EFFECT OF TREATED RUM DISTILLERY EFFLUENTS ON THE DISTRIBUTION AND SURVIVAL OF POTENTIAL BACTERIAL PATHOGENS

by Terry C. Hazen
Microbial Ecology Laboratory
Department of Biology
College of Natural Sciences
University of Puerto Rico
Rio Piedras, Puerto Rico
00931

Project No. G930-03
Grant Agreement No. 14-08-0001-G930

FINAL TECHNICAL REPORT
TO
U.S. DEPARTMENT OF THE INTERIOR
WASHINGTON, D.C. 20240

The research on which this report is based was financed in part by the U.S. Department of the Interior, as authorized by the Water Research and Development Act of 1978 (P.L. -95-467).

Contents of this publication do not necessarily reflect the views and policies of the U.S. Department of the Interior, nor does mention of trade names or commercial products constitute their endorsement by the U.S. Government.

February 1987

LIST OF SYMBOLS AND ABBREVIATIONS

ALK	Alkalinity, mg $CaCO_3$ l^{-1}
BOD	Biological Oxygen Demand, mg l^{-1}
CC	Coulter Counter Counts
CFU	Colony Forming Units
CHLA	Chlorophyll A, mg l^{-1}
COD	Chemical Oxygen Demand, mg l^{-1}
HARD	Hardness, mg $CaCO_3$ l^{-1}
MT	Rum Effluent Treatment Level
NO3	Nitrates, mg l^{-1}
PO4	Phosphates, mg l^{-1}
SAL	Salinity, parts per thousand
SC	Sugar Concentration, % Brix
SO4	Sulfates, mg l^{-1}
T	Time, hours
TDC	Total Direct Cell Counts, cells ml^{-1}
TEMP	Temperature, ^oC
TP	Total Phosphorus, mg l^{-1}
TURB	Turbidity, % Transmitance
VC	Viable Cell Counts, CFU ml^{-1}
WTEMP	Temperature, ^oC
%ACT	Percentage Active Cells
[]	Concentration, %

TABLE OF CONTENTS

ACKNOWLEDGEMENTS..iii

LIST OF SYMBOLS AND ABBREVIATIONS........................ v

LIST OF TABLES..vii

LIST OF FIGURES... ix

ABSTRACT.. xi

INTRODUCTION.. 1

ORGANISM OF STUDY....................................... 4

MATHERIALS AND METHODS.................................. 11

STUDY SITE.. 11

WATER QUALITY ANALYSIS.................................. 12

BACTERIOLOGICAL ANALYSIS................................ 12

CELL ACTIVITY AND TOTAL CELL COUNT....................... 15

SURVIVAL STUDIES.. 16

DATA ANALYSIS... 18

RESULTS... 20

WATER QUALITY... 20

BACTERIAL COMMUNITIES................................... 21

MICROCOSMS.. 22

BACTERIAL SURVIVAL IN AQUARIA........................... 26

DISCUSSION.. 27

CONCLUSIONS... 34

LITERATURE CITED.. 36

TABLES 41

FIGURES... 67

LIST OF TABLES

Table 1. Characteristics for the Identification of Vibrio cholerae 41

Table 2. Water Quality in Untreated Rum Distillery Effluents....... 42

Table 3. Water Quality in Treated Rum Distillery Effluents......... 43

Table 4. Correlation Matrix of Water Quality in Rum Effluents...... 44

Table 5. Aerobic and Facultative Anaerobic Bacterial Populations in Rum Effluents........ 45

Table 6. Shannon-Weaver's Diversity Index in Rum Distillery Effluents........ 46

Table 7. 5 Factorial, ANOVA on Microcosm Culturable Counts......... 47

Table 8. 5 Factorial, ANOVA on Microcosm Total Cell Counts......... 48

Table 9. 5 Factorial, ANOVA on Microcosm Percent Active Cells...... 49

Table 10. 5 Factorial, ANOVA on Microcosm Percent Respiring Cells 50

Table 11. Viable Densities of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents............. 51

Table 12. Viable Densities of Vibrio cholerae ATCC 25872 at Various Rum Effluent Concentrations Incubated at Selected Temperatures...... 52

Table 13. Viable Densities of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations............... 53

Table 14. Total Counts of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents................ 54

Table 15. Total Counts of Vibrio cholerae ATCC 25872 at Various Rum Effluent Concentrations Incubated at Selected Temperatures......... 55

Table 16. Total Counts of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations.................. 56

Table 17. Percent Activity of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents.............. 57

Table 18. Percent Activity of *Vibrio cholerae* ATCC 25872 at Various Rum Effluent Concentrations Incubated at Selected Temperatures...... 58

Table 19. Percent Activity of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations................ 59

Table 20. Percent Respiration of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents 60

Table 21. Percent Respiration of *Vibrio cholerae* ATCC 25872 at Various Effluent Concentrations Incubated at Selected Temperatures 61

Table 22. Percent Respiration of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations 62

Table 23. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Coulter Counter Counts.................................. 63

Table 24. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Total Cell Counts.. 64

Table 25. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Percent Activity... 65

Table 26. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Percent Respiration...................................... 66

LIST OF FIGURES

Figure 1. Serological Nomenclature used presently to describe and serologically group Vibrio cholerae 67

Figure 2. Map showing Arecibo Study Site.......................... 69

Figure 3. Diagram of Methane Digester Model....................... 71

Figure 4. Densities of Bacterial Populations in Rum Effluents..... 73

Figure 5. Total Cell Counts of Bacterial Populations in Rum Effluents... 75

Figure 6. Percent Activity of Bacterial Populations in Rum Effluents... 77

Figure 7. Densities of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents................ 79

Figure 8. Densities of Vibrio cholerae ATCC 25872 at Various Effluent Concentrations Incubated at Selected Temperatures.................. 79

Figure 9. Densities of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations.................. 79

Figure 10. Total Cell Counts of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents............. 81

Figure 11. Total Cell Counts of Vibrio cholerae ATCC 25872 at Various Effluent Concentrations Incubated at Selected Temperatures......... 81

Figure 12. Total Cell Counts of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations............... 81

Figure 13. Percent Activity of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents............. 83

Figure 14. Percent Activity of Vibrio cholerae ATCC 25872 at Various

Effluent Concentrations Incubated at Selected Temperatures......... 83

Figure 15. Percent Activity of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations............... 83

Figure 16. Percent Respiration of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents..... 85

Figure 17. Percent Respiration of *Vibrio cholerae* ATCC 25872 at Various Effluent Concentrations Incubated at Selected Temperatures.. 85

Figure 18. Percent Respiration of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations....... 85

Figure 19. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Coulter Counter Counts................................ 87

Figure 20. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Total Cell Counts...................................... 89

Figure 21. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Percent Activity...................................... 91

Figure 22. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Percent Respiration................................... 93

ABSTRACT

Yazmin Alicia Rojas Nieves

DISTRIBUTION AND SURVIVAL OF POTENTIALLY PATHOGENIC BACTERIA IN TREATED AND UNTREATED RUM DISTILLERY EFFLUENTS

Thesis under the direction of Terry C. Hazen, Ph.D., Professor, Department of Biology, University of Puerto Rico.

The efficiency of the methane anaerobic digestion treatment on rum distillery effluents and it's capacity to change populations of potentially pathogenic bacteria was determined. It was found that BOD, COD and limiting nutrient concentrations were equal or higher in treated effluents than in untreated rum effluents. Also, viable bacteria were negatively correlated with sulfates and sugar concentration. Total bacterial densities were higher in treated effluents, however, a large proportion of these bacteria were anaerobic methane bacteria responsible for early effluent transformation. There was no difference on species diversity between treatment levels. *Aeromonas* and *Citrobacter* were the main genera of the aerobic and facultative anaerobic communities, since they are also the acetic acid producers of the digestion process. *Vibrio cholerae* was inoculated to microcosms with treated and untreated effluent under controlled conditions. Temperature and pH were the most critical parameters influencing bacterial density and metabolism. *Vibrio cholerae* in treated rum distillery effluents had lower densities and metabolic activity. However, *Vibrio cholerae* submitted to a survival study

under non-controlled conditions at both treatment levels showed no significative differences in survival or activity. Moreover, survival rates were higher than those earlier reported for ambient waters. It was concluded that methane anaerobic digestion treatment fails to significantly reduce BOD and COD levels in rum distillery effluents in terms of it's effect on the environment, and there is no difference in treated and untreated effluents in terms of growth and survival of potentially pathogenic bacteria.. Thus methane digestion treatment alone was shown to be insufficcient in reducing the potential hazard of rum distillery effluents to the environment and/or public health.

INTRODUCTION

Industrial effluents are a primary source of water contamination in near-shore coastal areas of Puerto Rico. One of the most rapidly expanding industries in Puerto Rico during the past 20 years has been rum distillation. Effluents from rum distillation (mostos or slops) cause drastic changes in water temperature, dissolved oxygen, pH, content of inorganic and organic nutrients, and chlorophyll A concentration (Biamon and Hazen, 1983). Ensenada de Boca Vieja, next to San Juan harbor in Puerto Rico is the site of the world's largest rum distillery. The waters of this cove, until recently, received 1.4 x 10^6 liters per day of untreated effluent from this distillery (Costle, 1979).

Rum distillery effluents are a hot, odorous, reddish brown and viscous mixture of molasses fermentation residues and the by products of the alcohol distillation process. Also, mostos contains water from cooling towers, fermentation vats, and distillery cleaning (Sheehan and Greenfield, 1980). The mostos forms a complex mixture, with an organic fraction of free amino acids, aldehydes, aromatic compounds, proteins, simple sugars and polysaccharides, which has a pH near 4.7 and a BOD close to 32,000 mg O_2 l^{-1}. The inorganic fraction of rum slops has heavy metals, minerals, and high concentrations of nitrogen and phosphorus salts (Costle, 1979). The effluent plume outfall normally generates an anoxic and acidic environment, which is toxic to many forms of marine life (Gonzalez et al., 1979).

Biamon and Hazen (1983) reported high densities of bacterial pathogens, including *Vibrio cholerae* in the effluent plume (>10^4 CFU ml^{-1}). Highest densities of these bacteria were measured closest to the effluent outfall, and background counts (>200 m upcurrent) were always less than 10 CFU ml^{-1}. The bacteria present were shown to be chemotactically attracted to rum slops, which also increased their respiration, and metabolic activity (Fuentes et al., 1983). Therefore, rum distillery effluent can be an important source of potentially pathogenic bacteria in near-shore coastal waters by increasing survival mechanisms from human and non-human sources (Hazen et al., 1982).

Until recently, the two rum distillers on the north coast of Puerto Rico have been pumping untreated effluents directly into coastal waters. The largest of these recently began an anaerobic digestion treatment combined with a new deep ocean outfall as a contamination prevention method. The other one has not used as yet any water treatment method. They pump their effluent directly onto the beach. However, the efficiency of the anaerobic digester on rum effluents has not been showed.

The anaerobic digestion method is one of the most successful methods in sewage treatment plants in terms of reduction of aerobic bacteria and regaining of energy. Large scale anaerobic digesters are used for treatment of very high BOD (Biological Oxygen Demand) industrial effluents. The process combines reduction of BOD in the treated effluents with production of methane, which can be used later

as fuel. Methane production also raises the pH and temperature to levels where only methanogenic and thermophilic bacteria can live. Successful use of this treatment method needs close control of such parameters as pH, temperature and carbon concentration, since extreme conditions can change methane production for other by-products, which can be extremely toxic (Atlas and Bartha, 1981).

Need to determine the capacity of the anaerobic digestion process to alter densities of potentially pathogen bacteria, such as *Vibrio cholerae* and it's role on aquatic natural flora is real. A descriptive study focusing on the distribution and density of these bacterial populations in treated effluents is the primary goal of this proposal. Also, an attempt to establish relationships between water quality and bacterial density will be made. Finally, survival studies concerning the effect of environmental factors on bacterial populations will be performed.

The specific aims of this study are the following:

1) Quantitative evaluation of physical-chemical parameters that affect growth and survival of *Vibrio cholerae* in treated rum effluents;

2) Establishment of a microcosm for predicting bacterial densities in rum slops according to environmental factors.

ORGANISM OF STUDY

Vibrio cholerae (Pacini 1854) was the first *Vibrio* sp. to be discovered, since it was isolated and characterized by Robert Koch in 1882 (Koch, 1883). It is a gram negative, non-spore forming, facultative anaerobic rod, 0.5-0.8 um in width and 1.4-2.6 um in length, with a single polar flagellum (present only on liquid media). Colonies are commonly convex, creamy, smooth and glistening with a typical greenish or red-bronze iridescence (Baumann et al., 1984; Shewan and Veron, 1974).

Koch's first definition of *Vibrio cholerae* was broadened and actually two major biotypes are now recognized. Koch's isolate belongs to the most ancient, now referred as the classical biotype. The other biotype, the El Tor biotype was named from an isolate from Mecca pilgrims at El Tor quarantine station on the Sinai peninsula in 1905. This biotype was first classified as a new species *Vibrio El Tor* but recently it was recognized and incorporated as a biotype of *V. cholerae* , differing from the classical biotype by hemolysin production (Colwell, 1970; Kaper et al., 1979; Roberts et al., 1982).

In 1935, Gardner and Venkatraman distinguished serologically between *V. cholerae* isolates based on structural differences in heat stable components of cell wall, which is called the O-antigen group (Roberts et al., 1982). Classical and El Tor biotypes were classified in the O:1 group, which was extended later by Sakazaki et al. (1970) to include 3 serological varieties: Ogawa, Inaba and Hikojima. Also,

there were strains that do not share antigenic properties of O:1 group but belong to V. cholerae (Colwell, 1970). These are called the non-agglutinable vibrios or non-O1's and are serologically grouped by Sakazaki into 60 serovars (Shimada and Sakazaki, 1977). Serology of V. cholerae is better illustrated on Figure 1.

The O:1 group is the primary agent of pandemic cholera, with 36,840 cases reported in 1981 (CDC, 1982). Most cases belonging to this group had been isolated from such places as Australia (Rogers et al., 1980), Bangladesh (CDC, 1980) and the United States (CDC of 1980b, 1981 and 1982). Is the only group that can produce the cholera enterotoxin, responsible for such symptoms as abdominal cramps, vomiting and sudden onset of an explosive bloody watery diarrhea (CDC of 1980 and 1981). Infection occurs by ingestion of contaminated water or food with a minimum infectious dose of 10^2 organisms per ml (Levine et al., 1983), although a dose of 10^4 to 10^8 can routinely induce the infection in humans (Davis and Sizemore, 1982). The bacteria goes through gastric acids, host defense barriers and penetrates the coliform-free proximal small intestine where they attach to enterocytes via chemotactic mechanisms. As Vibrio cholerae associates with small intestine it begins to elaborate cholera enterotoxin. This toxin irreversibly activates the adenylate cyclase system producing excess amounts of cyclic AMP. Elevated levels of cAMP modify the nature of cell membranes, resulting in outside secretion of electrolytes by crypt cells and cells on villus sides, and decreased adsorption by villus tip cells. The excessive loss of electrolytes by

crypt cells leads to severe dehydration, acidosis, renal shut down, shock and death (Levine et al., 1983).

Non-O1 *V. cholerae* are suggested to be O:1 groups that lost the group 1 antigen. Some of these strains have a toxin closely related to cholera enterotoxin and can cause cholera-like outbreaks that range from mild diarrhea to severe cholera (CDC, 1979). Most environmental isolates of *V. cholerae* belong to this group, and seem to be cosmopolitan, having been reported in Australia (Desmarchelier and Reichelt, 1982), England (West and Lee, 1982), Japan (Kodama et al., 1984), and the USA (CDC, 1979; Hood et al., 1983; Kenyon et al., 1984; Roberts et al., 1982). According to the Center for Disease Control, of 26 isolates reported in clinical cases, 50% were isolated from feces of patients with gastroenteritis, 35% were isolated from other body fluids and tissues, such as sputum, blood, and gall bladder, almost 100% have a history of recent shellfish ingestion, and 73% live in coastal states near brackish waters (Kaper et al., 1979).

Although O:1 strains have been found mostly in humans, and non-O1 strains belong mostly to the environment, there are no remarkable differences between them. Recently, DNA-DNA homology studies of 157 Australian isolates with biochemical properties of *V. cholerae* were made (Desmarchelier and Reichelt, 1981). The phenotypic analysis showed a high degree of phenotypic homology among clinical and environmental strains, despite serological classification or source. Twenty-nine of these isolates were later used in a genetic homology study (Desmarchelier and Reichelt, 1982). The results showed greater

water environments, far from fecal contamination sources (Hood et al., 1981; Lee et al., 1982; West and Lee, 1982). Moreover, Motes et al. (1983) isolate Vibrio cholerae serotype Ogawa from Florida estuaries and found that bacterial densities between fecal contaminated and uncontaminated waters were non distinguishable.

This bacterium could be also isolated from other sources instead of water. Most cholera cases in USA are related to ingestion of raw oysters and crabs, so shellfish and crustaceans are the second major source of infection (Davis and Sizemore, 1982; Hood et al., 1981; Hood et al., 1983; Huq et al., 1984). Other possible carriers are aquatic plants like water hyacinth (Spira et al., 1981), birds, and mammals (Lee et al., 1982; Rhodes et al., 1985). It was clearly showed that brackish waters are the major source, followed by crustaceans, shellfish, and sediments, in that order (Hood et al., 1983).

V. cholerae survival in environment is related to certain factors, ie., temperature, pH, salt concentration, amount of nutrients, bacterial contamination. Distribution surveys in Florida and Kent yield the highest concentrations of V. cholerae when temperatures are between 20°C and 35°C (Hood et al., 1983; Singleton et al., 1982b) although it can survive above 9°C (West and Lee, 1982). V. cholerae exhibits seasonality in coastal habitats. From seven coastal sites in California a 5- to 56-fold increase in non-01 V. cholerae during the summer of 1983 compared to the winter of the same year (Kenyon et al., 1984). In England, V. cholerae strains can be detected only from May to November (West and Lee, 1982). In both cases

increase in V. cholerae numbers are associated with increasing water temperatures, suggesting this is an extremely important parameter of density.

Salinity and nutrient concentrations also influence growth and viability of V. cholerae in estuarine systems. Microcosm studies showed that better growth patterns were obtained with moderate salinity, i.e., between 15 and 25 parts per thousand (Singleton et al., 1982b). This was corroborated by Hood et al. (1983), when they studied the distribution of V. cholerae in two Florida estuaries. Thus, salinity should be correlated with optimal nutrient concentration and pH to enhance better growth patterns on V. cholerae . Singleton et al. (1982a) found in earlier studies with microcosms that optimum nutrient concentration was 1,000 ug tryptone/liter. Bacteria under this concentration were starved, decreasing their cell volume by 85%, and divisional rate to assume a spherical coccoid form and steady division rate. However, if nutrient supplementation was restored, bacteria assumed their natural bacillary shape within 2 h and began division 5 h later. This is assumed to be a physiological mechanism to achieve survival in the environment (Baker et al., 1983).

