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DO FECAL INDICATOR BACTERIA MULTIPLY IN THE SOIL ENVIRONMENTS OF HAWAII?

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EXECUTIVE SUMMARY

Concentrations of fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) are routinely used by regulatory agencies to monitor the hygienic quality of recreational waters and to establish water quality standards. In using these fecal indicator bacteria as an index of water quality, it is generally assumed that (1) *there are no significant environmental sources of these bacteria* and (2) *the fecal indicator bacteria do not multiply in the environment*. However, studies conducted in Hawaii, Guam and Puerto Rico have demonstrated that all of the USEPA recommended microbial indicators of water quality are readily recoverable in high concentrations from environmental waters as well as environmental sources such as plants and soil. Based on the studies completed in Hawaii, we concluded that soil is the primary environmental source of fecal indicator in Hawaii and that rain is the mechanism by which these soil-bound fecal bacteria are transported in high concentrations into streams and rivers. We hypothesized that the soil-borne fecal bacteria must be multiplying in order to maintain a continuous population in the soil. However, the data we obtained did not clearly differentiate between persistence and actual multiplication of the fecal bacteria in soil. Demonstration that fecal indicator bacteria multiply in soil is critical because concentrations of fecal bacteria in environmental waters which reflect multiplication of fecal bacteria in the environment no longer can be associated with the degree of fecal contamination or the presence of many sewage-borne pathogens such as viruses and protozoa which cannot multiply in the environment.

The goals of this study were as follows: (1) to reconfirm the earlier findings that tropical environmental conditions such as in Hawaii favor the establishment of fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) in the soil environment, (2) to characterize the physical and biological factors in soil which control the survival and multiplication of fecal bacteria, (3) to obtain definitive data that fecal indicator bacteria can multiply in the soil under tropical conditions, (4) to propose a hypothesis for the establishment of fecal bacteria as a group of soil microorganisms and (5) to make an assessment and recommendation to UESPA with regard to recreational water quality standards for Hawaii and other tropical locations. The important findings of this project are summarized below.

High concentrations of fecal indicator bacteria can be readily recovered from stream waters and soils in Hawaii even in the absence of any significant fecal or sewage contamination of these natural environments. Fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) were recovered in locations throughout the island of Oahu which represented different environmental conditions and different types of soils. These results show that fecal indicator bacteria have established populations in all the soil environments of Oahu and can impact the environmental waters throughout the island of Oahu. These results support the hypothesis that fecal indicator bacteria can establish populations in the soil environment of Hawaii because the temperature, humidity and other properties of tropical soil are within the adaptive capabilities of fecal bacteria.

The identity of the fecal bacteria recovered from soil was confirmed as *E. coli* and six species of *Enterococcus* using established bacterial identification systems such as API 20E, API 20 Strep and Biolog system. The results of the Biolog system which tests the ability of isolates to metabolize 95 different carbon sources showed that the soil isolates of *E. coli* and enterococci were metabolically diverse. These results support the notion that most strains of fecal indicator bacteria can multiply in the soil environment and that the soil environment is not selecting for a mutant strain with special properties to enable them to multiply in the soil

Soil moisture was a physical characteristic of soil which controlled the concentrations of fecal bacteria in soil. Populations of *E. coli* were much more sensitive to drop in soil moisture than populations of enterococci. Moisture content of natural soil varies considerably and is one factor which determines the variable concentrations of fecal indicator bacteria in soil. Drop in soil moisture was shown to stress the fecal bacterial cells but enough of the cells survived to multiply when moisture and other conditions become favorable. Soil was shown to contain sufficient nutrients to support the growth of *E. coli* and enterococci. This conclusion was based on extracting nutrients from soil and showing that the fecal indicator bacteria can multiply on the soil extract growth medium.

Experiments to demonstrate that multiplication of fecal indicator bacteria occur in natural soil environment is difficult because the variables that occur under field conditions are too many. For better interpretation of results, experiments were conducted under laboratory, simulated filed and finally actual field conditions. The results under laboratory controlled conditions predicted the results obtained under simulated field and under field conditions. Multiplication of fecal bacteria in soil was shown by measuring its metabolic activity in soil. Moreover, many experiments showed that populations of pure cultures of fecal indicator bacteria added to soil or populations of fecal indicator bacteria indigenous to soil increased in concentrations in soil samples. Genetic evidence for the multiplication of *E. coli* in soil was provided by showing that populations of *E. coli* with genetic markers such as ability to produce light or to be resistant to antibiotic multiplied in soil samples.

