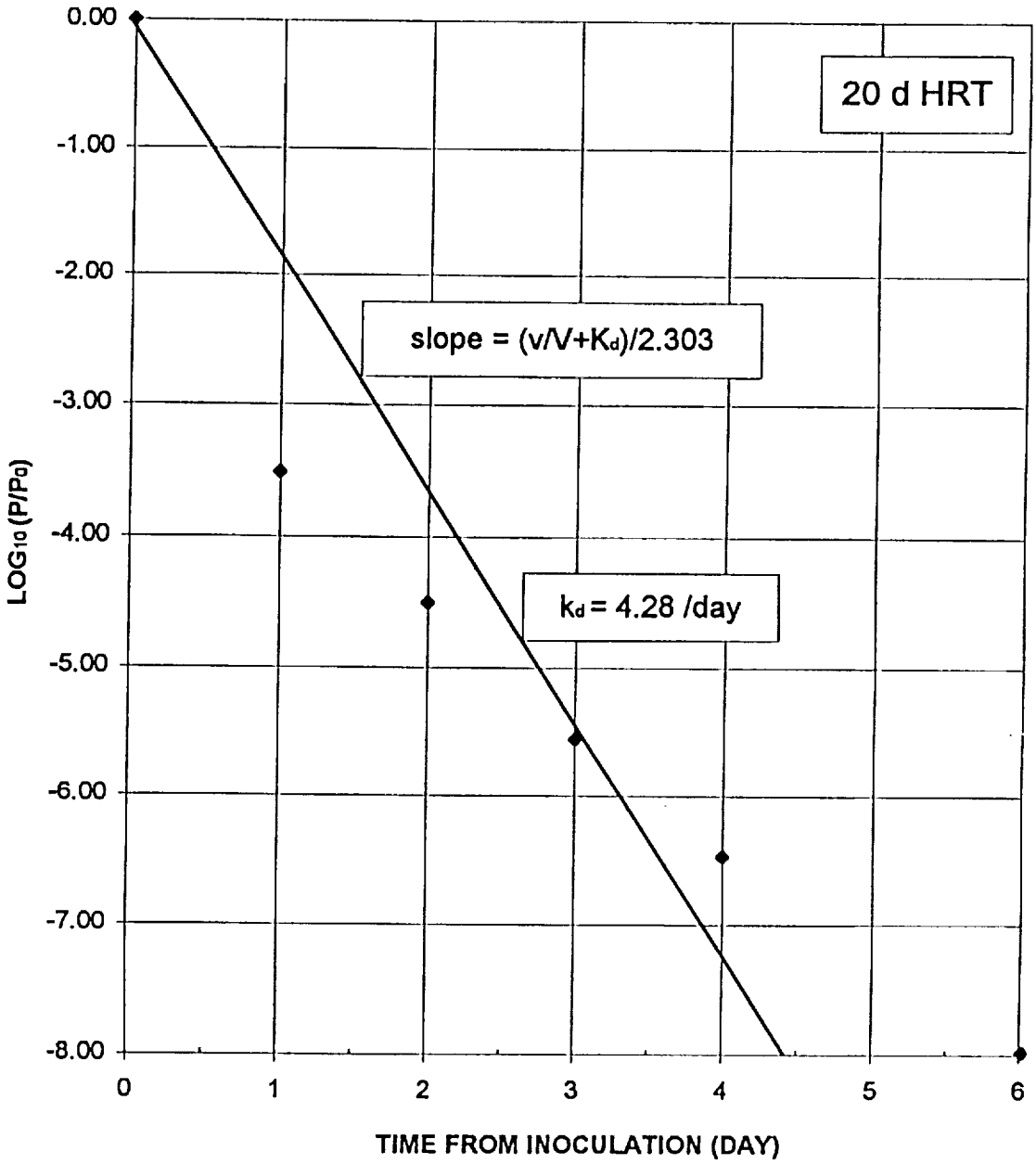

Figure IV-7. Decimal decay rate (k_d) of *Salmonella choleraesuis* in the single-dose study during continuous mesophilic anaerobic digestion under 20 days hydraulic retention time (HRT) and steady state

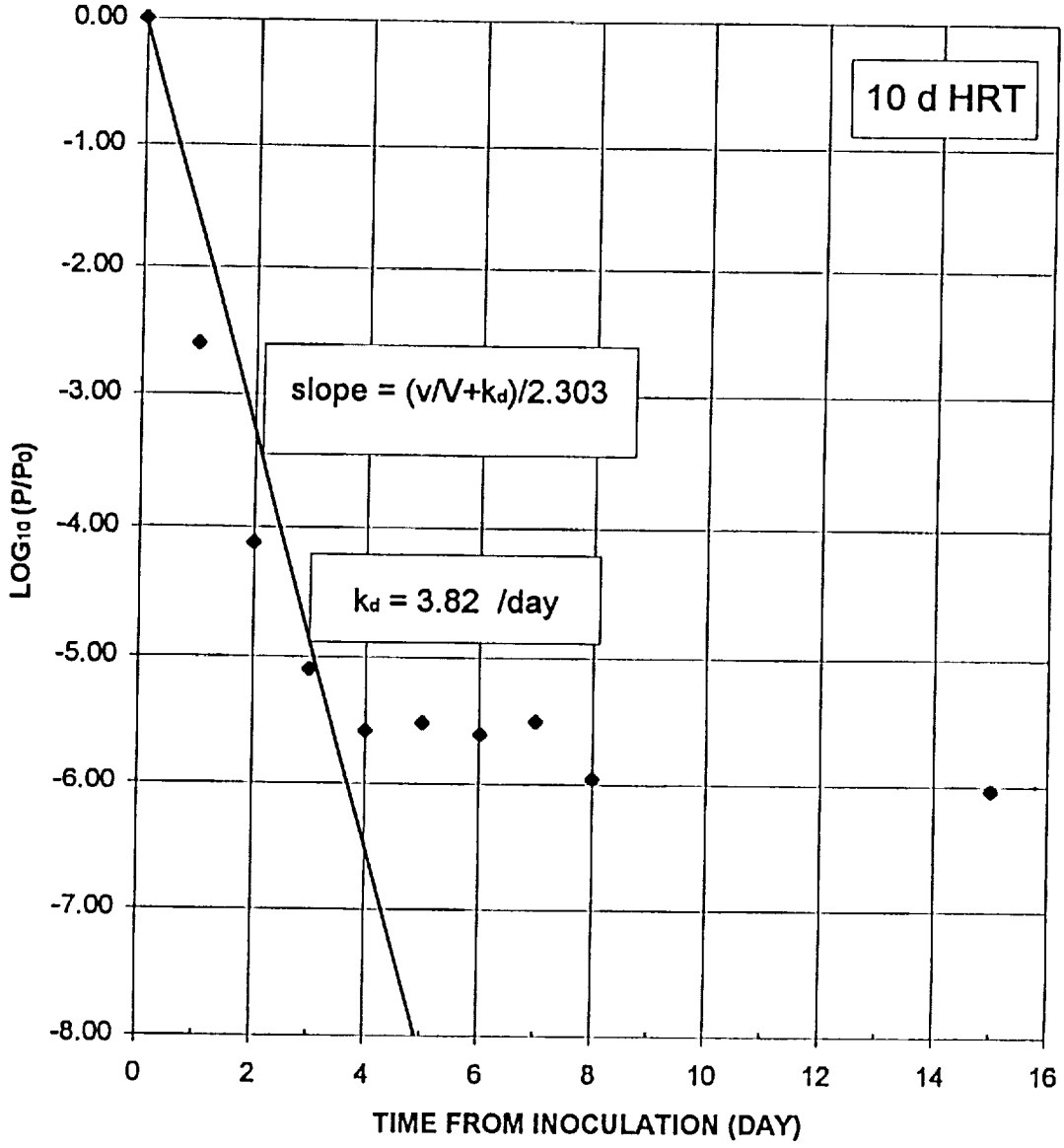

Figure IV-8. Decimal decay rate (k_d) of *Salmonella choleraesuis* in the single-dose study during continuous mesophilic anaerobic digestion under 10 days hydraulic retention time (HRT) and steady state