The pH is a extremely important factor since it regulates the ability of V. cholerae to grow and attach to other organisms, a important characteristic to survival. Microcosms with planktonic crustaceans as foreign organisms showed that moderate alkalinity (pH 8.5), combined with optimal salinity and temperature enhances maximum attachment and multiplication of Vibrio cholerae on marine animal

sources (Huq et al., 1984). These conditions are present in most brackish estuaries. Thus, the frequency of isolation, the lack of correlation with fecal contamination, and the growth of organisms in conditions typical of estuarine waters reenforces the hypothesis that *Vibrio cholerae* is an autochthonous member of the estuarine ecosystem (Baker et al., 1983; Colwell et al., 1981; Kaper et al., 1979; Lee et al., 1982; Singleton et al., 1982a).

MATERIALS AND METHODS

Study Site

A rum distillery on the northwest coast of Puerto Rico near Arecibo (18° 28' N, 66° 44' W) 50 miles west of San Juan was selected as the first study site (Fig. 2). The rum distillery and the near-by Barrio Obrero covers about 42 acres of a sub-humid region with an average temperature of $77.86^{o}F$ (Gonzalez et al., 1979). Beachfront near the distillery outfall is influenced by natural and man-associated activities, such as freshwater from rivers and storm drains, domestic sewage, and landfill operations. The discharge outfall dumps effluent directly on the beach a short distance below the cliff where the distillery is located. The effluent then moves along the shore in an easterly direction until it reaches coastal waters. The effluent then mixes and disperses forming a black plume which hugs the shore across several miles of beach and rocky areas along the coast. This distillery provided an excellent study site since its effluents lack any treatment.

The second study site was a methane digester model (Fig. 3). It is at the Quality Control Laboratory at the Engineering College (University of Puerto Rico, Mayaguez Campus). The digester is an anaerobic contactor type, with a full capacity of 1-gallon of treating material. The methanogenic seed bacteria is obtained from sewage and a stirring bar maintains seed and mostos continuously resuspended. Temperature and pH are kept steady with daily monitoring, other

parameters, such as BOD and COD are taking regularly. This model provides a continuous source of treated material from a carefully controlled method.

Water Quality Analysis

Temperature was measured *in situ* using a Keithley Model 870 Digital Thermometer (Keithley Instruments, Cleveland, OH). The pH was taken with a Corning Model 130 pH Meter (Corning Medical, Corning Glass Works, Medfield, MA). Alkalinity and hardness were also measured by standard methods (APHA, 1985) using Spectrokits (Bausch and Lomb, Rochester, NY). Salinity and sugar concentrations were measured using hand refractometers (American Optical). BOD and COD values were supplied by the Quality Control Lab. using standard methods (APHA, 1985). Water samples were collected in presterilized, Nalgene, black, polypropylene bottles and preserved by fixation with sulfuric acid, mercuric chloride, and zinc acetate for further analysis of turbidity, chlorophyll A, sulfates, nitrates, orthophosphates, and total phosphorus according to APHA Standard Methods (APHA, 1985). All water sample bottles were transported on ice to the laboratory and preserved at $4^{o}C$ until analized within 2 weeks of collection according to Standard Methods for Water and Wastewater Analysis (APHA, 1985).

Bacteriological Analysis

1. Viable Cell Count

Water samples were collected by grab sampling and 20 ml were

incubated 24 h at $37^{o}C$ in 80 ml of alkaline peptone water (APW-sodium chloride 1.0 g, bacto-peptone 1.0 g, 100 ml distilled water; the pH was adjusted to 8.6 with 1N NaOH and sterilized by autoclaving). This is to achieve a 1:5 dilution. Aliquots from this solution were diluted (0.5 ml through 10^{-6}ml) in sterilized distilled water. Volumes of 0.5 ml of APW sample and dilutions were filtered through 0.45 um pore size, 47 mm diameter, HA type, membrane filters (Millipore Corp., Bedford, MA). Filters then were placed on thiosulfate-citrate bile-sucrose (TCBS) agar (Difco), and incubated for 24 h at $37^{o}C$. After incubation, yellow, sucrose fermenting colonies were considered presumptive Vibrio cholerae and all colonies growing on TCBS were recorded as total presumptive vibrio counts. The yellow colonies from countable plates were subcultured onto Trypticase Soy Agar (TSA)(BBL, Cockeysville, MD) with 1% NaCl. After Gram staining, those isolates which were gram-negative and rod shaped were tested for oxidase (filter paper method), motility (tube method), sensitivity to 2,4-diamino 6,7-diisopropylpteridine phosphate (O/129) using 150 ug/ml discs, lactose oxidation/fermentation (O/F), growth without NaCl, and biochemical properties using the API 20-E system (Analytab Products, Plainview, NY). Those tests were performed, as specified by Furniss & Donovan (1978) and West et al. (1982). Isolates that were gram-positive and cocci shaped were tested for catalase, oxygen requirements, and hemolysis on blood agar. Those isolates that were chain-like, catalase negative and facultative anaerobic were biochemically identified using the API 20-S system (Analytab Products,

Plainview, NY). The control strain used was *Vibrio cholerae* ATCC 25872 (O. Felsenfeld 280 NAG) and *Vibrio cholerae* ATCC 14035 (NCTC 8021, El Tor biotype). Both strains were obtained directly from the American Type Culture Collection. All isolates and control strains were maintained in culture on TSA (BBL, Cockeysville, MD) 1% NaCl.

2. Direct *Vibrio cholerae* Count

Direct *V. cholerae* counting was made by Fluorescent Antibody (FA) Direct Counting, according to Xu et al. (1982). A 1-ml sample was passed through a 0.2 um pore size, 25 mm diameter, polycarbonate membrane filter (Nucleopore Corp., Pleasanton, CA) prestained with Sudan Black (1:15,000) for 24 h. The membrane was placed onto a small petri dish (35 x 10 mm), and the cells were fixed to the membrane surface by incubation at 55°C for 10 min. Two drops of rhodamine isothiocyanate (RITC)-conjugated bovine serum albumin (BSA)(BBL, Cockeysville, MD) at a dilution of 1:5 were placed on the surface of the membrane, and spreaded uniformly over the entire surface with a cover slip. The membrane was incubated at 35°C for 30 min in a moist chamber. After incubation, the cover slip was removed and the membrane was rinsed with and then soaked for 10 min in 0.1M phosphate buffered saline (PBS- sodium chloride 8.5 g, potassium phosphate monobasic (KH_2PO_4) 1.5 g, and sodium phosphate dibasic (Na_2HPO_4) 9.1 g in 1 liter distilled water, adjusted to pH 7.3 with 1.0 N NaOH). The membrane was air-dried, and two drops of *Vibrio cholerae* Antiserum Poly (1:32)(Difco) were placed onto the membrane surface and covered with a glass cover slip. The membrane was again

incubated at 35°C for 30 min under humid conditions. After incubation, the membrane was again rinsed with and then soaked in PBS for 10 min. Then two drops of FA Rabbit Globulin Antiglobulin [Goat] serum (1:160)(Difco) were added to the membrane surface, and incubated at 35°C for 30 min under humid conditions. Again, the membrane was rinsed and soaked in PBS for 10 min. Finally, a drop of mounting fluid, pH 9.0 (Difco) was placed on each filter, covered with a glass cover slip and examined under 100x immersion oil lens of epifluorescent microscope Model 2071 (American Optical Corp., Buffalo, NY).

Cell Activity and Total Cell Count

Total cell counts were determined by acridine orange direct count (AODC) staining as described by Hobbie et al. (1977). Simultaneously the percent of respiring bacteria was measured in terms of the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) by the electron transport chain during respiration to form INT-formazan which appears as intracellular red granules under brightfield microscope, as described by Zimmerman et al. (1978). Ten ml samples were incubated with 1 ml of 0.2% INT *in situ* for 20 minutes in dark conditions. Then the reaction was stopped by adding 0.1 ml of 37% formaldehyde. An aliquot of this sample (1 ml) was filtered through a sterile, gridded 0.2 um pore size, 47 mm diameter, polycarbonate membrane filter (Nucleopore Corp., Pleasanton, CA). This membrane was previously stained in a Sudan Black B (1:15,000) solution.

One ml of 0.1% acridine orange solution was added to the filter and allowed to stand for 2 min to stain the bacteria, the remaining stain solution was removed by filtration. The filter was then allowed to dry and examined under 100x immersion oil objective lens with an epifluorescent microscope Model 2071 (American Optical Corp., Buffalo, NY). Red (active) and green (quiescent) fluorescing bacteria were counted on ten fields. The number of total cells per ml of sample was determined as the sum of the mean of red and green bacteria by the number of fields per filter. The percentage of active bacteria was determined from the ratio of red to red plus green cells. Respiring cells, with red granules, were counted on a light field microscope. The percentage of respiring bacteria was determined by the ratio of the number of red granuled bacteria to the total number of cells per milliliter. The same membrane and fields were used for determinations of a single sample.

Survival Studies

1. Microcosms

Microcosms were used to determine environmental parameters on rum slops that influence growth of *Vibrio cholerae* , as described by Singleton et al. (1982a, 1982b). Screw-capped 250 ml flasks were cleaned with 0.0250 M potassium dichromate, rinsed 10 times with distilled deionized water, and sterilized by autoclaving. The microcosms consisted of 50 ml of rum slops of different treatment levels, which were brought to appropiate pH, mostos concentration and

temperature. Mostos concentrations used were 25%, 50%, and 100%; pH determined were 4.6, 6.6, and 8.6; incubated at 25, 35 and 45°C . Combinations of the three parameters were made for each mostos treatment level for a total of 54 different microcosms. Mostos were sterilized by membrane filtration and added aseptically to each flask. The strain used for this study was Vibrio cholerae ATCC 25872 because most environmental strains belong to the non-01 group. The strain was incubated 24 h at 35°C in 100 ml of TSB (Difco). Cells were collected by centrifugation and washed 5 times with a sterile 0.85% (wt/vol) saline solution. After the final washing, a stock suspension was prepared in sterile saline and inoculated to microcosms at an initial concentration of 4×10^{2} cells per ml. All microcosms were incubated for 4 days at designated temperatures with constant agitation (125 rpm) in a shaker-water bath. Samples were collected aseptically daily with 1-ml pipettes. Total viable bacteria were counted by viable counting methods. Microcolonies were counted with the aid of a membrane filter stain (MFS- bromocresol green 100 mg, methyl red 20 mg, isopropyl alcohol 100 ml, sterilized by filtration). Direct (AODC), and metabolic (INT) counts were also done.

2. Diffusion Chambers

Pure cultures of Vibrio cholerae ATCC 25872 were grown in T_1N_1 broth. The broth consists of Trypticase (pancreatic digest of casein)(BBL, Baltimore, MD) 10.0g, sodium chloride 10.0g and 1 liter distilled water, adjusted to pH 7.2 with 1 N NaOH, and sterilized by autoclaving. T_1N_1 was incubated for 24 h at

$35^{o}C$. The cells were harvested by centrifugation and washing in filtered-sterilized phosphate buffered saline (pH 7.0). The number of cells were determined with a Model ZM Coulter Counter (Coulter Electronics, Hialeah, FL) and adjusted to 10^{8} cells per ml. The final suspension was placed in each chamber before immersion at the site. Chambers were a modification (Biamon and Hazen, 1983; Carrillo et al., 1985; West and Lee, 1982) of the MSU-DME chamber (Mc Feters and Stuart, 1972). These lucite diffusion chambers had a capacity of 100 ml and a total diffusion surface area of 16,515 mm^{2}. The chambers have O-rings and direct sampling port to reduce contamination and leakage. Diffusion surfaces were created by placing 0.45 umpore size, 142 mm diameter, nylon reinforced, Versapore membrane filters (Gelman Co., Ann Arbor, MI) on each chamber side previously greased with silicon. Chambers were suspended just below the surface in half-filled 5 gallon aquaria containing rum slops of different treatment levels. Four chambers were used for each site. One ml samples were taken from each chamber with sterile syringes at regular intervals for 72 h. Half of each sample was fixed with 10% phosphate-buffered formalin (pH 7.0) and refrigerated for later counting with an Coulter Counter. The other 0.5 ml sample was incubated with INT for 20 min at _in situ_ temperature and then fixed with 37% formaldehyde for further determination of total direct cell count, percent activity and percent respiring cells.

Data Analysis

Statistical analysis was performed with programs developed for an

Apple II computer and NIH Prophet Computer system. Multiple correlation and regression was made to correlate data against bacterial densities. A five way factorial analysis of variance without replication was performed to test the differences between established parameters and bacterial densities. Differences between time and site of collection during chamber studies were determined using the two factor analysis of variance (FANOVA). Differences between time and site of collection during sampling period were determined using a one factor analysis of variance (ANOVA). The data was made more homoscedastic by transformation with log (x+1). Any statistical probability equal or greater than 0.05 was considered significant (Zar, 1984).

RESULTS

Water Quality

The Arecibo distillery was sampled four times for an eight-month period, since distilling and discharging follows an eight week cycle. The first two samples were from the discharge cooling column, remaining samples were taken just at the plume outfall after being diluted and discharged. Sampling was stopped, resulting from distillery closure after being purchased by competitor. Treated samples were collected after being submitted to a methane digestion treatment process for one month. Measurements of water quality parameters were obtained from both untreated and treated rum distillery effluents (Tables 2 and 3). Values for chlorophyll A, orthophosphates, sulfates, total phosphorus, and turbidity were lower on raw than on treated mostos. Water temperature and pH were always higher in raw mostos. BOD and COD remained unchanged before and after methane digester treatment. Changes were observed in individual parameters at both treatment levels before and after mostos has been discharged. Both treatment levels registered drops in chlorophyll A, nitrates, phosphates, sugar concentrations, and total phosphorus after discharging. In raw mostos, alkalinity, salinity, sulfates, and water temperature were lower after dilution and discharge, while pH increased. Treated effluent values for outfall diluted samples show an increase at the outfall point and decrease with dilution in parameters, such as alkalinity, salinity, and sulfates. A multiple correlation was done to compare water quality

with bacterial densities (Table 4). Viable counts were significantly positively correlated with pH, and negatively correlated with sulfates, sugar concentration and turbidity ($P<0.05$). Direct counts were significantly positively correlated with bacterial viable densities. Percent activity of the bacteria was correlated significantly with viable densities and total direct counts.

Bacterial Communities

Differences between sites were not significant for viable bacterial densities. No viable counts were detected from undischarged mostos samples (Table 2 and 3, Figure 4). Samples taken from discharged mostos show higher viable counts when the mostos is untreated. Significant differences were found in total direct cell counts by site ($F=2.90$, $df=1$ and 6, $P<0.009$). Higher densities of bacteria were found in the raw than in the treated undischarged mostos. However, this difference was greatly decreased in both the raw and treated discharged mostos (Figure 5). Microbial cell activity was not significantly greater in raw mostos than in treated mostos in most cases (Figure 6).

A characterization of the aerobic and facultative anaerobic bacterial populations in rum effluents was performed, 36 isolates from untreated mostos and 49 isolates from treated mostos were obtained (Table 5). From the total isolates from raw mostos, 75% were gram-negative rods. Further identification with the API 20-E system reveals *Aeromonas hydrophila* (22.22%), *Citrobacter freundii* (13.89%),

Enterobacter cloacae (11.11%) and Pseudomonas cepacia (11.11%). Other bacteria found were Klebsiella pneumoniae , Proteus vulgaris and Serratia odorifera 1 . Gram-positive enterococci comprised the remaining 25% of raw mostos community with 16.67% of Streptococcus faecium , and minor numbers of S. faecalis and Staphylococcus sp., according to API 20-S system. From the treated mostos, 85.71% of the total strains isolated were gram-negative rods. API 20-E system identifies Citrobacter freundii (24.49%), Proteus mirabilis (14.29%), and Aeromonas hydrophila (12.24%). CDC Groups VE-1 , Enterobacter agglomerans , E. cloacae , Klebsiella pneumoniae , Pseudomonas cepacia and Serratia liquefaciens were also identified. Again, gram-positive enterococci formed the remaining 14.28% of total isolates. All the gram-positive cocci were in the Streptococcus genus, with S. faecium (12.24%) and S. faecalis (2.04%). Shannon-Weaver's diversity index showed no difference in non-anaerobe species diversity between both rum effluent treatment levels (Table 6). No Vibrio spp. were isolated from rum effluents for either treatment. Results were confirmed by fluorescent antibody microscopy.

Microcosms

Daily sampling of each microcosm for a 4-day period was made. Data was statistically analyzed by a 5-factor ANOVA without replications (Tables 7-10). Culturable colony counts were significantly different over time ($F=68.15$, $df=3$ and 24, $P<0.0001$). The maximum population was observed at 48 h, data from this sampling

was used for report illustration (Tables 11 and 12, Fig. 7 and 8). Temperature (F=293.2, 74.09, 21.57, and 386.0) and pH (F=76.61, 22.67, 12.59, and 30.6) were established as significant factors influencing population densities and metabolic activity (df=2 and 24, P<0.0001 for all cases). Culturable colony counts were significantly influenced by treatment level (F=311.9, df=1 and 24, P<0.0001) in which higher bacterial densities were always obtained from untreated effluents. Comparisons between temperature and pH (Table 11) show significant differences in bacterial densities associated with treatment (F=15.75, df=4 and 24, P<0.0001). Culturable colony counts increased with temperature to 35°C (Figure 7). An increasing effect was observed with pH, however at 35°C differences between pH 6.6 and 8.6 were not significant. Colonies growing at 25°C and 45°C at any pH did not show characteristic *Vibrio cholerae* appearance. They grew as extremely tiny microcolonies and were difficult to detect and count. Microcosms of different rum effluent concentrations incubated at selected temperatures (Table 12) show significant differences in colony densities associated with treatment level and temperature (F=72.17, df=2 and 24, P<0.0001). The highest densities were seen at 35°C with no significant differences owed to concentration, however, extreme temperatures in raw effluents show some decrease in densities when mostos concentration was increased (Fig. 8). Treated effluent colonies, as in Fig. 7 show a decrease in culturable colony counts associated with increase in temperature. Colony counts at various pH incubated at selected concentrations were significantly different

(F=3.26, df=4 and 24, P<0.029) with remarkable differences in pH according to the treatment level (F=40.19, df=2 and 24, P<0.0001; Table 13). Figure 9 shows higher densities in untreated effluents with an increasing effect with decreasing effluent concentration. Also, higher pH had the highest viability. No differences were seen in treated effluents. In culturable colony counts, untreated effluent densities were higher by more than two orders of magnitude.

