A Biochemical Predictor of Performance during Mesophilic Anaerobic Fermentation of Starch Wastewater

by

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Abstract

The aim of this study was to determine the potential of biochemical parameters, such as enzyme activity and adenosine triphosphate (ATP) levels, as monitors of process performance in the Upflow Anaerobic Sludge Blanket (UASB) reactor utilizing a starch wastewater. The acid and alkaline phosphatase activity and the ATP content of the UASB sludge were measured in response to changes in flow rate and nutrient loading. Conventional parameters of process performance, such as gas production, acetic acid production, COD, phosphorus, nitrogen and suspended solids loadings and % COD removal were also monitored. The response of both biochemical and conventional parameters to changing process conditions was then compared. Alkaline phosphatase activity exhibited the highest activity over the entire study period. A high suspended solids loading was observed to upset the system in terms of gas production, acetic acid production and % COD removal. The initial rate of increase in alkaline phosphatase activity following an increase in loading was four times as great during process upset than under conditions of good performance. The change in enzyme activity was also more sensitive to process upset than changes in acetic acid production. The change in ATP content of the sludge with time suggested that enzyme activity was changing independently of the actual viable biomass present. The bacterial composition of the anaerobic sludge granules was similar to that of other sludge bed systems, at the light and scanning electron microscope level. Isolated serum bottle cultures produced several acids involved in anaerobic carbohydrate metabolism. The overall performance of the UASB system indicated that higher loadings of soluble nutrients could have been tolerated by the system.

Acknowledgements

I would like to thank Dr. M. S. Manocha and Dr. M. Dickman for their support throughout this project. I would also like to thank the staff of CCIW and WTC and especially Dr. E. R. Hall and Dr. S. Rao for their invaluble assistance throughout this project.

Dedication

I would like to dedicate this work to all my family and friends but especially to Kathy, for keeping things in perspective and on track.

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Total Chemical Oxygen Demand	TCOD
Soluble Chemical Oxygen Demand	SCOD
Total Kjeldahl Nitrogen	TKN
Soluble Total Kjeldahl Nitrogen	STKN
Total Phosphorus	TP
Total Suspended Solids	TSS
Volatile Suspended Solids	VSS
Adenosine Triphosphate	ATP
P-Nitrophenol Phosphate	PNP
Upflow Anaerobic Sludge Blanket	UASB

INTRODUCTION

The extent of waste stabilization and methane production in several anaerobic treatment systems is being tested at the Wastewater Technology Centre in Burlington. One biological treatment system which shows potential in this area is the upflow anaerobic sludge blanket (UASB system, Figure 1).

The UASB system is a modified version of the contact process and houses its bacterial population within a dense "blanket" of granular or flocculated sludge. A particular waste is pumped up through the sludge blanket and suitable substrates are metabolized by the bacteria. "Acid" forming bacteria first reduce a substrate to a number of volatile fatty acids. The fatty acids are in turn metabolized to carbon dioxide and methane by methanogenic bacteria (Figure 2). The anaerobic fermentation which takes place in the UASB system is sensitive to influent variables, environmental conditions and biological and operational parameters. Digesters often respond slowly once they are upset, therefore the best operation is obtained by preventing upsets from occurring (Pause, 1983).

Graef and Andrews (1974) have pointed out that it is difficult to specify the threshold level above which a digester will fail, however, the trends established during changes in performance are often more useful than absolute numbers.

The inadequacy of available parameters for design and operation of anaerobic treatment systems has long been recognized. Many parameters used to monitor the anaerobic digestion process are inadequate because they do not relate to

Figure 1. Schematic of the 1100 l Upflow Anaerobic Sludge Blanket Reactor (Hall, 1980).

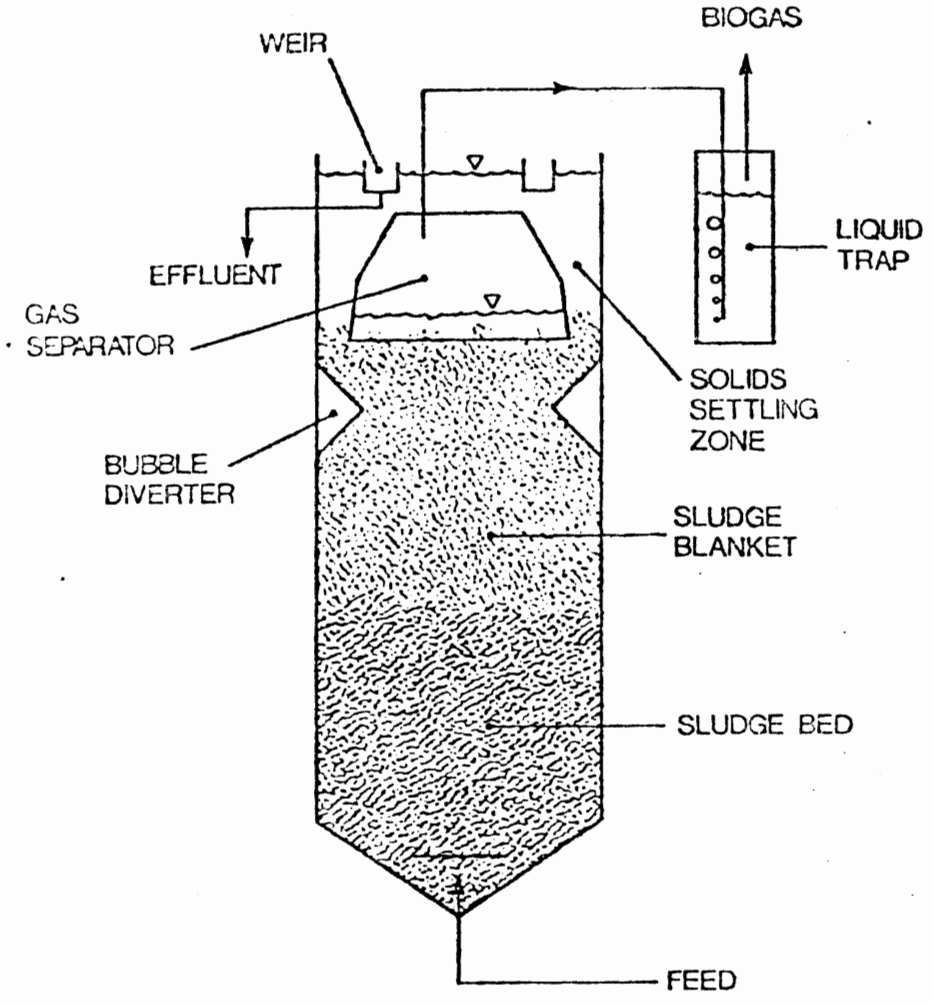
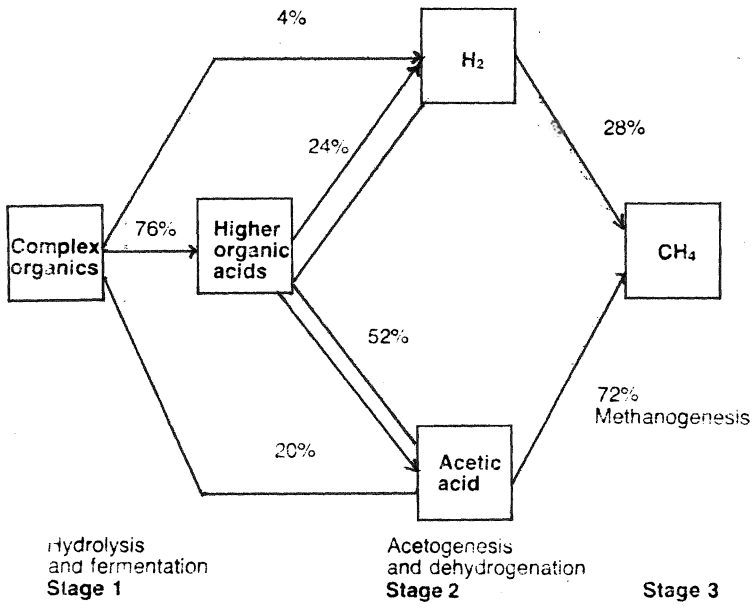


Figure 2. Schematic of anaerobic biological waste stabilization pathways (Speece, 1983).



viable bacterial mass but only to generalized mass (Pause, 1983). According to Ford et al. (1966) the monitoring tests applicable to anaerobic waste treatment are:

1. Chemical parameters: pH, alkalinity, volatile acids;
2. Unit operational efficiency : BOD or COD reduction;
3. Biological population density: V.S.S. (volatile suspended solids), enumeration techniques;
4. Biological oxidative capacities: sludge yields, gas production (methane and hydrogen);
5. Biochemical parameters: DNA, RNA, ATP, dehydrogenase, polysaccharides and lipid.

The relationship between monitoring tests for anaerobic treatment processes is outlined in Figure 3.

Good Performance vs. Process Upset

Good performance for a set flow rate is characterized by:

(a) High % COD removal

(b) High Gas Production

(c) Low volatile acid levels

Process upset is characterized by:

(a) A marked reduction in % COD removal

(b) A marked reduction in gas production

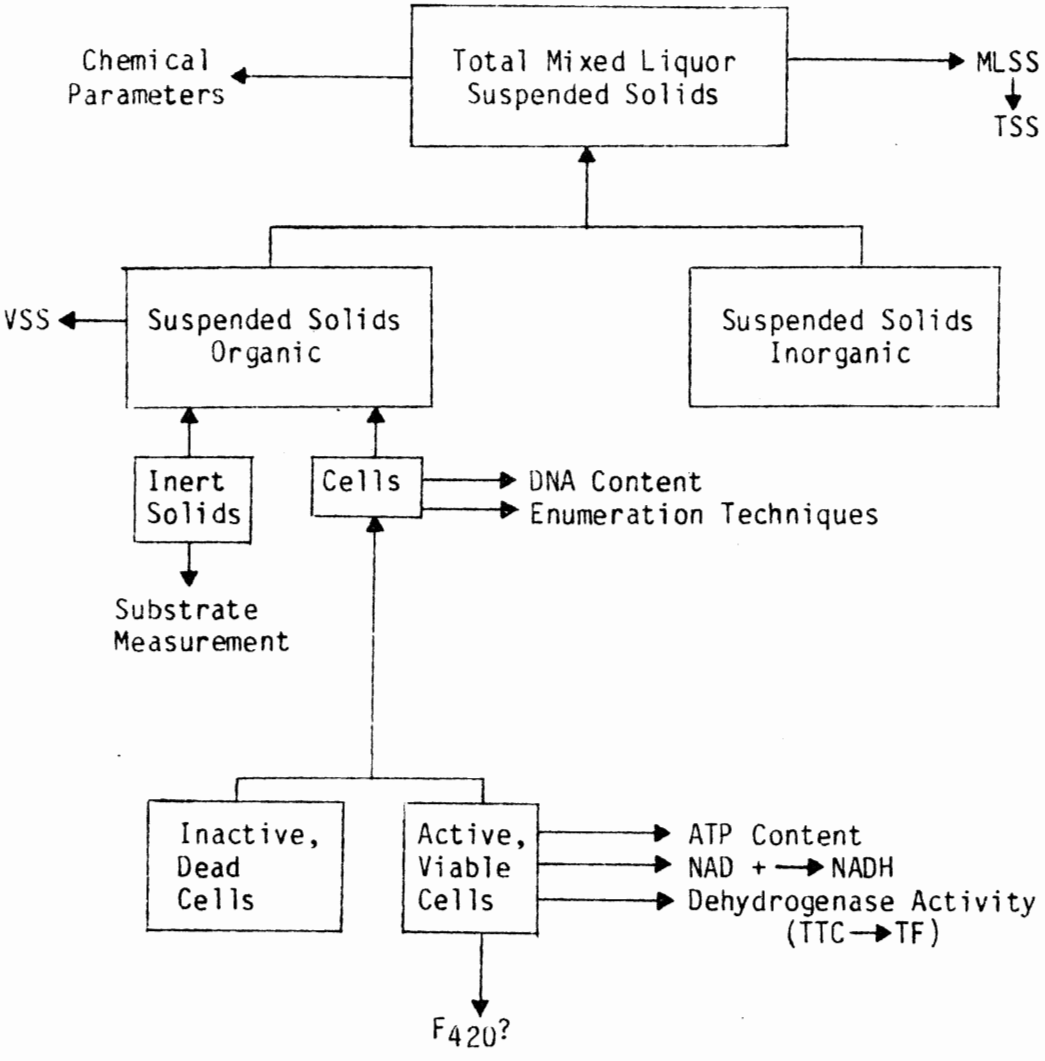
(c) High levels of volatile fatty acids (VFA)

(d) limiting concentrations of C,P or N

(e) Loss of active biomass due to solids washout

In light of the shortcomings of the current monitoring tests, several biochemical parameters have been applied to the determination of active biomass and metabolic activities. In 1981 a study by Ashley and Hurst utilizing raw sewage wastewater, concluded that "the determination of acid and alkaline

Figure 3. Monitoring tests for anaerobic treatment processess (Pohland & Kang, 1979).



phosphatase levels in anaerobic digestors offers a biochemical check on the stability of the digestion process, reflecting changes in the population of non-methanogenic organisms and gives warning of instability of the process and perhaps its subsequent failure in advance of conventional tests which examine end products of metabolism." In addition to this, Bull et al. (1983) in a study utilizing a chemically defined medium have shown that alkaline phosphatase activity in an anaerobic fluidized bed reactor changes with organic loadings.

The purpose of this study is to monitor the behavior of acid and alkaline phosphatase activity in the sludge of the Upflow Anaerobic Sludge Blanket reactor utilizing a starch wastewater. This investigation was undertaken;

(a) To determine whether phosphatase activity changes in response to changes in nutrient loadings and flow rates.

(b) To determine whether phosphatase activity may be used to predict process upset in the UASB system.

The hypotheses tested were as follows;

Null Hypothesis (1): Phosphatase activity in the sludge of the UASB system is not sensitive to changes in flow rate and loading.

Predictions from Null: If the null hypothesis is true then enzyme activity should remain unchanged even with changes in loading.

Null Hypothesis (2): Phosphatase activity is not a useful monitor of process upset in the UASB system.

Predictions from the Null:

If phosphatase activity is not a useful monitor of process upset then;

(a) Changes in alkaline phosphatase activity should not occur prior to process upset.

(b) Changes in activity should not show a high sensitivity to changes in input conditions.

In addition the ATP content of the sludge will be used as the measure of viable biomass in the system. The viable biomass will also be characterized in serum bottle cultures with the aid of light and scanning electron microscopy and gas/liquid chromatography. Such an experimental design will allow the comparison of phosphatase enzyme activity and ATP content to conventional monitoring tests during process upset. The usefulness of these biochemical parameters as a measure of process upset in the UASB system can then be determined.

LITERATURE REVIEW

During the last decade interest in anaerobic biotechnology has grown considerably, especially in the use of the process for industrial wastewater treatment and in bioconversion of biomass to methane. This surge of interest has translated into numerous treatability studies of various industrial wastewaters and the development of unique and novel treatment systems (Speece, 1983). Some organics amenable to anaerobic biotechnology are shown in Table 1 and various reactor configurations for anaerobic biotechnology are shown in Figure 4.

Anaerobic treatment processes are generally grouped into four types based on reactor configuration. These include the conventional, contact, anaerobic filter and attached film expanded bed process (Pause, 1983). The microbial biomass responsible for anaerobic biotechnology is "packaged" in these different process configurations. When the anaerobic growth occurs in large agglomerations described as "granular" sludge, this forms the "packaging" for the Upflow Anaerobic Sludge Blanket (UASB) system.

Upflow anaerobic sludge blanket technology has been developed primarily in the Netherlands where Central Sugar Manufacturing (CSM) have operated two full scale systems for the reduction of sugar beet waste. The success of the UASB system at CSM has led to the construction of several additional full scale systems and at the University of Wageningen, pilot scale studies are being conducted into the treatment of yeast, slaughterhouse and municipal wastewaters (Hall,1980). Table 2 shows several of

Table 1: Organics amenable to anerobic biotechnology

(Speece, 1983)

Acetaldehyde	Formic acid	Isopropyl alcohol	Giant kelp
Acetic anhydride	Fumaric acid	Propionate	Animal wastes
Acetone	Glutamic acid	Propylene glycol	Cheese whey
Acrylic acid	Glutaric acid	Protocatechuic acid	Pear wastes
Adipic acid	Glycerol	Resorcinol	Pectin wastes
Aniline	Hexanoic acid	*Sec*-butanol	Meat packing
1-amino-2-propanol	Hydroquinone	*Sec*-butylamine	Corn milling
4-amino butyric acid	Isobutyric acid	Sorbic acid	Dairy
Benzoic acid	Isopropanol	Syringaldehyde	Brewery
Butanol	Lactic acid	Syringic acid	Rum distillery wastes
Butyraldehyde	Maleic acid	Succinic acid	Wine distillery wastes
Butylene glycerol	Methanol	*Tert*-butanol	Guar gum wastes
Catechol	Methyl acetate	Vanillic acid	Water-soluble polymers
Cresol	Methyl acrylate	Vinyl acetate	Bean blanching
Crotonaldehyde	Methyl ethyl ketone	Corn	Pulp mill evaporate
Crotonic acid	Methyl formate	Potato	Coking mill
Diacetone gulusonic acid	Nitrobenzene	Sugar cane	H_2-CO pyrolysis
Dimethoxy benzoic acid	Pentaerythritol	Bagasse	Wool scouring
Ethanol	Pentanol	Peat	Tannery wastes
Ethyl acetate	Phenol	Wood	Yeast
Ethyl acrylate	Phthalic acid	Corn stover	Heat-treated activated sludge
Ferulic acid	Propanal	Straw	
Formaldehyde	Propanol	Water hyacinths	

Figure 4. Reactor configuations for biotechnology (Speece, 1983).