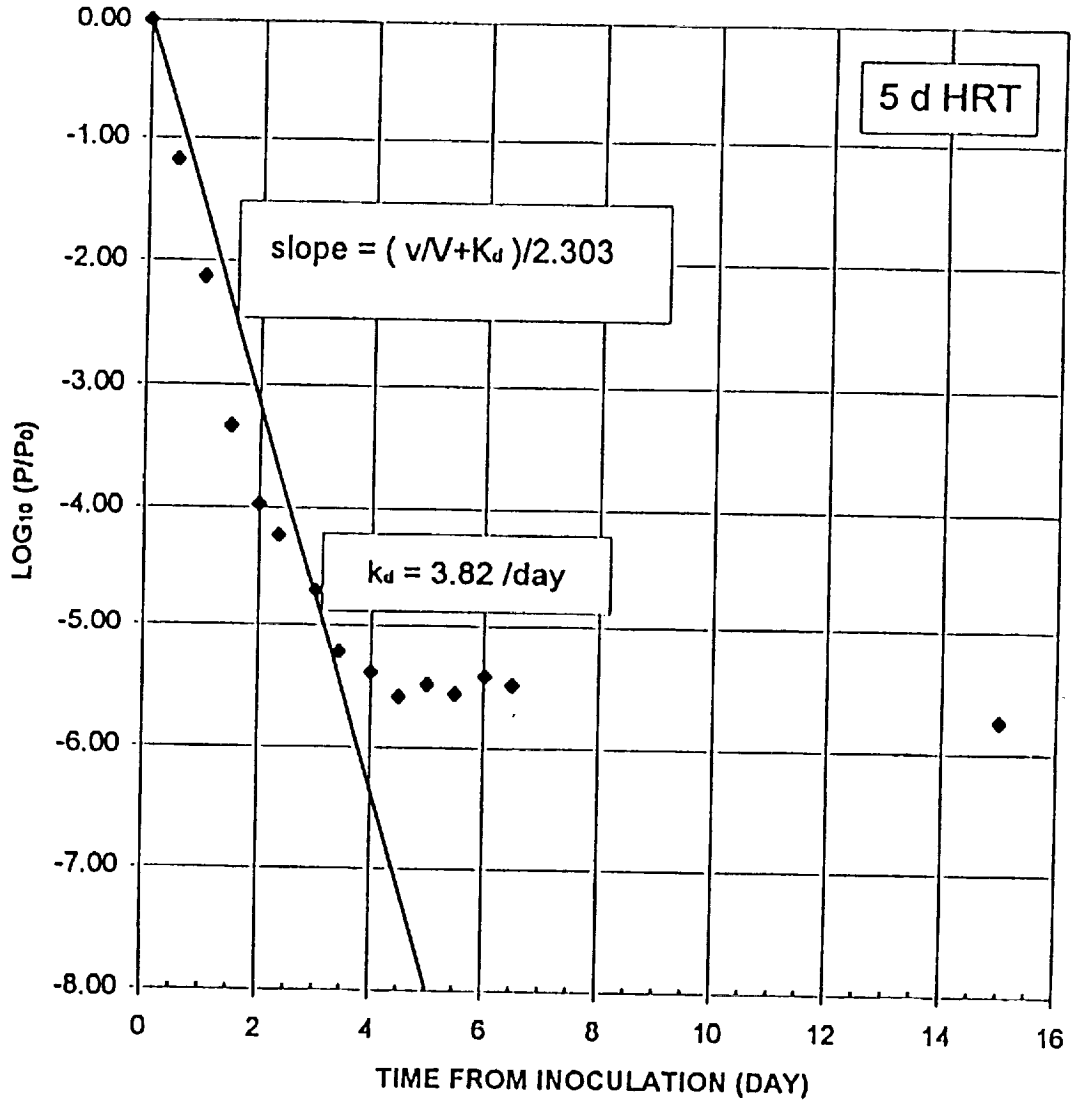

Figure IV-9. Decimal decay rate (k_d) of *Salmonella choleraesuis* in the single-dose study during continuous mesophilic anaerobic digestion under 5 days hydraulic retention time (HRT) and steady state

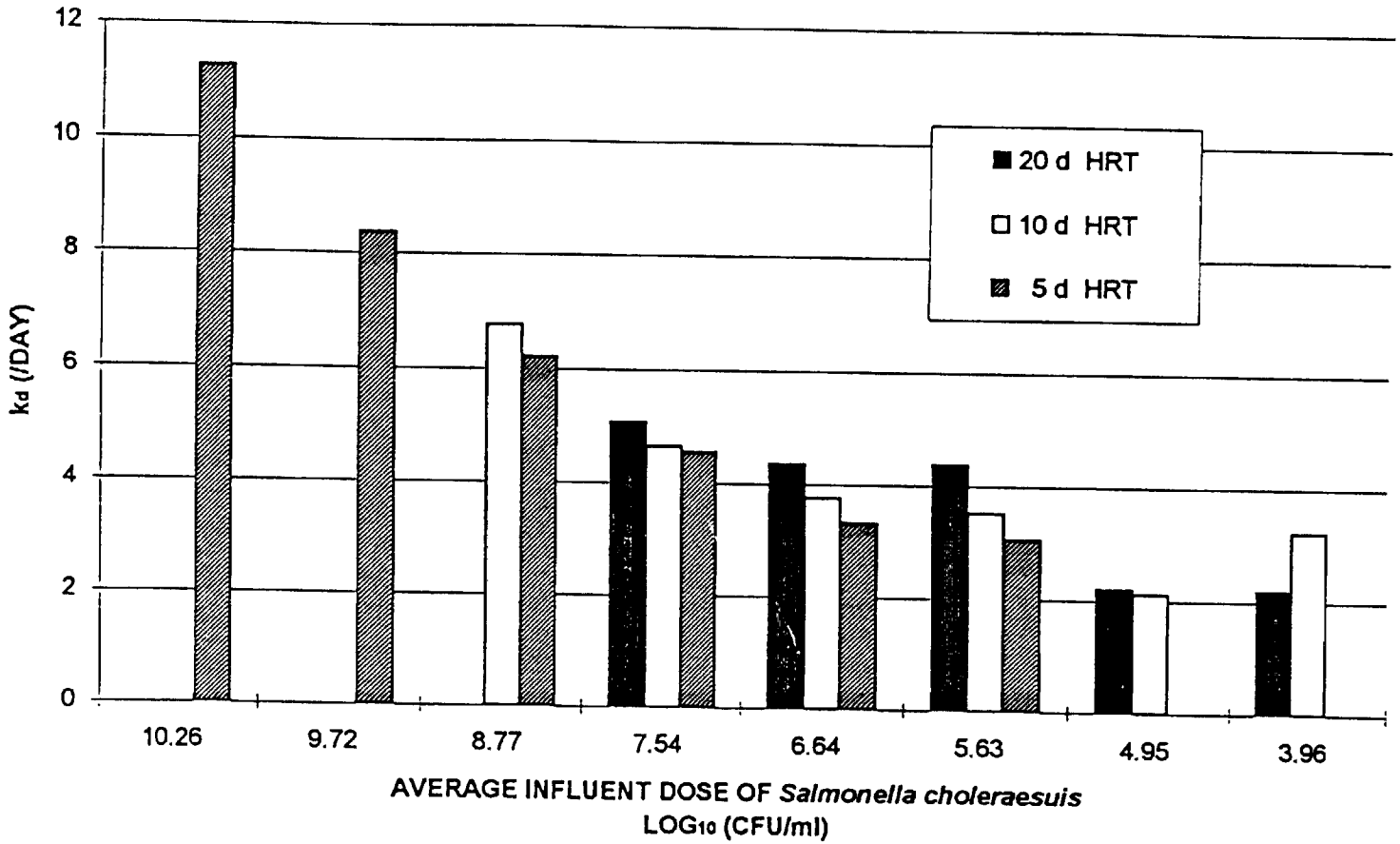

Figure IV-10. Influent dose of *Salmonella choleraesuis* and decimal decay rate constant (k_d) in the multi-dose study during continuous mesophilic anaerobic digestion under various HRT (Mean pH = 7.48; Mean biogas = 123 ml/interval)

Table IV-7. Decimal decay rate constant (k_d) *Salmonella choleraesuis* in the multi-dose study during continuous mesophilic anaerobic digestion under various HRT (Mean pH = 7.48; Mean biogas = 123 ml/interval)

Average	20 d HRT			10 d HRT			5 d HRT		
Influent (CFU/ml)	Influent (CFU/ml)	Effluent (CFU/ml)	k_d (/day)	Influent (CFU/ml)	Effluent (CFU/ml)	k_d (/day)	Influent (CFU/ml)	Effluent (CFU/ml)	k_d (/day)
1.8E+10	NA	NA	NA	NA	NA	NA	1.8E+10	1.2E+08	11.27
5.2E+09	NA	NA	NA	NA	NA	NA	5.2E+09	7.2E+07	8.37
5.9E+08	NA	NA	NA	9.6E+08	1.0E+06	6.77	2.2E+08	8.9E+06	6.22
3.5E+07	5.3E+07	1.9E+03	5.08	3.3E+07	4.8E+05	4.65	3.6E+07	3.0E+06	4.55
4.4E+06	1.6E+06	2.4E+02	4.35	4.2E+06	8.9E+04	3.75	4.5E+06	6.6E+05	3.32
4.3E+05	8.4E+05	1.2E+02	4.38	2.9E+05	7.9E+03	3.52	5.6E+05	1.1E+05	3.05
8.9E+04	2.1E+04	2.3E+02	2.21	8.9E+04	9.7E+03	2.12	NA	NA	NA
9.1E+03	2.3E+03	2.6E+01	2.19	9.10E+03	4.40E+02	3.22	NA	NA	NA

Biogas production and pH remained relatively stable throughout this digestion also with a mean pH of 7.48 and mean biogas production of 123 ml/interval which contained a mean of 71% methane.