Total direct cell counts were significantly influenced by treatment (F=115.7, df=1 and 24, P<0.0001) combined with temperature, pH, and concentration (F=5.26, df=8 and 24, P<0.001). Total cell densities, temperature, and pH comparisons (Table 14) showed significant differences in temperature and pH parameters combined (F=29.28, df=4 and 24, P<0.0001). Also, an effect of pH on treatment level influenced cell densities significantly (F=33.6, df=2 and 24, P<0.0001). Higher densities were shown at 35°C (Fig. 10) at pH 6.6 and 8.6. No remarkable differences could be seen at other temperatures or treatment levels. Temperature and concentration influenced significantly cell densities (F=3.61, df=4 and 24, P<0.019; Table 15) and was greatly correlated with treatment level (F=2.97, df=4 and 24, P<0.04). Combined effect of treatment level and temperature is shown in Figure 11 (F=73.68, df=2 and 24, P<0.0001) with higher densities in untreated effluents at 35°C and lower concentrations (25 and 50%). Extreme temperatures and treated rum effluents showed similar densities. Total cell counts at various pH at selected concentrations (Table 16) showed significant effect of treatment with pH (F=33.6, df=2

and 24, $P<0.0001$) and pH with concentration ($F=6.03$, df=4 and 24, $P<0.002$) on total densities. An increasing effect was observed on total densities directly proportional to pH in raw effluents (Figure 12). Similar tendencies were observed in treated effluents with an overall density decrease at intermediate concentrations.

The percent of active cells in the microcosms were influenced by treatment combined with temperature, pH and concentration in the same way as total cell counts. Table 17 shows significant differences on activity resulting from combined influence of treatment level, temperature and pH ($F=4.51$, df=4 and 24, $P<0.007$). Specific differences were shown on Figure 13, as percent activity increases with temperature and pH ($F=3.50$, df=4 and 24, $P<0.022$) on treated effluents. Table 18 shows the effect of temperature and concentration ($F=3.51$, df=4 and 24, $P<0.022$). Activity was related to temperature more than concentration or treatment level (Fig. 14). The activity of microcosms at various pH incubated at selected effluent concentrations (Table 19) shows no significant differences. However, some decrease in activity with increasing effluent concentrations was observed in treated mostos (Figure 15).

Percent of respiring cells at various pH incubated at selected temperatures (Table 20) show significant differences caused by temperature ($F=386.0$, df=2 and 24, $P<0.0001$) and pH ($F=30.6$, df=2 and 24, $P<0.0001$). An increasing effect of respiration with increasing temperature and pH was observed with untreated effluents (Figure 16), although higher rates were observed in treated effluents. Same effect

was noted on Figure 17 illustrating relationships between temperature and effluent concentration. This effect was showed to be significant ($F=4.90$, df=4 and 24, $P<0.005$; Table 21). Effluent concentration and pH ($F=4.96$, df=4 and 24, $P<0.005$) influenced by treatment ($F=2.93$, df=8 and 24, $P<0.02$) were significant in respiring cell populations on microcosms (Table 22). Treated effluents percentage respiration was almost 10% higher than untreated effluents, as shown on Figure 18.

Bacterial survival in aquaria

Densities of *Vibrio cholerae* as measured by Coulter Counter decreased significantly with time ($F=3.81$, df=10 and 22, $P<0.01$). No significant differences by treatment were detected (Table 23, Fig. 19). Total direct cell counts, percent of active cells and percent of respiring cells were determined according to the methods of Zimmerman et al. (1978) and Hobbie et al. (1977). Total direct cell counts of *Vibrio cholerae* showed a significant decrease by time ($F=6.98$, df=10 and 22, $P<0.001$) but not by treatment (Table 24, Figure 20). However, cell counts were more steady on treated effluent, while untreated effluent decreased more sharply. Percent active cells was significantly different by time ($F=3.48$, df=10 and 22, $P<0.02$) but not by treatment level (Table 25, Figure 21). There was no significant differences by treatment of percent respiring cells of *Vibrio cholerae* (Table 26). However, a significant difference by time was observed ($F=7.66$, df=10 and 22, $P<0.001$; Figure 22).

DISCUSSION

Rum distillery effluents showed extremely high values for BOD and COD. These values are not higher than those previously reported for molasses, but are significantly higher than those reported for other types of distillery effluents, such as grains and wine (Sheehan and Greenfield, 1980); and other high organic wastewaters, such as potato-starch, retting and slaughterhouse effluents (La Riviere, 1977). Also, other limiting nutrients, such as phosphorus and sulfur increase with treatment on rum distillery effluents, indicating possible failure of the treatment process. A decrease in nitrates, phosphorus and sugar concentrations was observed in discharged samples of both treatments. Just after discharge, fermentation wastes were mixed with cooling tower and distillery cleaning waters. Thus, discharged mostos is really a watery stillage on which most salt, sugar and nutrient concentrations were decreased by dilution and not by any treatment, and on which can be found conditions as reported in previous studies (Biamon and Hazen, 1983; Hazen et al., 1982; A. Lopez, M.S. Thesis, 1982; N. Perez-Rosas, M.S. Thesis, 1984). However, dilution may be advantageous to anaerobic treatment particularly when large inorganic salt concentrations are great (Sheehan and Greenfield, 1980). Dilution is necessary to overcome inhibition of the digestion process by toxic ions, which can decrease the %BOD removal. In fact, BOD removal increased as organic loading of the digester was decreased (Sheehan and Greenfield, 1980).

Viable bacteria were positively correlated with pH. Culturable

colonies increased as pH approaches neutral. Viable bacteria correlated negatively with sulfates. High concentrations of sulfates are common in molasses, which enhances microbial activity. However, under anaerobic conditions sulfates can be turned into sulfide, whose toxic effect is increased when pH drops below 6.5 (Henze and Harremoes, 1983). Sulfate was also inhibiting above 6750 mg l^{-1}, so dilution will decrease sulfate levels in stillage which can be reduced into sulfide ions and inhibit the digestion process (Sheehan and Greenfield, 1980). Viable bacteria also correlates negatively with the sugar concentration. Tosteson and Hale (1979) argue that bacteria growing on mostos has a substrate utilization efficiency maximum at which bacterial populations proliferate. Levels above this maximum inhibits bacterial growth. The maximum is found at extremely diluted mostos concentrations. Crude mostos, thus, inhibits most bacterial growth (Fig. 4). The inhibition, however, is in viability and is influenced by other factors, such as extreme temperatures and low pH.

Positive correlations between total cell counts and percent bacterial activity seems to be indirect, although activity could be enhanced by sulfate concentrations (Henze and Harremoes, 1983). A treatment effect is seen on which most total bacterial densities were found on the treated mostos, although this effect could be decreased by dilution (Figure 5). Thus high densities could be attributed to the anaerobic methane producing bacteria of the digestion process present and the mesophilic temperatures which made less severe the effect of some toxics present under anaerobic conditions in mostos (Sheehan and

Greenfield, 1980). An increase in microbial activity, without respiration was observed in treated mostos simultaneously. This suggests that anaerobic bacteria could be responsible for the early detoxification of the anoxic mostos in the environment which leads to the establishment of other algal and bacterial populations previously found (Biamon and Hazen, 1983). This asseveration is supported by Hale and Tosteson (1979) which characterized bacteria that could detoxify the slops either by metabolizing of inhibitors or by inactivation with extracellular materials if enough time is supplied.

Aerobic microbial communities in untreated rum distillery effluents are dominated by Aeromonas , Citrobacter , Enterobacter and Pseudomonas . Aeromonas is the main genus, almost doubling it's presence over the rest of the community. This phenomena was previously reported by Biamon and Hazen (1983) in marine waters receiving rum distillery effluents. No reports have been made until now of such isolations in crude mostos. Treated rum effluents, however, have no dominant species. The essential change is the decline of Aeromonas as a completely dominant species and the rise of Proteus to co-dominate the community together with Citrobacter . This is a direct result of the anaerobic environment of the digester with high sulfur concentrations due to protein digested and a great turbidity, which are negative influences on Aeromonas spp. Also is influenced by the absence of other populations, such as cyanobacteria which can help on the detoxifying process. Since they were found in both treatments together with some enterococci, they were not considered as

allochthonous. Hale and Tosteson (1979) also reported that these bacteria could metabolize polysaccharides and peptidic materials found in fermentation wastes, such as mostos. It should be stated that they are the functional acetic acid producers of the treatment process, which accounts for 70% of the methane produced from organic matter in a methane anaerobic digester (de Haast and Britz, 1986). Biamon and Hazen (1983), Hazen et al. (1982) and N. Perez-Rosas (M.S. Thesis, UPR, 1984) have isolated Vibrio spp. especially Vibrio cholerae from marine waters receiving rum effluents. However, no Vibrio spp. were isolated from crude mostos, even with a proper selective media. Also, no Vibrio cholerae had been detected using fluorescent antibody techniques. This suggests that Vibrio spp. originate in the coastal waters and are not autochthonous in the mostos community.

It is known that under extreme conditions in pH, temperature and nutrient availability pathogenic bacteria such as Vibrio cholerae cannot survive (Roberts and Seidler, 1984). These are also the critical parameters for the proper performance of an anaerobic digester. However, if these factors are brought to optimal conditions, Vibrio cholerae are able not only to grow, but survive in this environment. Temperature seems to have the greatest effect on the growth of Vibrio cholerae , where densities decrease with increasing temperature. The most dramatic effect is on the culturable colonies, where methane digestion seems effective in reducing viability, especially at 45°C and pH below 6.6. Previous studies on common aquatic environments by Finkelstein (1973) and Guthrie and Scovill

(1984) showed no recovery under these conditions. This was not the case in rum effluents, where tiny microcolonies could be observed even at 24 hours after inoculation. The extremely nutrient rich mostos makes *Vibrio cholerae* reach highest populations in half the time reported previously for microcosm studies (Singleton et al., 1982a, 1982b).

The effect of treatment with temperature and pH is diminished on total cell counts. Moreover, only at 35°C is the expected density increase with pH observed. Extreme temperatures show a similar effect of lowering densities with increasing pH. Cells growing at 25°C and 45°C showed coccoid morphology, with some increase in cell number, indicating a stress status on the populations. Baker et al. (1983) reports decrease in cell volume of *Vibrio cholerae* associated with increase in cell number as a survival mechanism to nutrient deprivation. This is to allow increase in cell numbers without changes in biomass. However, other stressing conditions could trigger the survival system. Baker et al. (1983) also argues that these coccoid cells are more resistant to cold temperatures. Thus *Vibrio cholerae* could survive in a nearly dormant stage under suboptimal temperature and nutrient concentrations, waiting for suitable conditions for recovery. Moreover, conditions currently present in rum effluents do not allow *Vibrio cholerae* to reach the viable but non-culturable stage (Colwell et al., 1985) found in most stressed bacteria. The hypereutrophic environment allow high recovery of injured bacteria in half time required for most ambient waters.

Activity of Vibrio cholerae in terms of protein synthesis decreases with treatment. However, it shows more defined response patterns to environmental factors than untreated rum effluents. Even at extreme temperatures, activity rises with increasing pH. On the other hand, the pH effect may be masked by the effluent concentration. As stated earlier, sulfate enhances activity, but under anaerobic conditions it could be turned to sulfide which is toxic. So, high sulfide concentrations in treated mostos could be responsible for neutralizing the increase in pH and activity enhancing effect. Heterotrophic activity and respiration also decrease in treated effluents. Incorporation of INT is clearly associated with temperature, since pH, as in the percentage activity could be masked by effluent concentration.

Survival of Vibrio cholerae in the diffusion chambers did not show significant differences by treatment. Both Coulter Counter cell counts and total direct cell counts showed an initial increase in density followed by gradual significant decrease by time. Tosteson and Hale (1979) reported that in the presence of mostos, most cells tend to form aggregates. That could explain the 4 log difference between the Coulter cell counts and the direct total cell counts. Bacteria in untreated mostos decreased more rapidly than cells in treated effluents. Metabolism followed a similar pattern. This was different from the microcosm survival studies in which treated effluents showed a significant decrease in all density and metabolic parameters. Only the strictly control on certain critical parameters of the microecosystems

differs both studies. Thus, diffusion chambers resemble more natural environments. Survival rates were also higher than those reported for V. cholerae in ambient waters, even after 3 days in the low pH, high carbohydrate mostos. It was reported by Fuentes et al. (1983) that Vibrio cholerae shows a strong positive chemotaxis and ability to oxidize rum effluents. This is supported by the high percentage of respiring bacteria which decreases only after 48 hours. Thus pathogenic bacteria like Vibrio cholerae are able not only to grow, but multiply, regardless of the biological treatment used.

This study has showed that the anaerobic digestion treatment fails to control bacterial populations and metabolism. It is possible that the high suspended solids present in mostos overloads digester capacity performance. Under proper control of critical parameters of digestion, especially temperature and pH, outbreaks of potentially pathogenic bacteria like Vibrio cholerae could be significantly limited. Without this, and in the present conditions there is no difference in treated and untreated rum distillery effluents in terms of growth and survival of potentially pathogenic bacteria. The methane anaerobic treatment should be considered mainly as an energy recovering method and not as a vehicle for reduction of environmental impact on high organic wastewater effluents. This reduction could be achieved only by more advanced secondary and tertiary treatment systems. Further research for an optimal treatment method for rum distillery effluents is still needed.

CONCLUSIONS

1. Treated rum distillery effluents yielded higher critical limiting nutrients than untreated rum distillery effluents.
2. Viable bacteria was positively correlated with pH, and negatively correlated with sulfates, turbidity, and sugar concentrations.
3. Higher bacterial densities were found in treated rum distillery effluents, and were related to methane bacteria essential to the digestion process.
4. Total bacterial densities were positively correlated with activity.
5. Anaerobic bacteria were responsible for initial detoxification of mostos.
6. Microbial aerobic and facultative anaerobic communities in untreated mostos are dominated by *Aeromonas*, *Citrobacter*, *Enterobacter* and *Pseudomonas* genera, with *Aeromonas* as the community leader.
7. Microbial aerobic and facultative anaerobic communities in treated mostos are co-dominated by *Aeromonas*, *Citrobacter* and *Proteus*, without dominance.
8. Microbial species diversity was the same for treated and untreated effluents.
9. No *Vibrio* spp. were found in rum distillery effluents at either treatment levels.
10. Densities of viable *Vibrio cholerae* under controlled conditions were lower in treated rum effluents.

11. Temperature and pH had great impact on _Vibrio cholerae_ growth and survival in rum distillery effluents. Mesophilic temperatures and high pH supported the highest densities.

12. _Vibrio cholerae_ could survive in a nearly dormant stage under extreme rum effluent conditions in either treatment level. A viable but non-culturable stage was not observed.

13. An increase in activity was always related with methane digestion treatment.

14. _Vibrio cholerae_ survival in diffusion chambers did not show significant differences associated with treatment.

15. Survival rates for _Vibrio cholerae_ in rum effluents were always higher than those reported for tropical freshwater and marine environments.

16. Anaerobic methane digestion treatment failed to reduce critical limiting nutrients in rum distillery effluents. This failure could be attributed to improper management of the treatment process, which under proper control could limit growth of pathogenic bacteria like _Vibrio cholerae._

17. At present, there is no difference between treated and untreated rum distillery effluents in terms of growth and survival of potentially pathogenic bacteria, and it's potential effect on the environment.

LITERATURE CITED

American Public Health Association. 1985. Standard Methods for the Examination of Water and Wastewater. 16th Edition. American Public Health Association, Washington, DC.

Atlas, R.M., and R. Bartha. 1981. Ecological aspects of biodeterioration control; soil, water and waste management. In: R.M. Atlas and R. Bartha (ed.) Microbial Ecology: Fundamentals and Applications. 1rst ed. Addison-Wesley Publishing Co., Reading, Mass.

Baker, R.M., F.L. Singleton, and M.A. Hood. 1983. Effects of nutrient deprivation on Vibrio cholerae . Appl. Environ. Microbiol. 46:930-940.

Baumann, P., A.L. Furniss, and J.V. Lee. 1984. Genus I. Vibrio Pacini 1854, 411. In:N.R. Krieg and J.G. Holt (ed.). Bergey's Manual of Systematic Bacteriology, Vol 1. The Williams & Wilkins Co. Baltimore.

Biamon, E.J., and T.C. Hazen. 1983. Survival and distribution of Aeromonas hydrophila in near-shore coastal waters of Puerto Rico receiving rum distillery effluent. Water Res. 17:319-326.

Carrillo, M., E. Estrada, and T.C. Hazen. 1985. Survival and enumeration of fecal indicators Bifidobacterium adolescentis and Escherichia coli in a tropical rain forest watershed. App. Environ. Microbiol. 50:468-476.

Center for Disease Control. 1979. Non-01 Vibrio cholerae infections-Florida. Morbidity and Mortality Weekly Report. 28:571-577.

Center for Disease Control. 1980a. Multiply antibiotic-resistant O-Group 1 Vibrio cholerae -Bangladesh. Morbidity and Mortality Weekly Report. 29:109-110.

Center for Disease Control. 1980b. Cholera-Florida. Morbidity and Mortality Weekly Report. 29:601.

Center for Disease Control. 1981. Cholera-Texas. Morbidity and Mortality Weekly Report. 30:389-390.

Center for Disease Control. 1982. Cholera, 1981. Morbidity and Mortality Weekly Report. 31:481-488.

Colwell, R.R. 1970. Polyphasic taxonomy of the Genus Vibrio : numerical taxonomy of Vibrio cholerae , Vibrio parahaemolyticus , and related Vibrio species. J. Bacteriol. 104:410-433.

Colwell, R.R., R.J. Seidler, J. Kaper, S.W. Joseph, S. Garges, H. Lockman, D. Maneval, H. Bradford, N. Roberts, E. Remmers, I. Huq, and A. Huq. 1981. Occurrence of Vibrio cholerae serotype 0:1 in Maryland and Louisiana estuaries. Appl. Environ. Microbiol. 41:555-558.

Colwell, R.R., P.R. Brayton, D.J. Grimes, D.B. Roszak, S.A. Huq, and L.M. Palmer. 1985. Viable but non-culturable Vibrio cholerae and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technology 3:817-820.

Costle, D.M. 1979. Effect of distillery wastes on the marine environment. United States Environmental Protection Agency, Washington, D.C.

Davis, J.W., and R.K. Sizemore. 1982. Incidence of Vibrio species associated with blue crabs (Callinectes sapidus) collected from Galveston Bay, Texas. Appl. Environ. Microbiol. 43:1092-1097.

de Haast, J., and T.J. Britz. 1986. Characterization of aerobic and facultative anaerobic bacteria from the liquid phase of an anaerobic fixed-bed digester treating a cheese whey substrate. Microb. Ecol. 12:173-179.

Desmarchelier, P.M., and J.L. Reichelt. 1981. Phenotypic characterization of clinical and environmental isolates of Vibrio cholerae from Australia. Curr. Microbiol. 5:127-130.