The experimental results indicated that fecal bacteria (fecal coliform, *E. coli*, enterococci) were capable of establishing and multiplying in sterile soil. However, in natural soil which contains indigenous microflora, the multiplication of the fecal bacteria was not noticeable until excess nutrients were added to the soil indicating that other indigenous microorganisms that are more numerous and better adapted to the soil conditions were out-competing fecal bacteria for usable energy sources in the soil. Despite this disadvantage, the fecal indicator bacteria has developed a strategy for survival which best fits into the feast and famine model, a scheme for survival used by many other types of bacteria. For most of the time, fecal indicator are in a famine state and do not multiply but remain metabolically ready to multiply when nutrients become available at low dose or following some events at high dose (feast). It should be noted that in the strategy for survival, success is measured in ensuring that the population survives in that environment

over a long period of time. Thus, rapid growth is not essential for the maintenance of a stable population.

In conclusion, although fecal indicator bacteria represent a small fraction of the microbial population in soil, their concentration in soil and environmental water is significant because it nullifies the two assumptions (that *there are no major environmental sources of these bacteria, and the fecal bacteria do not multiply in the environment*) used in the application of the USEPA recommended recreational water quality standards. Thus, there is a need to use an alternate microbial indicator to effectively monitor the quality of recreational waters in Hawaii and other tropical locations. Studies conducted in Hawaii indicate that *Clostridium perfringens,* an alternative fecal indicator bacteria, can be used to establish recreational water quality standards in Hawaii because there is no significant environmental source of *C. perfringens* and the concentrations of this fecal bacteria in environmental waters correlates well with pollution with sewage.

CHAPTER 1

INTRODUCTION TO STUDY

I. Use of Fecal Indicators to Establish Recreational Water Quality Standards

The Clean Water Act (P.L. 92-500) was passed in 1972 as a federal law to ensure that every state meets the same minimal standard of treatment for wastewater and to ensure that the treated wastewater will not cause harmful pollution of environmental waters. The United States Environmental Protection Agency (USEPA) was established as the federal agency to implement the goals of the Clean Water Act. To meet these goals, USEPA established guidelines and regulations for minimal treatment of wastewaters and instituted water quality standards on the use of those environmental waters. For waters classified for recreational (swimming) use, USEPA identified fecal-borne microbial pathogens as the contaminants in water with greatest potential for transmitting diseases. The primary source of fecal-borne pathogens is feces of human and warm blooded animals. The diseases that are transmitted as a result of ingestion of waters contaminated with these pathogens are referred to as waterborne diseases.

Ideally, water should be tested for pathogens to determine whether the water is contaminated with pathogens. However, this is not practical nor feasible because there are numerous and different types fecal-borne pathogens (bacteria, viruses, protozoans) and special methods are required to detect different pathogens. Moreover, the diagnostic tests for pathogens are expensive, inefficient or not available. Finally, even if a water sample is tested and determined to be negative for some pathogens, one cannot conclude that the water is free of all other pathogens. As a result, waters are not monitored for pathogens and USEPA has established recreational water quality standards based on the concentrations of bacteria that are normally present in feces in high concentrations. The theoretical basis for use of fecal-borne bacteria as an index of water quality is that the recovery of such bacteria from a water sample indicates that the water is contaminated with feces. Moreover, increasing concentration of fecal indicator bacteria in the water represents increasing contamination with feces and the greater likelihood that fecal-borne pathogens are present in the water. The ideal criteria for selecting a suitable group of fecal indicator bacteria to establish water quality standards have previously been reported by Dutka (1973) and can be summarized as follows.

- It must be consistently present in feces and at higher concentrations than fecal-borne pathogens.
- It must not multiply outside the human intestinal tract.
- It must be equal to or more resistant than pathogens to environmental conditions and to disinfection.
- It must be detected in water by simple and reliable methods.