IV.4.6 Inactivation of *Salmonella choleraesuis* during batch mesophilic anaerobic digestion

A rapid decline in the viable count of *Salmonella choleraesuis* during batch mesophilic anaerobic digestion is shown in Figure IV-11 and Table IV-8. The viable count of *Salmonella choleraesuis* declined rapidly within the first 2-4 days after inoculation. This rapid decline was followed by a period of equilibrium where the indicator bacteria remained at 10^2 CFU/ml until the 15th day from the inoculation. The viable counts of effluent correlated with the incubation time suggesting a moderate negative relationship (r = - 0.39).

The decimal decay rate (k_d) of the *Salmonella choleraesuis* during batch mesophilic digestion is shown in Figure IV-12.

The viable counts of effluent correlated with the biogas suggesting a strong negative relationship (r = - 0.85). A rapid decline in the viable population of the indicator bacteria was reflected in a large volume of biogas production; as biogas production declined, so did the viable population of the indicator bacteria in the digester.

The pH values remained relatively stable throughout batch mesophilic digestion with a mean value of 6.90. The viable counts and the pH value did not show a relationship (r = - 0.07). The mean methane gas percentage was 71%.

IV.4.7 The multi-drug resistant bacteria other than *Salmonella* sp. isolated from the effluent

Multi-drug resistant strains of *Pseudomonas* were isolated from the effluent throughout the study. The two most frequently occurring species were *P. aeruginosa* and *P. fluorescens*.

IV.4.8 The presence of the indicator bacteria on the pall rings

The biofilms on the pall rings were examined at the conclusion of the study. Indicator bacteria were also cultured from the pall rings.

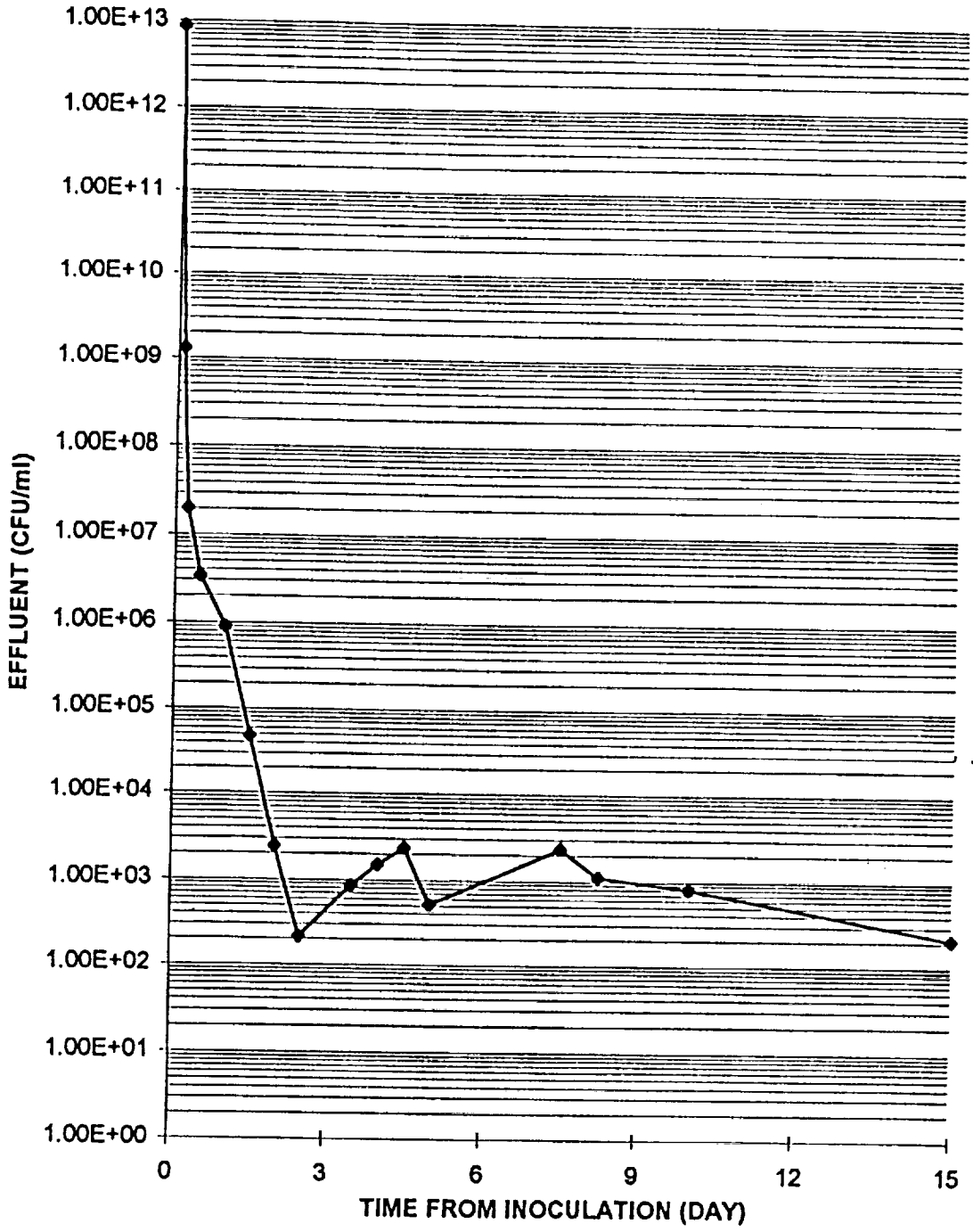

Figure IV-11. The inactivation of *Salmonella choleraesuis* during batch mesophilic anaerobic digestion

Table IV-8. The viable count of *Sallmonella choleraesuis*, biogas production, and pH value during the batch style mesophilic anaerobic digestion

Time (Day)	Effluent (CFU/ml)	Log(p/p$_0$)	Biogas ml/interval	Biogas cumulative (ml)	pH
0	8.60E+12	0.00			
0.125	1.30E+09	-3.82	100	100	6.89
0.25	2.00E+07	-5.63	48	148	6.85
0.5	3.30E+06	-6.42	30	178	6.85
1	8.80E+05	-6.99	18	196	6.85
1.5	4.70E+04	-8.26	12	208	6.83
2	2.40E+03	-9.55	4	212	6.92
2.5	2.10E+02	-10.61	0	212	6.86
3.5	8.50E+02	-10.01	1	213	6.93
4	1.51E+03	-9.75	0	213	6.98
4.5	2.34E+03	-9.56	0	213	6.88
5	5.13E+02	-10.22	1	214	7.05
7.5	2.34E+03	-9.56	1	215	6.90
8.25	1.10E+03	-9.89	1	216	6.97
10	8.00E+02	-10.03	1	217	6.99
15	2.10E+02	-10.61	0	217	6.84