Desmarchelier, P.M., and J.L. Reichelt. 1982. Genetic relationships among clinical and environmental isolates of Vibrio cholerae from Australia. Curr. Microbiol. 7:53-57.

Finkelstein, R.A. 1973. Cholera. CRC Crit. Rev. Microbiol. 2:553-623.

Fuentes, F.A., E.J. Biamon and T.C. Hazen. 1983. Bacterial chemotaxis to effluent from a rum distillery in tropical near-shore coastal waters. Appl. Environ. Microbiol. 46:1438-1441.

Furniss, A.L., and T.J. Donovan. 1976. The Gram negative non-sporing rods: 2. Vibrios. In: C.H. Collins and P.M. Lyne (ed). Microbiological Methods. 4th ed. Butterworths, London.

Gardner, A.D., and K.V. Venkatraman. 1935. The antigens of the cholera group of vibrios. J. Hygiene. 35:262-282.

Gonzalez, J.G., P.M. Toshioka, R.J. Zimmerman, J.M. Lopez, M. Hernandez-Avila, J.N. Suhayda, H.H. Roberts, D. Cruz Baez, D. Pesante and A.T. Velazco. 1979. Biological effects of rum slops in the marine environment. United States Environmental

Protection Agency, Washington, D.C.

Guthrie, R.K., and M.A. Scovill. 1984. Recovery of *Escherichia coli* and *Vibrio cholerae* from aquatic microcosms. Water Res. 18:1055-1057.

Hale, D.R., and T.R. Tosteson. 1979. The utilization of rum slops by marine bacteria. II. Characterization of efficient strains. J. Agr. University of Puerto Rico. LXIII:189-194.

Hazen, T.C., L.J. Prieto, A.J. Lopez-Torres, and E.J. Biamon. 1982. Survival and activity of fecal coliform bacteria in near-shore coastal waters. Simposio de Recursos Naturales, Commonwealth of Puerto Rico, Department of Natural Resources. San Juan, P.R.

Henze, M., and P. Harremoes. 1983. Anaerobic treatment of wastewater in fixed film reactors-a literature review. Water Sci. Tech. 15:1-101.

Hobbie, J.E., R. J. Daley, and S. Jasper. 1977. Use of Nucleopore filters for counting bacteria by fluorescence microscopy. App. Environ. Microbiol. 33:1225-1228.

Hood, M.A., G.E. Ness, and G.E. Rodrick. 1981. Isolation of *Vibrio cholerae* serotype O:1 from the eastern oyster *Crassostrea virginica* . Appl. Environ. Microbiol. 41:559-560.

Hood, M.A., and G.E. Ness. 1982. Survival of *Vibrio cholerae* and *Escherichia coli* in estuarine waters and sediments. Appl. Environ. Microbiol. 43:578-584.

Hood, M.A., G.E. Ness, G.E. Rodrick, and N.J. Blake. 1983. Distribution of *Vibrio cholerae* in two Florida estuaries. Microb. Ecol. 9:65-75.

Huq, A., P.A. West, E.B. Small, M.I. Huq, and R.R. Colwell. 1984. Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio cholerae* Serovar O:1 associated with live copepods in laboratory microcosms. Appl Environ. Microbiol. 48:420-424.

Kaper, J., H. Lockman, R.R. Colwell, and S.W. Joseph. 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. Appl. Environ. Microbiol. 37:91-103.

Kenyon,J.E., D.R. Piexoto, B. Austin, and D.C. Gillies. 1984. Seasonal variation in numbers of *Vibrio cholerae* (Non-O1) isolated from California coastal waters. Appl. Environ. Microbiol. 47:1243-1245.

Koch, R. 1883. Der zweite Bericht der deutschen cholera-commission. Dtsch. Med. Wochenschr. 9:743.

Kodama, H., Y. Gyobu, N. Tokuman, I. Okada, H. Uetake, T. Shimada, and R. Sakazaki. 1984. Ecology of Non-01 Vibrio cholerae in Toyama Prefecture. Microbiol. Inmunol. 28:311-325.

La Riviere, J.W.M. 1977. Microbial Ecology of Liquid Waste Treatment. In: M. Alexander (ed.) Advances in Microbial Ecology. Vol 1. Plenum Press, New York, NY.

Lee, J.V., D.J. Bashford, T.J. Donovan, A.L. Furniss & P.A. West. 1982. The incidence of Vibrio cholerae in water,animals and birds in Kent, England. J. Appl. Bacteriol. 52:281-291.

Levine, M.M., J.B. Kaper, R.E. Black, and M.L. Clements. 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiological Reviews. 47:510-550.

Lopez-Torres, A.J. 1982. Ecology of Klebsiella pneumoniae in aquatic habitats of Puerto Rico. M.S. Thesis. University of Puerto Rico, Rio Piedras.

MacDonell, M.T., and R.R. Colwell. 1984. Identical 5S rRNA nucleotide sequence of Vibrio cholerae strains representing temporal, geographical, and ecological diversity. Appl. Environ. Microbiol. 48:119-121.

McFeters, G.A., and D.G. Stuart. 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane filter chambers. Appl. Microbiol. 24:805-811.

Motes, M.L., S.R. Zywno, A. DePaola, R.E. Becker, and M.W. Presnell. 1983. Isolation of Vibrio cholerae Serotype Ogawa from a Florida estuary. Appl. Environ. Microbiol. 45:321-322.

Perez-Rosas, N. 1983. Distribution and survival of Vibrio spp. in tropical freshwater and marine environments. M.S. Thesis. University of Puerto Rico, Rio Piedras.

Rhodes, J.B., D. Schweitzer, and J.E. Ogg. 1985. Isolation of non-01 Vibrio cholerae associated with enteric disease of hervibores in Western Colorado. J. Clin. Microbiol. 22:572-575.

Roberts, N.C., R.J. Siebeling, J.B. Kaper, and H.B. Bradford Jr. 1982. Vibrios in the Louisiana Gulf Coast Environment. Microb. Ecol. 8:299-312.

Roberts, N.C., and R.J. Seidler. 1984. Methods for monitoring vibrios in the environment. In: R.R. Colwell (ed.) Vibrios in the environment. lrst ed. John Wiley & Sons, New York, NY.

Rogers, R.C., R.G.C.J. Cuffe, Y.M. Cossins, D.M. Murphy, and A.T.C. Bourke. 1980. The Queensland cholera incident of 1977. 2. The epidemiological investigation. Bulletin of the World Health

Organization. 58:665-669.

Sakazaki, R., K. Tamura, C.Z. Gomez, and R. Sen. 1970. Serological studies on the cholera group of vibrios. Jap. J. Med. Sci. Biol. 23: 13-20.

Sheehan, G.J., and P.F. Greenfield. 1980. Utilization, treatment and disposal of distillery waste water. Water Res. 14:257-277.

Shewan, J.M., and M.M. Veron. 1974. Genus I. Vibrio Pacini 1854, 411. In: R.E. Buchanan and N.E. Gibbons (ed). Bergey's Manual of Determinative Bacteriology. 8th ed. The Williams & Wilkins Co., Baltimore.

Shimada, t., and R. Sakazaki. 1977. Additional serovars and inter-O-antigenic relationships of Vibrio cholerae. Jap. J. Med. Sci. Biol. 30:275-277.

Singleton, F.L., R.W. Atwell, M.S. Jangi, and R.R. Colwell. 1982a. Influençe of salinity and organic nutrient concentration on survival and growth of Vibrio cholerae in aquatic microcosms. Appl. Environ. Microbiol. 43:1080-1085.

Singleton, F.L., R.W. Atwell, M.S. Jangi, and R.R. Colwell. 1982b. Effects of temperature and salinity on Vibrio cholerae growth. Appl. Environ. Microbiol. 44:1047-1058.

Spira, W.W., A. Huq, Q.S. Ahmed, and Y.A. Saeed. 1981. Uptake of Vibrio cholerae Biotype eltor from contaminated water by water hyacinth (Eichornia crassipes). Appl. Environ. Microbiol. 42:550-553.

Tosteson, T.R., and D.R. Hale. 1979. The utilization of rum slops by marine bacteria. I. Isolation of efficient strains. J. Agr. University of Puerto Rico. LXIII:180-188.

West, P.A. & J.V. Lee. 1982. Ecology of Vibrio species, including Vibrio cholerae in natural waters of Kent, England. J. Appl. Bacteriol. 52:435-448.

Xu, H., N. Robers, F.L. Singleton, R.W. Atwell, D.J. Grimes and R.R. Colwell. 1982. Survival and viability of nonculturable Escherichia coli and Vibrio cholerae in the estuarine and marine environment. Microb. Ecol. 8:313-323.

Zar, J.H. 1983. Biostatistical Analysis. 2nd ed. Prentice-Hall, Englewoood Cliffs, NJ.

Zimmerman, R.R., R. Iturriaga, and J. Becker-Birck. 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. App. Environ. Microbiol. 36:926-935.

Table 1. Characteristics for the Identification of *Vibrio cholerae*[a]

Criteria	Type of Reaction
Gram-negative, rod	+
Motility	+
Oxidase	+
ONPG	+
Arginine dihidrolase	-
Lysine decarboxylase	+
Ornithine decarboxylase	+
Citrate utilization	+
H_2S utilization	-
Urease	-
Tryptophane deaminase	-
Indole production	+
Voges-Proskauer	+
Gelatin hydrolisis	+
Acid from:	
D-glucose	+
D-sucrose	+
D-inositol	-
D-arabinose	-
Lactose	-
Growth in 0% NaCl	+
0/129 at 150 ug/ml	S

[a] Abbreviations: +=positive reaction, -=negative reaction, S=sensitive.

Table 2. Water Quality in Untreated Rum Distillery Effluents

	MAR[a]	MAY[a]	JUL	SEP	MEAN ± SE
ALK	4000	4000	2000	2000	3000 ± 577
BOD	27500	27100	27700	27700	27500 ± 141
CHLA	0	14062	6.19	1.28	3517 ± 3514
COD	82300	83200	82700	82300	82625 ± 213
HARD	8000	8000	2000	18000	9000 ± 3316
NO3	796	1072	27	19	478 ± 269
PH	4.67	4.92	6.39	7.48	5.87 ± 0.66
PO4	28.28	34.86	2.06	0.60	16.45 ± 8.84
SAL	60	50	14	16	35 ± 12
SO4	6648	7835	668	669	3955 ± 1912
SC	6.00	5.00	3.00	3.50	4.38 ± 0.69
TP	72.57	113.95	1.91	1.22	47.41 ± 27.79
TURB[b]	97.0	93.5	39.0	84.5	78.50 ± 13.43
WTEMP	125	125	39.5	31.1	80.2 ± 26.0
VC[c]	0.00	0.00	19.00	14.55	8.59 ± 5.07
TDC[d]	.367	.237	59.5	34.8	23.73 ± 14.43
%ACT	99.73	70.54	78.05	64.41	78.18 ± 7.71

[a] Undischarged rum effluents.

[b] TURB x 10^2.

[c] VC x 10^7.

[d] TDC x 10^7.

Table 3. Water Quality in Treated Rum Distillery Effluents

	DEC[a]	APR[a]	JUN[a]	AUG	OCT	MEAN ± SE
ALK	6000	2000	2000	4000	2000	3200 ± 200
BOD	26900	28000	28000	27100	28000	27600 ± 247
CHLA	16875	9375	27364	61	15	10738 ± 5220
COD	82800	81100	83200	83100	83200	82680 ± 402
HARD	12000	8000	8000	8000	4000	8000 ± 1265
NO3	1563	796	929	239	32	712 ± 271
PH	5.30	7.00	4.64	4.61	4.68	5.25 ± 0.46
PO4	11.7	134.6	63.5	36.6	29.5	55.2 ± 21.5
SAL	50	40	50	60	20	44 ± 7
SO4	2194	7275	10808	12357	2541	7035 ± 2077
SC	5.00	5.00	5.00	5.00	2.00	4.40 ± 0.60
TP	16	146	100	111	35	82 ± 24
TURB[b]	97.0	68.5	91.0	86.5	98.0	88.2 ± 5.4
WTEMP	25	25	25	25	25	25 ± 0.00
VC[c]	.0235	37	.104	1.3	8000	1607 ± 1598
TDC[d]	ND	24.4	378.0	18.8	60.8	120.5 ± 86.3
%ACT	ND	54.84	58.44	67.02	81.58	66.47 ± 5.41

[a]Undischarged rum effluents.

[b]TURB x 10^2.

[c]VC x 10^3.

[d]TDC x 10^7.

ND= not determined.

Table 4. Correlation Matrix of Water Quality in Rum Effluents

	ALK	BOD	CHLA	COD	HARD	NO3	PH	PO4	SAL	SO4	TP	TURB	WTEMP	SC	VC	AODC	%ACT
ALK	1																
BOD	-.779	1															
CHLA	.16	-.028	1														
COD	.078	-.321	.271	1													
HARD	.264	-.222	.163	-.185	1												
NO3	.57	-.417	.784	.092	.235	1											
PH	-.31	.293	-.079	-.535	.446	-.122	1										
PO4	-.128	.425	.309	-.578	-.099	.237	.131	1									
SAL	.655	-.483	.41	.083	.106	.648	-.504	.318	1								
SO4	.159	-.092	.109	-.068	-.164	.019	-.572	.408	.625	1							
TP	.057	.054	.346	-.211	-.125	.330	-.201	.819	.651	.707	1						
TURB	.383	-.262	.075	.106	.207	.204	-.625	-.051	.418	.402	.123	1					
WTEMP	.217	-.308	.05	.145	-.022	.36	-.104	-.123	.404	-.012	.227	.113	1				
SC	.59	-.394	.336	-.285	.263	.596	-.23	.385	.875	.602	.59	.3	.4	1			
VC	-.393	.385	.06	.071	.103	-.183	.51	.019	-.437	-.563	-.213	-.605	-.406	-.559	1		
AODC	.021	.034	.417	.201	.703	.391	.347	.033	.126	-.46	.075	-.304	.221	-.079	.715	1	
%ACT	.094	-.022	.243	.292	.077	.354	.221	-.085	.164	-.493	.01	-.319	.439	-.049	.565	.944	1

P<0.05 when R>0.532.

Table 5. Aerobic and Facultatively Anaerobic Bacterial Populations in Rum Effluents

	Untreated mostos		Treated mostos	
Species	Number	% Identified	Number	% Identified
Aeromonas hydrophila	8	22.22	6	12.24
CDC Groups VE-1	0	0.00	1	2.04
Citrobacter freundii	5	13.89	12	24.49
Enterobacter agglomerans	0	0.00	5	10.20
Enterobacter cloacae	4	11.11	4	8.16
Klebsiella pneumoniae	1	2.78	3	6.12
Proteus mirabilis	0	0.00	7	14.29
Proteus vulgaris	1	2.78	0	0.00
Pseudomonas cepacia	4	11.11	1	2.04
Serratia liquefaciens	0	0.00	2	4.08
Serratia odorifera 1	1	2.78	0	0.00
Staphylococcus sp.	2	5.56	0	0.00
Streptococcus faecalis	1	2.78	1	2.04
Streptococcus faecium	6	16.67	6	12.24
Unidentified	3	8.33	1	2.04
TOTAL	36		49	

Table 6. Shannon-Weaver's Diversity Index in Rum Distillery Effluents

Treatment Level	Diversity Index	Variance
Untreated	0.9386	0.00202701
Treated	0.9526	0.00180856

Student's t Test for Evenness Between Treatment Levels:

$t = -0.2262$
df= 60

$t_{0.05(2)60} = 2.0000$

Table 7. 5 Factorial, ANOVA on Microcosm Culturable Counts[a]

Variable	Sum of Squares	DF	Mean Square	F	P
Temp.	162.7020	2[b]	81.3510	293.20	.0001
MT	86.5572	2	86.5572	311.90	.0001
Temp-MT	40.0519	2	20.0259	72.17	.0001
PH	42.5121	2	21.2561	76.61	.0001
Temp-PH	38.1076	4	9.5269	34.33	.0001
MT-PH	22.3054	2	11.1527	40.19	.0001
Temp-MT-PH	17.4801	4	4.3700	15.75	.0001
[]	2.8157	2	1.4078	5.07	.015
Temp-[]	2.1847	4	0.5462	1.97	.132
MT-[]	0.1531	2	0.0765	0.28	.761
Temp-MT-[]	0.2530	4	0.0633	0.23	.92
PH-[]	3.6191	4	0.9048	3.26	.029
Temp-PH-[]	2.0182	8	0.2523	0.91	.525
MT-PH-[]	0.6662	4	0.1666	0.60	.666
Temp-MT-PH-[]	4.5528	8	0.5690	2.05	.083
T	56.7314	3	18.9105	68.15	.0001
Temp-T	29.6001	6	4.9333	17.78	.0001
MT-T	7.9376	3	2.6459	9.54	.0005
Temp-MT-T	11.5308	6	1.9218	6.93	.0005
PH-T	6.6710	6	1.1118	4.01	.006
Temp-PH-T	15.5770	12	1.2981	4.68	.001
MT-PH-T	1.4213	6	0.2369	0.85	.542
Temp-MT-PH-T	7.0045	12	0.5837	2.10	.001
[]-T	0.8989	6	0.1498	0.54	.773
MT-[]-T	2.7896	6	0.4649	1.68	.17
Temp-[]-T	2.6709	12	0.2226	0.80	.645
MT-Temp-[]-T	1.6903	12	0.1409	0.51	.889
PH-[]-T	1.2692	12	0.1058	0.38	.958
MT-PH-[]-T	1.6272	12	0.1356	0.49	.902
Temp-PH-[]-T	4.5032	24	0.1876	0.68	.828

[a] Original values are in Log CFU/ml.

[b] Error df=24.

Table 8. 5 Factorial, ANOVA on Microcosm Total Cell Counts[a]

Variable	Sum of Squares	DF	Mean Square	F	P
Temp.	41.4808	2[b]	20.7404	74.09	.0001
MT	32.3908	1	32.3908	115.70	.0001
Temp-MT	41.2508	2	20.6250	73.68	.0001
PH	12.6942	2	6.3471	22.67	.0001
Temp-PH	32.7847	4	8.1962	29.28	.0001
MT-PH	18.8115	2	9.4058	33.60	.0001
Temp-MT-PH	1.2380	4	0.3095	1.11	.377
[]	2.0565	2	1.0282	3.67	.041
Temp-[]	4.0412	4	1.0103	3.61	.019
MT-[]	1.4105	2	0.7053	2.52	.102
Temp-MT-[]	3.3208	4	0.8301	2.97	.04
PH-[]	6.7565	4	1.6891	6.03	.002
Temp-PH-[]	5.1115	8	0.6389	2.28	.056
MT-PH-[]	4.0700	4	1.0175	3.64	.019
Temp-MT-PH-[]	11.7763	8	1.4720	5.26	.001
T	5.7508	3	1.9169	6.85	.002
Temp-T	6.0435	6	1.0072	3.60	.011
MT-T	1.8446	3	0.6149	2.18	.115
Temp-MT-T	1.6504	6	0.2651	0.98	.459
PH-T	0.6160	6	0.1027	0.37	.893
Temp-PH-T	2.4022	12	0.2002	0.72	.723
MT-PH-T	1.6555	6	0.2759	0.99	.457
Temp-MT-PH-T	6.4045	12	0.5337	1.91	.086
[]-T	1.9492	6	0.3249	1.16	.359
MT-[]-T	6.5363	6	1.0894	3.89	.007
Temp-[]-T	3.8045	12	0.3170	1.13	.381
MT-Temp-[]-T	5.1707	12	0.4309	1.54	.178
PH-[]-T	6.6177	12	0.5515	1.97	.076
MT-PH-[]-T	5.2547	12	0.4379	1.56	.169
Temp-PH-[]-T	7.0161	24	0.2923	1.04	.458

[a] Original values are in Log cell counts/ml.