Initially, 1000 total coliform bacteria/100 ml was the recreational water quality standard in the US. In 1972, USEPA for the first time established a national recreational water quality standard and recommended that the standard be set at 200 fecal coliform/100 ml because fecal coliform bacteria were more specifically associated with feces than total coliform bacteria. However, these fecal coliform standards were not based on valid epidemiological studies and evidence to demonstrate that the risk to waterborne diseases would proportionately increase as the concentrations of these bacteria in the water increased could not obtained.

To address the limitation of the fecal coliform standard, USEPA conducted long-term, intensive and well designed epidemiological and water quality studies (Cabelli, 1981; Dufour, 1984) which resulted in the following two significant conclusions: (1) concentrations of total and fecal coliform bacteria in recreational waters could not be used to predict incidences of waterborne diseases and (2) concentrations of other indicator bacteria such as enterococci in marine waters and enterococci and *Escherichia coli* in fresh waters could be used to predict incidences of waterborne diseases associated with recreational waters known to be contaminated with a sewage source. As a result of those studies, USEPA established a new set of recreational water quality standards in 1986 based on concentrations of *E. coli* and enterococci. For marine waters, the recommended standard was a geometric mean of 35 enterococci/100 ml and for freshwater the standard was a geometric mean of either 126 *E. coli*/100 ml or 33 enterococci/100 ml. All states were urged to implement the new standards that were based on risk levels. However, in 1990, the state of Hawaii adopted a more stringent recreational water quality standard of 7 enterococci/100 ml for its marine waters, but retained the old standard of 200 fecal coliform/100 ml for the island freshwaters.

II. Inherent Problems in Applying the Water Quality Standards to Freshwater Streams in Hawaii and Other Tropical Islands

The inherent problems in applying the USEPA recommended water quality standards of 1986 to freshwater streams in Hawaii and other tropical islands has been extensively emphasized in studies conducted in Hawaii and elsewhere. Studies conducted in Hawaii (Fujioka and Byappanahalli, 1996; Fujioka, 1983; Fujioka and Shizumura, 1985; Fujioka et al., 1988; Hardina and Fujioka, 1991), in Guam (Fujioka, 1989) and in Puerto Rico (Bermudez and Hazen, 1988; Hazen, 1988; Toranzos, 1991) indicated that the USEPA recreational water quality standards may not be applicable to tropical freshwater environments because the following two criteria used in establishing national recreational water quality standards may not be applicable to Hawaii and other tropical islands.

A. The only significant sources of fecal indicator used in establishing recreational water quality standards are feces of human/warm blooded animals and sewage

This criterion is important because in the assessment of environmental water quality, it is assumed that there is no significant environmental source of indicator bacteria other than those from feces or sewage. However, data have been obtained to show that in tropical islands such as Hawaii, fecal bacteria (fecal coliform, *E. coli*, fecal stereptococci, enterococci) are consistently present in high concentrations in freshwater streams and storm drains in the absence of known sources of fecal contamination. Moreover, these fecal indicator bacteria have also been recovered in high concentrations in natural soil conditions in Hawaii and on some plants along stream beds. Thus, environments in tropical islands are significant sources of fecal indicator bacteria and the detection of such bacteria in environmental waters do not necessarily indicate that the water is contaminated with fecal matter. Therefore, the above criterion does not appear to be applicable in evaluating the quality of freshwaters in tropical environments.

B. Indicator bacteria used in establishing recreational water quality standards should not multiply in the environment

This criterion is especially important because the concentrations of indicator bacteria in environmental waters are used to assess the presence and expected concentrations of fecal-borne pathogens in that water sample. However, it is known that bacterial indicators used in recreational water quality standards have the potential to multiply outside the human intestinal tract whereas, most of the human intestinal pathogens (viruses, protozoa) cannot multiply in the environment. The consistently high concentrations of fecal indicator bacteria recovered from the streams and soils of Hawaii (Fujioka and Byappanahalli 1996; Fujioka, 1983; Fujioka and Shizumura, 1985; Fujioka et al., 1988; Hardina and Fujioka, 1991) and Guam (Fujioka, 1989) strongly suggest that these indicator bacteria are able to multiply in soil under natural conditions. However, at present there is no substantial evidence to indicate that fecal bacteria can multiply in soil under natural conditions. If indicator bacteria are multiplying in the soil environment, the concentrations of indicator bacteria recovered from streams of Hawaii can no longer be used to predict the presence of pathogens in those streams. Moreover, if multiplication of indicator bacteria in the soil environment can be proven, it will explain the widespread occurrence of indicator bacteria in freshwater streams of tropical islands. Thus, it is critical that studies be conducted to ascertain whether fecal indicator bacteria can or cannot multiply in natural soil under tropical conditions.