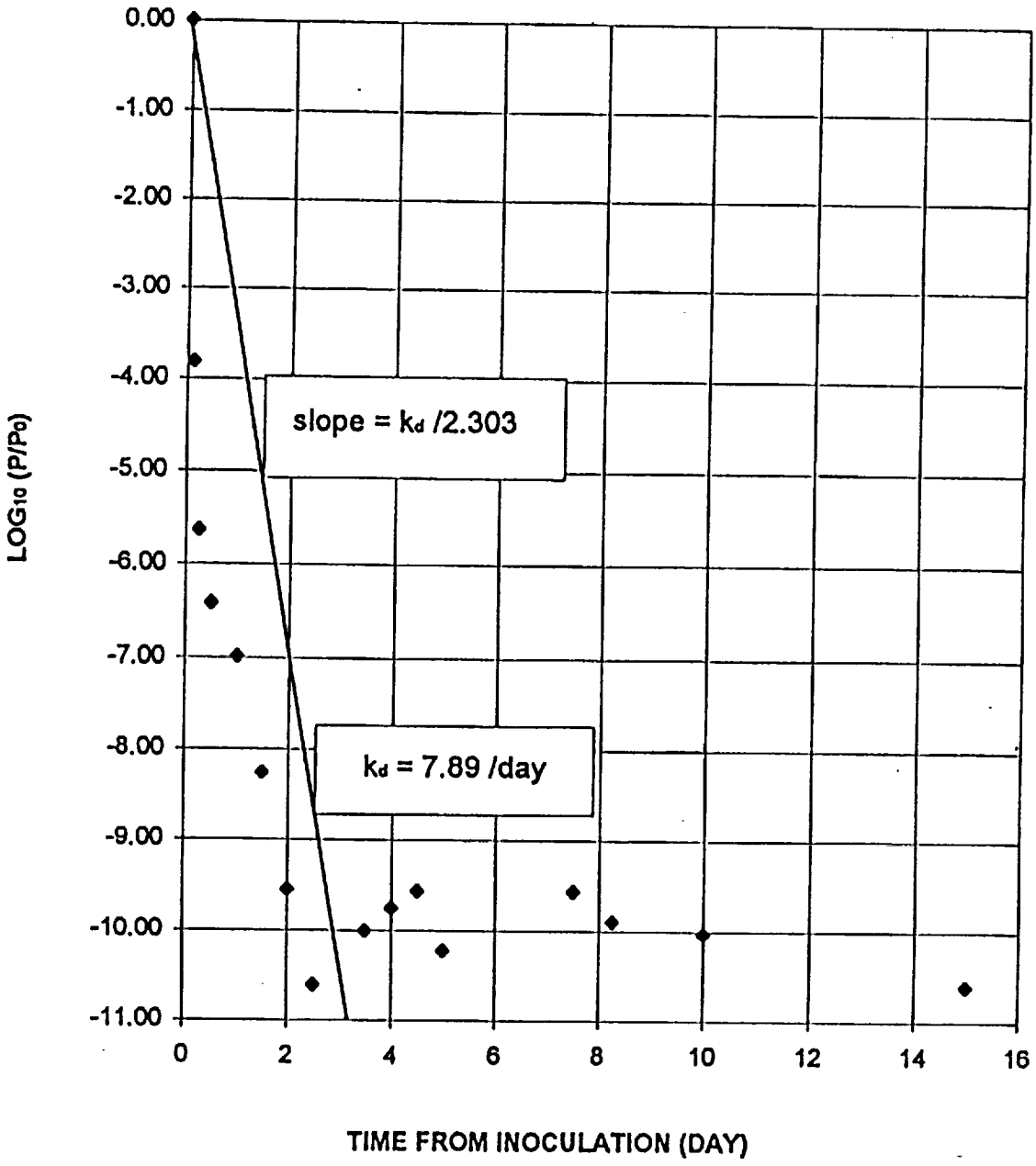

Figure IV-12. The decimal decay rate constant (kd) of *Salmonella choleraesuis* during batch mesophilic anaerobic digestion

IV.5 DISCUSSION

Salmonella choleraesuis are facultatively anaerobic organisms capable of using biochemical systems which are metabolically active in the absence or presence of oxygen. However, during anaerobic decomposition, volatile fatty acids and other toxic metabolites produced have an inhibitory or bactericidal effect on their survival. Thus, the bacteria tend to be inactivated during anaerobic digestion (Goepfert & Hicks, 1969).

This study confirmed previous reports indicating rapid declines in the viable number of pathogenic bacteria during mesophilic anaerobic digestion (Carrington, 1978, 1980; Carrington et al., 1982; Kearney et al., 1993, 1994). Rapid declines in viable numbers of indicator bacteria occurred within the first 2 - 4 days following inoculation during both continuous and batch digestion in this study. Kearney et al. (1993) reported rapid declines of *S. typhimurium* in the first 4-7 days, while Carrington et al. (1982) found a rapid decline of *S. duesseldorf* within 3 days. Variations are probably mainly due to the differences in sensitivities of the indicator bacteria as well as in their nutrients.

The rapid declines in viable numbers of *S. choleraesuis* during the initial digestion for continuous and batch studies in this study were found to be strongly related to the type of the digestion but not to the hydraulic retention time (HRT). The viable counts of *S. choleraesuis* declined more rapidly during batch digestion than during continuous digestion. The declines was also reflected by higher k_d values obtained during batch anaerobic digestion than in continuous digestion. Similar results were obtained by Sorlini et al. (1987) who reported a greater reduction (-4 $\log_{10}$) of coliform bacteria during batch anaerobic digestion than during continuous anaerobic digestion (-1 or 2 $\log_{10}$). Olsen and Larsen (1987) reported similar findings in their study of the inactivation of pathogenic bacteria where lower T_{90} (decimation time, the time taken for viable counts of a population to decrease by one logarithmic unit, which is equivalent to a 90% reduction) values were obtained during batch digestion than during continuous digestion. Additionally, Kearney et al. (1993) reported T_{90} to be higher during the continuous anaerobic digestion than during batch anaerobic digestion at 25 d HRT. Possible explanations for our findings appear to be related to the availability of nutrients within the system as well as to competition for nutrients between the indicator bacteria and the digesting flora (Tappouni, 1984). *Salmonella* species are capable of utilizing carbohydrates and therefore must compete with other acidogenic and acetogenic bacteria for these nutrients. Therefore, the rapid decline in viable numbers after inoculation may have been due to an inadequate supply of available nutrients to support viable populations of 10^7 CFU/ml bacteria (Kearney et al., 1994). During continuous digestion, feedings occurred at a frequency as determined by the HRT, but during batch anaerobic digestion the flora was not fed after their first feeding, and hence, the cells were unable to get their required nutrients and declined more rapidly as a result.

The rapid declines in viable numbers of *S. choleraesuis* during initial digestion were also found to be strongly related to the initial dose of indicator bacteria added to the digester. Contrary to the study by Carrington et al. (1982), the present study showed k_d, at the same HRT, to be greater when there was a high initial dose of indicator bacteria in the feed than when there was a low initial dose of indicator bacteria in the feed. This may due to more competition occurring in a large bacteria population than in a small bacteria population.

As also noted by Kearney et al. (1993) and El-Abagy & El-Zanfaly (1984), following rapid decline, a period of equilibrium was observed during batch digestion as well as during continuous digestion at HRT of 10 days and 5 days. After 2-4 days of rapid decline, a point was reached where the rapid decline stopped and residual viable populations of approximately 10^2 CFU/ml were maintained for at least 15 days from inoculation. Bacteria are known to adhere to organic matter and can therefore utilize nutrients released during the breakdown of the organic matter. In an earlier study, adhesion of bacteria to suspended particulate solids in sewage was shown to increase the viability of the bacteria (Bar-Or, 1990). Therefore, it is possible that the residual viable populations were supported by the slow release of nutrients from the breakdown of organic matter, and the utilization of substrates released from dying and lysed cells within the anaerobic digester (Kearney et al., 1993). Additionally, there may have been sufficient nutrients to support viable populations of 10^2 CFU/ml although insufficient amounts for support of 10^7 CFU/ml.

The bacteria can exist for an extended period because of a long period of equilibrium. In this study, bacteria survived in the continuous digestion at HTR of 10 days and 5 days as well as batch digestion for at least 15 days. Willinger and Thiemann (1982) using an initial dose of 10^6 - 10^8 CFU/ml of *S. typhimurium* found survival for 14 to 21 days from inoculation. Gadre et al. (1986) reported an antibiotic resistant strain of *S. typhimurium* with an initial dose of 10^5 CFU/ml to be totally eliminated in 9 days. Variations in survivability are probably due to differences in the composition of the fermenting mass, nutrient content and sensitivity of the indicator bacteria.

Biogas production and pH remained relatively constant throughout continuous digestion. However, during batch digestion, viable numbers of indicator bacteria present were strongly affected by biogas production. There was a rapid decline of the viable populations of indicator bacteria when there was a large volume of the biogas produced; while there was a slow decline in the viable population of the indicator bacteria when a lesser amount of biogas was produced. Tappouni (1984) observed similar findings; however, Kearney et al. (1993) observed opposite trends. A combination of factors may be responsible for the decline in viable populations of both the indicator bacteria and digesting flora during batch digestion. Unlike continuous digestion, there was no input of fresh nutrients nor was there removal of accumulated toxic metabolites which created a highly unfavorable environment for bacterial growth.

Biogas production was observed to occur at HRT as low as 5 days. Carrington et al. (1982) reported that when the mean retention period of the digesters at 35°C was reduced to 6 days, the rate of biogas production declined progressively.

Constant pH values were obtained during our batch digestion study while Kearney et al. (1993) reported pH becoming more alkaline with time (7.5-7.9).

Pseudomonas sp., especially *P. aeruginosa*, were frequently isolated from the effluent of the digesters throughout the study. These organisms are widely distributed in nature, very often encountered in water, and are resistant to a wide variety of antibiotics including those used in this study. *Pseudomonas* are usually aerobic, but in the absence of oxygen, they are capable of using the H_2 which existed in the anaerobic digesters as their alternative energy source (Figure IV-2). Willinger and Thiemann (1982) also isolated these bacteria and reported *P. aeruginosa* to be persistent in slurries of pig and poultry at least 28 days. In further studies, supplementing the media with novobiocin will be helpful in inhibiting its growth (Krieg & Holt, 1984).

It can be concluded that indicator bacteria may be maintained at a constant population level for an extended period of time following a rapid decline within the first 2-4 days during mesophilic anaerobic digestion. In CELSS, because of residual bacterial flora, it is recommended that further treatment of the digested slurry occur to reduce the risk of contamination by salmonellae. Decimal decay rates (k_d) are (1) affected by the concentration of indicator bacteria in the feed; (2) related to the type of mesophilic anaerobic digestion; and (3) not significantly affected by the HRT.