[b] Error df=24.

Table 9. 5 Factorial, ANOVA on Microcosm Percent Active Cells

Variable	Sum of Squares	DF	Mean Square	F	P
Temp.	9947.03	2[a]	4973.52	21.57	.0001
MT	1988.45	1	1988.45	8.62	.007
Temp-MT	1025.35	2	512.68	2.22	.13
PH	5805.14	2	2902.60	12.59	.0005
Temp-PH	3226.72	4	806.58	3.50	.022
MT-PH	15.23	2	7.62	0.03	.968
Temp-MT-PH	4154.78	4	1038.70	4.51	.007
[]	1225.42	2	612.71	2.66	.091
Temp-[]	3240.55	4	810.14	3.51	.022
MT-[]	348.04	2	174.02	0.75	.481
Temp-MT-[]	1996.50	4	499.13	2.17	.104
PH-[]	2186.67	2	546.67	2.37	.081
Temp-PH-[]	3683.69	8	460.46	2.00	.091
MT-PH-[]	253.26	4	63.31	0.27	.891
Temp-MT-PH-[]	5992.98	8	749.12	3.25	.012
T	656.12	3	218.71	0.95	.433
Temp-T	2157.29	6	359.55	1.56	.202
MT-T	413.99	3	138.00	0.60	.622
Temp-MT-T	1233.19	6	205.53	0.89	.517
PH-T	587.19	6	97.87	0.42	.855
Temp-PH-T	3549.37	12	295.78	1.28	.29
MT-PH-T	1508.67	6	251.44	1.09	.396
Temp-MT-PH-T	1473.07	12	122.76	0.53	.872
[]-T	915.44	6	152.57	0.66	.681
MT-[]-T	791.20	6	131.87	0.57	.749
Temp-[]-T	2974.57	12	247.88	1.08	.421
MT-Temp-[]-T	1600.27	12	133.36	0.58	.838
PH-[]-T	2354.39	12	196.20	0.85	.602
MT-PH-[]-T	2310.20	12	192.52	0.84	.616
Temp-PH-[]-T	3646.92	24	151.95	0.66	.843

[a] Error df=24.

Table 10. 5 Factorial, ANOVA on Microcosm Percent Respiring Cells

Variable	Sum of Squares	DF	Mean Square	F	P
Temp.	46257.12	2[a]	23128.56	386.00	.0001
MT	78.49	1	78.49	1.31	.264
Temp-MT	42.23	2	21.12	0.35	.707
PH	3667.00	2	1833.50	30.60	.0001
Temp-PH	258.78	4	64.69	1.08	.389
MT-PH	122.66	2	61.33	1.02	.374
Temp-MT-PH	368.37	4	92.09	1.54	.223
[]	646.43	2	323.21	5.39	.012
Temp-[]	229.48	4	57.37	0.96	.449
MT-[]	102.76	2	51.37	0.86	.437
Temp-MT-[]	1173.32	4	293.33	4.90	.005
PH-[]	1189.77	4	297.44	4.96	.005
Temp-PH-[]	1405.10	8	175.64	2.93	.02
MT-PH-[]	266.37	4	66.59	1.11	.374
Temp-MT-PH-[]	802.56	8	100.32	1.67	.156
T	743.88	3	247.95	4.14	.017
Temp-T	309.14	6	51.52	0.86	.538
MT-T	391.12	3	130.37	2.18	.117
Temp-MT-T	379.55	6	63.26	1.06	.416
PH-T	399.88	6	66.65	1.11	.384
Temp-PH-T	1151.11	12	95.93	1.60	.158
MT-PH-T	165.56	6	27.59	0.46	.83
Temp-MT-PH-T	619.13	12	51.59	0.86	.593
[]-T	301.25	6	50.21	0.83	.553
MT-[]-T	123.05	6	20.51	0.34	.907
Temp-[]-T	583.15	12	48.60	0.81	.637
MT-Temp-[]-T	548.46	12	45.71	0.76	.681
PH-[]-T	539.60	12	44.97	0.75	.692
MT-PH-[]-T	686.94	12	57.25	0.96	.513
Temp-PH-[]-T	1556.36	24	64.85	1.08	.424

[a] Error df=24.

Table 11. Viable Densities of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents; Mean ± SE[a]

	Temp.	pH	Untreated[b]	Treated[b]
24 HOURS	25	4.6	0.00 ± 0.00[c]	0.00 ± 0.00
		6.6	0.00 ± 0.00	0.00 ± 0.00
		8.6	3.69 ± 0.05	0.00 ± 0.00
	35	4.6	1.79 ± 0.17	1.65 ± 0.16
		6.6	3.72 ± 0.16	1.35 ± 0.21
		8.6	3.19 ± 0.34	1.58 ± 0.18
	45	4.6	0.43 ± 0.22	0.48 ± 0.25
		6.6	0.16 ± 0.16	0.23 ± 0.23
		8.6	0.20 ± 0.20	0.00 ± 0.00
48 HOURS	25	4.6	2.03 ± 0.10	1.91 ± 0.01
		6.6	2.66 ± 0.46	1.99 ± 0.10
		8.6	4.00 ± 0.19	2.03 ± 0.05
	35	4.6	1.53 ± 0.21	1.32 ± 0.02
		6.6	4.28 ± 0.44	1.24 ± 0.24
		8.6	4.59 ± 0.15	1.32 ± 0.24
	45	4.6	0.96 ± 0.18	1.02 ± 0.21
		6.6	1.25 ± 0.09	0.74 ± 0.16
		8.6	0.62 ± 0.48	0.26 ± 0.26
96 HOURS	25	4.6	4.59 ± 0.06	1.22 ± 0.13
		6.6	4.48 ± 0.13	1.59 ± 0.14
		8.6	5.40 ± 0.05	2.33 ± 0.84
	35	4.6	1.61 ± 0.20	1.09 ± 0.07
		6.6	4.81 ± 0.12	1.99 ± 1.03
		8.6	7.03 ± 0.68	3.23 ± 0.37
	45	4.6	0.67 ± 0.11	0.42 ± 0.06
		6.6	0.48 ± 0.25	0.79 ± 0.06
		8.6	0.89 ± 0.15	0.46 ± 0.24

[a] Initial population was aproximately 4×10^2 cells/ml.

[b] Values are means from all concentrations tested.

[c] All units are in Log CFU/ml.

Table 12. Viable Densities of *Vibrio cholerae* ATCC 25872 at Various Rum Effluent Concentrations Incubated at Selected Temperatures; Mean ± SE[a]

Time	Temp.	%[]	Untreated[b]	Treated[b]
24 HOURS	25	25	1.25 ± 1.25[c]	0.00 ± 0.00
		50	1.25 ± 1.25	0.00 ± 0.00
		100	1.29 ± 1.20	0.00 ± 0.00
	35	25	3.09 ± 0.81	1.33 ± 0.15
		50	2.82 ± 0.57	1.76 ± 0.08
		100	2.81 ± 0.40	1.49 ± 0.22
	45	25	0.36 ± 0.18	0.20 ± 0.20
		50	0.20 ± 0.20	0.23 ± 0.23
		100	0.23 ± 0.23	0.28 ± 0.28
48 HOURS	25	25	3.38 ± 0.63	2.05 ± 0.07
		50	2.76 ± 0.56	1.93 ± 0.08
		100	2.55 ± 0.61	1.94 ± 0.03
	35	25	3.50 ± 1.19	1.14 ± 0.10
		50	3.24 ± 0.90	1.63 ± 0.13
		100	3.65 ± 0.91	1.14 ± 0.09
	45	25	1.34 ± 0.11	0.47 ± 0.23
		50	0.87 ± 0.32	1.08 ± 0.18
		100	0.62 ± 0.33	0.48 ± 0.28
96 HOURS	25	25	4.93 ± 0.25	2.35 ± 0.84
		50	4.68 ± 0.31	1.29 ± 0.16
		100	4.86 ± 0.31	1.49 ± 0.09
	35	25	4.57 ± 1.66	2.54 ± 0.89
		50	5.00 ± 1.76	2.08 ± 0.91
		100	3.88 ± 1.35	1.68 ± 0.74
	45	25	0.40 ± 0.20	0.60 ± 0.17
		50	0.57 ± 0.30	0.39 ± 0.21
		100	0.80 ± 0.18	0.68 ± 0.10

[a] Initial population was aproximately 4 x 10^2 cells/ml.

[b] Values are means of all pH tested.

[c] All units are in Log CFU/ml.

Table 13. Viable Densities of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations; Mean ± SE[a]

	pH	%[]	Untreated[b]	Treated[b]
24 HOURS	4.6	25	0.49 ± 0.49[c]	0.67 ± 0.40
		50	0.82 ± 0.55	0.54 ± 0.54
		100	0.91 ± 0.60	0.93 ± 0.56
	6.6	25	1.46 ± 1.23	0.35 ± 0.35
		50	1.28 ± 1.28	0.82 ± 0.51
		100	1.15 ± 1.15	0.42 ± 0.42
	8.6	25	2.74 ± 1.07	0.51 ± 0.51
		50	2.16 ± 1.11	0.64 ± 0.64
		100	2.18 ± 1.11	0.43 ± 0.43
48 HOURS	4.6	25	1.54 ± 0.34	1.31 ± 0.36
		50	1.51 ± 0.32	1.55 ± 0.17
		100	1.47 ± 0.39	1.39 ± 0.24
	6.6	25	3.12 ± 1.02	1.27 ± 0.45
		50	2.40 ± 0.57	1.53 ± 0.25
		100	2.66 ± 1.10	1.17 ± 0.44
	8.6	25	3.56 ± 1.01	1.09 ± 0.60
		50	2.96 ± 1.35	1.55 ± 0.40
		100	2.69 ± 1.35	0.98 ± 0.55
96 HOURS	4.6	25	2.08 ± 1.35	0.82 ± 0.27
		50	2.37 ± 1.11	0.84 ± 0.22
		100	2.14 ± 1.29	1.01 ± 0.28
	6.6	25	3.34 ± 1.37	2.27 ± 0.93
		50	3.11 ± 1.57	1.13 ± 0.25
		100	3.32 ± 1.24	0.97 ± 0.19
	8.6	25	4.47 ± 2.01	2.41 ± 0.98
		50	4.77 ± 2.04	1.74 ± 1.15
		100	4.09 ± 1.51	1.31 ± 0.95

[a] Initial population was aproximately 4 x 10^2 cells/ml.

[b] Values are means from all temperatures tested.

[c] All units are in Log CFU/ml.

Table 14. Total Counts of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents; Mean ± SE[a]

	Temp.	pH	Untreated[b]	Treated[b]
24 HOURS	25	4.6	5.9504 ± 0.1149[c]	5.8259 ± 0.1486
		6.6	5.7263 ± 0.2295	5.5507 ± 0.1531
		8.6	5.7026 ± 0.2865	5.8088 ± 0.0917
	35	4.6	5.6119 ± 0.1052	5.6678 ± 0.1987
		6.6	7.7321 ± 0.2459	5.5788 ± 0.0580
		8.6	8.6869 ± 0.0945	5.8518 ± 0.4623
	45	4.6	5.8223 ± 0.4645	6.5743 ± 0.0497
		6.6	6.4431 ± 0.0313	6.3159 ± 0.2409
		8.6	6.6263 ± 0.0742	6.3241 ± 0.0554
48 HOURS	25	4.6	6.0021 ± 0.0759	6.3669 ± 0.0071
		6.6	6.0119 ± 0.1538	5.9513 ± 0.1672
		8.6	6.6319 ± 0.5237	5.6760 ± 0.1123
	35	4.6	6.1655 ± 0.2535	5.5312 ± 0.1439
		6.6	7.7582 ± 0.2853	5.9174 ± 0.8189
		8.6	9.5291 ± 0.0368	6.4860 ± 0.4331
	45	4.6	6.3668 ± 0.5057	6.9713 ± 0.0254
		6.6	6.2887 ± 0.4186	6.5714 ± 0.2929
		8.6	6.7086 ± 0.0482	5.8186 ± 0.2649
96 HOURS	25	4.6	5.7955 ± 0.2295	6.1007 ± 0.1675
		6.6	6.1702 ± 0.4125	5.8240 ± 0.0712
		8.6	7.0586 ± 0.3806	4.5374 ± 2.2706
	35	4.6	7.5910 ± 0.9241	6.1007 ± 0.0238
		6.6	8.5086 ± 0.0739	6.7125 ± 1.1979
		8.6	9.9176 ± 0.2820	6.5774 ± 0.8228
	45	4.6	6.0322 ± 0.5619	6.8314 ± 0.1719
		6.6	6.4432 ± 0.0908	6.3002 ± 0.4968
		8.6	6.2964 ± 0.2357	6.1456 ± 0.1463

[a] Initial population was aproximately 4 x 10^2 cells/ml.

[b] Values are means from all concentrations tested.

[c] All units are in Log cells/ml.

Table 15. Total Counts of *Vibrio cholerae* ATCC 25872 at Various Rum Effluent Concentrations Incubated at Selected Temperatures; Mean ± SE[a]

Time	Temp.	%[]	Untreated[b]	Treated[b]
24 HOURS	25	25	5.8259 ± 0.1864[c]	5.5220 ± 0.3142
		50	5.9114 ± 0.2266	5.4815 ± 0.1077
		100	5.6420 ± 0.2483	5.5151 ± 0.4680
	35	25	7.3519 ± 0.9885	5.5431 ± 0.0869
		50	7.4936 ± 0.9493	5.4349 ± 0.0956
		100	7.1853 ± 0.8096	6.1203 ± 0.3514
	45	25	6.2703 ± 0.2783	6.4660 ± 0.0816
		50	6.0191 ± 0.4740	6.2292 ± 0.2261
		100	6.6019 ± 0.1100	6.5190 ± 0.0597
48 HOURS	25	25	6.0245 ± 0.1430	5.8991 ± 0.2120
		50	6.1658 ± 0.3047	6.0444 ± 0.1294
		100	6.4556 ± 0.4154	6.0508 ± 0.3138
	35	25	7.9288 ± 0.8457	6.4008 ± 0.4610
		50	7.9960 ± 0.9536	5.4258 ± 0.1718
		100	7.5280 ± 0.1334	6.1079 ± 0.5974
	45	25	6.6261 ± 0.1969	6.3431 ± 0.4159
		50	6.1864 ± 0.2962	6.2078 ± 0.4150
		100	6.5692 ± 0.5558	6.8104 ± 0.2322
96 HOURS	25	25	6.1822 ± 0.3705	6.1517 ± 0.4096
		50	5.8586 ± 0.3394	6.3116 ± 0.2163
		100	6.9835 ± 0.4479	3.9989 ± 2.0005
	35	25	9.5408 ± 0.1695	6.9913 ± 1.0998
		50	8.3214 ± 1.2887	6.4260 ± 0.8874
		100	8.4884 ± 0.5698	5.3838 ± 0.0116
	45	25	5.9875 ± 0.3107	5.6226 ± 0.1900
		50	6.0969 ± 0.3454	5.9283 ± 0.3773
		100	6.6873 ± 0.2554	6.7264 ± 0.2458

[a] Initial population was aproximately 4 x 10^2 cells/ml.

[b] Values are means from all pH tested.

[c] All units are in Log cells/ml.

Table 16. Total Counts of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations; Mean ± SE[a]

	pH	%[]	Untreated[b]	Treated[b]
24 HOURS	4.6	25	5.6213 ± 0.1072[c]	5.9126 ± 0.3138
		50	5.5803 ± 0.2711	5.9096 ± 0.3554
		100	6.1831 ± 0.2662	6.2456 ± 0.1910
	6.6	25	6.8836 ± 0.5719	5.0306 ± 0.2877
		50	6.6381 ± 0.7118	5.5350 ± 0.1646
		100	6.3798 ± 0.5000	5.8798 ± 0.3142
	8.6	25	6.9193 ± 0.8767	5.9213 ± 0.2233
		50	7.2058 ± 0.8355	5.7009 ± 0.2862
		100	6.8664 ± 0.9444	6.3625 ± 0.2483
48 HOURS	4.6	25	6.2253 ± 0.1902	6.2660 ± 0.3938
		50	5.9185 ± 0.1824	6.1307 ± 0.5382
		100	6.7236 ± 0.3365	6.4728 ± 0.3212
	6.6	25	6.9740 ± 0.4111	6.4488 ± 0.4441
		50	6.8510 ± 0.7371	5.8399 ± 0.2165
		100	6.2339 ± 0.5400	6.1513 ± 0.5435
	8.6	25	7.3625 ± 1.0951	5.9282 ± 0.2894
		50	7.5786 ± 0.9617	5.7074 ± 0.0722
		100	7.9258 ± 0.8439	6.3450 ± 0.5133
96 HOURS	4.6	25	6.7686 ± 1.2205	5.9733 ± 0.3849
		50	5.6626 ± 0.1796	6.1515 ± 0.3548
		100	6.9874 ± 0.3843	6.2186 ± 0.5220
	6.6	25	7.1239 ± 0.7707	7.2198 ± 1.0035
		50	6.8114 ± 0.8888	5.6152 ± 0.1709
		100	7.1868 ± 0.6280	5.9923 ± 0.3731
	8.6	25	7.4847 ± 1.1779	6.5630 ± 0.2107
		50	7.8028 ± 1.3307	6.8992 ± 0.6882
		100	7.9851 ± 0.8381	3.8982 ± 1.9683

[a] Initial population was aproximately 4×10^2 cells/ml.

[b] Values are means from all temperatures tested.

[c] All units are in Log cells/ml.