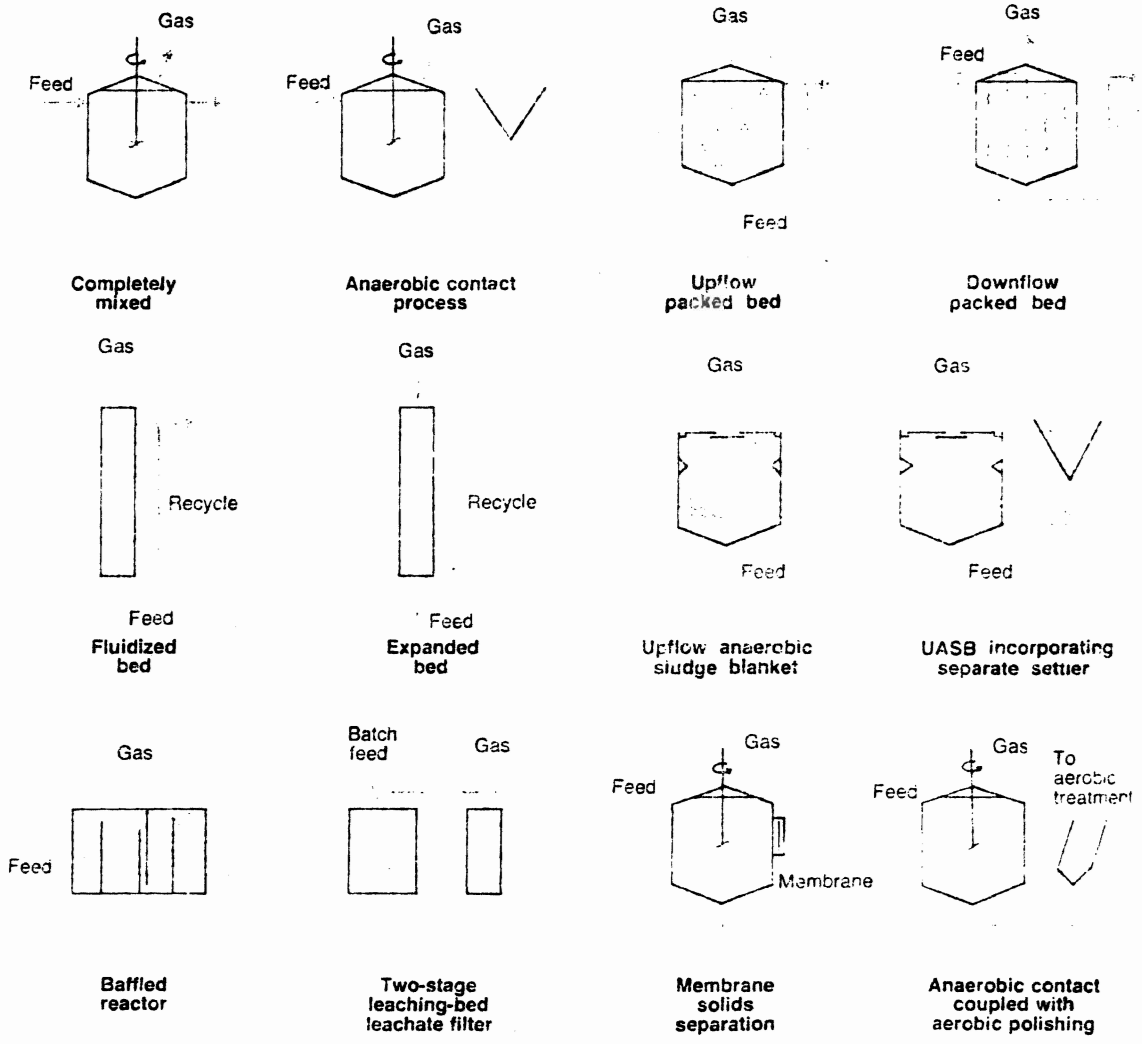


Table 2: Several treatability studies utilizing the UASB system.

Waste	UASB reactor (m3)	COD range (mg/liter)	Treatment efficiency(%)	Reference
Heat treated sludge	1.1	4000-10000	80-90	Hall (1980)
Synthetic	1.1	2000-4000	70-80	Hall (1980)
Liquid sugar	6.0	4000-6000	92-95	Lettinga(1980)
Beet sugar	200	4000-5200	87-95	Lettinga(1980)
Beet sugar	800	3000	88	Lettinga(1980)
Potato waste	6.0	2000-5000	95	Lettinga(1980)

treatability studies employed to date utilizing the UASB system. At the Wastewater Technology Centre in Burlington the USAB system has not been used to date to treat a high carbohydrate waste.

As shown in Table 2, the efficiency of the UASB system is quite high for a wide range of industrial wastewaters. In order to maximize the efficiency of anaerobic treatment processes it is necessary to monitor the system closely and avoid process upset. The lack of a single indicator which will reliably signal an imbalance among the major bacterial populations necessitates that a number of indicators be considered simultaneously (McCarty, 1964). Due to the biological nature of the anaerobic systems, recent emphasis has focused on the application of enzyme activity and ATP methodology as monitors of process performance.

Enzyme Activity as a Parameter of Anaerobic Process Performance

Enzymes mediate all major reactions in a cell and are especially of interest for waste treatment processes because they catalyze reactions related to substrate utilization. From a waste stabilization standpoint, Agardy et al.(1963) suggested that under conditons of stress, such as changes in organic loading, pH, temperature, alkalinity etc.; anaerobic biomass should adjust rapidly to the new environment particularly with respect to enzyme production and activity. This hypothesis was tested in a study by Agardy et al.(1963) in which the proteolytic enzyme activity of a bench scale reactor was measured over a 28 week period. During this period the organic loading was increased from 0.28 to 2.7 lbs. COD/day/lb. V.S.S. at two week

intervals. The results showed that a rapid rise in volatile acid levels preceeded all other indices of fermentation failure except enzyme activity increase which was at least seven times that of the increase in volatile acid concentration.

Thiel and Hattingh (1967) examined the activity of several hydrolytic enzymes during the adaptation of an anaerobic bench scale reactor to a synthetic substrate. The overall phosphatase enzyme activity of the digestor increased slightly during the acclimatization period but was much lower than the increases in amylase, protease and cellobiase activities. However, recent studies have indicated that phosphatase enzyme activity may serve as a useful monitor of process upset in anaerobic wastewater treatment systems.

Phosphatase enzymes catalyze the hydrolytic cleavage of phosphoric acid esters and can be divided into two groups, alkaline and acid phosphatases depending on their pH optima. Table 3 shows the substrates hydrolyzed by alkaline phosphatase of*Escherichia coli*. Phosphatases may function as enzymes of nutrition in digesting sludge degrading or partially hydrolyzing complex phosphate-rich molecules (such as phospholipids, nucleotide phosphates, hexose and pentose phosphate) to render them assimilable by the bacteria.

Phosphatase enzymes are located in bacteria in the periplasmic space of the cell. The location of these enzymes also lends itself to the hypothesis that phosphatases may be involved in the incorporation of phosphate into the cell (Reid and Wilson,1971).

Table 3. Reaction velocity and Michaelis constant for *Escherichia coli* alkaline phosphatase and various substrates (Heppel et al. 1962).

Substrate	Relative reaction velocity	$K_m \times 10^3$
5′-AMP	1.0	1.1
PP_i	1.0	1.4
PPP_i	0.9	1.7
3′-AMP	1.0	1.7
ApAp	0.6	
ATP	1.05	1.4
dATP	1.05	
dGTP	1.05	
UDP	1.0	1.3
5′-UMP	0.85	2.2
dCTP	1.05	
Ribose-5-P	0.7	1.1
β-Glycerol-P	0.9	0.7
Ethanolamine-P	0.7	0.8
Glucose-1-P	0.8	0.7
Glucose-6-P	0.9	1.7
Histidinol-P	0.8	1.0
dTTP	1.0	
p-Nitrophenyl-P	1.0	3.0
Polymetaphosphate[a]	0.9	
poly C	0.6	
poly I	0.6	

Although bacterial phosphatases are assumed to be constitutive, Horiuchi (1959) and Torriani (1960) have shown that when inorganic phosphate became limiting in the growth medium of certain aerobic strains of E.coli, there resulted a high level of alkaline phosphatase activity. Leventhal (1963) has also shown that the formation of alkaline phosphatase occurs in the presence of orthophosphate. The results of Ashley and Hurst (1981, 1982) have shown that phosphatase enzymes of a mixed anaerobic system are constitutive. Changes in enzyme activity were attributed to changes in the total "acid" forming population under stressful conditions. Their results also indicated that changes in the enzyme activity preceeded changes in conventional parameters such as volatile fatty acid production, gas production and pH. The work of Bull et al. (1983) corroborates these results. In the latter study a bench scale anaerobic fluidized bed reactor was subjected to loadings of 6, 12 and 18 g COD/m3/day. Alkaline phosphatase activity changed with loading but was highest at the intermediate COD loading. The former studies have examined phosphatase enzyme levels at the bench scale level. To date, there has been no report in the literature of any study dealing with the use of phosphatase activity as a monitor of process performance in the UASB system at the pilot plant or full scale level.

As noted by Ashley and Hurst (1981) the changes in phosphatase activity were constitutive and due to a corresponding change in the number of viable bacteria present.

Enumeration of viable bacteria through plate count techniques is cumbersome, time consuming and subject to a large amount of error due to the nature of anaerobic "granular" sludge. This has led investigators to seek a more reliable and accurate measure of the viable biomass present in a system.

ATP as a Measure of Viable Biomass

Adenosine triphosphate (ATP) is a universal constituent of living cells. ATP is non-conservative in dead cells and is directly proportional to the amount of living material present and is therefore of value in assessing the potential activity of a mixed microbial culture such as exists in anaerobic systems.

The bulk of the research to date has dealt with the possibility of using ATP for control of aerobic wastewater treatment systems, such as the activated sludge process. Levein et al. (1975) in a study of a 20 (mgd) sewage treatment plant revealed a close coupling between sludge ATP content and metabolic activity of the sludge. Kung et al. (1980) examined the applicability of ATP methodology as a measure of active biomass in activated sludge and also in rotating biological contactors (RBC) for the first time. The authors concluded that the previous use of substrate utilization and other conventional parameters to estimate biomass concentration will gradually be replaced by the use of ATP methodology. This is due to the fact that their results showed the method to be more accurate, reliable and less time consuming than other estimates of viable biomass. The use of ATP as a measure of the viable biomass in

anaerobic systems is not without limitations however. Perhaps the most significant of these is the fact that certain anaerobic organisms possess multiple catabolic pathways by which ATP may be produced. If a mixed substrate is used, different metabolic pathways may be used at different times resulting in fluctuating levels of ATP in the bacterial cell (Thauer et al., 1977).

To date the literature suggests that although ATP methodology has been applied to full scale aerobic systems, there has been no aplication to the UASB system. The application of enzyme methodology to anaerobic systems and in particular phosphatase enzyme activity has been documented. However there have been no applications of phosphatase methodology to the UASB system at the pilot plant level. The application of phosphatase enzyme activity and ATP methodology as monitors of UASB performance is a new and as yet untested form of process control.

MATERIALS AND METHODS

Sludge Sampling

Sludge samples were obtained from the sludge blanket of the UASB system housed in the Wastewater Technology Centre, Burlington. A graduated tygon hose was used to siphon the sludge from a specified depth into a sample bottle. A solids meter (Ultrasonic suspended solids meter-model 500 Markland Specialty Engineering Ltd.) was used to determine the position of the solids/liquid interface. The "upper" bed sample was taken six inches below the interface and the lower bed sample taken 30 inches below the upper bed sample. The position of the solids/liquid interface was checked before each sampling period.

Preparation of Cell Free Sludge Extracts

Cell free sludge extracts were prepared according to the method of Thiel and Hattingh (1967). A 40ml sample of sludge was transferred to a glass centrifuge tube cooled in an ice bath to 4°C. The sludge was then homogenized for 15 minutes in an ultrasonic apparatus (Sonicator, Model,Biosonick II Bromwell Scientific, Rochester N.Y.). After homogenization the sludge particles were removed by centrifugation at 40,000 g for 30 minutes at 4°C using an IEC B-60 Ultracentrifuge. The clear supernatant was stored at 4°C for subsequent phosphatase enzyme activity determination.

Phosphatase Enzyme Assay

Acid and alkaline phosphatase assays were performed using a method based on that of Ashley and Hurst (1981). To 0.5 ml of

substrate (1% w/v p-nitrophenol phosphate) 1.5 ml of buffer and 1 ml of sonicated sludge supernatant were added. The controls consisted of the above to which 1 ml of heat inactivated supernatant was added. All tubes were run in duplicate at 37°C for 60 minutes. After incubation 1.0 ml was removed from each tube and added to 2 ml of 0.2M NaOH. After 30 minutes samples were assayed on a spectrophotometer and the absorbance converted to ug PNP hydrolysed/hr./ml supernatant. Final enzyme activity was expressed as units/mg V.S.S. (1 unit = 1 ug PNP hydrolysed/hr/ml supernatant).

The optimum pH range of phosphatase activity was determined in sonicated and unsonicated sludge supernatants by incubation with assay buffers in the pH range 3 - 11. The buffers used were 0.2M Na citrate/citric acid (Sigma), pH 3 - 6; 0.2M Imidazole (Sigma), pH 6.5 - 7.0; 0.2M 2-amino-2methyl-1propanol (BDH), pH 7.5 - 10.5.

Optimum sonication time was determined by sonicating sludge samples for 5 - 60 minute intervals.

Optimum incubation time at the respective pH optima was determined by incubation of samples for intervals of 0.1 to 2.0 hours in a water bath held at 37°C.

Total and Volatile Suspended Solids

A known volume of sludge was placed in a previously weighed aluminum tray and dried for 24 hours in an oven at 100°C. The tray and sample were then cooled in a dessicator for 30 minutes and weighed. The sample was combusted at 500°C for

15 minutes, in a muffle furnace, cooled and weighed again. The weight of the sample dried at 100°C provided the total suspended solids weight per ml of sludge. The difference in weight of the dried sample and combusted sample provided the volatile suspended solids per ml of sludge.

Volatile Fatty Acids (VFA) Analysis

Effluent samples were filtered through glass fibre filters and acidified to pH 2 with 50% v/v H_2SO_4. Effluent volatile fatty acids were analyzed by direct aqueous injection of acidified samples into a 1/8" by 8' Teflon column, packed with 60-80 mesh Chromosorb 101. Volatile fatty acid standards consisted of 0.25 g each of acetic acid, propionic acid, iso-butyric acid and butyric acid in a total volume of 1000 ml. Analyses were run isothermally at 130°C on a gas chromatograph equipped with a flame ionization detector (Carle Model 211) and an automatic sampler (Varion Model 8040). The detector and injection port were held at 200°C. The carrier gas was nitrogen at a flow rate of 40 ml/min. The hydrogen flow rate was 20 ml./min. while the air flow was held at 200 ml./min. Peak heights were integrated automatically to provide the relative VFA concentration in mg/l.

The method used to detect volatile fatty acid end products in anaerobic serum bottle cultures was adapted from Bulletin 748G, Supelco Inc. Cultures were acidified, extracted with ether and the ether extracts chromatographed. For ether extraction, 1 ml of ethyl ether was added to the acidified culture. The ether

and culture were mixed by inverting the sample 20 times. The mixture was centrifuged briefly to break the ether - culture emulsion. The sample was placed in the freezing compartment of a refrigerator until the aqueous phase was frozen. The ether layer was then poured into a small test tube and anhydrous Na_2SO_4 added to equal approximately one half the volume of ether in the tube. The sample was allowed to stand for at least 10 minutes before being chromatographed. The volatile fatty acid standard used contained 1 meq each of acetic, formic, propionic, isobutyric, n-butyric, iso-valeric, n-valeric, isocaproic, n-caproic, heptanoic (Supelco). The samples were run in a programmed mode on a gas chromatograph equipped with a flame ionization detector (Hewlitt-Packard Model 5700) and a 6' by 1/4" glass column packed with 15% SP-1220/1% H_3PO_4on 100/120 Chromosorb W AW. The column temperature was increased from 100°C to 160°C at a rate of 32°C/min. The detector temprature was held at 200° C . The carrier gas was nitrogen maintained at a flow rate of 56 ml/min. The hydrogen and air flow rates were 56 ml/min. and 300 ml/min respectively.

Gas Production

The total gas production was measured on a wet test meter (Fisher Model 401) and recorded daily.

ATP Extraction and Assay

Extraction

ATP was extracted from sludge samples utilizing the TCA diluted method adapted from Thore (1981a). The TCA/EDTA

extracting solution (10%/4mM) contained 10.0 g of trichloroacetic acid (Sigma) and 0.149g EDTA (Sigma) dissolved in 100 ml distilled water. The Tris/EDTA buffer solution (0.1M, pH 7.75) contained 6.06g of Tris (hydroxy methyl) amino ethane (Sigma) and 0.372g EDTA (Sigma) dissolved in distilled water, titrated to pH 7.75 with acetic acid, and made up to 500 ml with distilled water. A volume of 1.0 ml of sludge was mixed with an equal volume of 10% TCA/4mM EDTA solution. The mixture was placed on ice and mixed frequently for 15 minutes. A volume of 8.0 ml of Tris/EDTA solution was then added, mixed and the mixture frozen immediately in liquid nitrogen. Samples were frozen at -18°C until they were assayed.

The method used for ATP assay was a modification of the luciferase assay. The ATP is measured by use of the enzyme luciferase extracted from Photinus pyralis (american firefly). In the presence of luciferin and magnesium ion, the enzyme catalyzes the breakdown of ATP to adenosine monophosphate (AMP). This reaction is accompanied by light emission and the intensity is proportional to the amount of ATP when all other reactants are present in excess. The ATP Monitoring Reagent (luciferase) contained the lyophilized reagent (LKB reagents) reconstituted in 5.0 ml of distilled water. The reconstituted reagent was frozen at -18°C in 0.5 aliquots until required. The ATP Standard contained the lyophilized standard (LKB reagents) reconstituted in 10 ml of distilled water. This provided a final stock solution of 1.0×10^{-5}M ATP. The reconstituted vial contents were divided into 1.0 ml aliquots and

stored at -18°C.

Prior to the actual assay, a frozen sludge sample, ATP monitoring sample and ATP standard sample were thawed quickly and placed on ice. The sludge sample was placed in a bench top centrifuge and spun for three minutes. The clear supernatant was poured off and placed on ice. The ATP standard was diluted to 10^{-6} M or lower depending on the concentration of the extracted sample.

The following were combined in a cuvette and placed in the luminometer; 0.8 ml Tris/EDTA buffer, 0.1 ml luciferase enzyme and 0.1 ml sample. The reactants were mixed quickly and the light output recorded over six minutes. A volume of 0.1 ml of ATP standard was then added and the output recorded again. All samples were run in duplicate.

The formula used for calculation of ATP (uM) =

$$\frac{\text{ATP sample (mV)}}{\text{ATP std. (mV)}} \times \text{Molarity ATP std.} \times \text{Volume ATP std.} \times \text{dilution factor}$$

where std. = standard

mV = millivolt light output

Scanning Electron Microscopy

The method used for scanning electron microscopy of sludge granules was adapted from Alleman et. al. (1982) and Osatake et. al. (1980).