III. Soil as a Habitat for Microorganisms in General and Fecal Indicator Bacteria in Particular

Soil is made of a dynamic combination of variously sized organic and mineral particulate matter, living organisms and their nonliving remains. Soil has been described as the most complex microbiological medium by researchers (Metting, 1985; Stotzky, 1972). The predominant microflora in soil include, algae, bacteria, fungi, protozoa and viruses. The main focus of this section is to evaluate soil as a potential habitat for fecal indicator bacteria to persist and to multiply over time. In addition, the following section will also emphasize how the climatic and the soil conditions of Hawaii can select a sub-population of fecal indicator bacteria to establish and persist in Hawaii's soil environment .

A. Geographic location

The Hawaiian islands are located in the sub-tropical region of the Pacific Ocean and the islands extend from 18° 54' to 28° 15' north latitude and from 154° 40' to 178° 15' west longitude (Morgan, 1996). Geographically speaking, the Hawaiian islands are the most isolated groups of islands in the world. Of the 132 islands, only 8 islands (Hawaii, the Big island, Kahoolawe, Kauai, Lanai, Maui, Molokai, Niihau and Oahu) are inhabited. The temperatures are generally warmer throughout the year. Average temperatures for the warmest and coolest months differ only by less than 9°F for all locations below 5000'. During summer months, temperatures at most locations close to sea level generally do not exceed 90°F. Although the geographical area of the state is about 6500 square miles, there is tremendous diversity of climates. For instance, the climate can range from desert type to wettest spot on earth (Waialeale, Kauai) and from incessant warm weather to periods of snow.

B. Soil diversity in Hawaii

Soils are defined as the products of the interactions between climate and the geologic formations of the earth's crust (Wambeke, 1992). Soil formation is basically a weathering process of rocks that results from physical, chemical and biological forces. The primary step in soil formation is the erosion of the parent material. In the Hawaiian islands, the parent material (***basaltic rock***) is distinctly different from that (***granitic rock rich in mineral quartz***) found on the mainland United States (Morgan, 1996). The nature of the parent material as well as the conditions under which soils are formed in Hawaii have resulted in Hawaiian soils being characteristically different from those of the continental United States. The soils in Hawaii are in many places very deep, rich in iron and aluminum, free of quartz sand and highly permeable (Morgan, 1996). Moreover, Hawaii is the first state in the nation to classify its soils according to the new classification scheme proposed by the US Soil Conservation Service in 1960. To illustrate the uniqueness of Hawaii in terms of soil diversity, one can find 8 of the 10 soil orders on the island of Oahu (refer map in **Figure 1.1**) which has a geographical area of only 608 square miles.

However, on the mainland, it may not be possible to see such soil diversity in any state or region of comparable geographical area.

C. Acclimatization of fecal indicator bacteria to Hawaii's soils

Although soil as a habitat for microorganisms has been extensively studied, the extent of microbial diversity in soil is not well understood. Among soil microorganisms (culturable), bacteria are the most numerous. For instance, culturable bacteria alone from a temperate mollisol in Midwestern North America can range from 10^8-10^9/g soil (**Table 1.1**). Moreover, it is largely speculated that about one to five percent of all microorganisms on earth have been classified and named (ASM 1994; Ward et al., 1992; Hawksworth and Mound, 1991). As many as 4000 bacterial species may occur in a gram of soil (Tate, 1997). Such being the case, the sheer diversity and counts of bacterial communities alone in the soil are difficult to comprehend.

The counts of microorganisms and their aggregated biomass can vary significantly within and among soils. Some of the soil related factors such as soil type, particle size distribution, and aggregate stability are known to influence the range of microbial diversity estimates in soil (Richaume et al., 1993). In summary, soil as a medium for living organisms has the potential to support the growth of diverse and innumerable microflora.