Table 17. Percent Activity of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents; Mean ± SE[a]

	Temp.	pH	Untreated[b]	Treated[b]
24 HOURS	25	4.6	68.43 ± 12.97	80.80 ± 9.17
		6.6	54.48 ± 19.17	39.22 ± 9.24
		8.6	75.22 ± 10.15	64.38 ± 7.82
	35	4.6	80.78 ± 2.14	66.68 ± 11.61
		6.6	70.11 ± 19.85	57.76 ± 2.81
		8.6	87.04 ± 4.82	80.83 ± 6.05
	45	4.6	70.81 ± 10.52	96.10 ± 0.55
		6.6	97.12 ± 0.56	93.75 ± 2.68
		8.6	92.09 ± 4.82	95.09 ± 0.37
48 HOURS	25	4.6	57.38 ± 17.49	53.66 ± 17.38
		6.6	74.92 ± 11.61	59.31 ± 10.12
		8.6	96.92 ± 0.88	70.13 ± 7.52
	35	4.6	92.05 ± 2.02	73.51 ± 7.52
		6.6	68.09 ± 15.75	81.49 ± 5.56
		8.6	88.71 ± 0.32	86.82 ± 6.54
	45	4.6	87.22 ± 6.14	79.40 ± 0.59
		6.6	87.24 ± 4.19	84.49 ± 3.99
		8.6	85.16 ± 3.12	87.62 ± 6.79
96 HOURS	25	4.6	57.07 ± 11.11	58.79 ± 13.16
		6.6	67.34 ± 7.70	58.42 ± 3.51
		8.6	96.16 ± 0.58	64.93 ± 32.49
	35	4.6	89.82 ± 0.58	63.61 ± 9.58
		6.6	55.22 ± 10.17	76.23 ± 3.91
		8.6	73.67 ± 10.23	89.09 ± 4.44
	45	4.6	75.65 ± 16.71	79.45 ± 2.09
		6.6	81.62 ± 11.33	67.64 ± 12.25
		8.6	86.38 ± 6.01	86.03 ± 6.72

[a] Initial population was aproximately 4 x 10^2 cells/ml.

[b] Values are means from all concentrations tested.

Table 18. Percent Activity of *Vibrio cholerae* ATCC 25872 at Various Rum Effluent Concentrations Incubated at Selected Temperatures; Mean ± SE[a]

	Temp.	%[]	Untreated[b]	Treated[b]
24 HOURS	25	25	74.10 ± 15.56	65.46 ± 10.40
		50	63.39 ± 17.56	49.70 ± 15.08
		100	65.65 ± 10.88	71.58 ± 13.78
	35	25	82.25 ± 1.77	67.92 ± 5.71
		50	62.76 ± 15.49	60.22 ± 7.67
		100	92.92 ± 4.27	77.12 ± 12.31
	45	25	95.31 ± 2.29	95.66 ± 0.97
		50	86.89 ± 10.12	93.63 ± 2.57
		100	77.81 ± 12.05	95.67 ± 0.34
48 HOURS	25	25	86.46 ± 10.51	79.81 ± 0.62
		50	61.48 ± 21.59	53.12 ± 3.87
		100	81.27 ± 9.12	50.18 ± 15.77
	35	25	74.34 ± 14.95	82.95 ± 11.81
		50	80.42 ± 9.26	76.68 ± 1.17
		100	94.09 ± 2.98	82.19 ± 4.07
	45	25	85.80 ± 5.28	78.23 ± 2.13
		50	91.44 ± 2.10	87.76 ± 4.48
		100	82.38 ± 3.64	85.52 ± 5.49
96 HOURS	25	25	71.15 ± 18.61	80.12 ± 10.20
		50	77.81 ± 9.04	61.33 ± 18.18
		100	71.62 ± 12.32	40.68 ± 20.75
	35	25	80.64 ± 9.84	71.66 ± 12.79
		50	60.43 ± 15.71	80.03 ± 9.47
		100	77.65 ± 6.73	77.24 ± 4.18
	45	25	80.66 ± 10.96	79.08 ± 7.95
		50	77.15 ± 17.43	87.85 ± 10.58
		100	85.83 ± 5.44	88.19 ± 5.07

[a] Initial population was aproximately 4×10^2 cells/ml.

[b] Values are means from all pH tested.

Table 19. Percent Activity of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations; Mean ± SE[a]

	pH	%[]	Untreated[b]	Treated[b]
24 HOURS	4.6	25	71.72 ± 14.33	78.04 ± 8.56
		50	72.67 ± 3.11	71.70 ± 14.72
		100	75.64 ± 10.34	93.84 ± 3.96
	6.6	25	90.97 ± 5.47	66.95 ± 15.73
		50	52.78 ± 22.37	57.37 ± 19.86
		100	82.94 ± 14.21	86.39 ± 14.37
	8.6	25	88.95 ± 4.21	84.05 ± 5.20
		50	87.60 ± 4.96	74.47 ± 11.48
		100	77.80 ± 12.05	81.78 ± 12.26
48 HOURS	4.6	25	67.92 ± 5.75	73.11 ± 6.85
		50	67.97 ± 22.06	71.96 ± 6.72
		100	84.77 ± 6.03	61.50 ± 20.05
	6.6	25	74.93 ± 15.91	84.40 ± 4.14
		50	73.13 ± 11.23	71.64 ± 13.75
		100	82.20 ± 8.61	69.25 ± 8.11
	8.6	25	87.78 ± 4.89	83.48 ± 6.64
		50	92.24 ± 3.22	73.96 ± 10.65
		100	90.77 ± 2.75	67.14 ± 6.03
96 HOURS	4.6	25	72.84 ± 18.91	66.79 ± 10.37
		50	67.59 ± 13.71	60.55 ± 14.80
		100	82.12 ± 8.53	74.50 ± 3.28
	6.6	25	66.99 ± 7.02	71.16 ± 5.79
		50	65.28 ± 16.84	60.15 ± 8.65
		100	71.90 ± 10.99	70.99 ± 10.44
	8.6	25	92.62 ± 2.51	92.92 ± 3.92
		50	82.51 ± 13.05	88.51 ± 7.99
		100	81.09 ± 7.22	58.62 ± 29.43

[a] Initial population was aproximately 4 x 10^2 cells/ml.

[b] Values are means from all temperatures tested.

Table 20. Percent Respiration of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Temperatures; Mean ± SE[a]

	Temp.	pH	Untreated[b]	Treated[b]
24 HOURS	25	4.6	7.06 ± 2.79	6.65 ± 1.29
		6.6	10.35 ± 4.33	6.02 ± 3.09
		8.6	20.50 ± 5.09	13.31 ± 1.21
	35	4.6	16.97 ± 7.09	14.65 ± 2.15
		6.6	11.33 ± 6.70	15.17 ± 5.09
		8.6	18.82 ± 11.44	15.28 ± 4.88
	45	4.6	32.96 ± 9.03	30.85 ± 1.75
		6.6	49.95 ± 3.68	43.07 ± 3.09
		8.6	56.66 ± 7.15	51.04 ± 3.69
48 HOURS	25	4.6	5.05 ± 1.10	3.90 ± 1.50
		6.6	7.40 ± 2.46	5.24 ± 1.70
		8.6	11.79 ± 0.38	15.17 ± 2.42
	35	4.6	4.28 ± 1.82	14.40 ± 2.58
		6.6	21.68 ± 11.89	8.31 ± 2.26
		8.6	22.81 ± 6.31	28.42 ± 2.48
	45	4.6	29.85 ± 1.97	40.22 ± 7.56
		6.6	41.15 ± 0.70	58.56 ± 2.24
		8.6	48.86 ± 3.50	52.53 ± 3.27
96 HOURS	25	4.6	3.23 ± 1.13	2.85 ± 0.21
		6.6	5.46 ± 2.76	6.29 ± 2.98
		8.6	6.11 ± 1.67	10.62 ± 6.53
	35	4.6	5.79 ± 0.50	11.25 ± 4.37
		6.6	14.91 ± 5.63	10.79 ± 2.75
		8.6	21.64 ± 5.09	18.47 ± 5.07
	45	4.6	34.82 ± 5.88	38.07 ± 11.73
		6.6	27.35 ± 5.81	33.69 ± 7.37
		8.6	44.43 ± 11.89	42.72 ± 6.35

[a] Initial population was aproximately 4×10^2 cells/ml.

[b] Values are means from all concentrations tested.

Table 21. Percent Respiration of Vibrio cholerae ATCC 25872 at Various Effluent Concentrations Incubated at Selected Temperatures; Mean ± SE[a]

	Temp.	%[]	Untreated[b]	Treated[b]
24 HOURS	25	25	8.59 ± 5.17	10.67 ± 2.20
		50	9.07 ± 2.22	6.50 ± 4.06
		100	20.26 ± 5.13	8.80 ± 1.83
	35	25	18.72 ± 10.78	15.00 ± 5.13
		50	9.62 ± 3.36	14.18 ± 2.40
		100	18.77 ± 9.28	15.92 ± 4.64
	45	25	52.21 ± 12.04	41.57 ± 6.15
		50	50.63 ± 0.63	43.29 ± 7.59
		100	52.21 ± 12.04	41.57 ± 6.15
48 HOURS	25	25	7.51 ± 2.78	9.32 ± 3.99
		50	7.89 ± 1.92	7.20 ± 5.37
		100	8.83 ± 2.69	7.75 ± 1.73
	35	25	13.97 ± 7.61	17.35 ± 6.87
		50	14.99 ± 7.76	18.24 ± 7.49
		100	19.81 ± 12.83	15.55 ± 4.36
	45	25	42.14 ± 4.95	51.66 ± 4.50
		50	36.43 ± 4.52	45.42 ± 10.57
		100	41.30 ± 7.33	54.21 ± 2.75
96 HOURS	25	25	5.57 ± 2.04	10.57 ± 6.04
		50	3.90 ± 0.94	4.33 ± 2.53
		100	5.34 ± 2.85	4.86 ± 3.45
	35	25	16.62 ± 5.21	17.12 ± 4.55
		50	7.02 ± 2.31	16.65 ± 3.75
		100	18.05 ± 6.73	6.73 ± 1.39
	45	25	40.24 ± 1.10	49.54 ± 6.44
		50	37.95 ± 14.05	25.13 ± 3.25
		100	28.41 ± 6.72	39.82 ± 7.05

[a] Initial population was aproximately 4 x 10^2 cells/ml.

[b] Values are means from all pH tested.

Table 22. Percent Respiration of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations; Mean ± SE[a]

	pH	%[]	Untreated[b]	Treated[b]
24 HOURS	4.6	25	14.93 ± 7.69	16.83 ± 6.28
		50	21.09 ± 14.49	19.61 ± 8.32
		100	20.96 ± 5.39	15.72 ± 6.99
	6.6	25	21.40 ± 17.45	21.12 ± 13.21
		50	21.41 ± 14.37	16.55 ± 10.91
		100	28.82 ± 7.60	26.59 ± 9.92
	8.6	25	43.18 ± 15.04	29.30 ± 9.73
		50	26.82 ± 12.58	27.82 ± 15.31
		100	25.98 ± 13.66	22.51 ± 12.17
48 HOURS	4.6	25	14.07 ± 9.99	23.65 ± 11.78
		50	13.22 ± 7.20	13.53 ± 6.99
		100	11.89 ± 8.21	21.33 ± 14.18
	6.6	25	19.21 ± 11.65	23.30 ± 18.61
		50	18.19 ± 10.78	23.78 ± 18.68
		100	32.83 ± 10.78	25.01 ± 14.59
	8.6	25	30.33 ± 11.16	31.40 ± 9.17
		50	27.91 ± 9.00	33.55 ± 9.27
		100	25.21 ± 14.23	31.17 ± 14.49
96 HOURS	4.6	25	17.36 ± 11.33	27.95 ± 17.43
		50	10.49 ± 6.36	12.99 ± 6.99
		100	15.98 ± 12.73	11.12 ± 7.58
	6.6	25	19.84 ± 10.50	18.01 ± 10.77
		50	10.67 ± 7.06	12.18 ± 5.54
		100	17.26 ± 3.29	20.58 ± 11.02
	8.6	25	25.90 ± 9.55	31.27 ± 8.16
		50	27.75 ± 19.21	20.94 ± 6.12
		100	18.55 ± 7.59	19.60 ± 15.62

[a] Initial population was aproximately 4 x 10^2 cells/ml.

[b] Values are means from all temperatures tested.

Table 23. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Coulter Counter Counts, Mean ± SE[a]

Time (h)	Untreated	Treated
0	5.6500 ± .0781	5.5455 ± .1689
3	5.9858 ± .1063	5.6726 ± .1657
6	5.8584 ± .1095	5.6734 ± .1303
12	5.8824 ± .1373	5.8139 ± .1585
18	5.9646 ± .2475	5.7891 ± .0321
24	5.5498 ± .1258	5.7810 ± .1886
30	5.4878 ± .1517	5.5499 ± .0469
36	5.3675 ± .1596	5.5564 ± .0755
48	5.3941 ± .1530	5.5840 ± .1079
60	5.4354 ± .1738	5.2904 ± .0893
72	5.5882 ± .2466	5.4557 ± .1733

[a] Units are in Log CC/ml.

Table 24. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Total Direct Cell Counts, Mean ± SE[a]

Time (h)	Untreated	Treated
0	9.5358 ± .1132	9.4376 ± .1277
3	8.7838 ± .1896	8.2336 ± .1484
6	8.6902 ± .1292	8.2674 ± .1223
12	8.7289 ± .1009	8.3688 ± .0847
18	8.9337 ± .2913	8.5526 ± .1231
24	8.7415 ± .2395	8.3006 ± .1675
30	8.5296 ± .2064	8.3332 ± .1599
36	8.1168 ± .2426	8.0914 ± .0988
48	8.1403 ± .0666	8.2969 ± .1831
60	7.8430 ± .2547	8.0859 ± .1438
72	7.8623 ± .2690	8.2689 ± .0491

[a] Units are in Log cells/ml.

Table 25. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Percent Activity, Mean ± SE

Time (h)	Untreated	Treated
0	78.80 ± 6.00	66.19 ± 7.16
3	87.26 ± 4.14	87.32 ± 3.09
6	94.53 ± 3.10	84.67 ± 4.24
12	87.12 ± 7.81	90.32 ± 2.57
18	76.76 ± 5.71	78.48 ± 6.63
24	89.04 ± 1.94	88.32 ± 3.09
30	88.57 ± 3.38	91.25 ± 2.38
36	83.21 ± 2.54	86.24 ± 1.58
48	86.52 ± 1.51	93.75 ± 1.20
60	81.91 ± 3.10	86.66 ± 2.19
72	84.56 ± 5.24	85.37 ± 2.78

Table 26. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Percent Respiration, Mean ± SE

Time (h)	Untreated	Treated
0	15.36 ± 5.49	22.95 ± 3.94
3	42.55 ± 5.08	28.50 ± 4.43
6	46.72 ± 1.79	49.57 ± 1.96
12	43.83 ± 4.85	45.92 ± 3.12
18	41.64 ± 5.28	42.24 ± 5.97
24	51.52 ± 2.18	47.42 ± 4.26
30	32.64 ± 11.46	54.80 ± 2.23
36	45.17 ± 2.93	46.44 ± 0.38
48	53.84 ± 1.54	55.23 ± 6.03
60	29.68 ± 4.23	9.39 ± 0.78
72	8.96 ± 1.18	5.81 ± 0.69

Figure 1. Serological Nomenclature Used Presently to Describe and Serologically Group *Vibrio cholerae* (Taken from Roberts et al, 1982).

SEROLOGY OF VIBRIO CHOLERAE

epidemic V. cholerae

biotypes: "classical" El Tor

serotype: flagellar antigen unique to the species (a)

cell wall O-antigen

	Ogawa	Inaba	Hikojima	antigens other than A, B, C
	AB	AC	ABC	
Gardner - Venkatraman	O-antigen group I (O-1)			5 O-groups
Sakazaki	O-1			60 serovars
Smith	O-1			79 serotypes
				non O-1s

Figure 2. Map of Arecibo Study Site.

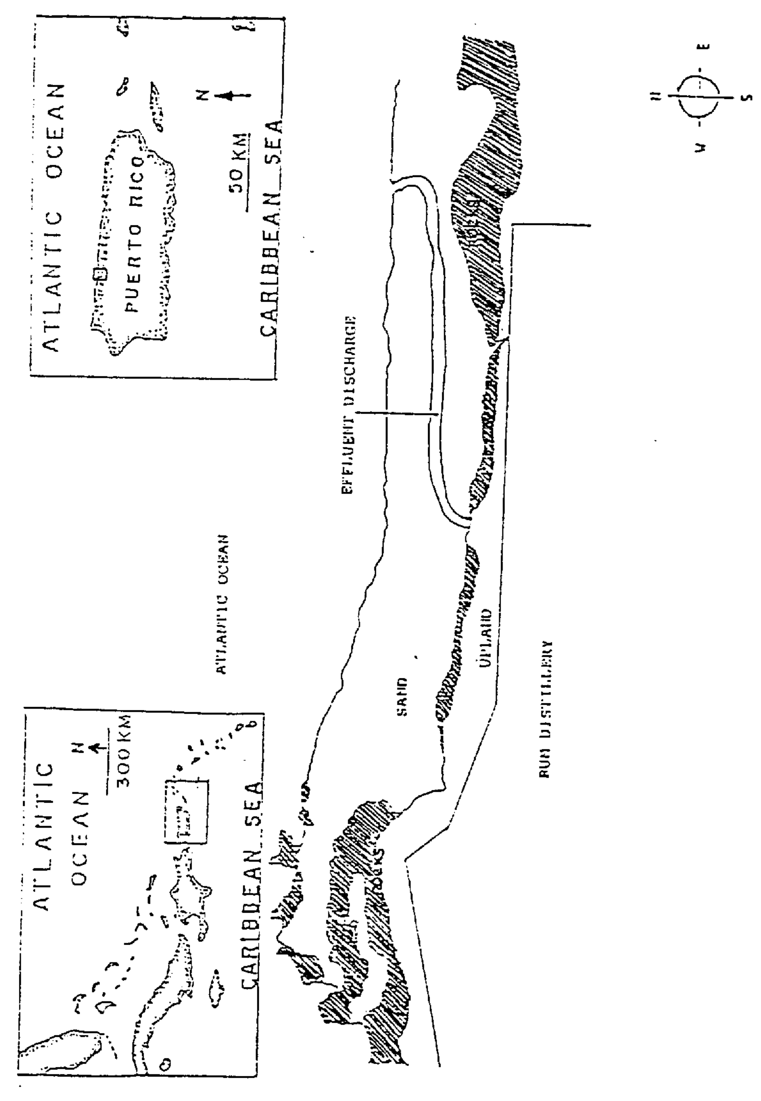
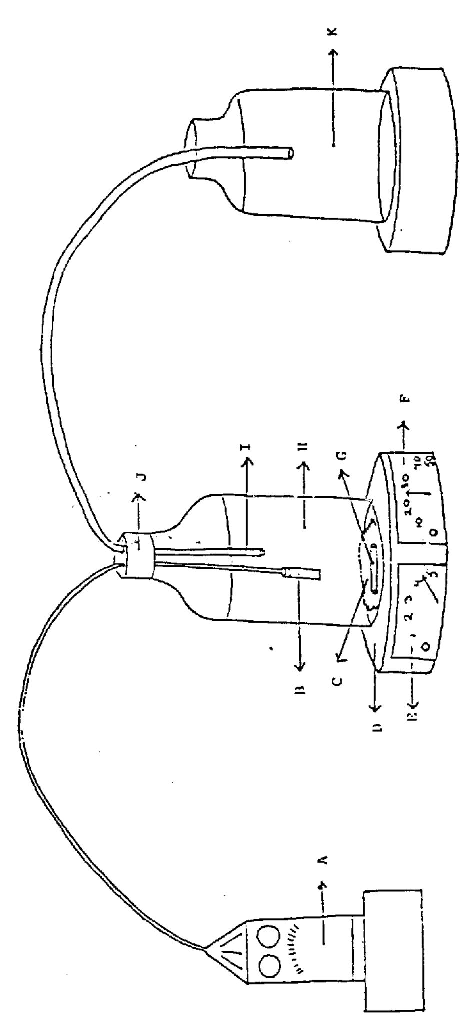

ATLANTIC OCEAN
PUERTO RICO
N
50 KM
CARIBBEAN SEA
ATLANTIC
OCEAN
N
300 KM
CARIBBEAN SEA
ATLANTIC OCEAN
EFFLUENT DISCHARGE
SAND
UPLAND
RUM DISTILLERY
ROCKS
N
S
E
W

Figure 3. Diagram of Methane Digester Model. Abbreviations: A= pH Meter, B= pH Probe, C= Methanogenic Bacteria, D= Magnetic Stirrer, E= Stirrer Controller (4.5 rpm), F= Temperature Controller (25°C), G= Stirring Bar, H= Rum Effluents, I= Gas Escape Tubing, J= Rubber Stopper, K= Distilled Water Escape System.