Sludge granules were fixed overnight in a buffered glutaraldehyde solution consisting of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.6. Subsequently the samples were rinsed for 10 minutes in buffer and dehydrated in a graded ethanol

series (30%, 50%, 75%, 100% ethanol) for 20 minutes at each concentration. The samples were held overnight in 100% ethanol and placed in 50% ether/ethanol for 20 minutes and finally in absolute ether. Following ether extraction the samples were frozen in aluminum beakers containing liquid nitrogen and placed under vacuum until all the nitrogen had evaporated. Each sludge granule was mounted on an aluminum block and sputter coated with gold in an argon atmosphere. Samples were then observed under the scanning electron microscope.

Chemical Oxygen Demand (COD)

This method was based on the fact that, under acid conditions, potassium dichromate can oxidize most organic compounds. A sample is refluxed with a standard dichromate solution in a sulphuric acid medium and excess dichromate is determined by titration with ferrous ammonium sulphate. The amount of oxidizable organic matter is proportional to the potassium dichromate consumed. The method is applicable to all types of samples. Biologically active samples were analyzed as soon as possible or acidified with sulphuric acid (2 ml concentrated acid /liter sample) and refrigerated. The reflux apparatus consisted of a flat bottom 500 ml boiling flask, with ground glass neck, and 300 mm condensor, heated on a small hot plate. The potassium dichromate (0.25N) solution contained 12.259 g potassium dichromate, $K_2Cr_2O_7$ primary standard grade previously dried at 103°C for 1 hour dissolved in distilled water and diluted to exactly 1000 ml.

The sulphuric acid reagent contained 22 g reagent grade silver sulphate, Ag_2SO_4 per 9 lb. bottle of reagent grade sulphuric acid (3-4 hours stirring required for dissolution). The ferroin indicator solution contained 1.485 g 1,10-phenanthroline monohydrate and 0.695 g ferrous sulphate $FeSO_4.7H_2O$ dissolved in distilled water and diluted to 100 ml. The ferrous ammonium sulphate (0.25N) solution contained 98 g ferrous ammonium sulphate, $Fe(NH_4)_2(SO_4)_2.\ 6H_2O$ dissolved in distilled water. A volume of 20 ml concentrated H_2SO_4 was added and diluted to 1000 ml. This solution was standardized each day against 0.25N $K_2Cr_2O_7$ as follows: 25.0 ml 0.250N potassium dichromate, 300 ml distilled water and 75 ml concentrated H_2SO_4 were added to a 500 ml flask. The mixture was cooled and 5-6 drops of ferroin indicator were added and titrated with ferrous ammonium sulphate to a reddish-brown endpoint.

$$\text{Normality} = \frac{0.25 \times 25.0}{\text{ml ferrous ammonium sulphate}}$$

To a 500 ml flask 0.4 g mercuric sulphate, 50 ml of sample and 25.0 ml potassium dichromate solution were added. A volume of 75 ml of sulphuric acid was added to the mixture and the flask connected to the condensor. The reaction mixture was then refluxed for one hour. The condensor was allowed to cool and a further 200 ml of distilled water added to the flask. The final solution was then titrated with ferrous ammonium sulphate, using 5-6 drops of ferroin indicator, to a reddish brown endpoint.

Total Nitrogen (TKN) and Total Phosphorus (TP)

An automated procedure involving the use of a continuouos digestor followed by a split manifold was used for the simultaneous determination of total phosphorus and total Kjeldahl nitrogen. The automated system consisted of a sampler, 2 proportioning pumps; a digestor, manifold, colourimeter, recorder and digital printer. In the case of phosphorus, digestion was followed by measurement of the phosphate produced utilizing the reduction of ammonium molybdate by amino-napthol sulphonic acid. In the case of nitrogen digestion, of the organic matter was followed by measurement of the ammonia produced using the Berthelot reaction in which the formation of a blue indophenol complex occurs when ammonia reacts with sodium phenate followed by the addition of sodium hypochlorite.

Total Phosphorus

The digestion mixture contained 3.0 g selenium dioxide, 10 ml concentrated perchloric acid and 900 ml concentrated sulphuric acid in a final volume of 1000 ml distilled water. The stock manganese diluent contained 3.076 g manganese sulphate in 1000 ml distilled water. The working manganese diluent contained 5 ml of stock solution diluted to 1000 ml with distilled water. The ammonium molybdate solution contained 30 g ammonium molybdate in 800 ml distilled water and diluted to 3 litres. To this solution 100 ml concentrated H_2SO_4 was added. The stock sulphonic acid reagent contained 120 g sodium bisulphite ($NaHSO_3$) and 4 g sodium sulphite (Na_2SO_3) in 800 ml warm distilled water.

To this 2 g of 1-amino-2-naphthol-4-suphonic acid were added and diluted to 1000 ml. The working sulphonic acid reagent was prepared by diluting 100 ml of the stock solution to 1000 ml with distilled water. The stock "P" standard (1000 mg/l P) contained 3.957 g sodium tripoly phosphate dissolved in 500 ml distilled water. To this 1 ml concentrated sulphuric acid was added and diluted to 1000 ml. Serial dilutions were prepared in the 0-30 mg/l range, for calibration purposes.

Total Nitrogen (TKN)

The digestion mixture used for TKN determination was the same as that for total phosphorus measurement. The stock alkaline phenol solution contained 270 ml of liquefied phenol in 500 ml of 5N sodium hydroxide. This mixture was diluted to 1000 ml with distilled water and stored in polyethylene bottles. The working alkaline phenol solution contained 500 ml alkaline stock solution diluted to a final volume of 1000 ml The stock and working manganese diluent used for TKN analysis were the same as those for total phosphorus measurement. The ammonia nitrogen standard (1000 mg/l) contained 4.717 g ammonium sulfate in a final volume of 1000 ml. 0.1N sulphuric acid. For calibration purposes, serial dilutions were prepared in the range 5 - 100 mg/l. The organic nitrogen standard (1000 mg/l) contained 5.362 g glycine in a final volume of 1000 ml. 0.1N sulphuric acid. For calibration purposes serial dilutions were prepared in the range 5 - 100 mg/l. All reagents were sampled and mixed automatically. The resulting nitrogen and phosphorous concentrations were

calculated automatically ~~and printed~~ in mg/l.

Preparation of Anaerobic Cultures

Anaerobic cultures of the bacteria found on the sludge granules were prepared using a serum bottle modification of the Hungate technique (Miller and Wolin, 1974). The growth medium for the anaerobic mixed culture consisted of fluid Thioglycollate medium prepared under anaerobic conditions. The Thioglycollate medium consisted of (g/l) yeast extract 5.0, tryptone 15.0, dextrose 5.5, sodium thioglycollate 0.5, sodium chloride 0.5, L-cysteine 0.5, resazurin .001 and "Ionoagar No. 2" 0.5. The method was adapted from Thiel and Hattingh (1967). The media was brought to a boil and cooled by purging with pure nitrogen gas for half an hour. All media was bottled and inoculated in a sealed gas bag previously evacuated and purged with nitrogen gas several times. The cooled media was drawn into the gas bag with the aid of a peristaltic pump and dispensed into serum bottles. The bottles were capped with butyl rubber stoppers and closed with a crimped aluminium seal. The serum bottles with media were then autoclaved for 20 minutes at 121°C , 15 psi and stored for 24 hours before use. Serum bottles were inoculated with sludge using a sterile hypodermic syringe and needle. Cultures were grown in an incubator at 37°C for a specified length of time.

Gram staining

The staining procedure used was as follows: a heat fixed smear of the bacterial culture was first prepared.The slide was

flooded with crystal violet for one minute and washed for five seconds. The slide was flooded with iodine, drained and flooded again for one minute. After washing and drying the slide, it was decolorized with 95% ethanol and rinsed. The slide was finally flooded with safranin for one minute, washed, dried and examined.

RESULTS

Sludge Characterization

Light Microscopy

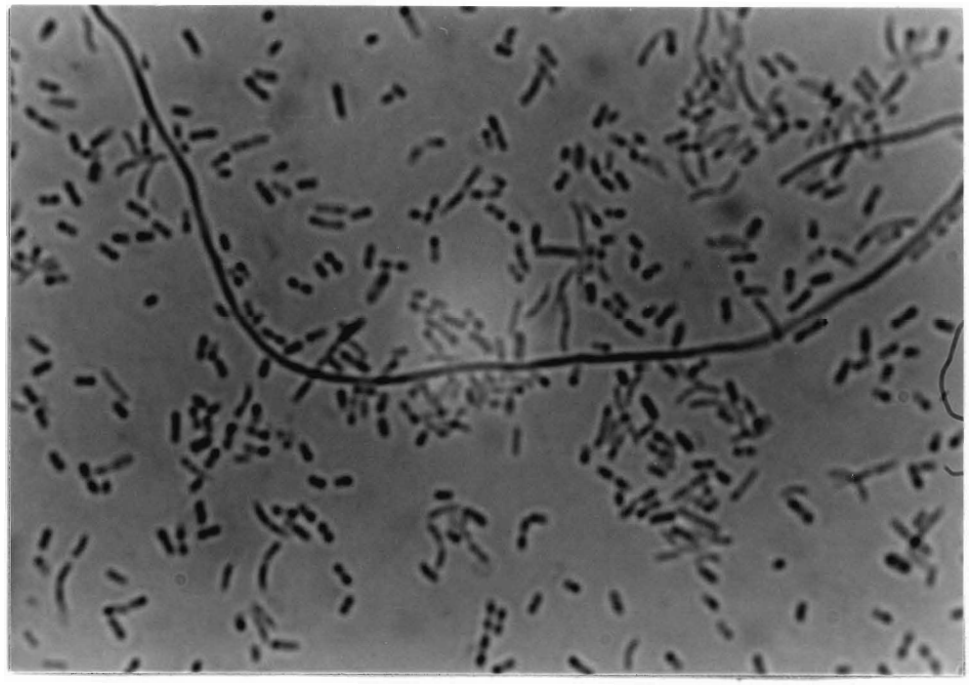
The light microscope analysis of the anaerobic bacteria cultured in fluid thioglycollate medium is shown in Figures 5-6. The main phenotypes observed were a short gram positive bacillus, 5 x 10 um and a gram positive filamentous colony 5 um in width and of irregular length. All bacterial colonies appeared to be motile in young cultures. Older cultures exhibited a characteristic "clumping", to form large masses of bacteria.

Scanning Electron Microscopy

Scanning analysis of the native sludge granules is shown in Figures 7 to 10. The matrix of each granule consisted of a dense mesh of filamentous bacteria (Figures 8&9). Other bacteria such as cocci and rods appeared to attach themselves to this matrix as seen in Figure 10.

Figure 5. A photomicrograph showing the rod shaped and filamentous bacteria in a anaerobic thioglycollate culture (magnification 200x)

Figure 6. A photomicrograph showing the clumping of rod shaped and filamentous bacteria in a anaerobic thioglycollate culture (magnification 200x).



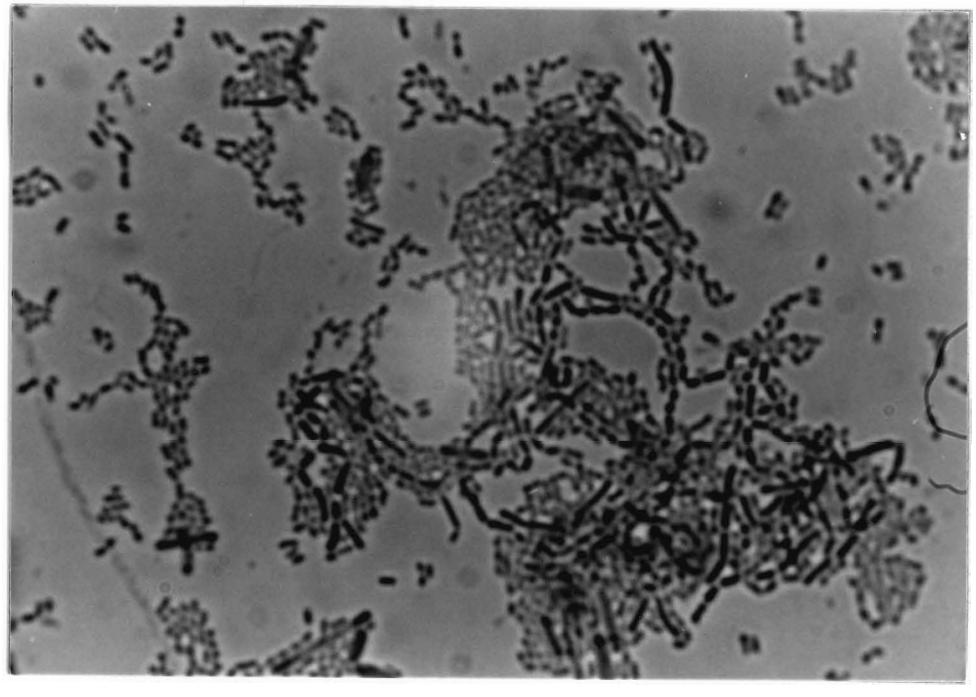
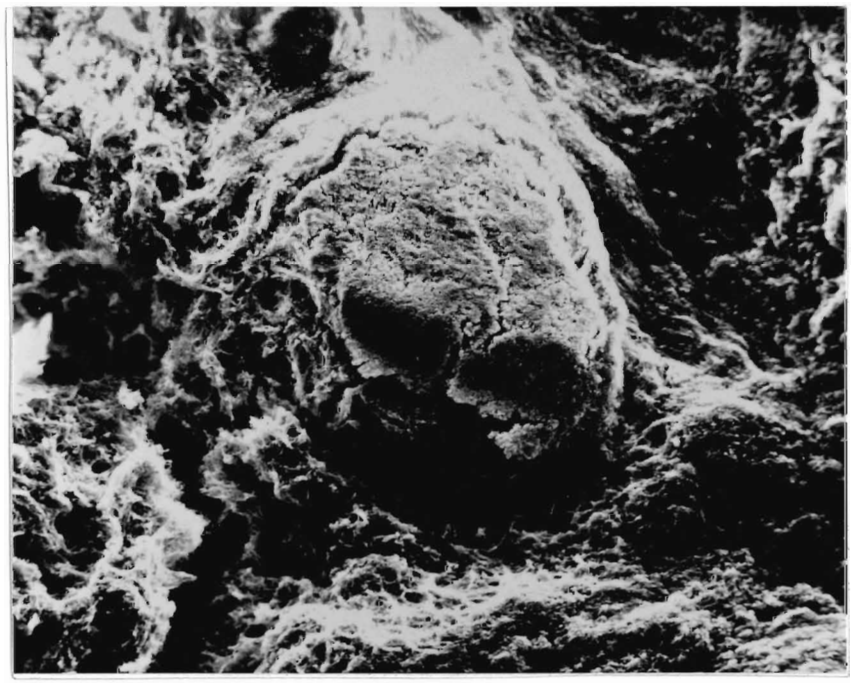


Figure 7. A scanning electron micrograph of a sludge granule from the UASB system (magnification 10x).

Figure 8. A scanning electron micrograph of a sludge granule from the UASB system (magnification 50x).



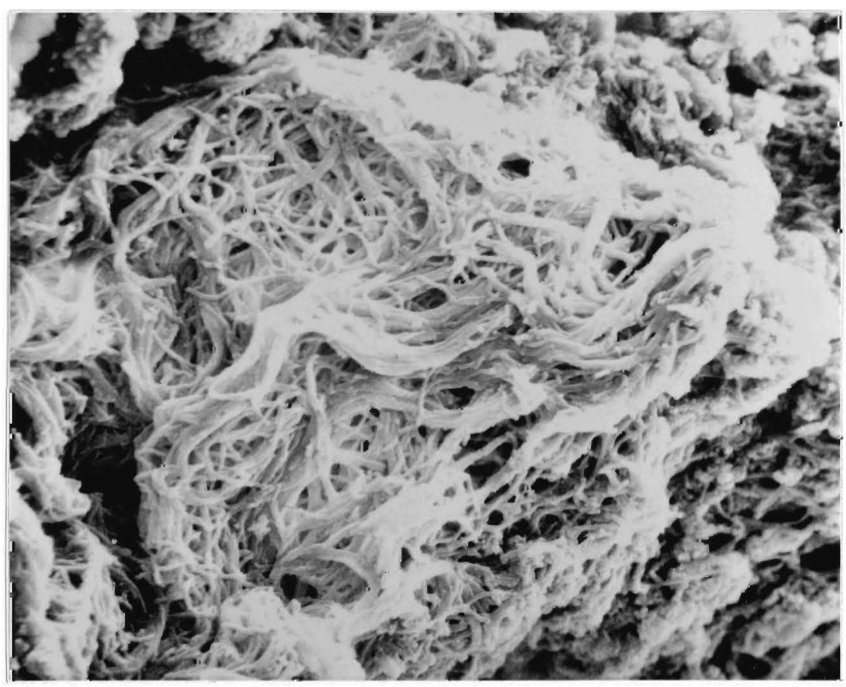
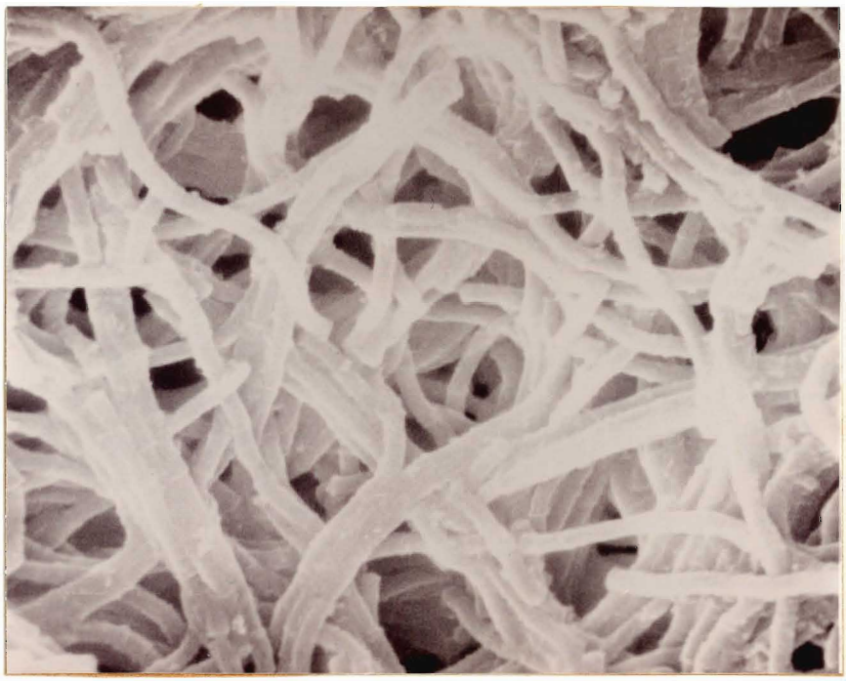
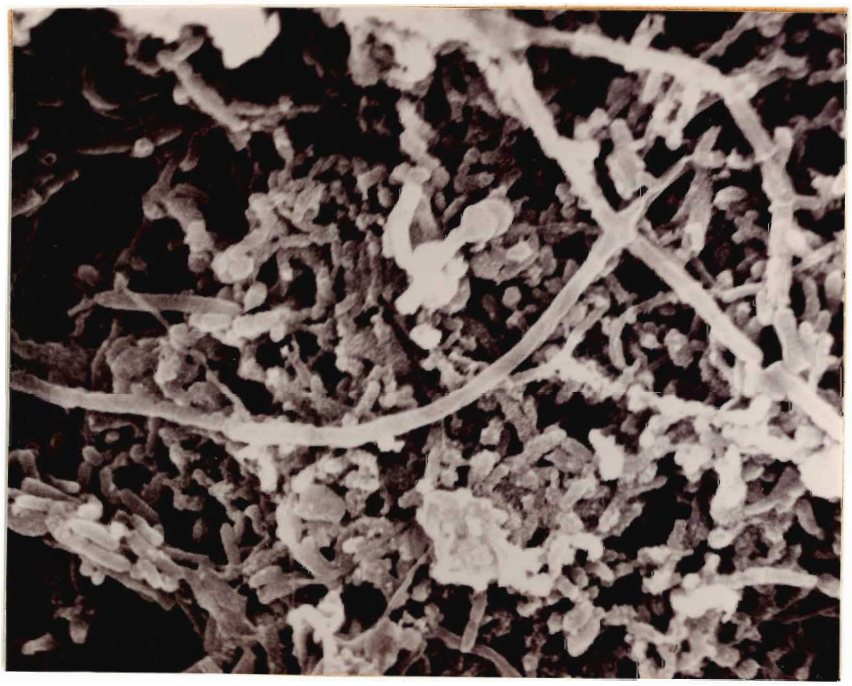


Figure 9. A scanning electron micrograph showing the filamentous matrix of a sludge granule (magnification 3200x).