Since the natural habitat of the fecal indicator bacteria is the gastrointestinal tract of human and warm-blooded animals, the current dogma is that these bacteria are not expected to survive and to multiply in natural habitats such as soil and environmental waters. However, the principle of microbial ecology states that microorganisms are very adaptable and will use all their capabilities to grow and establish themselves in any environment. Based on this principle, and the fact that fecal indicator bacteria such *as E. coli* have simple nutritional requirements and can grow well at 20-25°C, the growth and establishment of *E. coli* in the soil environments of tropical areas are reasonable expectation. A likely scenario is that fecal bacteria originally from human or animal feces were deposited on the soil and over a period of time, a sub-population of these bacteria not only survived in the soil, but also acclimatized to the new environment. Favorable environmental conditions such as year-round warm weather, high humidity, and occurrence of diverse soil types seem to have favored these bacteria to adapt to their new environment.

D. Multiplication of fecal indicator bacteria in soil

At present there is little evidence to suggest that fecal bacteria are able to multiply outside of their natural habitat. Available data indicate that these bacteria can survive for extended periods in stream sediments (Davies et al., 1995; Gary and Adams, 1985; Sherer et al., 1992; Stephenson and Rychert, 1982) and occasional increase in the concentrations of these bacteria in streamwaters is due to resuspension of the sediment-borne fecal bacteria into overlaying water (Gary and Adams, 1985; Stephenson and Rychert, 1982). However, there is no convincing evidence to demonstrate that sediment-borne fecal bacteria can eventually multiply under ambient conditions.

Soil's ability to support growth and multiplication of the various microflora is a well known fact. Therefore, it can be assumed that the soil environment has potential to support the growth of fecal bacteria. However, previous studies have not been designed to conclusively show that fecal bacteria can grow and multiply in the soil environment. For example, *E. coli* has been extensively used as a model organism in various soil-based experiments to understand genetic mechanisms such as conjugation and transduction in natural habitats (Krasovsky and Stotzky, 1987; Trevors and Oddie, 1986; Trevors and Starodub, 1987; Zeph et al., 1988) and to determine the fate of genetically engineered organisms under natural conditions (Devanas and Stotzky, 1986; Rattaray et al., 1990; Recorbet, 1992). There has been many studies conducted before to study indigenous microorganisms in soil. However, multiplication of fecal bacteria in soil has not been addressed because this hypothesis has not been proposed previously. Therefore, soil as a habitat for fecal indicator bacteria to persist and to multiply in this vastly studied, but less understood habitat needs further investigation.