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APPENDIX A
EXPERIMENTAL DATA FOR ANAEROBIC DIGESTERS

Table A.1
pH value at 20 days HRT

Date	Time (day)	pH inlet	pH 1#	pH 2#	pH 3#
7/13/94	0	8.28	7.43	6.44	7.36
7/15/94	2	8.26	7.80	7.03	7.79
7/17/94	4	7.74	7.43	6.78	7.37
7/19/94	6	7.98	7.40	6.92	7.31
7/21/94	8	8.08	7.45	7.37	7.37
7/23/94	10	6.00	7.51	7.47	7.43
7/25/94	12	8.02	7.61	7.56	7.59
7/27/94	14	6.41	7.66	7.90	7.94
7/28/94	15	6.00	7.62	7.56	7.53
7/30/94	17	6.34	7.58	7.54	7.47
8/01/94	19	6.45	7.59	7.62	7.57
8/03/94	21	6.71	7.60	7.60	7.55
8/04/94	22	6.57	7.62	7.58	7.52
8/06/94	24	6.81	7.59	7.65	7.62
8/08/94	26	5.71	7.77	7.77	7.76
8/10/94	28	5.85	7.85	7.83	7.86
8/12/94	30	4.23	7.93	7.91	7.97
8/14/94	32	5.75	7.94	7.90	7.97
8/15/94	34	6.38	8.06	7.96	7.99

Table A.2
pH value at 10 days HRT

Date	Time (day)	pH inlet	pH 1#	pH 2#	pH 3#
8/17/94	0	6.26	8.03	8.01	8.08
8/18/94	1	6.19	8.13	8.14	8.19
8/19/94	2	6.54	8.14	8.14	8.18
8/20/94	3	5.82	8.19	8.14	8.20
8/21/94	4	3.49	8.11	8.14	8.30
8/22/94	5	3.75	7.79	7.80	8.00
8/23/94	6	3.10	7.46	7.46	7.55
8/24/94	7	3.05	7.27	7.28	7.32
8/25/94	8	3.28	7.13	7.06	7.15
8/26/94	9	6.56	6.99	7.05	7.06
8/27/94	10	6.62	7.16	7.17	7.23
8/28/94	11	6.13	7.12	7.13	7.16
8/29/94	12	6.60	7.39	7.32	7.44
8/30/94	13	5.33	7.41	7.37	7.43
8/31/94	14	3.41	7.49	7.50	7.54
9/01/94	15	7.00	7.40	7.42	7.47
9/02/94	16	7.09	7.34	7.37	7.37
9/03/94	17	7.25	7.91	7.93	7.97
9/04/94	18	1.89	7.60	7.66	7.64
9/05/94	19	1.76	7.36	7.44	7.42

Table A.3
pH value at 5 days HRT

Date	Time (day)	pH inlet	pH 1#	pH 2#	pH 3#
9/06/94(1)	0	1.97	7.26	7.25	7.27
9/06/94(2)	0.5	1.99	7.10	7.10	7.12
9/07/94(1)	1.0	2.00	7.06	7.12	7.15
9/07/94(2)	1.5	1.92	7.04	7.04	7.05
9/08/94(1)	2.0	6.44	6.70	6.67	6.73
9/08/94(2)	2.5	6.73	6.81	6.79	6.78
9/09/94(1)	3.0	6.30	7.22	7.21	7.23
9/09/94(2)	3.5	1.99	7.34	7.32	7.40
9/10/94(1)	4.0	1.99	7.26	7.23	7.07
9/10/94(2)	4.5	2.00	7.15	7.14	7.13
9/11/94(1)	5.0	1.99	7.07	7.06	7.11
9/11/94(2)	5.5	1.97	7.07	7.00	7.06
9/12/94(1)	6.0	2.03	7.02	6.98	7.02
9/12/94(2)	6.5	1.79	6.73	6.69	6.73

Table A.4
Biogas production at 20 days HRT

Date	Time (day)	Biogas 1# (ml)	Biogas 2# (ml)	Biogas 3# (ml)
7/13/94	0	290	68	221
7/15/94	2	260	77	132
7/17/94	4	126	76	72
7/19/94	6	128	44	58
7/21/94	8	83	174	143
7/23/94	10	294	94	177
7/25/94	12	231	108	214
7/27/94	14	242	3	272
7/28/94	15	295	115	312
7/30/94	17	301	43	365
8/01/94	19	253	228	387
8/03/94	21	229	18	165
8/04/94	22	215	90	278
8/06/94	24	126	194	141
8/08/94	26	128	214	155
8/10/94	28	126	202	152
8/12/94	30	142	228	168
8/14/94	32	113	101	103
8/15/94	34	109	97	138

Table A.5
Biogas production at 10 days HRT

Date	Time (day)	Biogas 1# (ml)	Biogas 2# (ml)	Biogas 3# (ml)
8/17/94	0	77	72	85
8/18/94	1	63	64	98
8/19/94	2	59	58	79
8/20/94	3	30	32	45
8/21/94	4	142	195	189
8/22/94	5	225	252	283
8/23/94	6	319	315	344
8/24/94	7	333	363	374
8/25/94	8	456	443	492
8/26/94	9	412	407	437
8/27/94	10	323	338	379
8/28/94	11	207	333	341
8/29/94	12	144	200	200
8/30/94	13	200	313	188
8/31/94	14	311	350	371
9/01/94	15	346	248	148
9/02/94	16	149	125	116
9/03/94	17	96	106	98
9/04/94	18	203	168	156
9/05/94	19	150	175	163

Table A.6
Biogas production at 5 days HRT

Date	Time (day)	Biogas 1# (ml)	Biogas 2# (ml)	Biogas 3# (ml)
9/06/94(1)	0	93	104	131
9/06/94(2)	0.5	118	150	145
9/07/94(1)	1.0	148	168	148
9/07/94(2)	1.5	124	154	150
9/08/94(1)	2.0	90	92	118
9/08/94(2)	2.5	52	66	78
9/09/94(1)	3.0	66	78	88
9/09/94(2)	3.5	124	152	140
9/10/94(1)	4.0	136	137	162
9/10/94(2)	4.5	146	146	172
9/11/94(1)	5.0	124	146	140
9/11/94(2)	5.5	120	158	154
9/12/94(1)	6.0	156	156	154
9/12/94(2)	6.5	102	150	142

Table A.7
pH value and biogas production at 10 days HRT

Date	Time (day)	pH inlet	pH 1#	Biogas 1# (ml)
9/17/94	0	6.40	6.79	80
9/18/94	1	6.54	6.81	74
9/19/94	2	1.97	7.00	148
9/20/94	3	1.97	6.98	154
9/21/94	4	1.96	7.00	160
9/22/94	5	1.97	6.95	146
9/23/94	6	1.84	6.84	148
9/24/94	7	1.97	6.90	142
9/25/94	8	1.85	6.92	140
9/26/94	9	1.89	6.89	184
9/27/94	10	1.87	6.88	152
9/28/94	11	1.90	6.80	131
9/29/94	12	1.90	6.71	144
9/30/94	13	1.97	6.85	156
10/01/94	14	1.87	6.64	125
10/02/94	15	1.85	6.54	136

Table A.8
TOC concentration at 20 days HRT

Date	Time (day)	TOC inlet (mg/l)	TOC 1# (mg/l)	TOC 2# (mg/l)	TOC 3# (mg/l)
7/13/94	0	488.6	168.2	564.4	155.3
7/15/94	2	923.5	145.3	538.2	117.8
7/17/94	4	501.0	96.8	397.2	115.2
7/19/94	6	649.2	105.3	264.9	118.8
7/21/94	8	615.1	100.0	137.2	103.7
7/23/94	10	636.6	118.6	134.7	104.7
7/25/94	12	759.8	111.8	164.6	112.2
7/27/94	14	723.6	124.3	149.4	122.1
7/30/94	18	600.9	79.1	154.5	109.4
8/01/94	20	660.9	98.4	159.2	88.7
8/03/94	22	666.8	81.6	145.0	65.9
8/04/94	24	683.8	87.0	142.9	66.7
8/06/94	26	558.8	83.6	163.3	98.7
8/08/94	28	524.4	97.8	119.8	51.7
8/10/94	30	507.8	132.6	118.6	96.1
8/12/94	32	510.9	95.3	105.8	51.2
8/14/94	34	463.1	111.6	123.1	86.0

Table A.9
TOC concentration in 10 days HRT

Date	Time (day)	TOC inlet (mg/l)	TOC 1# (mg/l)	TOC removal (%)
9/25/94	8	752.7	50.1	93.42
9/26/94	9	759.1	68.3	91.00
9/27/94	10	524.1	48.9	90.67
9/28/94	11	488.1	52.3	89.28
9/29/94	12	539.2	62.2	88.46
9/30/94	13	458.1	65.1	85.79
10/01/94	14	575.3	68.5	88.09
10/02/94	15	523.6	67.0	87.20
10/03/94	16	498.7	68.3	86.30
10/04/94	17	465.3	58.2	89.21

Table A.10
TOC concentration at 5 days HRT

Date	Time (day)	TOC inlet (mg/l)	TOC 3# (mg/l)	TOC removal (%)
9/06/94(1)	0.0	506.5	56.0	88.94
9/06/94(2)	0.5	559.6	58.4	89.56
9/10/94(1)	4.0	539.4	41.8	92.25
9/10/94(2)	4.5	510.3	52.2	89.77
9/11/94(1)	5.5	518.0	34.6	93.32
9/12/94(1)	6.0	535.2	56.4	89.46
9/12/94(2)	6.5	540.5	54.7	89.88
9/13/94(1)	7.0	465.7	63.8	86.30