Figure 10. A scanning electron micrograph showing the attachment of a mixed bacterial community to the filamentous matrix of a sludge granule (magnification 2000x).





Gas Liquid Chromatography

The volatile acids produced by the mixed anaerobic culture grown in a fluid thioglycollate medium were analysed by gas/liquid chromatography. The volatile acid standard used in the assay is shown in Figure 11. The acids produced by the sludge culture were compared to the retention times of the various standards. The volatile acids produced under these conditions were acetic, propionic, butyric, isobutyric and valeric acid (Figure 12).

Phosphatase Enzyme Activity

The optimum assay conditions for sludge phosphatase activity were first determined. Acid and alkaline activity was then measured throughout the study period.

pH Optimum

Two pH optima were observed for phosphatase activity in sonicated sludge (Figure 13). The pH optimum in the acid region was taken as pH 4.8 while the pH optimum in the alkaline region was pH 9.9. There was negligable enzyme activity in the unsonicated sludge extract.

Optimum Incubation Time

The optimum incubation time for the assay of sludge phosphatase enzyme activity was taken to be 60 minutes (Figure 14). Both acid and alkaline preparations showed substrate limitation after 100 minutes of incubation at 37°C.

Figure 11. A chromatograph of the volatile fatty acid standard mixture containing 1 meq each of(a) formic, (b) acetic,(c) propionic,(d) isobutyric, (e) n-butyric, (f) iso-valeric, (g) n-valeric, (h) isocaproic, (i) n-caproic and (j) heptanoic acids.

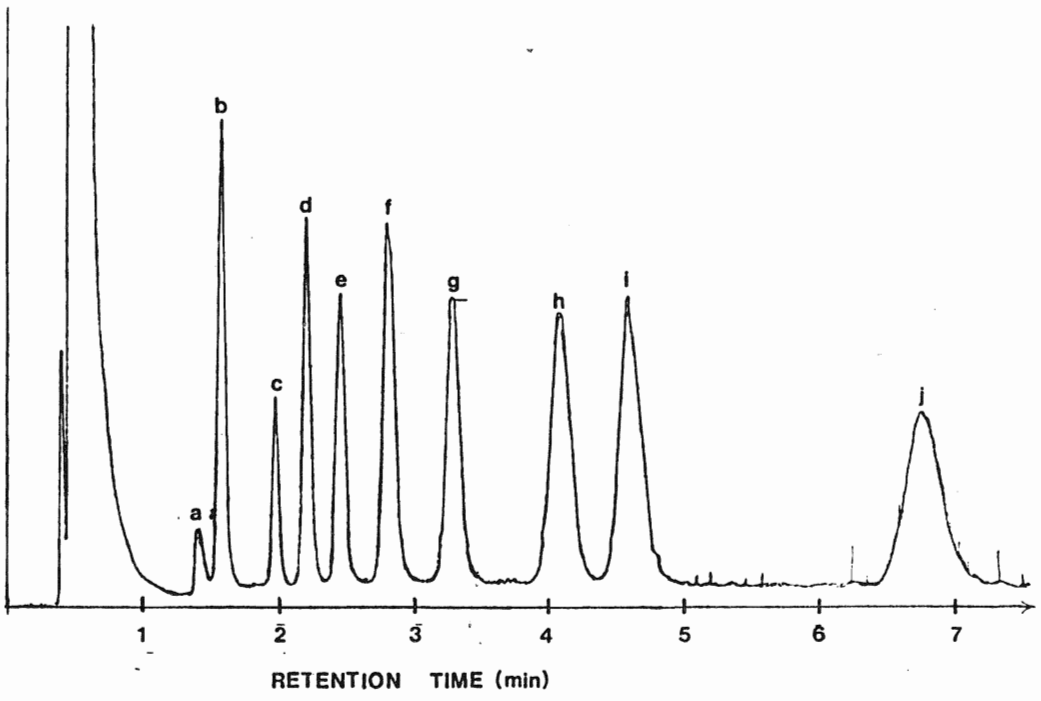

a
b
c
d
e
f
g
h
i
j
1
2
3
4
5
6
7
RETENTION TIME (min)

Figure 12. A chromatograph of the volatile acids, (b) acetic, (c) propionic, (d) isobutyric, (e) butyric, (g) valeric, produced by anaerobic serum bottle cultures of the UASB sludge.

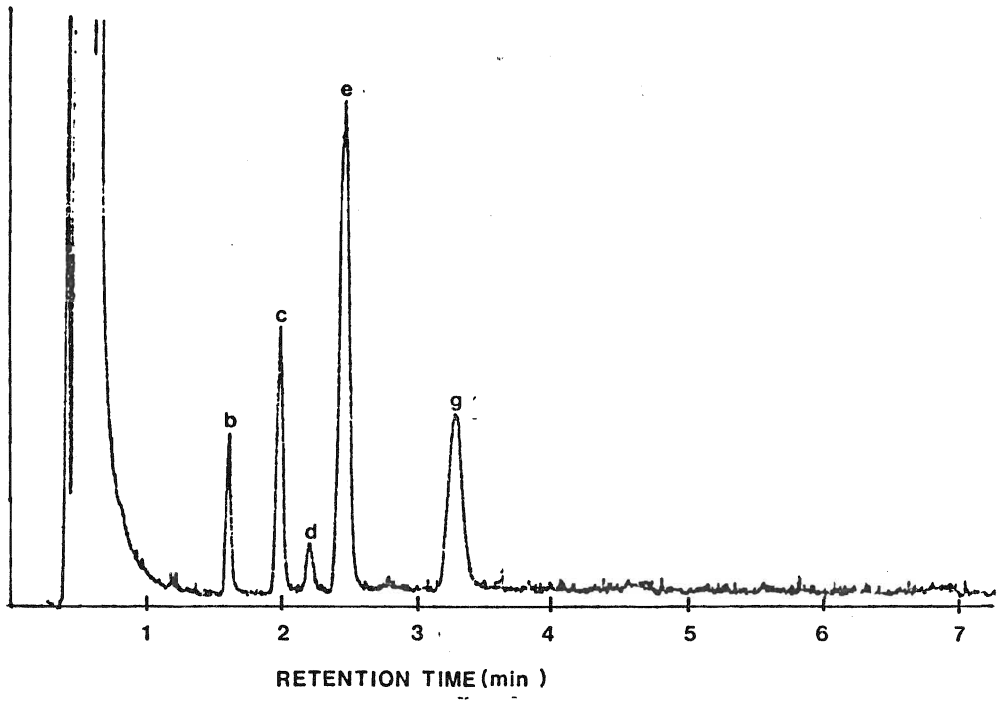

b
c
d
e
g
1
2
3
4
5
6
7
RETENTION TIME (min)

Figure 13. The pH optima of sonicated and unsonicated sludge phosphatase activity.

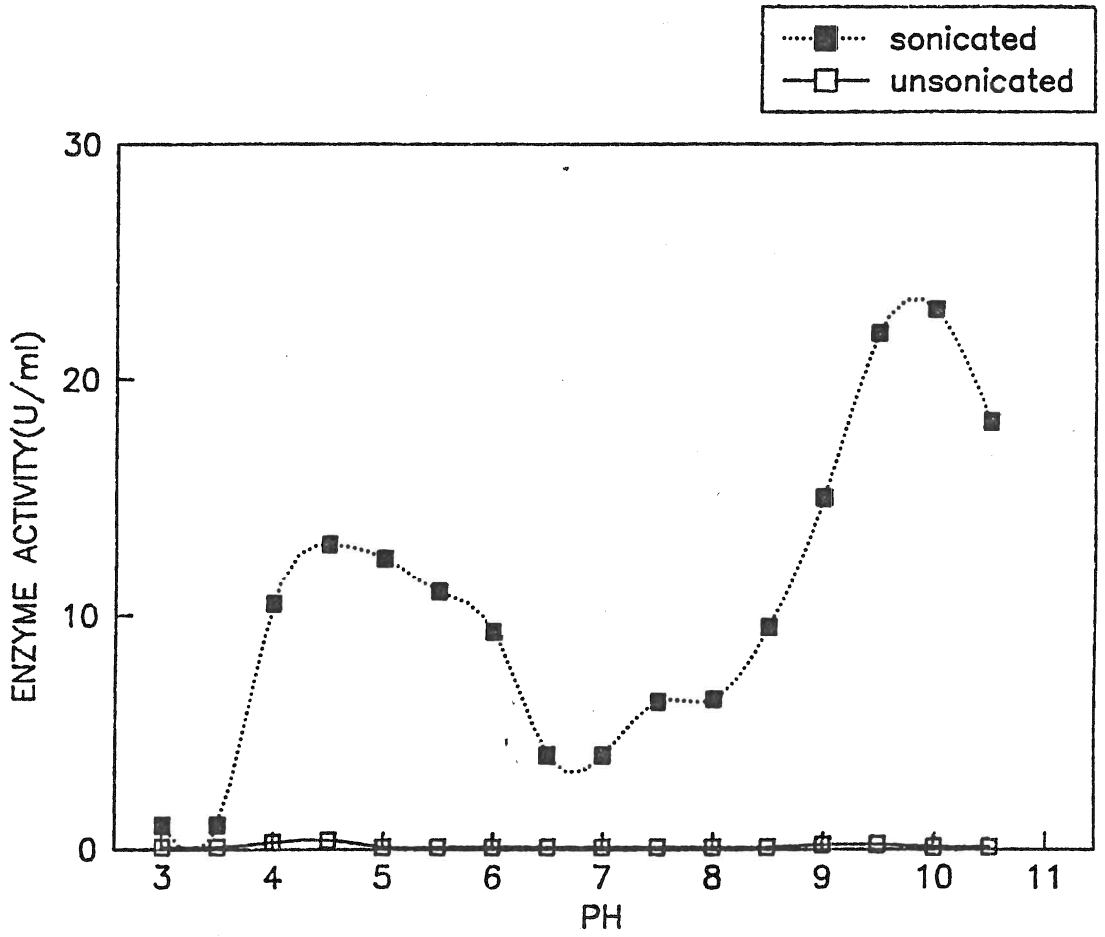
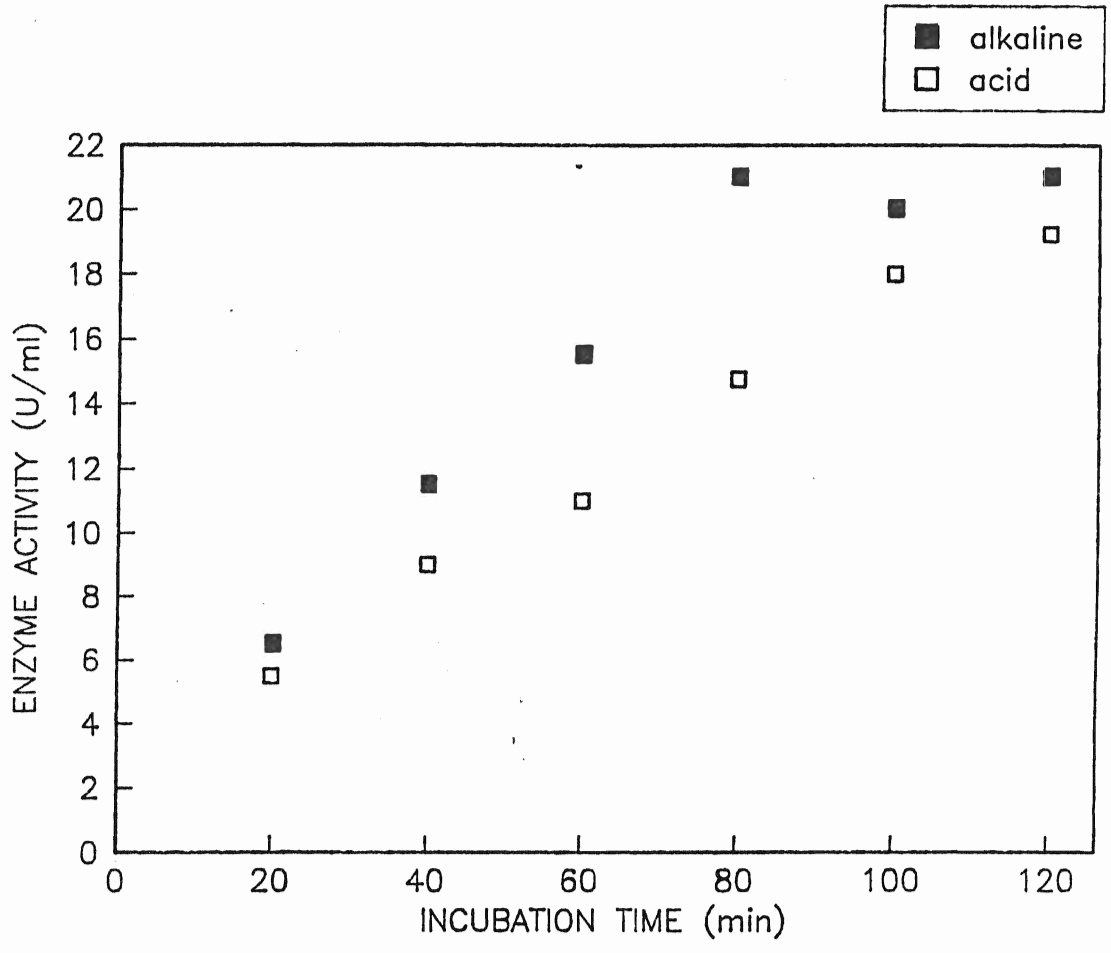

sonicated
unsonicated
30
20
10
0
ENZYME ACTIVITY(U/ml)
3
4
5
6
7
8
9
10
11
PH

Figure 14. The effect of incubation time on sludge acid and alkaline phosphatase activity at 37°C .


alkaline
acid
ENZYME ACTIVITY (U/ml)
0
2
4
6
8
10
12
14
16
18
20
22
0
20
40
60
80
100
120
INCUBATION TIME (min)

Optimum Sonication Time

The optimum sonication time for the release of sludge phosphatase activity was taken as 15 minutes for both acid and alkaline phosphatase (Figure 15). Alkaline activity was always higher and was released more rapidly than the acid activity.

Sludge Phosphatase Activity vs. Flow Rate

The sludge acid and alkaline phosphatase enzyme activity was measured in the upper and lower region of the sludge blanket (Figures 16 & 17). Both alkaline and acid phosphatase activity appeared to change with flow rate. Alkaline phosphatase activity was the highest throughout the entire study and showed the greatest response to a change in flow rate. The change in enzyme activity with time was analyzed in both the upper and lower region using the Kruskal-Wallace test. The results of this test show that only the alkaline phosphatase activity in the lower bed changed significantly at a 95% level of significance (Appendix A). The cumulative change in sludge alkaline phosphatase activity in the lower bed also showed a strong correlation with the cumulative COD loading using the Spearmann Rank coefficient of correlation (Appendix B).

ATP as a Measure of Viable Biomass

The ATP content of the anaerobic sludge was taken as the measure of active viable biomass throughout the study. The optimum extraction time for ATP in the anaerobic sludge was determined to be 15 minutes (Figure 18). The sludge ATP content was then measured in the upper and lower region of the sludge

Figure 15. The effect of sonication time on sludge acid and alkaline phosphatase activity.