A
B
C
D
E
F
G
H
I
J
K

Figure 4. Densities of Bacterial Populations in Rum Effluents. (Mean ± SE: □ Untreated Effluents; ▥ Treated Effluents; * Undischarged Effluents)

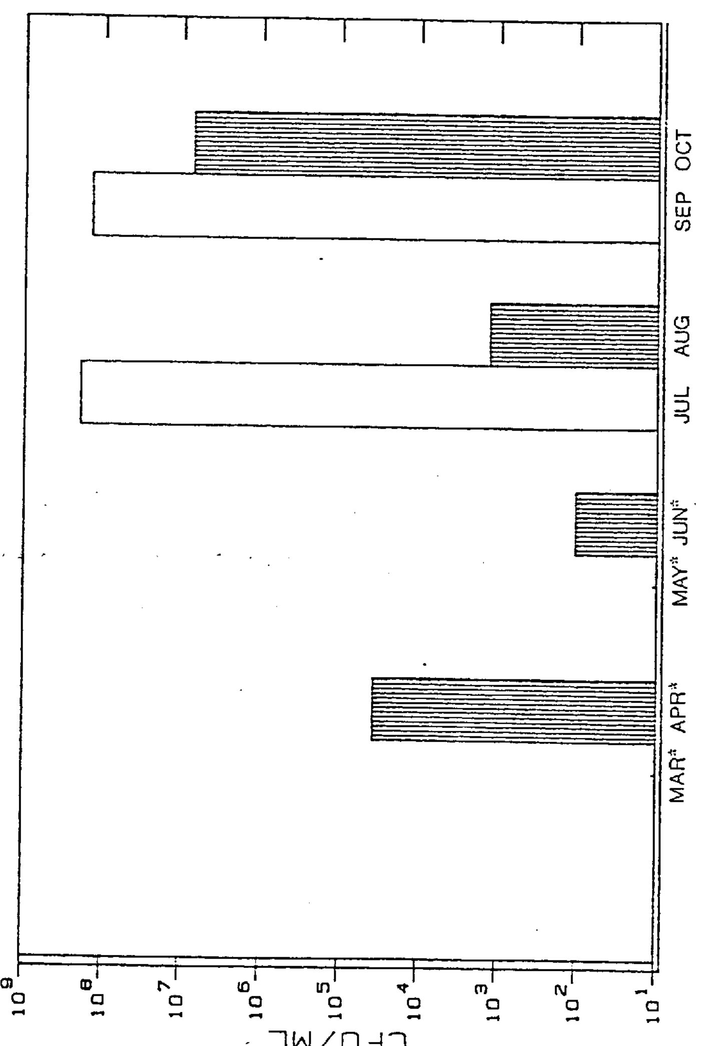
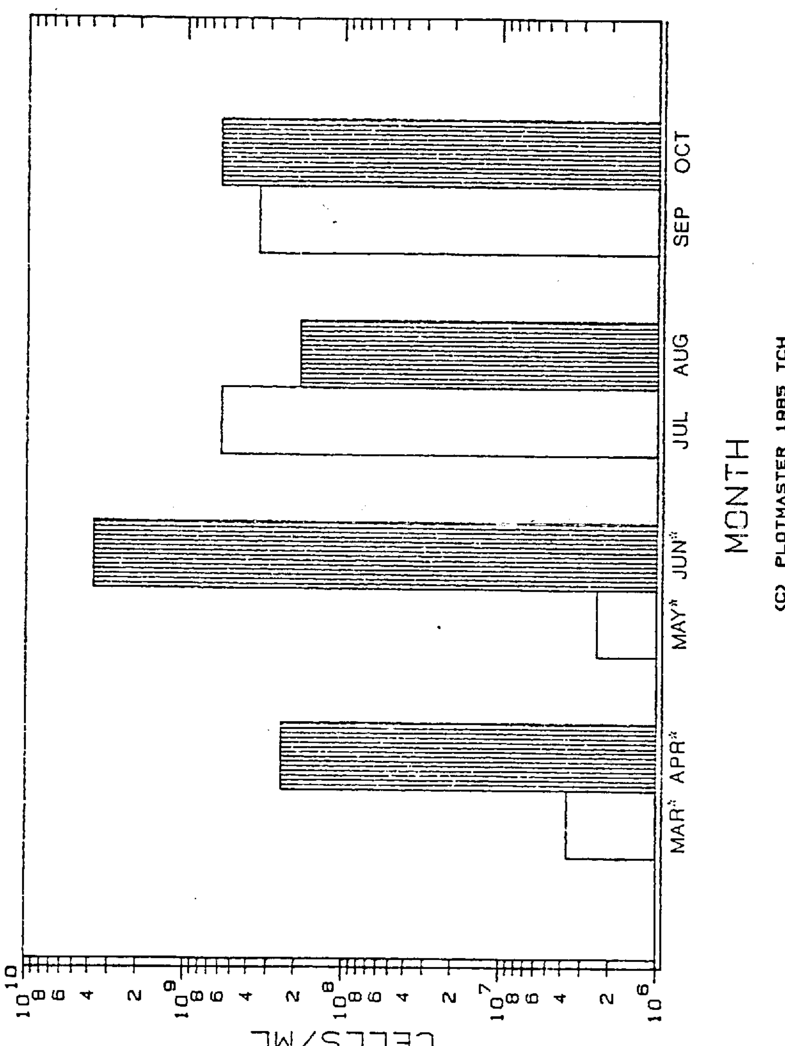
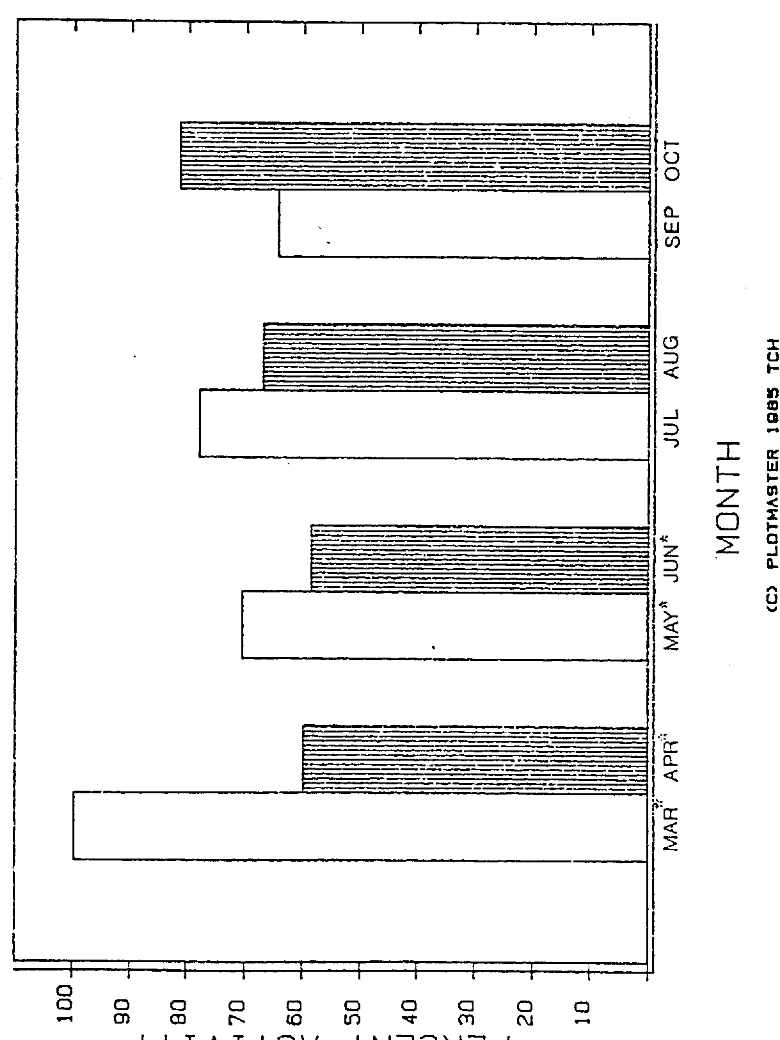

CFU/ML
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
MAR*
APR*
MAY*
JUN*
JUL
AUG
SEP
OCT
MONTH
(C) PLOTMASTER 1985 TCH

Figure 5. Total Cell Counts of Bacterial Populations in Rum Effluents. (Mean ± SE: ▭ Untreated Effluents; ▥ Treated Effluents; * Undischarged Effluents)



CELLS/ML
10^10
10^9
10^8
10^7
10^6
MAR*
APR*
MAY*
JUN*
JUL
AUG
SEP
OCT
MONTH
(C) PLOTMASTER 1985 TCH

Figure 6. Percent Activity of Bacterial Populations in Rum Effluents. (Mean $\pm$ SE: ▭ Untreated Effluents; ▥ Treated Effluents; * Undischarged Effluents)

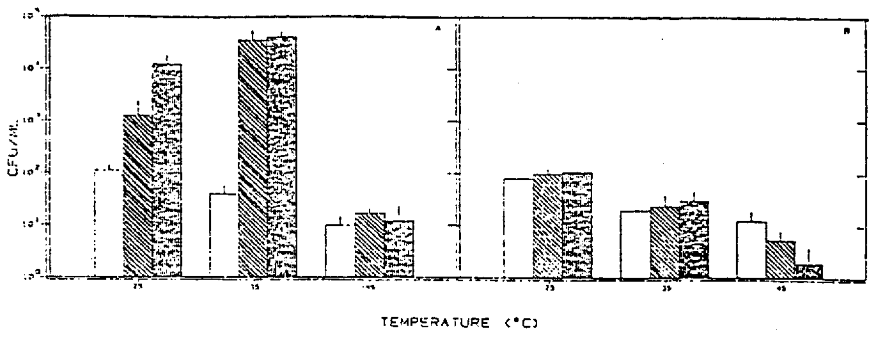


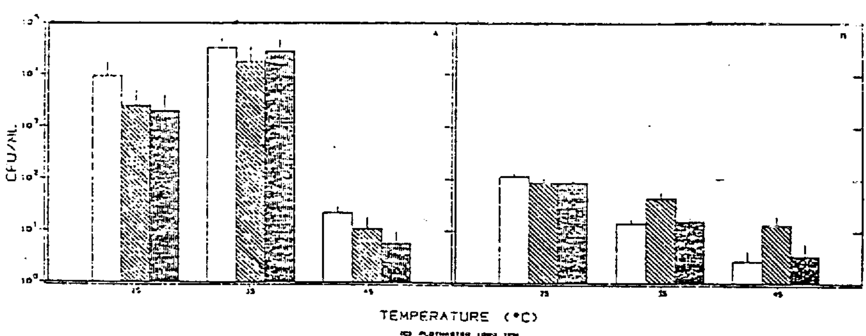
PERCENT ACTIVITY
100
90
80
70
60
50
40
30
20
10
MAR*
APR*
MAY*
JUN*
JUL
AUG
SEP
OCT
MONTH
(C) PLOTMASTER 1985 TCH

Figure 7. Densities of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents. (Mean $\pm$ SE: A= Untreated Effluents, B= Treated Effluents: pH 4.6; pH 6.6; pH 8.6)

Figure 8. Densities of *Vibrio cholerae* ATCC 25872 at Various Rum Effluent Concentrations Incubated at Selected Temperatures. (Mean $\pm$ SE: A= Untreated Effluents, B= Treated Effluents: 25%; 50%; 100%)

Figure 9. Densities of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations. (Mean $\pm$ SE: A= Untreated Effluents, B= Treated Effluents: 25%; 50%; 100%)


CFU/ML
TEMPERATURE (°C)


CFU/ML
TEMPERATURE (°C)

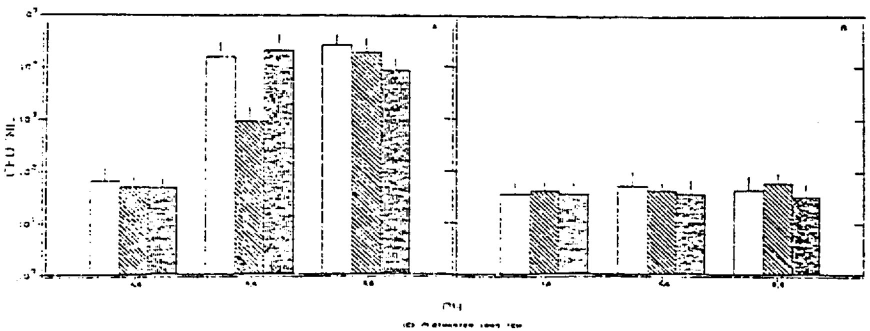
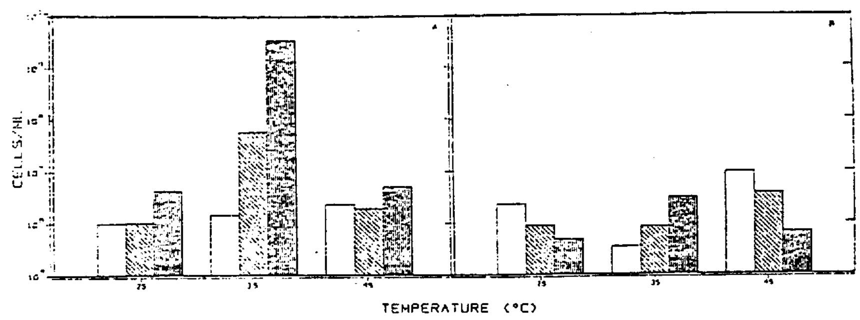
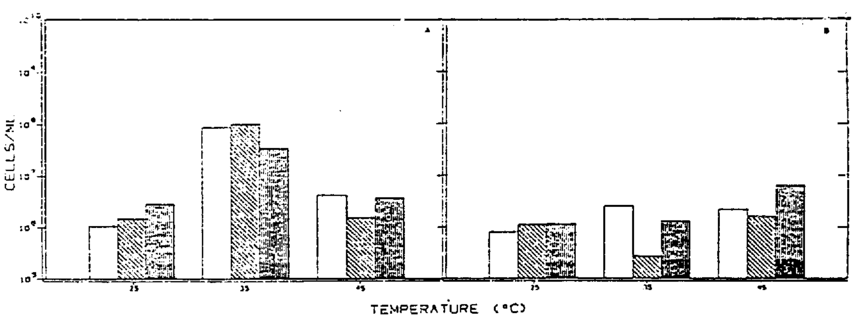

CFU/ML
pH

Figure 10. Total Cell Counts of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents. (Mean ± SE: A= Untreated Effluents, B= Treated Effluents: □ pH 4.6; ▨ pH 6.6; ▥ pH 8.6)

Figure 11. Total Cell Counts of Vibrio cholerae ATCC 25872 at Various Effluent Concentrations Incubated at Selected Temperatures. (Mean ± SE: A= Untreated Effluents, B= Treated Effluents: □ 25%; ▨ 50%; ▥ 100%)

Figure 12. Total Cell Counts of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations. (Mean ± SE: A= Untreated Effluents, B= Treated Effluents: □ 25%; ▨ 50%; ▥ 100%)


A
B
CELLS/ML
25
35
45
TEMPERATURE (°C)


A
B
CELLS/ML
25
35
45
TEMPERATURE (°C)

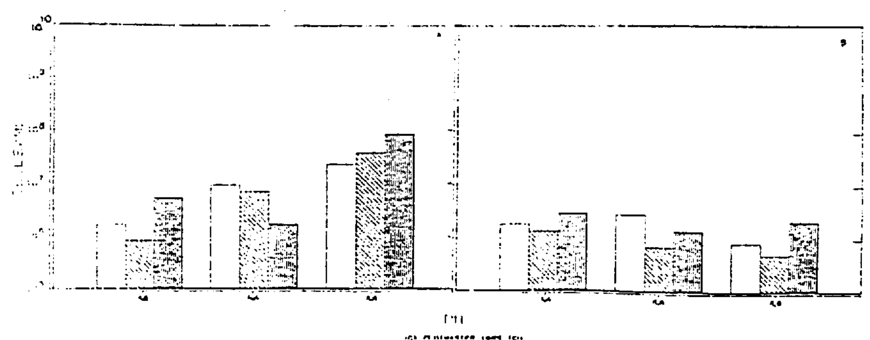
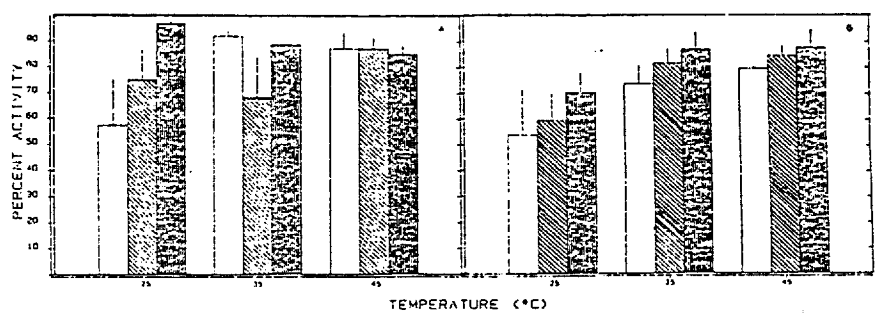
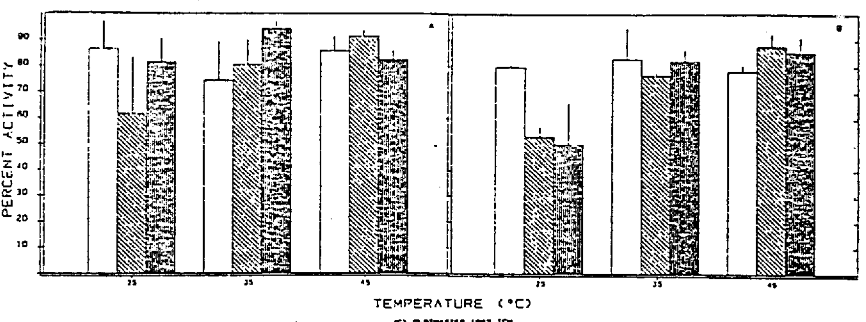