Table A.11
COD concentration at 20 days HRT

Date	Time (day)	COD inlet (mg/l)	COD 1# (mg/l)	COD 2# (mg/l)	COD 3# (mg/l)
7/13/94	0	1400	440	1550	470
7/15/94	2	2820	360	1540	380
7/17/94	4	2060	301	1560	391
7/19/94	6	2530	296	1020	398
7/21/94	8	2350	360	730	394
7/23/94	10	2460	494	740	412
7/25/94	12	2680	488	662	483
7/27/94	14	2780	478	612	498
7/28/94	16	2550	471	653	517
7/30/94	18	2430	361	653	363
8/03/94	22	2270	346	620	343
8/06/94	26	2110	389	673	343
8/10/94	30	1528	423	571	348
8/14/94	34	2750	682	824	767

Table A.12
COD concentration at 10 days HRT

Date	Time (day)	COD inlet (mg/l)	COD 3# (mg/l)	COD removal (%)
8/17/94	0	2880	720	75.00
8/21/94	4	2860	740	74.12
8/30/94	13	2390	920	61.51
8/31/94	14	2390	400	83.26
9/01/94	15	2390	640	73.22
9/03/94	17	2390	380	84.10
9/04/94	18	2150	380	82.32
9/05/94	19	1980	720	63.34

Table A.13
COD concentration at 5 days HRT

Date	Time (day)	COD inlet (mg/l)	COD 3# (mg/l)	COD removal (%)
9/06/94(1)	0	2210	630	71.49
9/06/94(2)	0.5	2820	960	65.96
9/09/94(1)	3.0	2450	990	59.59
9/10/94(1)	4.0	2290	880	61.57
9/10/94(2)	4.5	2760	720	73.91
9/11/94(2)	5.5	2780	350	87.41
9/12/94(2)	6.5	2250	990	56.00
9/13/94(1)	7.0	2560	780	69.53

Table 14-1* TC, IC, and TOC concentration at 20 days HRT

Date	Sample	TC (mg/l)	IC (mg/l)	TOC(mg/l)
7/13/94	Reactor 1#	0.4	272.2	168.2
	Reactor 2#	637.7	73.0	564.6
	Reactor 3#	430.5	275.2	155.3
	Inlet	708.4	219.8	488.6
7/15/94	Reactor 1#	432.8	287.5	145.3
	Reactor 2#	624.2	86.0	538.2
	Reactor 3#	410.7	283.9	117.8
	Inlet	1148.0	224.5	923.5
7/17/94	Reactor 1#	376.5	279.7	96.8
	Reactor 2#	517.1	119.9	397.2
	Reactor 3#	379.8	264.6	115.2
	Inlet	608.0	107.0	501.0
7/19/94	Reactor 1#	370.6	265.3	105.3
	Reactor 2#	417.3	152.4	264.9
	Reactor 3#	372.0	253.2	118.8
	Inlet	815.4	166.2	649.2
7/21/94	Reactor 1#	370.0	270.0	100.0
	Reactor 2#	372.2	235.0	137.2
	Reactor 3#	371.9	268.2	103.7
	Inlet	788.6	173.5	615.1
7/23/94	Reactor 1#	397.3	278.7	118.6
	Reactor 2#	387.1	252.4	134.7
	Reactor 3#	383.4	278.7	104.7
	Inlet	644.3	8.0	636.3
7/25/94	Reactor 1#	377.9	266.1	111.8
	Reactor 2#	400.2	235.6	164.6
	Reactor 3#	381.7	270.5	111.2
	Inlet	904.5	144.7	759.8
7/27/94	Reactor 1#	399.9	279.6	122.1
	Reactor 2#	403.8	275.6	124.3
	Reactor 3#	401.7	254.4	149.4
	Inlet	725.8	2.1	723.6
7/30/94	Reactor 1#	370.7	291.6	79.1
	Reactor 2#	414.1	259.6	154.5
	Reactor 3#	392.1	282.7	109.4
	Inlet	604.4	3.5	600.9

Note. * = Data which were collected during acclimation and were not used for the calculation of TOC removal efficiency.

Table A.14-2 TC, IC, and TOC concentration at 20 days HRT

Date	Sample	TC (mg/l)	IC (mg/l)	TOC(mg/l)
8/01/94	Reactor 1#	401.4	303.0	98.4
	Reactor 2#	422.7	263.5	159.2
	Reactor 3#	382.9	294.2	88.7
	Inlet	663.4	2.5	660.9
8/03/94	Reactor 1#	372.5	290.9	81.6
	Reactor 2#	409.2	264.2	145.0
	Reactor 3#	358.7	292.8	65.9
	Inlet	512.7	6.4	506.3
8/04/94	Reactor 1#	370.7	283.7	87.0
	Reactor 2#	422.0	279.1	142.9
	Reactor 3#	363.7	297.0	66.7
	Inlet	580.5	10.5	683.8
8/06/94	Reactor 1#	402.4	318.8	83.6
	Reactor 2#	402.9	24.0	163.3
	Reactor 3#	386.8	288.1	98.7
	Inlet	578.6	19.8	558.8
8/08/94	Reactor 1#	395.0	297.2	97.8
	Reactor 2#	397.3	277.5	119.8
	Reactor 3#	351.1	299.4	51.7
	Inlet	541.5	17.0	524.4
8/10/94	Reactor 1#	442.8	310.2	132.6
	Reactor 2#	411.1	292.6	118.6
	Reactor 3#	407.2	311.1	96.1
	Inlet	520.8	12.9	507.8
8/12/94	Reactor 1#	385.5	290.2	95.3
	Reactor 2#	382.3	276.5	105.8
	Reactor 3#	350.8	299.6	51.2
	Inlet	515.4	4.4	510.9
8/14/94	Reactor 1#	395.7	284.1	111.6
	Reactor 2#	398.3	275.2	123.1
	Reactor 3#	367.2	281.2	86.0
	Inlet	490.3	27.1	463.1
8/17/94	Reactor 1#	420.3	285.7	134.6
	Reactor 2#	406.2	277.1	129.1
	Reactor 3#	389.6	298.1	91.5
	Inlet	536.1	29.5	506.5

Table A.15
TC, IC, and TOC concentration at 10 day HRT

Date	Sample	TC (mg/l)	IC (mg/l)	TOC (mg/l)
8/21/94	Reactor 3#	410.7	292.6	118.1
	Inlet	1953.0	3.6	1949.0
8/22/94	Reactor 3#	534.4	282.9	251.5
	Inlet	1326.0	6.5	1319.0
9/01/94	Reactor 3#	585.5	455.4	130.1
9/03/94	Reactor 3#	655.5	506.2	149.3
9/04/94	Reactor 3#	617.9	497.5	120.4
	Inlet	545.0	5.5	539.4
9/05/94	Reactor 3#	555.0	458.7	96.3
9/15/94	Reactor 1#	411.0	328.8	82.2
	Reactor 2#	390.7	310.9	79.8
9/25/94	Reactor 1#	337.2	287.1	50.1
	Reactor 2#	382.1	330.5	51.6
	Inlet	758.8	6.0	752.8
9/26/94	Reactor 1#	363.5	295.2	68.3
	Reactor 2#	383.5	281.0	102.5
	Inlet	765.3	6.1	759.1
9/27/94	Reactor 1#	372.2	323.3	48.9
	Inlet	531.3	7.2	524.1
9/28/94	Reactor 1#	351.7	299.4	52.3
	Reactor 2#	411.5	329.6	81.9
	Inlet	495.4	7.9	488.1
9/29/94	Reactor 1#	387.5	525.3	62.2
	Inlet	547.2	7.9	539.2
9/30/94	Reactor 1#	290.3	225.2	65.1
10/01/94	Reactor 1#	354.8	286.2	68.5