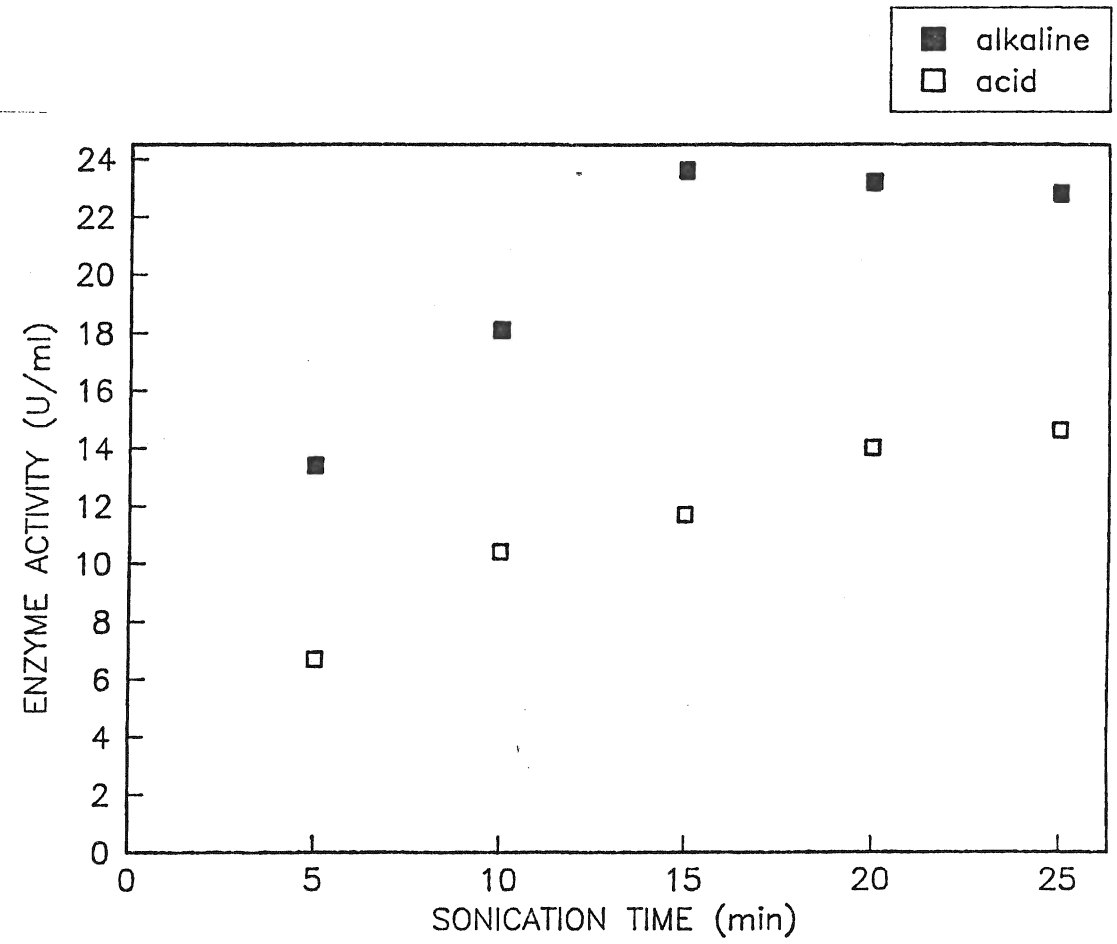
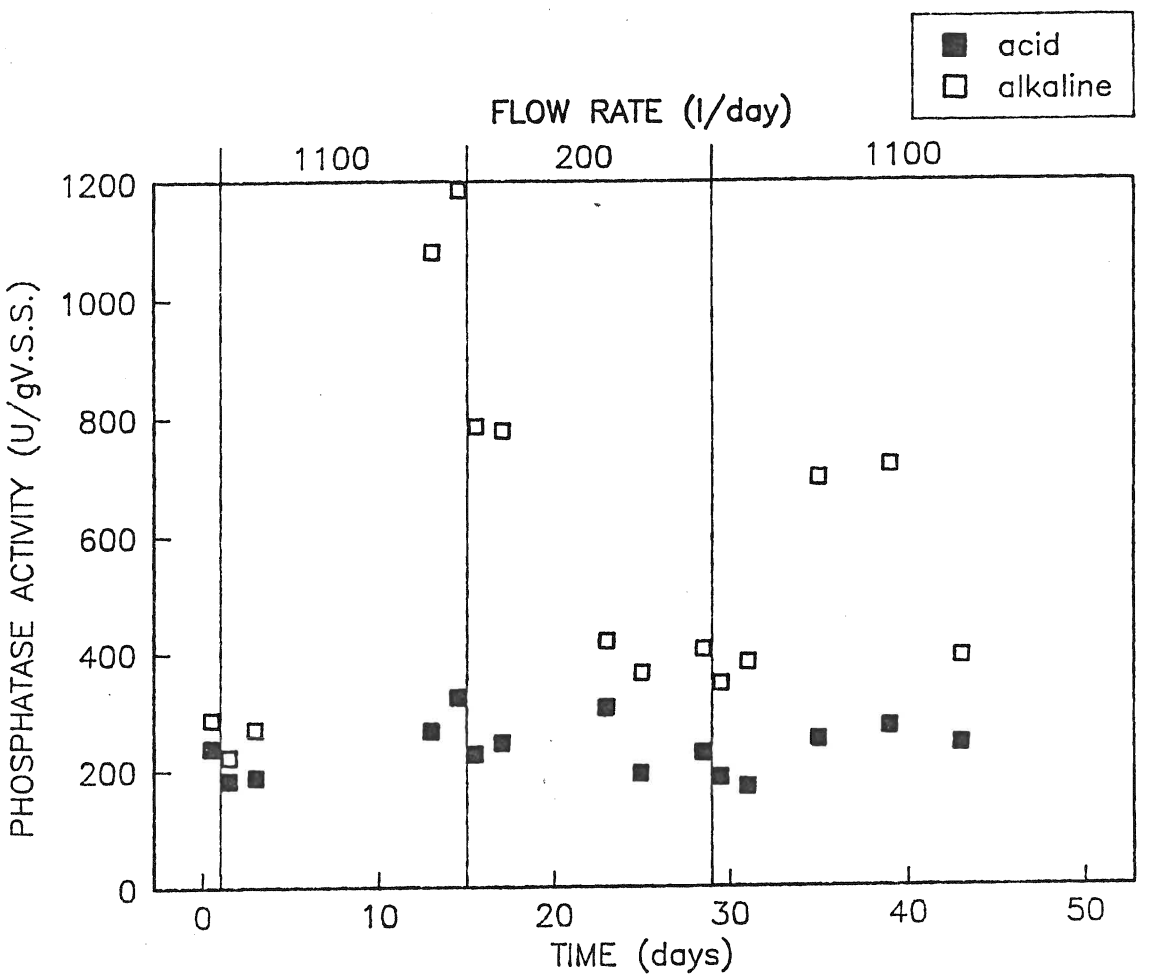

alkaline
acid
ENZYME ACTIVITY (U/ml)
0
2
4
6
8
10
12
14
16
18
20
22
24
0
5
10
15
20
25
SONICATION TIME (min)

Figure 16. Sludge acid and alkaline phosphatase activity (U/g V.S.S.) in the upper region of the sludge blanket vs time (days) and flow rate (l/day).


acid
alkaline
FLOW RATE (l/day)
1100
200
1100
PHOSPHATASE ACTIVITY (U/gV.S.S.)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
TIME (days)

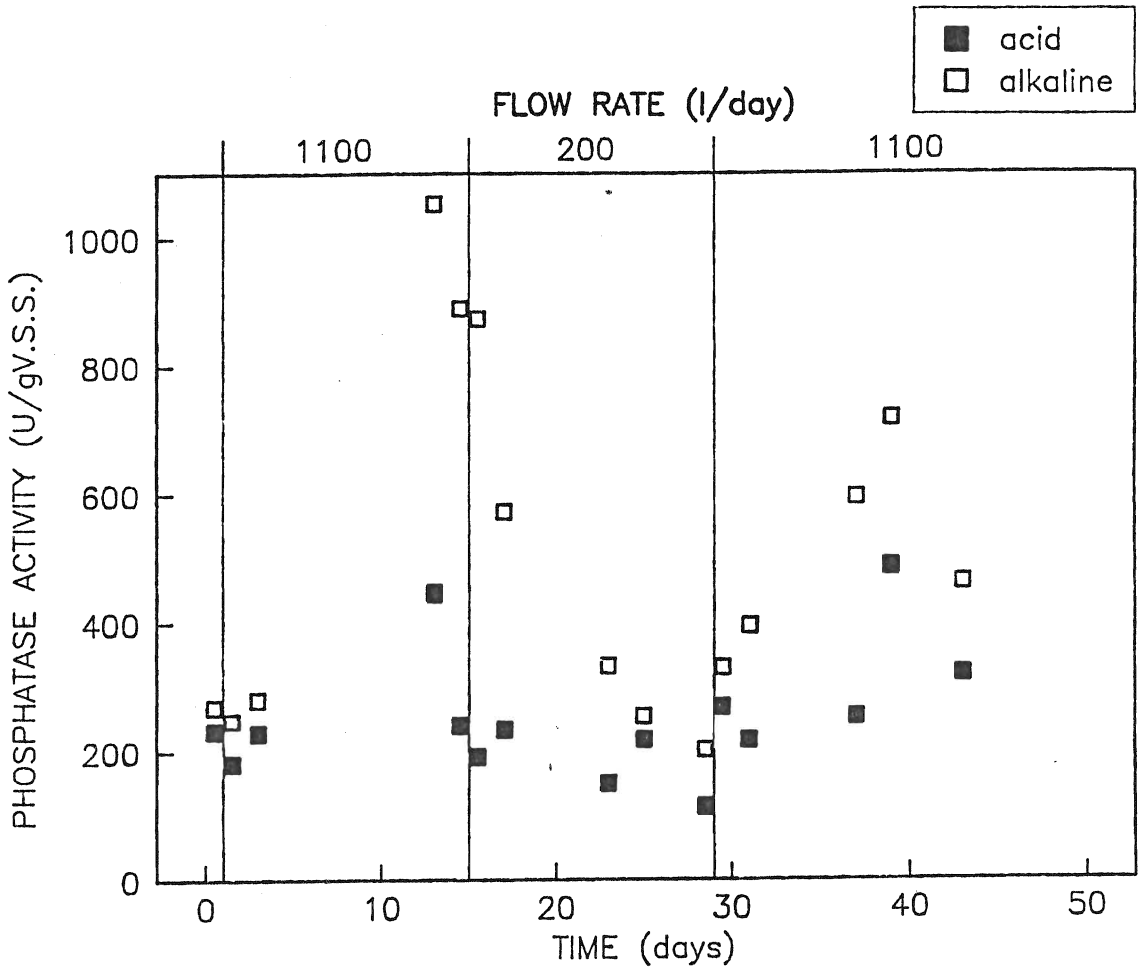
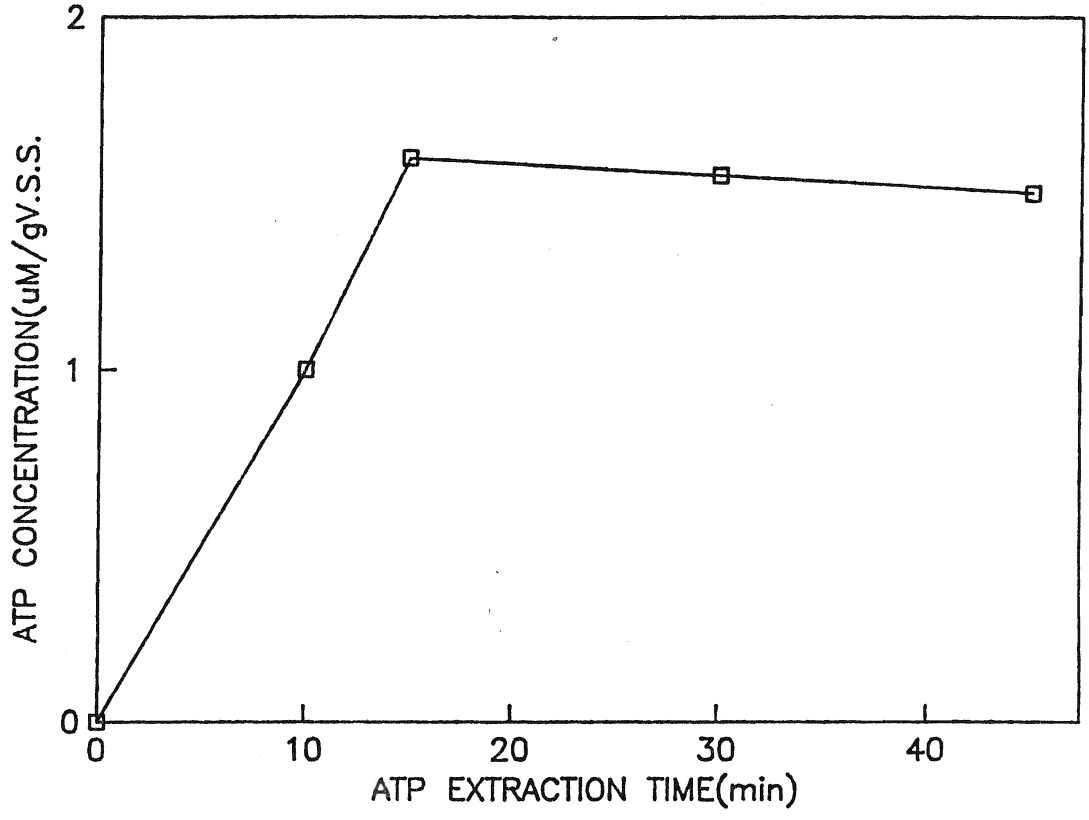

acid
alkaline
FLOW RATE (l/day)
1100
200
1100
PHOSPHATASE ACTIVITY (U/gV.S.S.)
0
200
400
600
800
1000
0
10
20
30
40
50
TIME (days)

Figure 18. The effect of extraction time on the sludge ATP yield (μM/ g V.S.S.).


2
1
0
ATP CONCENTRATION(uM/gV.S.S.
0
10
20
30
40
ATP EXTRACTION TIME(min)

blanket throughout the study (Figures 19 & 20). In both regions of the sludge blanket the sludge ATP content appeared to change with flow rate. However the change in ATP content with time was only significant at the 95% level in the lower bed using the Kruskal-Wallace test (Appendix C). ATP content showed a weak correlation with enzyme activity using the Spearmann rank coefficient of correlation (Appendix D). The cumulative COD loading showed an absolute correlation with cumulative ATP content (Appendix E).

In order to assess the performance of the UASB system throughout the study several monitoring tests were applied. The conventional monitoring tests used in this study were; gas production, acetic acid production, COD loading, phosphorus loading, nitrogen loading, effluent suspended solids concentration and % COD removal.

Flow and Recycle Rates

The flow and recycle rates used throughout the study are shown in Figure 21. The flow rate of nutrients to the reactor was increased from 200 l/day to aproximately 1100 l/day in the first stage of the study. The recycle rate was held at 200 l/day. In stage 2 the flow rate was lowered from 1100 l/day to 200 l/day. The recycle rate was maintained at 200 l/day. In stage 3 the flow rate was increased to 1100 l/day from 200 l/day while the recycle rate was increased from 200 l/day to 1000 l/day.

Figure 19. The sludge ATP concentration (μM/g V.S.S.) of the upper region of the sludge blanket vs time (days) and flow rate (l/day).

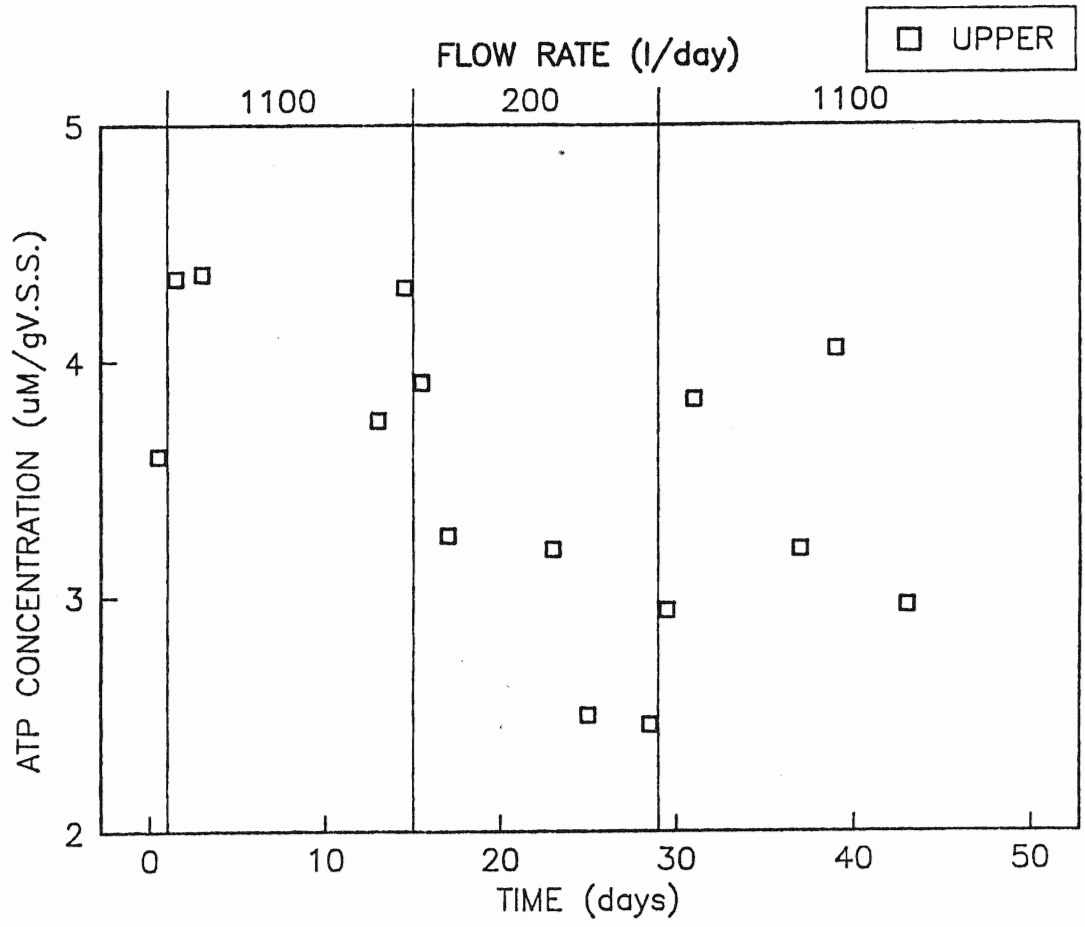

FLOW RATE (l/day)
UPPER
1100
200
1100
5
4
3
2
ATP CONCENTRATION (uM/gV.S.S.)
0
10
20
30
40
50
TIME (days)

Figure 20. The sludge ATP concentration (μM/g V.S.S.) of the lower region of the sludge blanket vs time (days) and flow rate (l/day).