B

Figure 13. Percent Activity of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents. (Mean $\pm$ SE: A= Untreated Effluents, B= Treated Effluents: pH 4.6; pH 6.6; pH 8.6)

Figure 14. Percent Activity of *Vibrio cholerae* ATCC 25872 at Various Effluent Concentrations Incubated at Selected Temperatures. (Mean $\pm$ SE: A= Untreated Effluents, B= Treated Effluents: 25%; 50%; 100%)

Figure 15. Percent Activity of *Vibrio cholerae* ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations. (Mean $\pm$ SE: A= Untreated Effluents, B= Treated Effluents: 25%; 50%; 100%)


PERCENT ACTIVITY
TEMPERATURE (°C)


PERCENT ACTIVITY
TEMPERATURE (°C)

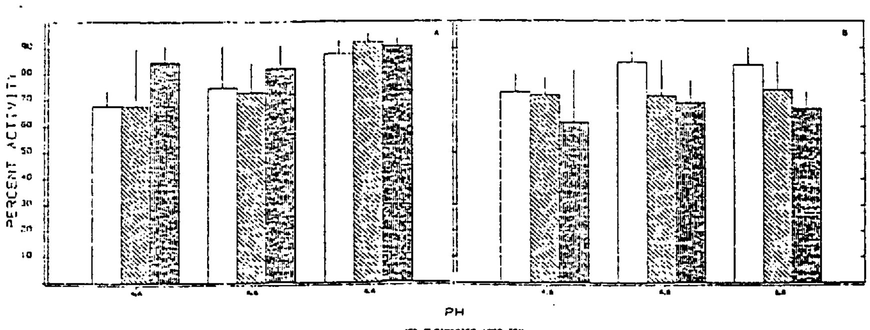
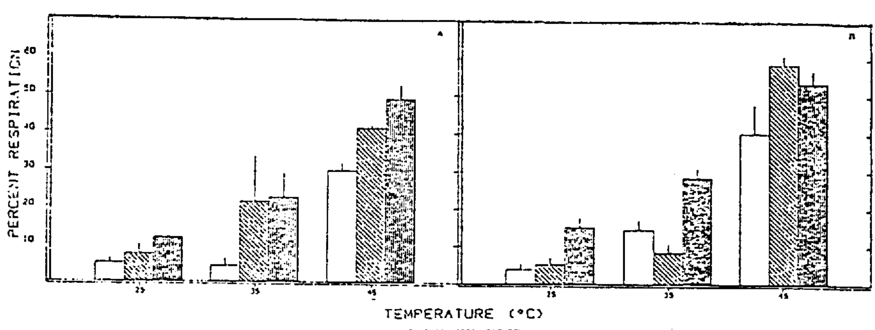
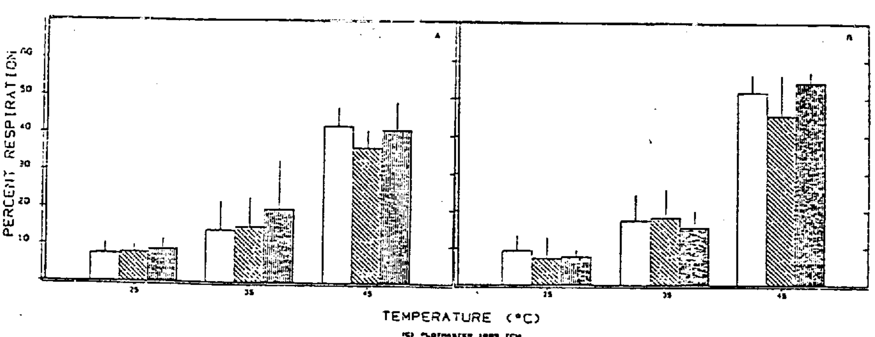

PERCENT ACTIVITY
PH

Figure 16. Percent Respiration of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Temperatures in Rum Effluents. (Mean ± SE: A= Untreated Effluents, B= Treated Effluents: ▭ pH 4.6; ▨ pH 6.6; ▨ pH 8.6)

Figure 17. Percent Respiration of Vibrio cholerae ATCC 25872 at Various Effluent Concentrations Incubated at Selected Temperatures. (Mean ± SE: A= Untreated Effluents, B= Treated Effluents: ▭ 25%; ▨ 50%; ▥ 100%)

Figure 18. Percent Respiration of Vibrio cholerae ATCC 25872 at Various pH Incubated at Selected Rum Effluent Concentrations. (Mean ± SE: A= Untreated Effluents, B= Treated Effluents: ▭ 25%; ▨ 50%; ▥ 100%)


PERCENT RESPIRATION
TEMPERATURE (°C)


PERCENT RESPIRATION
TEMPERATURE (°C)

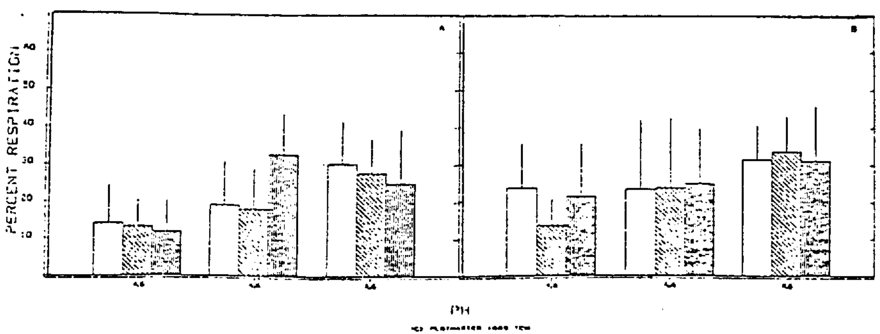
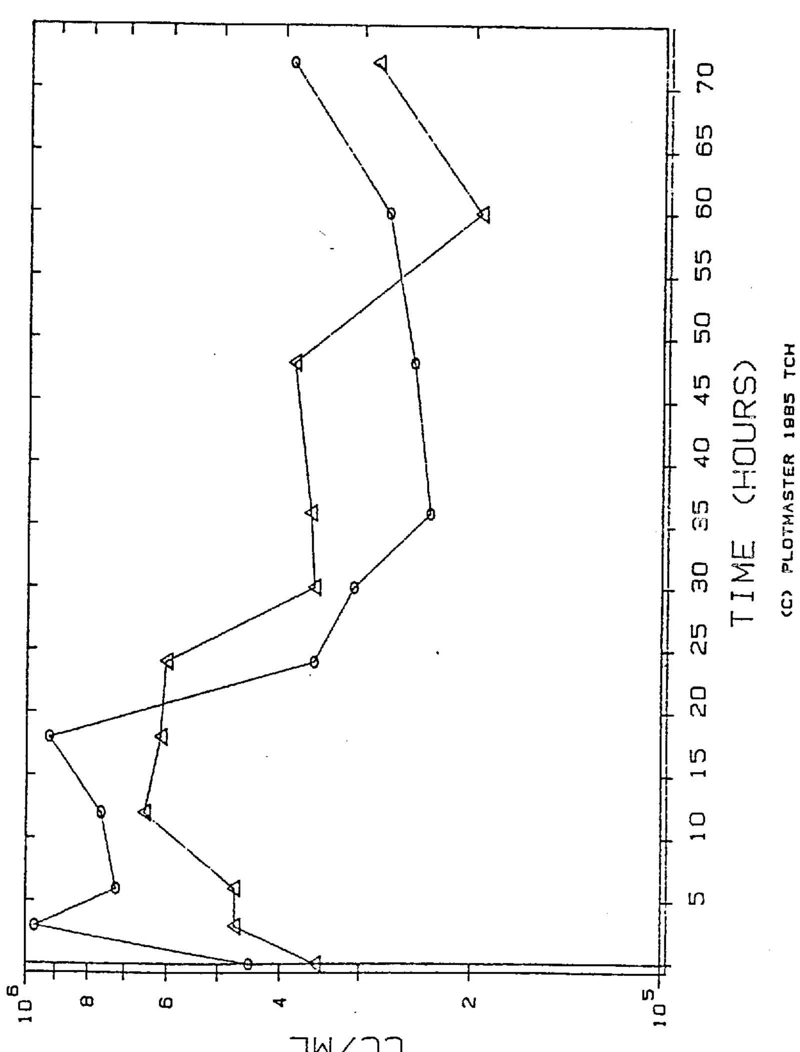

PERCENT RESPIRATION
PH

Figure 19. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Coulter Counter Counts. (Mean ± SE: ○ Untreated Effluents; △ Treated Effluents)



CC/ML
10^6
8
6
4
2
10^5
5
10
15
20
25
30
35
40
45
50
55
60
65
70
TIME (HOURS)
(C) PLOTMASTER 1985 TCH

Figure 20. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Total Cell Counts. (Mean ± SE: ○ Untreated Effluents; △ Treated Effluents)

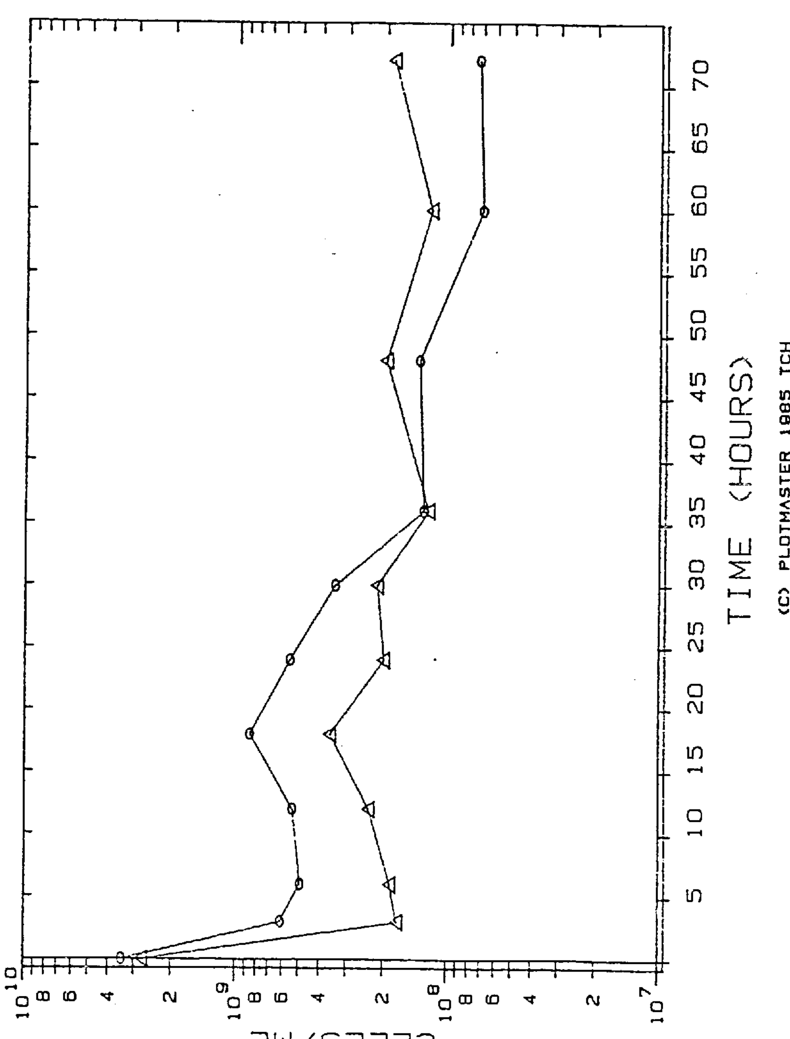

CELLS/ML
10^{10}
8
6
4
2
10^{9}
8
6
4
2
10^{8}
8
6
4
2
10^{7}
5 10 15 20 25 30 35 40 45 50 55 60 65 70
TIME (HOURS)
(C) PLOTMASTER 1985 TCH

Figure 21. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Percent Activity. (Mean ± SE: ○ Untreated Effluents; △ Treated Effluents)

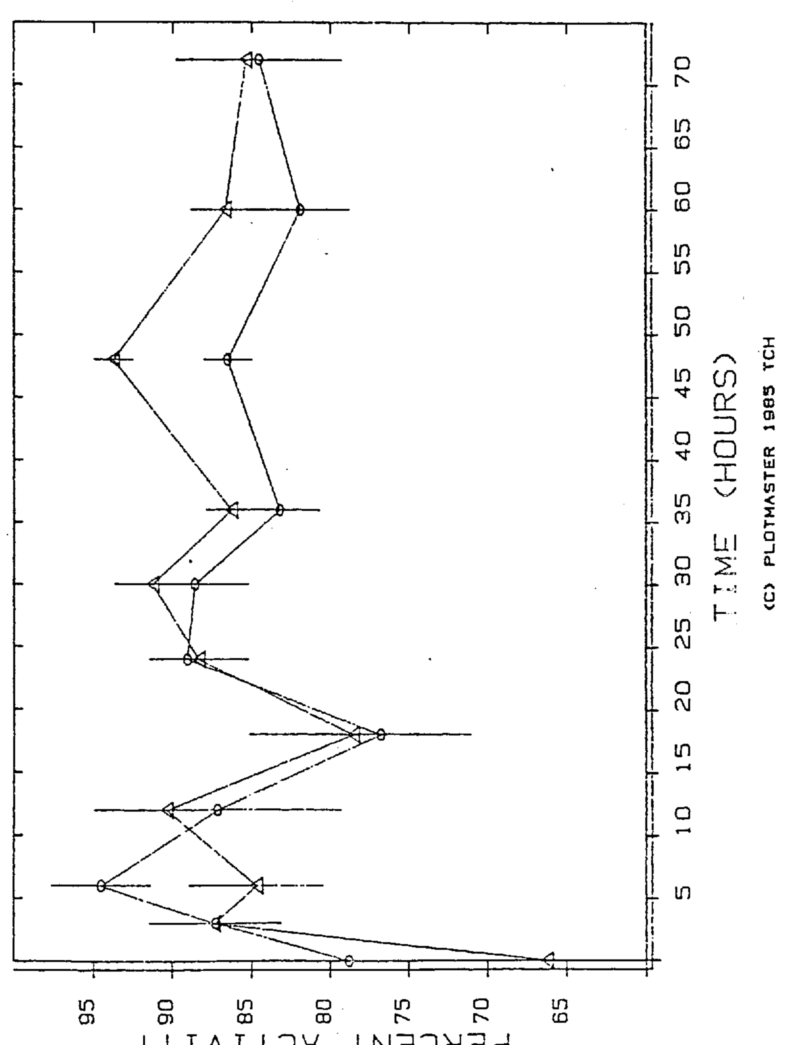
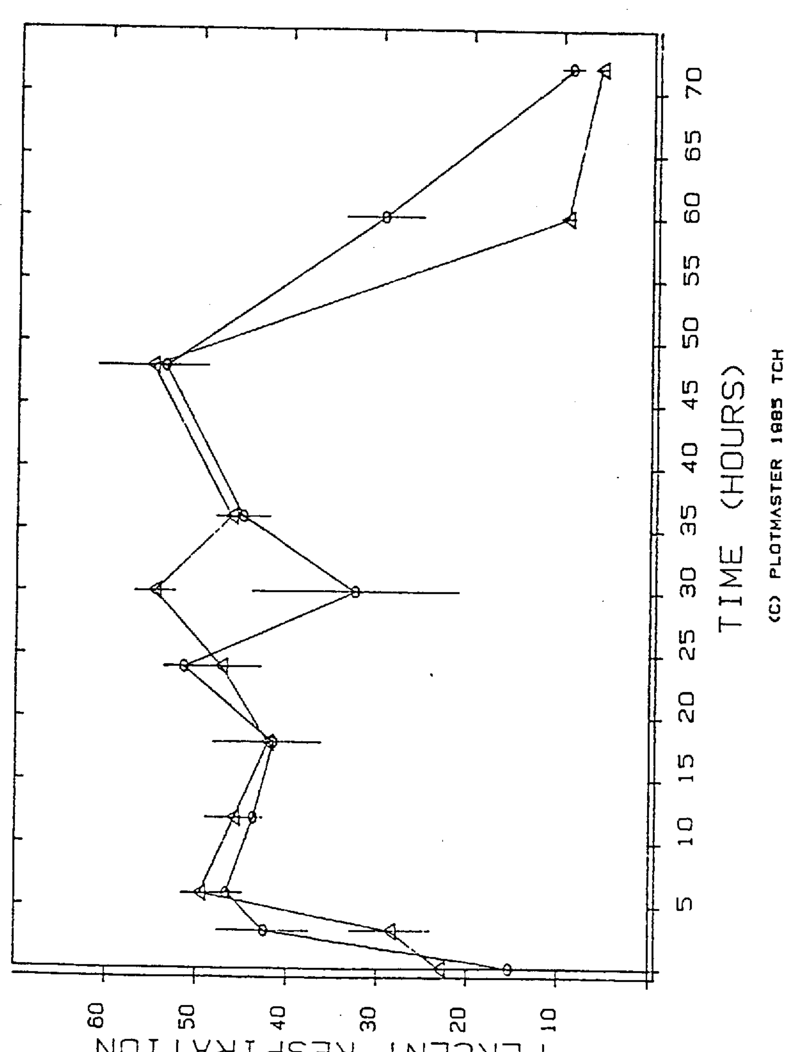

PERCENT ACTIVITY
95
90
85
80
75
70
65
TIME (HOURS)
5
10
15
20
25
30
35
40
45
50
55
60
65
70
(C) PLOTMASTER 1985 TCH

Figure 22. Survival of *Vibrio cholerae* ATCC 25872 in Rum Distillery Effluents; Percent Respiration. (Mean ± SE: ○ Untreated Effluents; △ Treated Effluents)


PERCENT RESPIRATION
60
50
40
30
20
10
TIME (HOURS)
5
10
15
20
25
30
35
40
45
50
55
60
65
70
(C) PLOTMASTER 1985 TCH