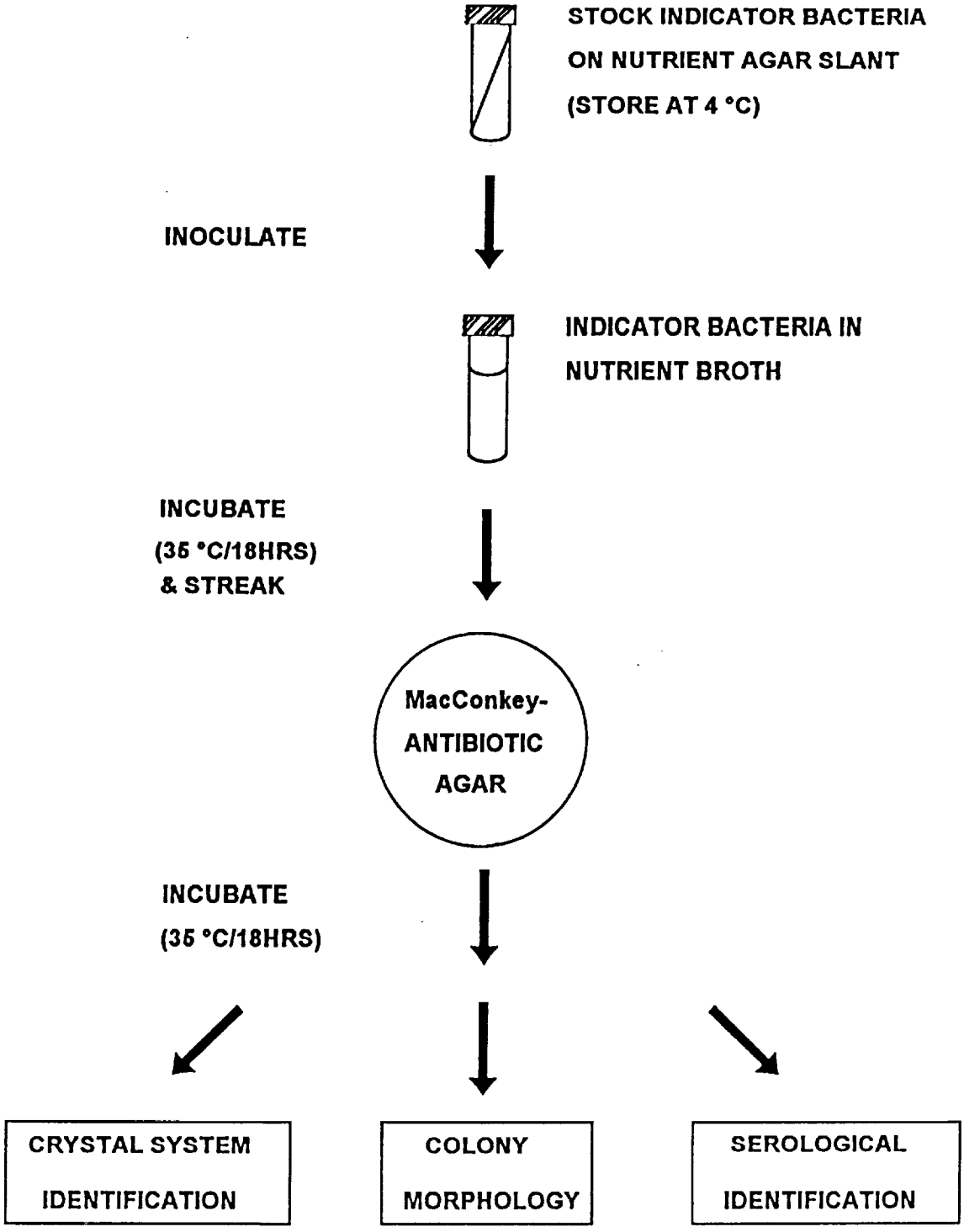
Table A.16
TC, IC, and TOC at 5 days HRT

Date	Sample	TC (mg/l)	IC (mg/l)	TOC (mg/l)
9/06/94(1)	Reactor 3#	480.3	424.3	56.0
9/06/94(2)	Reactor 3#	456.7	398.3	58.4
9/10/94(1)	Reactor 3#	401.9	360.1	41.8
9/10/94(2)	Reactor 3#	380.7	328.5	52.2
9/11/94(2)	Reactor 3#	341.3	307.6	34.6
9/12/94(1)	Reactor 3#	347.1	290.7	56.4
9/12/94(2)	Reactor 3#	347.0	292.3	54.7
9/13/94(1)	Reactor 3#	357.1	293.3	63.8
	Inlet	563.3	97.6	465.7

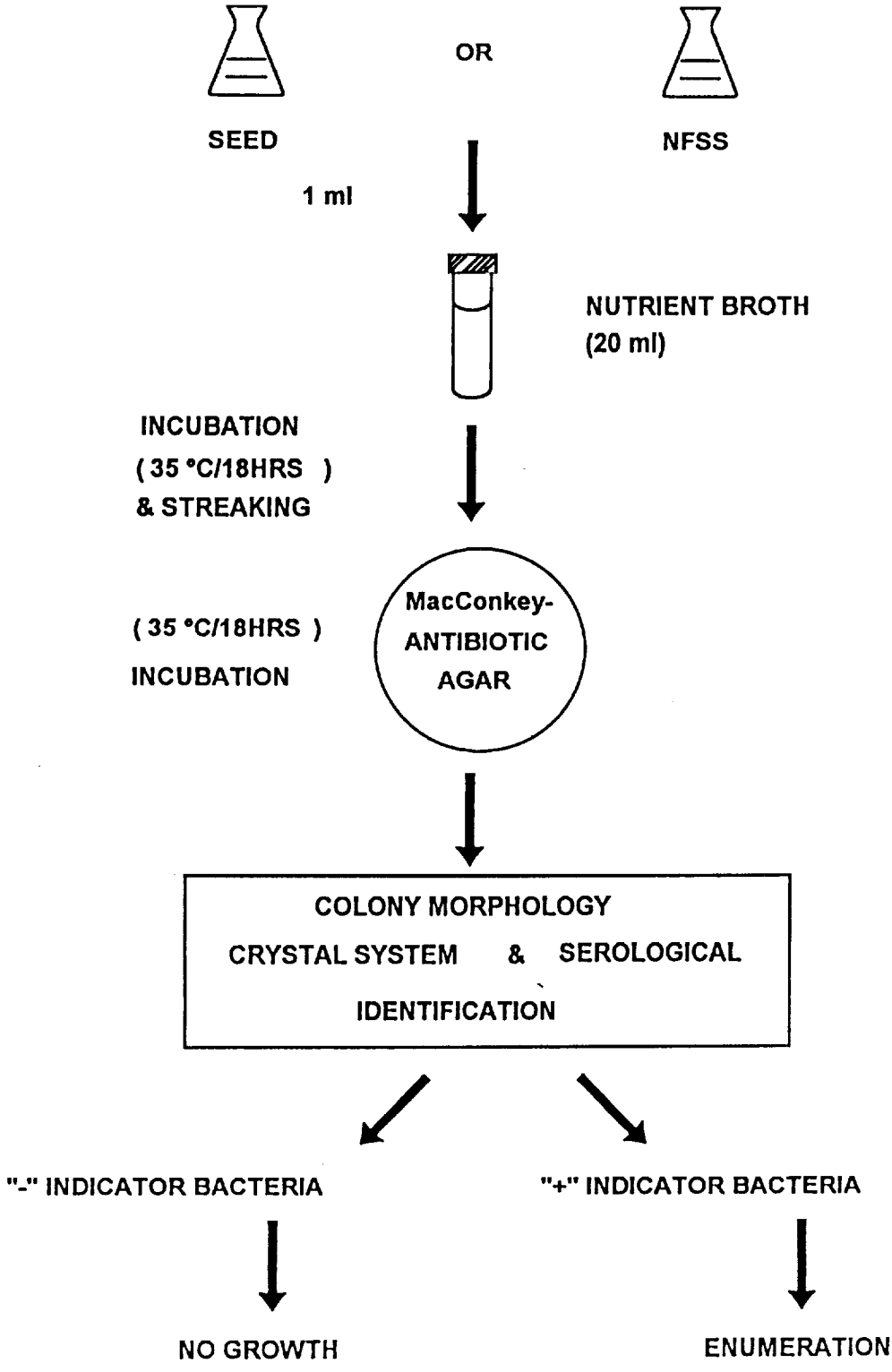
APPENDIX B

SCHEME OF EXPERIMENTAL PROCEDURES FOR EPIDEMIOLOGY STUDY

1. BACTERIAL QUALITY ASSURANCE



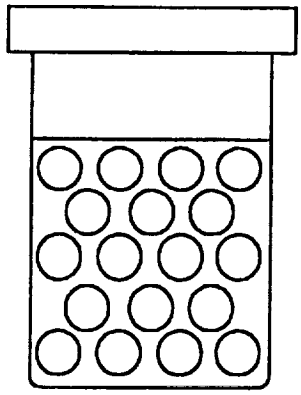
2. EXAMINATION OF WASTE WATER SEED & NFSS FOR PRESENCE OF INDICATOR BACTERIA



3. ESTABLISHMENT OF STEADY-STATE ANAEROBIC DIGESTION

FILL WITH

SEED SOLUTION



ANAEROBIC

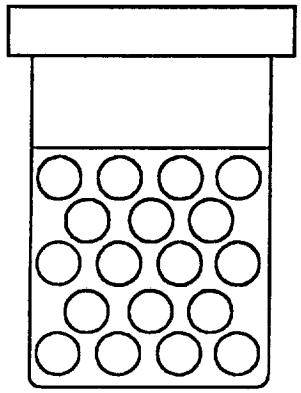
DIGESTER

(V = 3500ml)