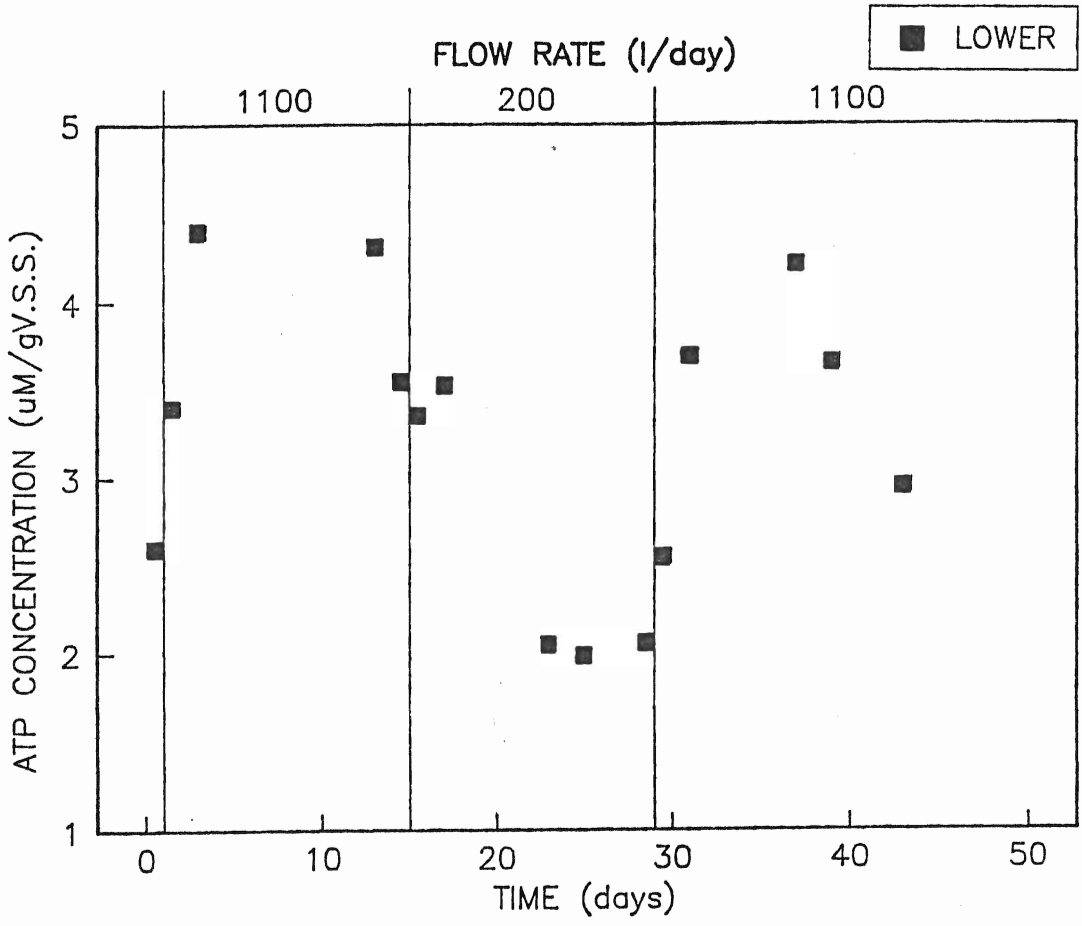
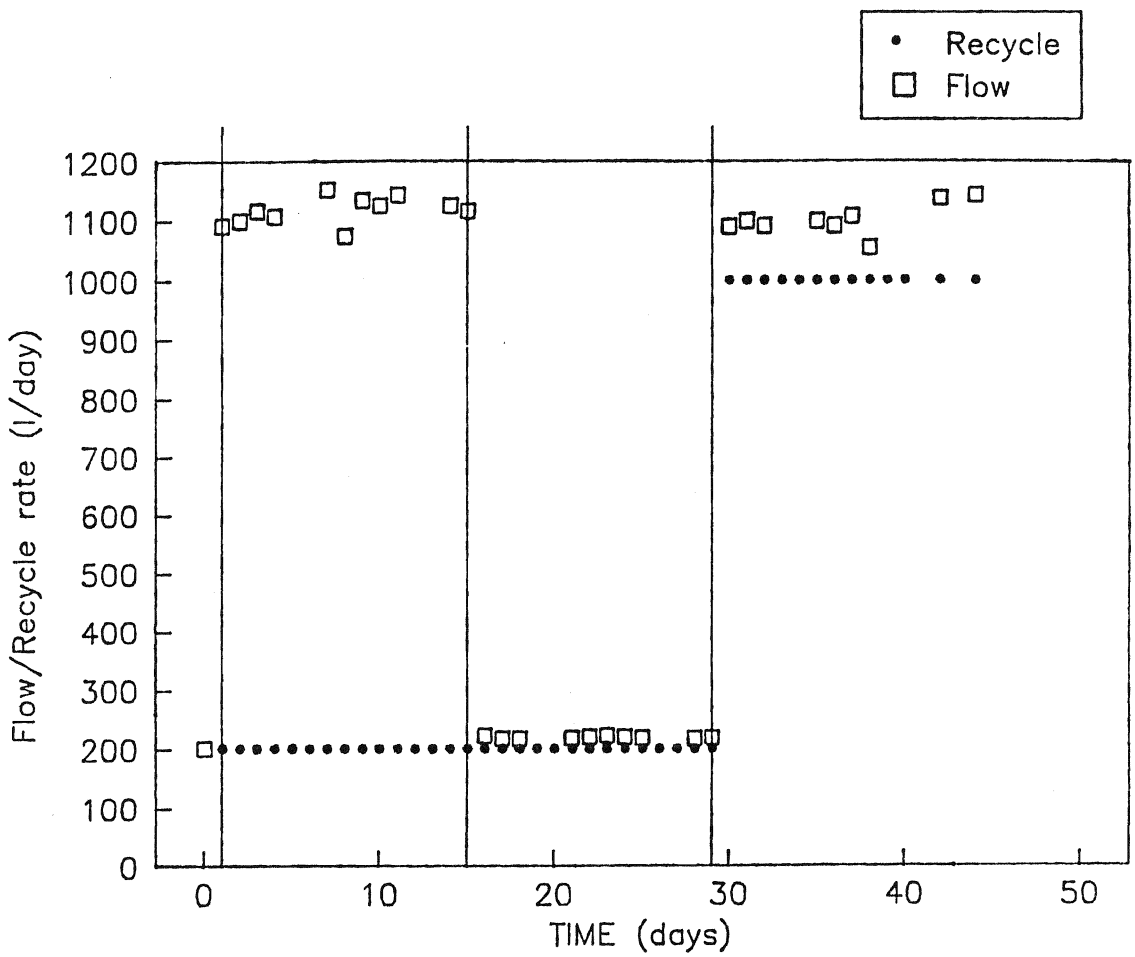

FLOW RATE (l/day)
1100
200
1100
LOWER
ATP CONCENTRATION (uM/gV.S.S.)
5
4
3
2
1
0
10
20
30
40
50
TIME (days)

Figure 21. The feed rate and recycle rate (l/day) vs time (days).


Recycle
Flow
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Flow/Recycle rate (l/day)
0
10
20
30
40
50
TIME (days)

COD Loading

The total and soluble COD loading is shown in Figure 22. In stage one the total and soluble COD loading was increased to approximately 11.5 and 10.5 kg COD/m3/day respectively. In stage 2 the COD loading was reduced to 2.0 and 1.5 kg/m3/day of total and soluble COD respectively. In stage 3 the total and soluble COD loading was again increased to approximately 7.0 and 6.5 kg COD/m3/day respectively.

Phosphorus Loading

The total and soluble phosphorus loading is shown in Figure 23. In stage one the total and soluble phosphorus loading was increased to approximately 12 and 8 g/m3/day, respectively. In stage 2 the phosphorus loading was reduced to approximately 5 and 4.5 g/m3 /day of total and soluble phosphorus, respectively. In stage 3 the total and soluble phosphorus loading was again increased to approximately 25 and 20 g/m3/day, respectively.

Nitrogen Loading

The total and soluble nitrogen loading is shown in Figure 24. In stage one, the total and soluble nitrogen loading was increased to approximately 110 and 100 g/m3/day respectively. In stage 2 the nitrogen loading was reduced to 25 and 20 g/m3/day of total and soluble nitrogen respectively. In stage 3 the nitrogen loading was again increased to approximately 150 and 130 g/m3/day of total and soluble nitrogen respectively.

Suspended Solids Loading

The feed suspended solids loading is shown in Figure 25.

Figure 22. The total COD loading (TCOD) and soluble COD loading (SCOD) in (g/m3/day) vs time (days) and flow rate (l/day).

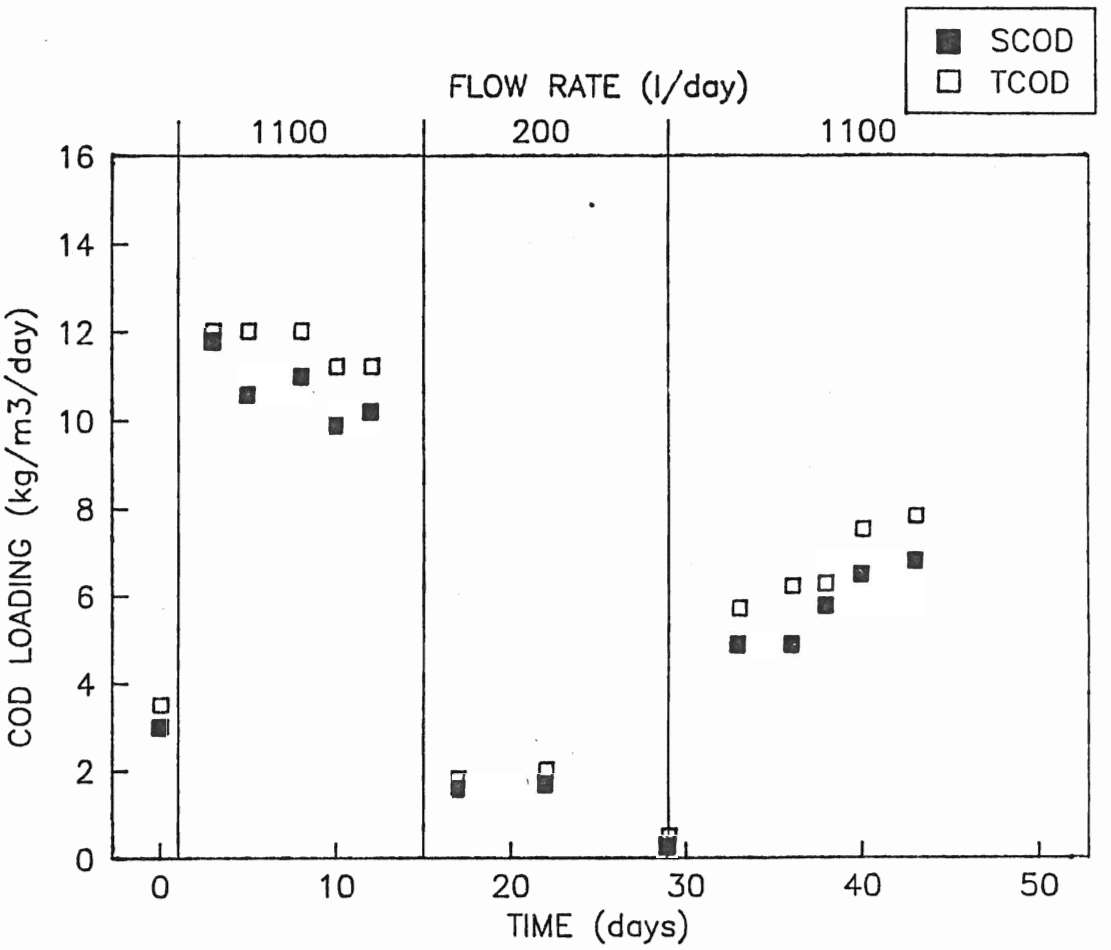

SCOD
TCOD
FLOW RATE (l/day)
1100
200
1100
COD LOADING (kg/m3/day)
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
TIME (days)

Figure 23. The total phosphorus loading (TP) and soluble phosphorus loading (STP) in (g/m3/day) vs time (days) and flow rate (1/day).

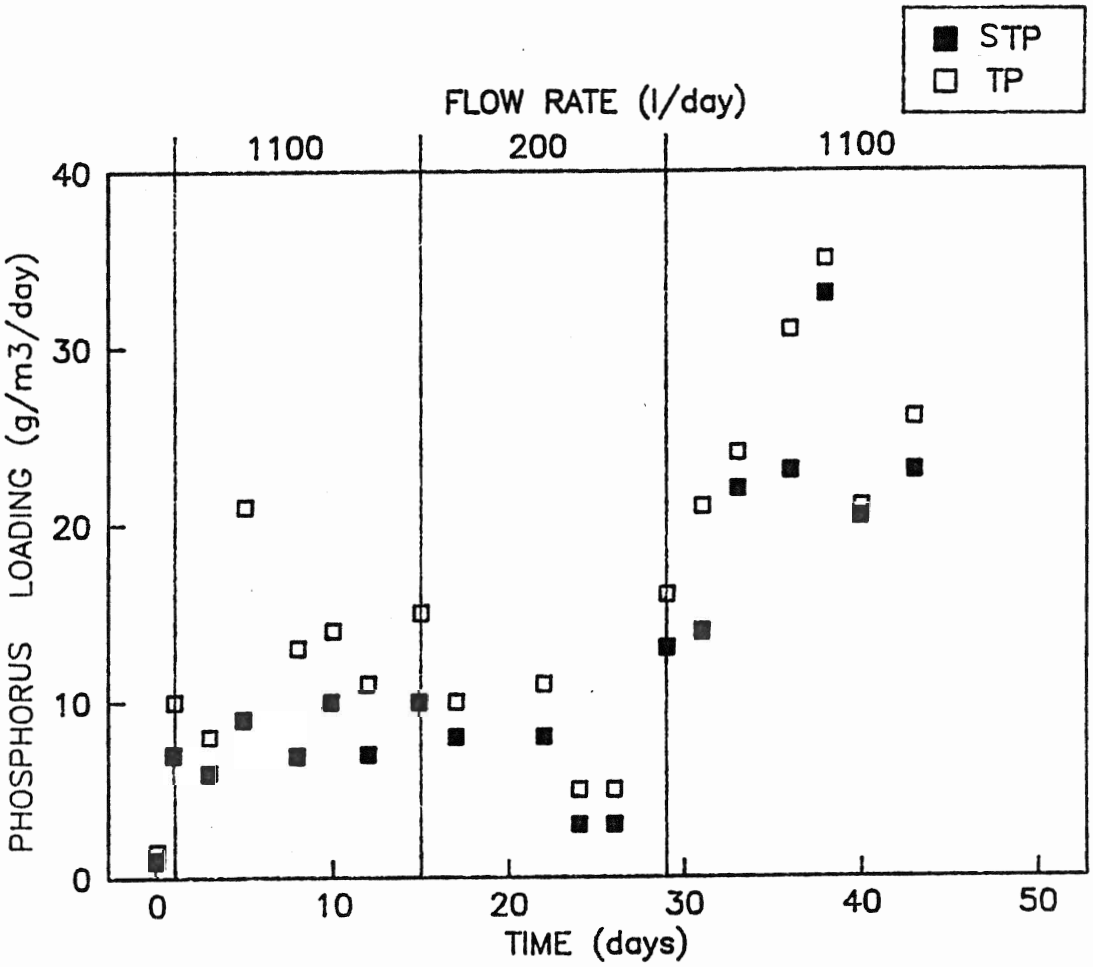

STP
TP
FLOW RATE (l/day)
1100
200
1100
PHOSPHORUS LOADING (g/m3/day)
0
10
20
30
40
0
10
20
30
40
50
TIME (days)

Figure 24. The total nitrogen loading (TKN) and the soluble nitrogen loading (STKN) in (g/m3/day) vs time (days) and flow rate (l/day).

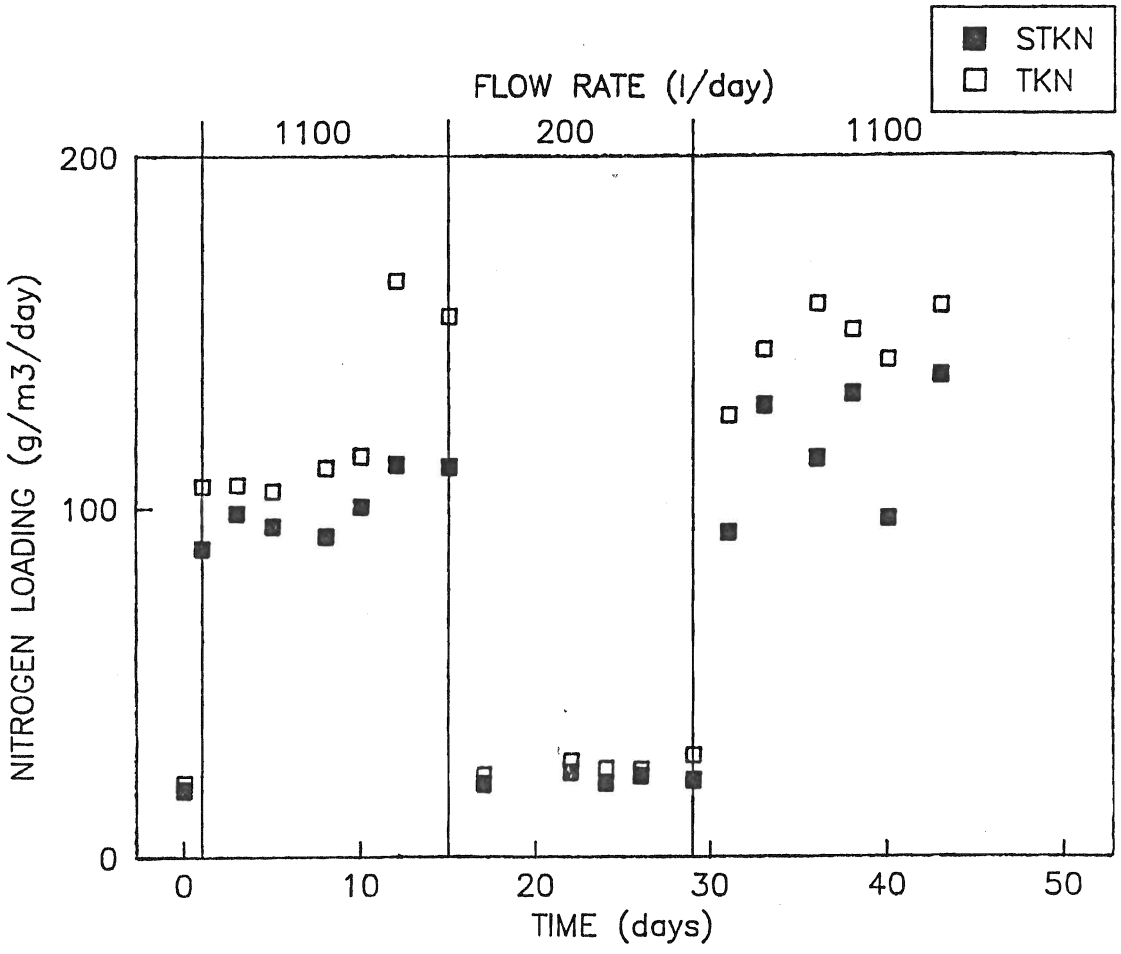
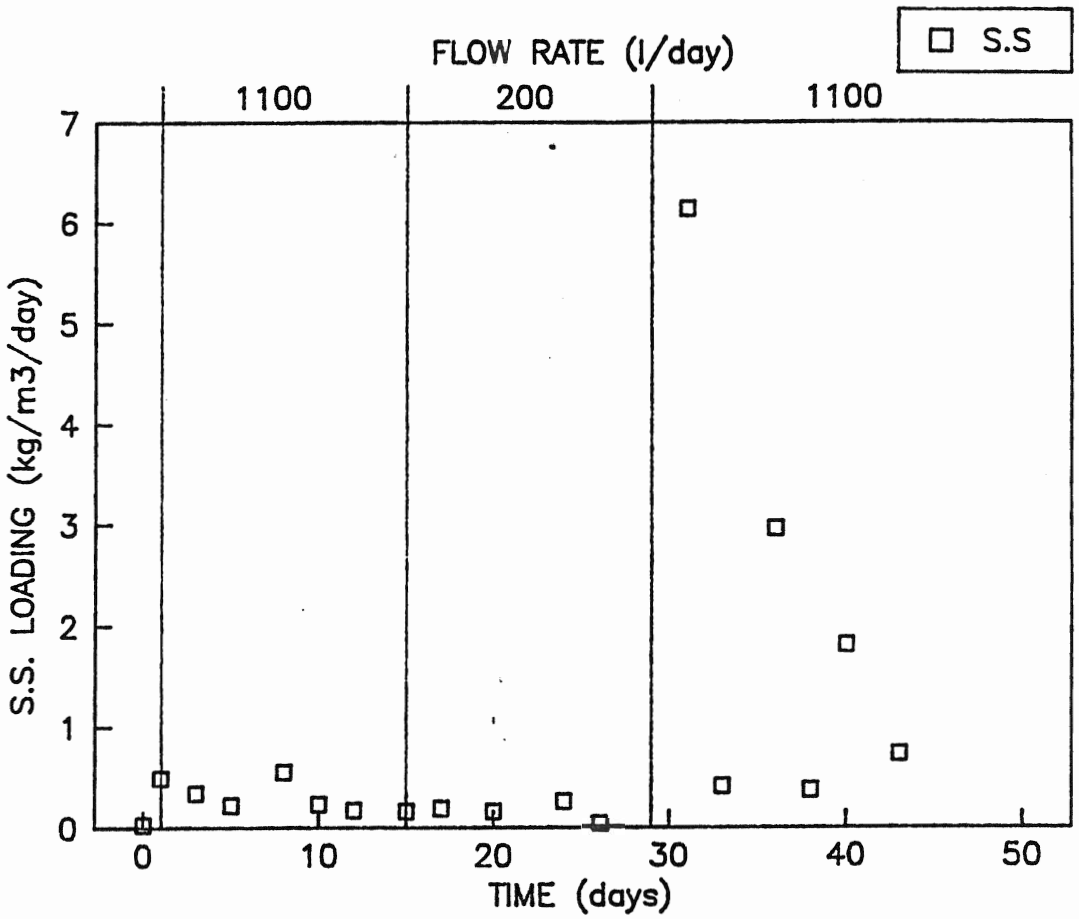

STKN
TKN
FLOW RATE (l/day)
1100
200
1100
200
100
0
NITROGEN LOADING (g/m3/day)
0
10
20
30
40
50
TIME (days)

Figure 25. The feed suspended solids loading (S.S.) vs time (days) and flow rate (l/day).


FLOW RATE (l/day)
1100
200
1100
S.S
S.S. LOADING (kg/m3/day)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
TIME (days)

For stages 1 and 2 the loading was approximately 0.5 kg/m3/day. In stage 3 however, the loading increased to an average of 2.5 kg/m3/day with an initial loading of 6.0 kg/m3/day. This high initial loading was only sustained for approximately 24 hours and fluctuated for the rest of the study period.

Gas Production

The total gas production over the entire study is shown in Figure 26. The total gas production in stages 1 and 2 was approximately 3600 and 1000 l/day respectively. Gas production in both these stages appeared to be stable. Total gas production in stage 3 was recorded as an average of 2500 l/day. However the gas production in stage 3 was unstable and declined over the entire 14 day period. The variability in gas production between stage 1 and stage 3 was significantly different using the Mood test for dispersion (Appendix F).

Effluent Acetic Acid Concentration

The effluent acetic acid levels were measured throughout the study (Figure 27). In stages 1 and 2 acetic acid production appeared to change with flow rate and was relatively stable. In stage 3 however acetic acid levels fluctuated to the greatest extent. This observation was confirmed statistically using the Mood test for dispersion (Appendix G). The results from this test show that the variability in acetic acid production was significantly higher in stage 3 when compared to acetic acid production in stage 1. The test was carried out with a 95% level of significance.

Figure 26. The total gas production (l/day) vs time (days) and flow rate (l/day).

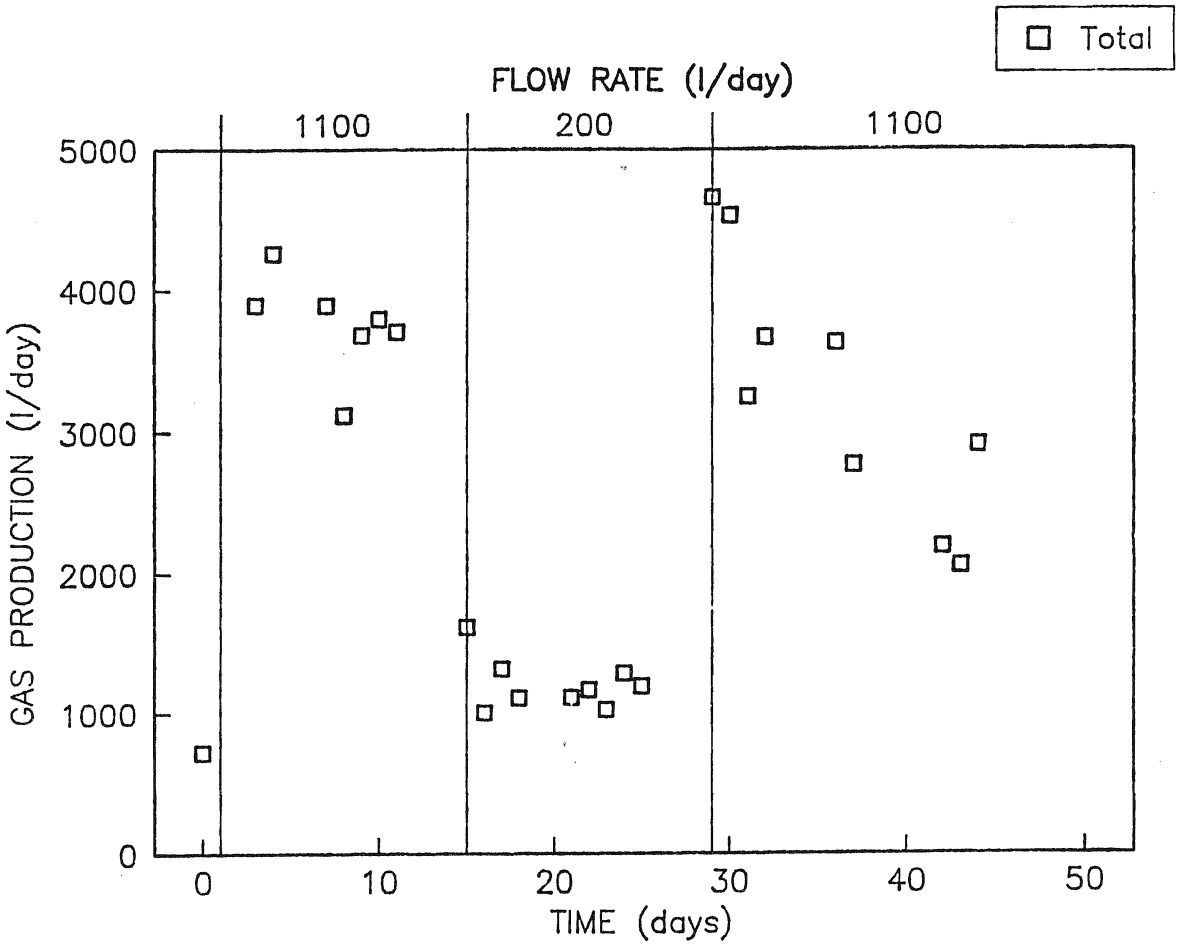
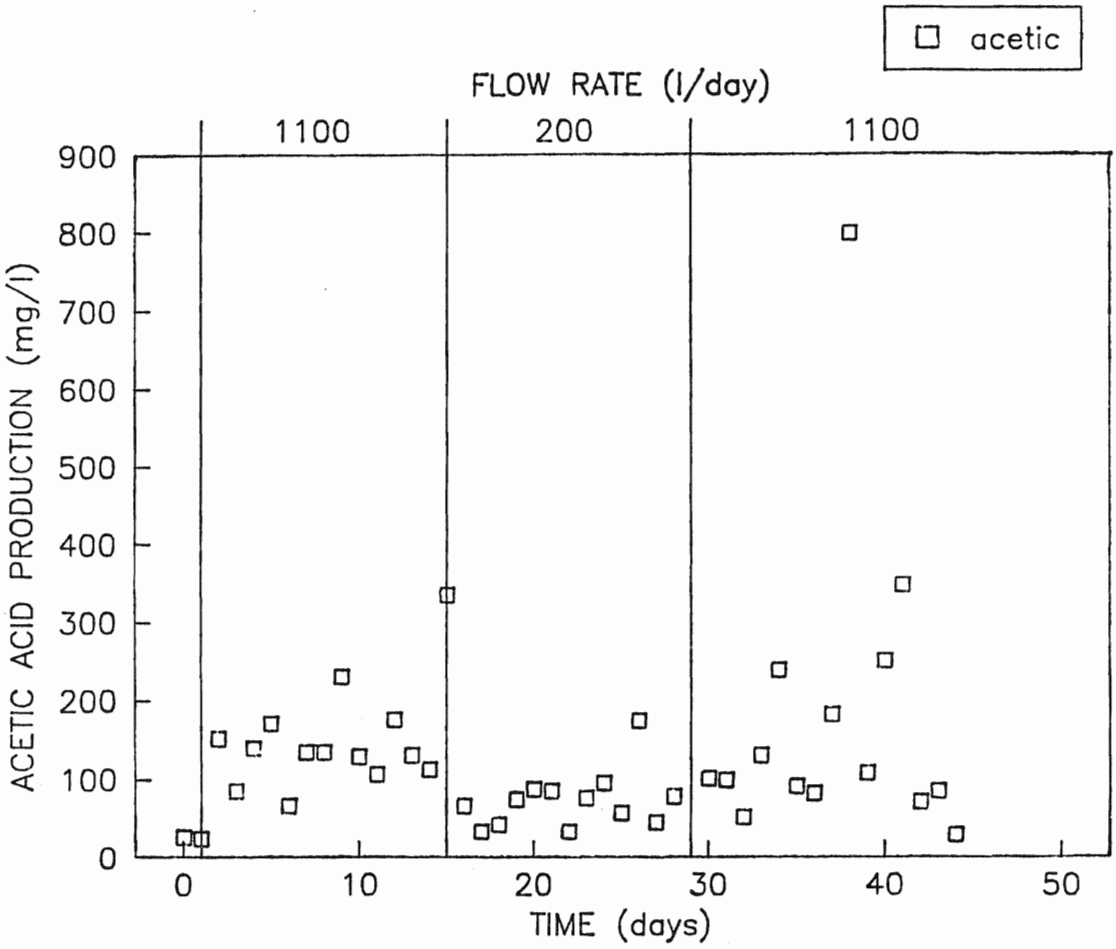

Total
FLOW RATE (l/day)
1100
200
1100
5000
4000
3000
2000
1000
0
GAS PRODUCTION (l/day)
0
10
20
30
40
50
TIME (days)

Figure 27. The effluent acetic acid concentration (mg/l) vs time (days) and flow rate (l/day).


acetic
FLOW RATE (l/day)
1100
200
1100
ACETIC ACID PRODUCTION (mg/l)
900
800
700
600
500
400
300
200
100
0
0
10
20
30
40
50
TIME (days)

Effluent Suspended Solids Concentration

The effluent suspended solids levels as monitored throughout the study are shown in Figure 29. Effluent suspended solids levels were highest in stage 3. This was probably due to the high suspended solids loading in this stage.

% COD Removal

The % COD removal throughout the study is shown in Figure 28. The total and soluble % COD removal in stage 1 remained at at over 90%. With a decrease in flow rate and loading in stage 2, the % COD removal became unstable. The total % COD removal dropped to 49% while the soluble % COD removal dropped 15% over a three day period. In stage 3 an increase in flow rate resulted in an immediate increase in soluble COD removal to 98%. However the total COD removal increased slowly over the entire period.

Figure 28. The % total COD removal (TCOD) and % soluble COD removal (SCOD) vs time (days) and flow rate (l/day).

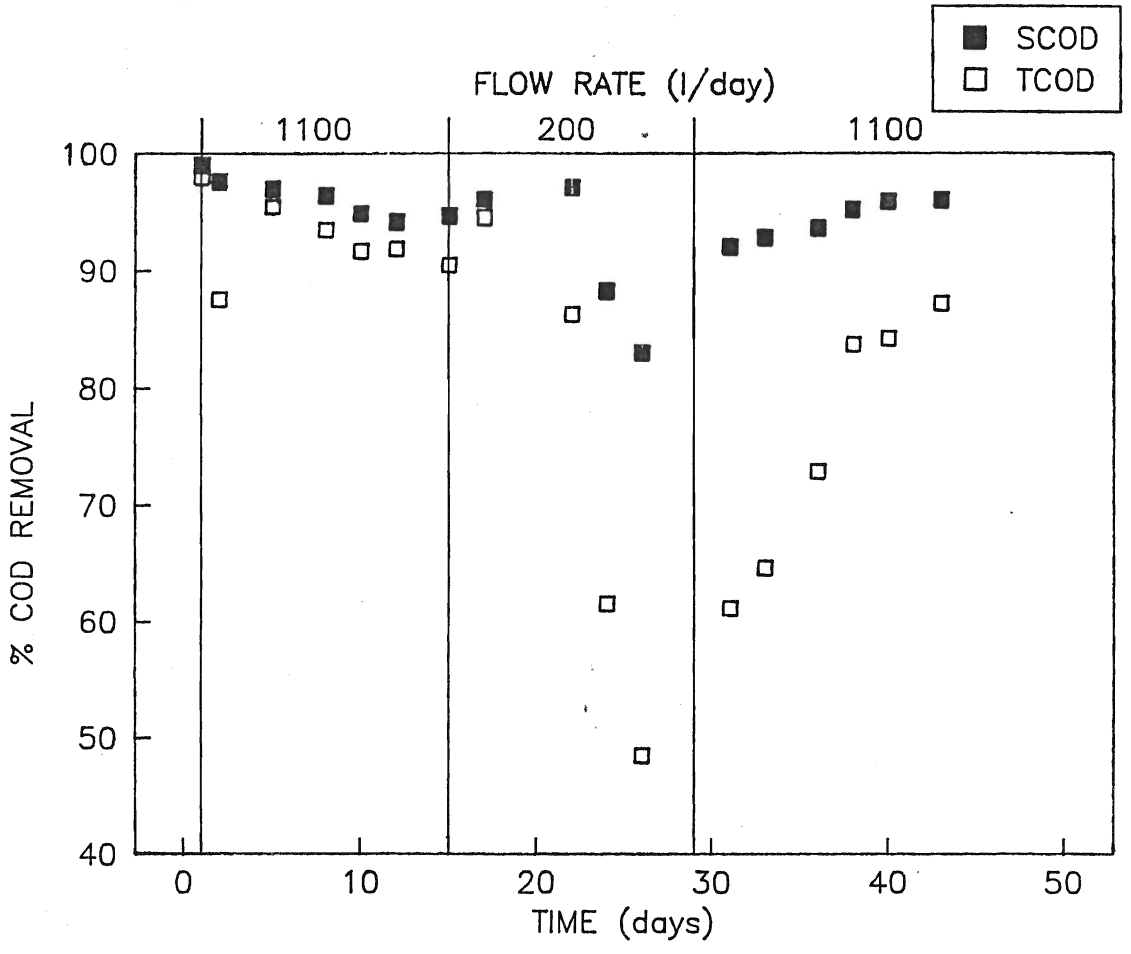
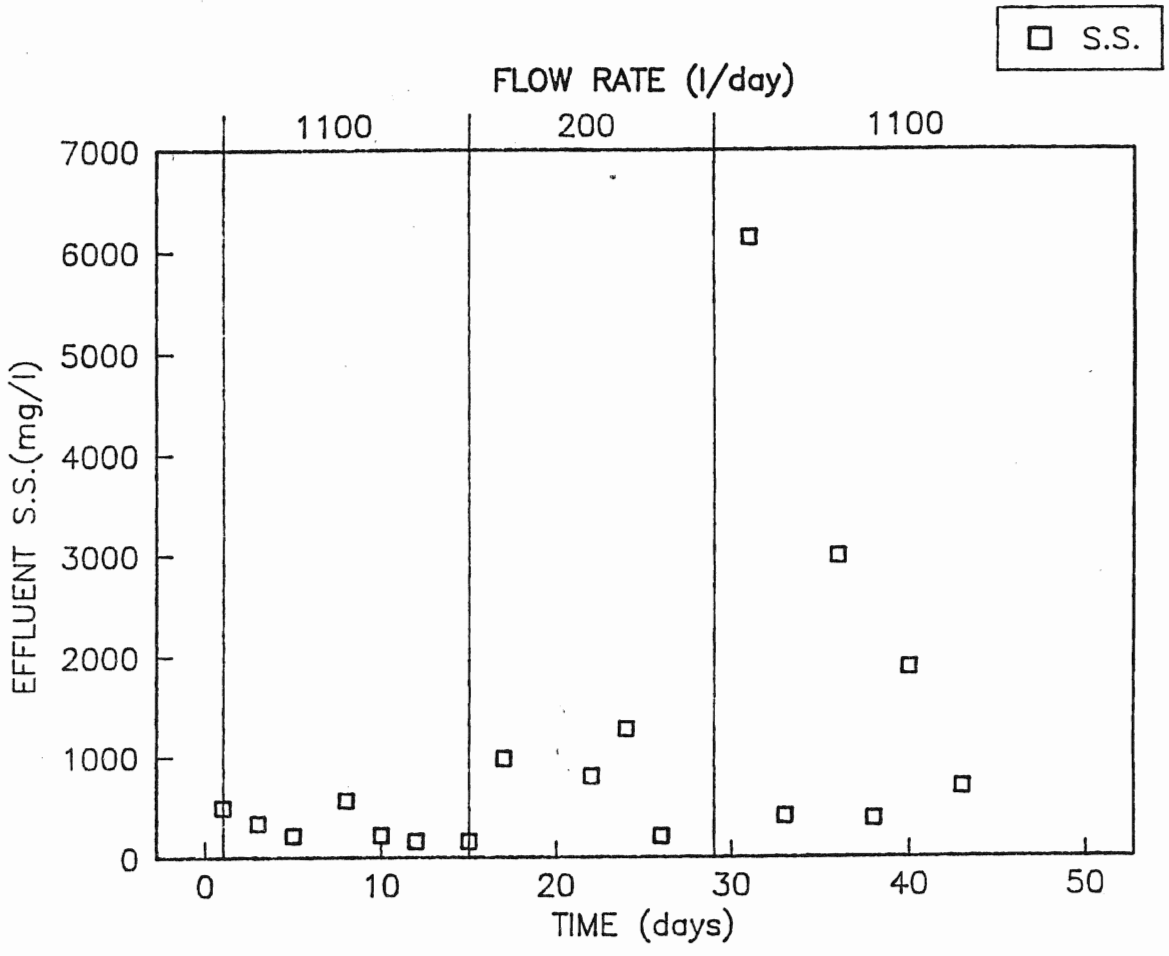

SCOD
TCOD
FLOW RATE (l/day)
1100
200
1100
100
90
80
70
60
50
40
% COD REMOVAL
0
10
20
30
40
50
TIME (days)

Figure 29. The effluent suspended solids concentrations (mg/l) vs time (days) and flow rate (l/day).