WITHDRAW AND

ADD 350ml NFSS

PER TIME INTERVAL

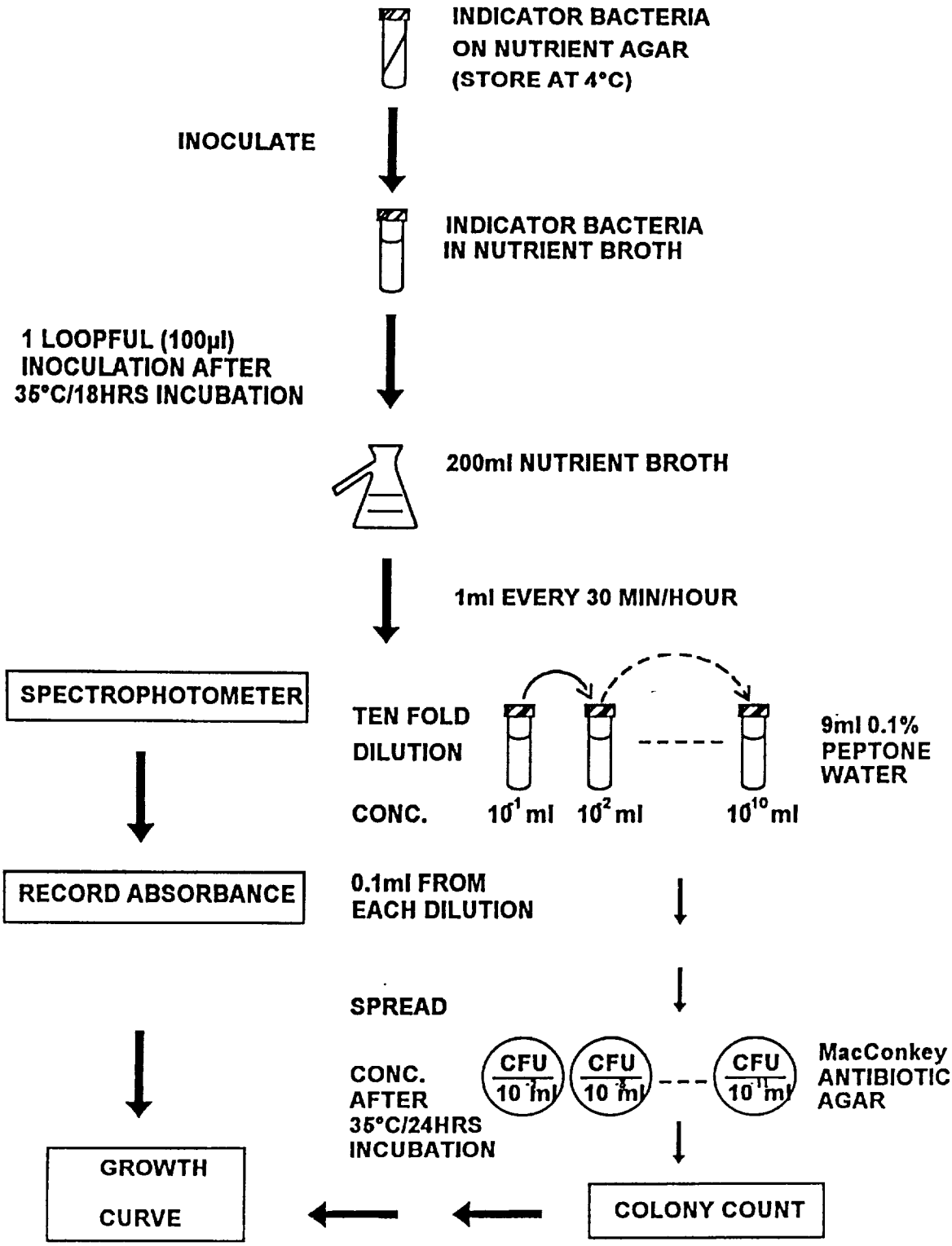


ANAEROBIC

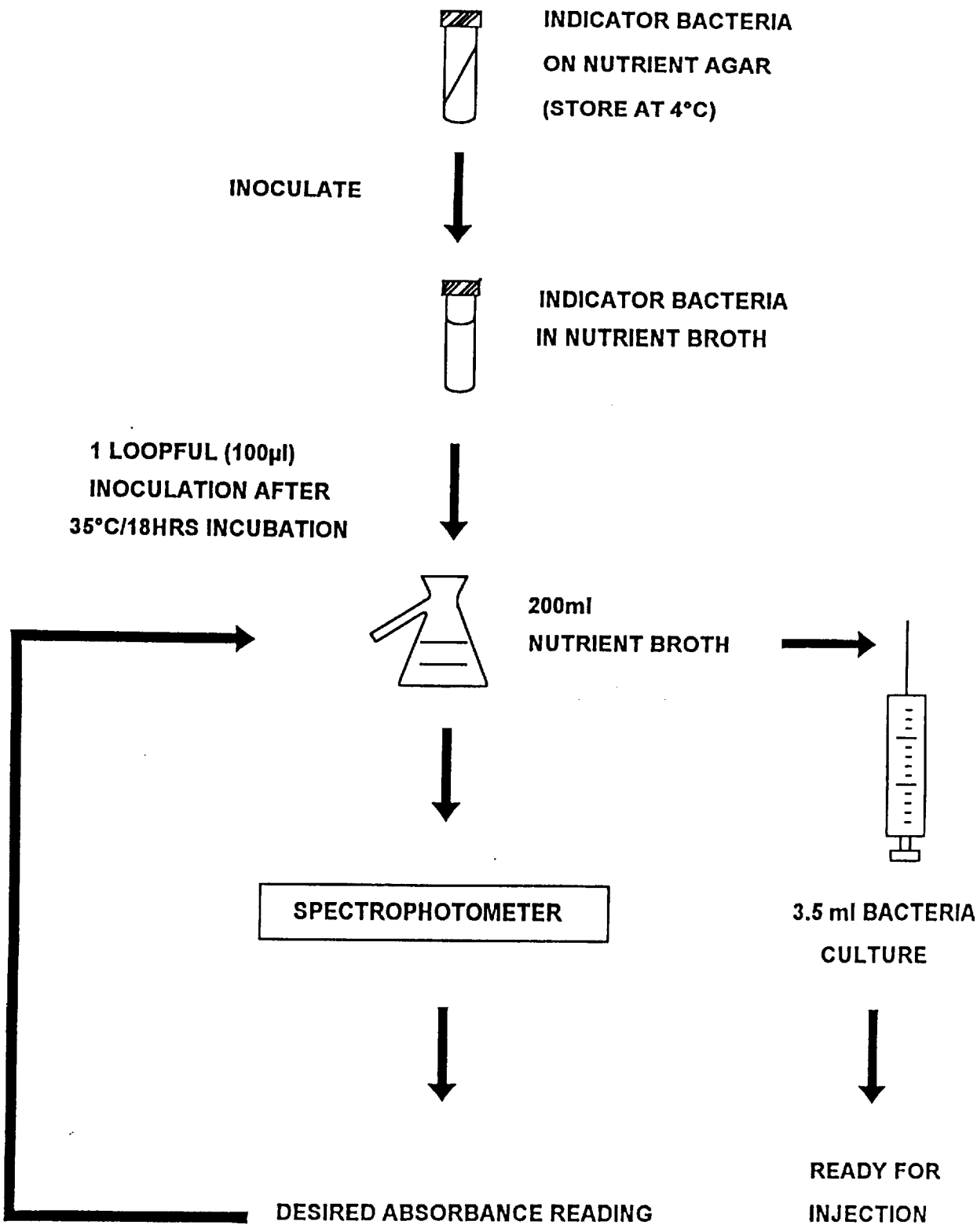
DIGESTER WITH

STEADY-STATE

4. ESTABLISHMENT OF BACTERIA GROWTH CURVE

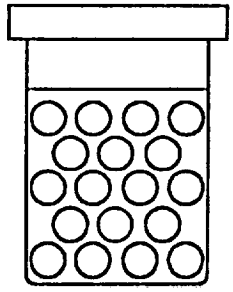


5. PREPARATION OF BACTERIAL SOLUTION



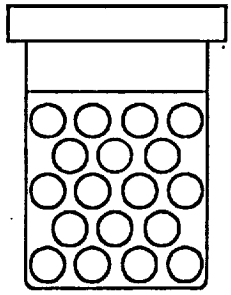
6. INJECTION OF INDICATOR BACTERIA

WITHDRAW
350 ml
SOLUTION



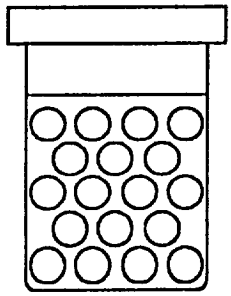
ANAEROBIC
DIGESTER
(V = 3500ml)

INJECTION
OF 3.5 ml
BACTERIA
CULTURE



ANAEROBIC
DIGESTER
(V = 3500ml)

FLUSH WITH
346.5 ml
NFSS



ANAEROBIC
DIGESTER
(V = 3500ml)

7. ENUMERATION OF THE INDICATOR BACTERIA