S.S.
FLOW RATE (l/day)
1100
200
1100
7000
6000
5000
4000
3000
2000
1000
0
EFFLUENT S.S.(mg/l)
0
10
20
30
40
50
TIME (days)

DISCUSSION & CONCLUSIONS

The acid and alkaline phosphatase enzyme activity of the Upflow Anaerobic Sludge Blanket system was found to change with flow rate and nutrient loading. The results in Figures 16 and 17 revealed that the overall sludge phosphatase activity displayed an upward trend when flow rate and COD loading were increased. These results are in accordance with those of Ashley and Hurst (1981) and Bull et al. (1983). The former study observed an increase in phosphatase activity with an increase in flow rate while the latter study indicated a similar increase in enzyme activity with an increase in COD loading. However only the change in alkaline phosphatase activity in the lower bed was significantly different in all three stages. This indicated that the alkaline phosphatase activity changed to a greater extent in the lower bed than in the upper bed for all three loading conditions. This observation could be due to the slow mixing of the incoming feed. As the lower bed sampling region was closer to the incoming feed port, the feed concentration was highest at this point. The bacteria located in this region were therefore exposed to a higher feed concentration than those in the upper bed. The more severe environmental conditions of the lower bed may have resulted in the higher levels of enzyme activity. The alkaline phosphatase activity was always greater than acid phosphatase activity under the assay conditions used and sonication was required to release the phosphatase activity of the granular sludge.

Unlike the results of Ashley and Hurst (1981), a single pH optimum was observed in the acid and in the alkaline region. The UASB sludge did not provide multiple pH optima but only a broad activity in the acid region and a narrower optimum range in the alkaline region. The difference in the number of pH optima observed was most likely due to a difference in the overall bacterial population of the two systems.

With regard to the cause of the changing enzyme activity, the results show a poor correlation between the change in enzyme activity and ATP levels. This implies that the enzyme activity and ATP were changing independently of each other to a certain extent. Ashley and Hurst (1981) indicated that changing enzyme levels were a measure of constitutive enzyme in the system. These results suggest that enzyme activity is not due to changes in the number of bacteria present alone, but may be induced. However ATP levels may have been affected by the overall metabolic state of the bacteria. This would further decrease the degree of association between enzyme activity and ATP. The ATP levels in the lower bed were found to change significantly with flow rate. These findings suggest that enzyme activity must have been affected in part by the growth or death of bacteria in the system.

With regard to process performance and enzyme activity, enzyme activity has been shown to be high in several systems when they were placed under stressful conditions (Agardy, et al. 1963; Thiel and Hattingh, 1977). More specifically, phosphatase activity in several systems increased rapidly under stressful

process conditions and preceeded changes in volatile acid levels, pH and gas production (Ashley and Hurst, 1981 and Bull et al., 1983). There was no indication of process upset in stage 1 in terms of % COD removal, gas production and acetic acid production. As shown in the results all monitors of performance appeared to be stable. This indicates that although the total COD loading was increased 300% stepwise in stage 1, the system was not under stress under these conditions. The lack of an immediate response in enzyme activity also supports the contention that the bacterial population was not stressed. Process upset in terms of total COD removal, gas production and acetic acid production was observed in stage 3. Even though the total COD loading was one-half that of stage 1, the initial feed suspended solids loading was 12 times as great. The simultaneous increase in effluent suspended solids levels and decline in % COD removal indicated that much of the feed suspended solids were not broken down in the system but passed through intact. Previous anaerobic digestor studies have shown that organic shock loading of digestors can result in a massive buildup in volatile acids with a consequent inhibition of methanogenic bacteria (McCarty, 1964). This did not occur in stage three due to the high flow rate and the brief period over which the organic shock loading took place. However the irregular loading and retention of solids in the system, did create an unstable acetic acid production in stage 3. As shown in the results, the acetic acid production in stage 3 was significantly more variable than in stage 1 where suspended solids loadings

were 12 times lower. The gas production was also significantly unstable and generally declined over the entire period. If the conditions in stage 1 are considered stable and compared to the unstable conditions in stage 3, then two factors become apparent. Firstly, the cause of process upset in stage 3 was probably due to the very high suspended solids loading. The results also show that the initial rate of increase in alkaline phosphatase activity was five times faster under the stressful conditions in stage 3 than in stage 1. In addition the change in enzyme activity was four times as sensitive as the corresponding change in acetic acid levels. These results are in agreement with those of Ashley and Hurst (1981) where the change in enzyme activity was several times more sensitive than changes in volatile acid levels. These results suggest that transient process upset in the UASB system induced through organic shock loading, may be detected more rapidly in changing alkaline phosphatase levels than in volatile acid levels.

In stage 2, acetic acid production and gas production appeared to be stable. The % COD removal should have remained high during this stage as the COD loading rate was approximately 6 times lower than in stage 1. However both soluble and total COD removal decreased rapidly after day 17. The reduction in total COD removal can be explained by observing the simultaneous increase in effluent suspended solids at this time. Gas production had previously been so high that solids may have failed to settle back into the system, and were then washed out. This loss of solids may have masked the actual total COD

removed. The decrease in soluble COD removal at the same time cannot be explained in terms of suspended solids loss. One possible explanation for the reduction in soluble COD removal could have been nitrogen limitation over this period. Gasser and Jeris (1969) have determined that 21 mg of nitrogen were required per gram of COD removed. This gives a COD:N ratio of 48:1. The SCOD/STKN ratio of the incoming feed was below the 48:1 ratio on days 22 and 26 when the lowest SCOD removal was observed (Appendix H). It has previously been shown that when the nitrogen and carbon ratios become unbalanced in anaerobic systems, the bacteria can switch the available energy to the maintenance of the cells. The available nitrogen will determine the amount of carbon utilized and consequently the ultimate COD removal rate (Hobson, et al.,1974). The fall in sludge ATP content at this time could also have suggested an overrall decrease in the metabolic rate of the bacteria which would also result in a drop in the SCOD removal rate. The decrease in enzyme activity during stage 2 was greater than the overall change in acetic acid levels. This is further evidence that enzyme activity is more sensitive to transient changes in performance than acetic acid production.

The three bacterial phenotypes observed in the fluid anaerobic cultures reflect that portion of the overall acid producing population able to grow under serum bottle assay conditions. However the fact that four major acids of digestion; acetic, propionic, butyric and isobutyric were produced suggests that these organisms could play a definite role in carbohydrate

metabolism in the UASB system. The mixed bacterial culture in this study was similar to that observed in a recent clarigestor study treating maize processing waste (Ross, 1983). The similarity in the bacterial populations is important if comparisions are to be made between systems treating similar wastes and in discerning which bacteria are actually involved in the metabolism of a particular waste. Although the bacteria present were not identified to the species level in this or the clarigestor study, several starch fermenting bacteria have been isolated in other studies. These include Bacteriodes spp and Clostridial spp, in particular, Clostridium butyricum. The dense filamentous matrix of the sludge granules observed in the scanning microscope results confirms the nature of the sludge granules observed in several other sludge blanket studies (Lettinga, 1980; Ross, 1983). The nature of the sludge granules in all these studies supports the view that where surfaces are not available, the bacteria grow in conglomerates with mutual attachment and intertwining (Hobson, 1980).

The limitations of an applied study such as this are numerous. In such a large system exact control of all the input parameters is at best difficult. The sampling of such a large system is also subject to sampling error when small samples are applied to the overall population. However if a better understanding and control of these systems is to be obtained an attempt must be made to decipher their fundamental characteristics. These characteristics will not be uncovered in

a single study but in the continuing application of new information as it becomes available. The results indicate that the UASB system could have tolerated much higher loading conditons. Future experiments could be designed to test the response of enzyme activity and process performance for more severe operating conditions.

Several conclusions can be made from this study:

1. Phosphatase enzyme activity does change significantly in response to flow rate and loading in the UASB system.
2. Alkaline phosphatase activity was higher than acid phosphatase activity under these conditions.
3. Alkaline enzyme activity does respond faster than changes in volatile acid levels when the process is upset.
4. The UASB system can withstand high COD loading rates for a protracted period of time providing the suspended solids loadings are low.

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Appendix A

The Kruskal-Wallace H test was used to test for significant change in the alkaline phosphatase activity with time, in the upper and lower bed of the UASB system.

Ho: There is no change in alkaline phosphatase activity with time for all three changes in flow rate.

Ha: There is a significant difference in the alkaline phosphatase activity with time for all three changes in flow rate.

Change in alkaline activity/unit time (days)

	Upper Bed	Rank	Lower Bed	Rank
Stage 1	- 70	2	- 210	2
	4	7	- 5	9
	60	12	61	14
	41	9	60	13
Stage 2	- 53	5	-1333	1
	-106	1	- 136	4
	- 62	3	- 85	5
	- 58	4	- 75	6
	- 46	6	- 52	7
Stage 3	427	14	- 196	3
	64	13	- 7.3	8
	44	10	33	12
	47	11	29	11
	17	8	- 0.9	10

Upper Bed

[Stage 1 = 2+7+12+9 = 30
[Stage 2 = 1+4+5+6+7 = 23
[Stage 3 = 3+8+12+4+10 = 44

$$H = \frac{12}{14 \times 15}\left(\frac{30^2}{4} + \frac{23^2}{5} + \frac{44^2}{5}\right) - 45$$

H = -3.97

$X^2_{.05}$ (2d.f.) = 5.99

since -3.97 < 5.99
fail to reject Ho

Lower Bed

[Stage 1 = 2+9+14+13 = 38
[Stage 2 = 5+1+3+4+6 = 19
[Stage 3 = 14+13+10+11+8 = 56

$$H = \frac{12}{14 \times 15}\left(\frac{38^2}{4} + \frac{19^2}{5} + \frac{56^2}{5}\right) - 45$$

H = 15.5

$X^2_{.05}$ (2d.f.) = 5.99

since 15.5 > 5.99
reject Ho

Appendix B

The Spearmann Rank coefficient of correlation was used to test the degree of association between the cumulative COD loading and the cumulative enzyme activity in the lower region.

Ho: There is no correlation between the cumulative COD loading and the cumulative enzyme activity in the lower region.

Ha: There is a correlation between the cumulative COD loading and the cumulative enzyme activity in the lower region.

Sample calculation for correlation in stage 3.

Cum. Enzyme Activity	Rank	Cum. COD loading	Rank
269	1	3.0	1
516	2	17.7	2
797	3	29.5	3
1849	4	40.1	4
2737	5	48.1	5

Rank Enzyme Activity = 1 2 3 4 5
Rank COD loading = 1 2 3 4 5
Difference = 0 0 0 0 0
$(\text{Difference})^2$ = 0 0 0 0 0

$r = 1$

Decision: since r = 1 we can reject Ho and conclude that there is an exact correlation between cumulative enzyme activity and cumulative COD loading.

Appendix C

Sample calculations to test for significant change in the sludge ATP content with time using the Kruskal-Wallace H test.

Ho: There is no significant change in the ATP content with time between stages 1,2 and 3.

Ha: There is a significant change in the ATP content with time between stages 1,2, and 3.

Change in ATP content/unit time (days)

	Upper Bed	Rank	Lower Bed	Rank
Stage 1	2.5	13	2.6	14
	0.3	11	0.6	12
	.01	6	0.13	8
	.05	7	.06	6.5
Stage 2	-1.3	1	-0.6	1
	-0.4	2	-0.006	5
	-0.12	5	-0.16	2
	-0.17	3	-0.14	3
	-0.13	4	-0.1	4
Stage 3	1.6	12	0.16	13
	3.02	14	0.54	11
	0.08	8	0.24	10
	0.12	10	0.15	9
	0.1	9	0.06	6.5

Upper Bed

[Stage 1 = 13+11+6+7 = 37
[Stage 2 = 1+2+5+3+4 = 15
[Stage 3 = 12+14+8+10+9 = 53

$$H = \frac{12}{14 \times 15}\left(\frac{37^2}{4} + \frac{15^2}{5} + \frac{53^2}{5}\right) - 45$$

$H = 0.24$

$X^2_{.05}$ (2d.f.) = 5.99
since 0.24 < 5.99 we fail to reject Ho.

Lower Bed

[Stage 1 = 14+12+8+6.5 = 40.5
[Stage 2 = 1+5+2+3+4 = 15
[Stage 3 = 13+11+10+9+6.5 = 49.5

$$H = \frac{12}{14 \times 15}\left(\frac{40.5^2}{4} + \frac{15^2}{5} + \frac{49.5^2}{5}\right) - 45$$

$H = 9.006$

$X^2_{.05}$ (2d.f.) = 5.99
since 9.006 > 5.99 we we reject Ho.

Appendix D

The Spearmann Rank coefficient of correlation was used to test the degree of association between the ATP concentration and enzyme activity in the lower bed.

Ho: There is no correlation between the ATP concentration and enzyme activity in the lower bed.

Ha: There is a correlation between the ATP concentration and enzyme activity in the lower bed.

Enzyme Activity	Rank	Rank	ATP concentration
269	4	5	2.61
247	2	8	3.41
281	5	15	4.40
1052	15	14	4.31
888	14	10	3.55
872	13	7	3.36
572	10	9	3.53
332	7	2	2.05
254	3	1	1.99
201	1	3	2.06
330	6	4	2.55
394	8	12	3.70
594	11	13	4.21
717	12	11	3.66
461	9	6	2.95

Rank Enz. Act.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Rank (ATP)	3	8	1	5	15	4	2	12	6	9	13	11	7	10	14
Difference	-2	-6	2	-1	-10	2	5	-4	3	1	-2	1	6	4	1
(Difference)^2	4	36	4	2	100	4	25	16	9	1	4	1	36	16	1

$\Sigma(\text{Difference})^2 = 466$

$$r = \frac{1 - 6(466)}{15(15 - 1)} = 1 - 0.83 = 0.17$$

Decision: since r= 0.17 we can not reject Ho and conclude that there is a very weak correlation between sludge ATP content and enzyme activity.

Appendix E

The Spearmann Rank coefficient of correlation was used to test the degree of association between the cumulative COD loading and the cumulative sludge ATP content in the lower bed.

Ho: There is no correlation between the cumulative COD loading and the cumulative sludge ATP content in the lower bed.

Ha: There is a correlation between the cumulative COD loading and the cumulative sludge ATP content in the lower bed.

Sample calculation for correlation in stage 3.

Cumulative COD loading	Rank	Cumulative (ATP)	Rank
269	1	2.55	1
516	2	6.25	2
797	3	10.46	3
1849	4	14.12	4
2737	5	17.07	5

Rank (ATP) = 1 2 3 4 5
Rank COD loading = 1 2 3 4 5
Difference = 0 0 0 0 0
$(\text{Difference})^2$ = 0 0 0 0 0

$(\text{Difference})^2 = 0$

$r = 1 - 0 = 1$

since r = 1 we can reject Ho and conclude that there is an exact correlation between (ATP) and Cumulative COD loading.

Appendix F

The Mood test for dispersion was used to determine if total gas production varied significantly between stages 1 and 3.

Ho:There was no significant variation in gas production between stages 1and 3.

Ha:There is a significant variation in gas production between stages 1and 3.

Stage 1	Joint Rank		Stage 3
3846	11	14	4654
4256	12	13	4527
3893	10	4	3248
3119	3	6	3669
3082	7	5	3633
3796	9	2	2765
3708	8	1	2184

Mean Rank = 15/2 =7.5

$M = (11 - 7.5)^2 + (12 - 7.5)^2 +$

$(10 - 7.5)^2 + (3 - 7.5)^2 +$

$(7 - 7.5)^2 + (9 - 7.5)^2 +$

$(8 - 7.5)^2$

$M = 56.05$

Decision for $n_1 = 7$, and $n_2 = 7$ and $\alpha = 0.05$, the critical value for M = 67.75. since 56.05 < 67.75 we can reject Ho and conclude that there was significant variability in gas production between stage 1 and stage 3.

Appendix G

The Mood test for dispersion was used to determine if acetic acid production varied significantly between stages 1 and 3.

Ho: There is no significant variation in acetic acid production between stages 1 and 3.

Ha: There is a significant difference in acetic acid production between stages 1 and 3.

Acetic Acid Concentration

Stage 1	Joint Rank		Stage 3
84	4	9	129
138	12	17	238
164	13	5	90
65	1	3	81
133	10	15	181
134	4	20	798
229	16	7	107
127	8	18	250
105	6	19	347
174	14	2	70

Mean Rank $= (N + 1)/2 = 10.5$

$$M = (4 - 10.5)^2 + (12 - 10.5)^2 + (13 - 10.5)^2 + (1 - 10.5)^2 + (10 - 10.5)^2 + (11 - 10.5)^2 + (16 - 10.5)^2 + (8 - 10.5)^2 + (6 - 10.5)^2 + (14 - 10.5)^2$$

$$M = 210.5$$

Decision for $n_1 = 10$, $n_2 = 10$ and $\alpha = 0.05$, the critical value for M = 220. since $210.5 < 220$ we can reject Ho and conclude that the dispersion in stage 1 is significantly less than that in stage 3.

Appendix H

The Mann-Whitney test was used to determine if there was a significant difference in the FCOD/FTKN ratio between stages 1 and 2.

Ho: There is no significant difference in the FCOD/FTKN ratio between stages 1 and 2.

Ha: There is a significant difference in the FCOD/FTKN ratio between stages 1 and 2.

FCOD/FTKN

Stage 1	Joint Rank		Stage 2
166	12	7	89
119	10	5	74
112	9	4	61
120	11	2	24
99	8	1	15
87	6	3	34

ζ Ranks for Stage 2 = 1+2+3+4+5+7 = 22

The critical region for $n_1 = 6$, $n_2 = 6$ and $\alpha C = 0.05$ is 28 - 40.

Decision: since 22 < 28 we can reject Ho and conclude that the FCOD/FTKN ration is significantly different in stage 2.