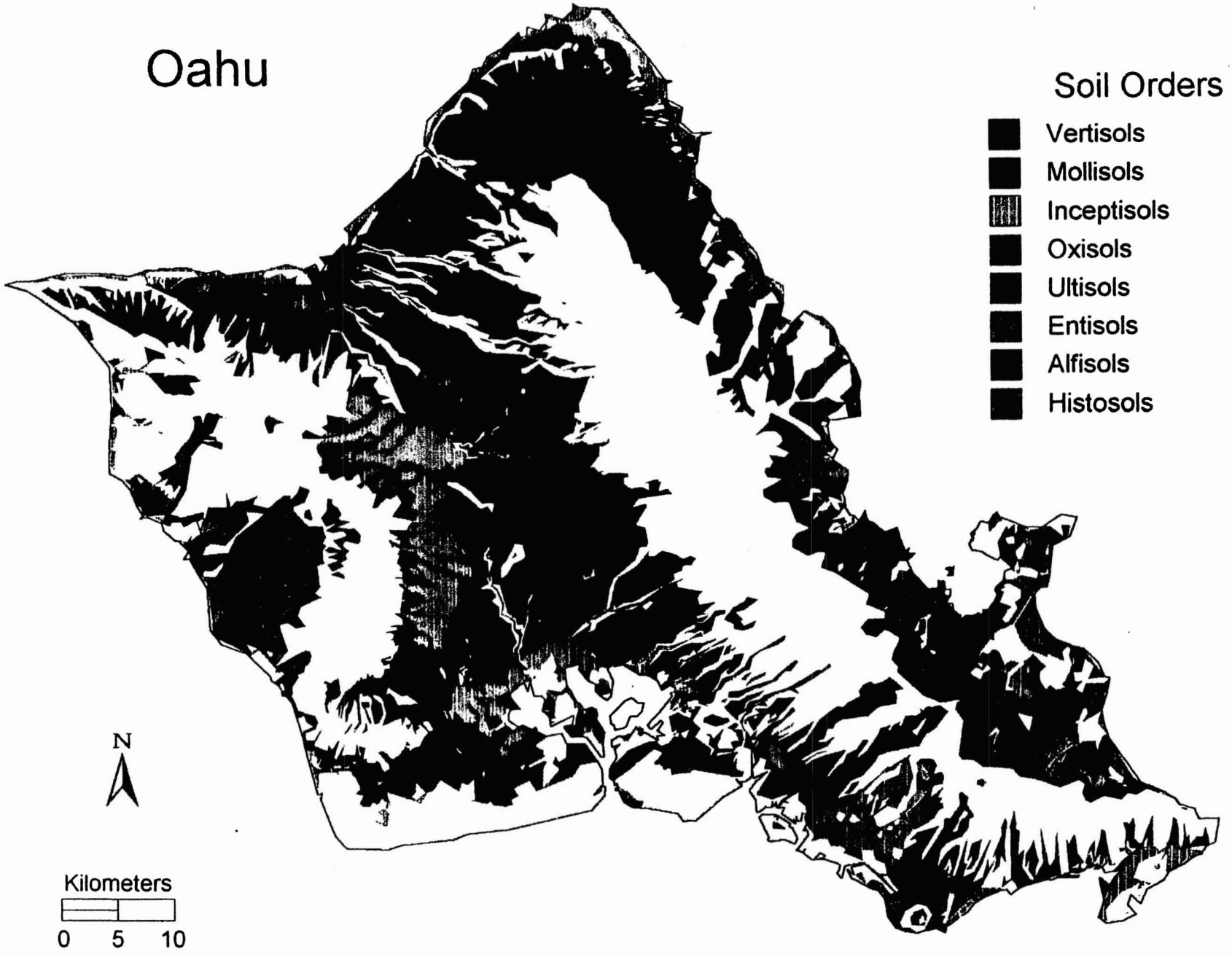


Figure 1.1. Various Soil Orders Found on the Island of Oahu.
(Photo Courtesy of P. Kilham, Department of Agronomy and

Table 1.1. Relative Counts and Approximate Biomass of the Soil Microorganisms in a Fertile Mollisol.

Organism	Numbers (per g)	Biomass (wet kg/ha)
Actinomycetes	10^7-10^8	300-3000
Bacteria	10^8-10^9	300-3000
Fungi	10^5-10^6	500-5000
Microalgae	10^3-10^6	10-1500
Protozoa	10^3-10^5	5-200

Source: Metting (1993)

CHAPTER 2

GOALS, OBJECTIVES AND EXPERIMENTAL DESIGN OF THE STUDY

I. Goals

We previously reported that the USEPA recreational water quality standards based on concentrations of fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) are not applicable in Hawaii because in Hawaii, the following two basic assumptions used in the interpretation of recreational water quality standards are not valid: *(1) the only source of fecal indicator bacteria is feces or sewage and there is no significant environmental source of these bacteria and (2) the fecal indicator bacteria do not multiply outside of their natural habitat.*

The goals of this study are as follows: (1) to reconfirm the earlier findings that tropical environmental conditions such as in Hawaii favor the establishment of fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) in the soil environment, (2) to characterize the physical and biological factors in soil which control the survival and multiplication of fecal bacteria, (3) to obtain definitive data that fecal indicator bacteria can multiply in the soil under tropical conditions, (4) to propose a hypothesis for the establishment of fecal bacteria as a group of soil microorganisms, and (5) to make an assessment and recommendation to USEPA with regard to recreational water quality standards for Hawaii and other tropical locations.

II. Objectives

To attain the goals of this study, the following questions (objectives) will be addressed.

- Is soil a natural environmental source of fecal indicator bacteria that are routinely recovered from freshwater streams in Hawaii?
- Can the soil fecal bacterial isolates be identified, confirmed and characterized by accepted cultural and biochemical methods?
- Does soil contain sufficient nutrients to support the growth of fecal indicator bacteria?
- What other soil factors control the survival and multiplication of fecal indicator bacteria in the soil environment of Hawaii?
- What microbial ecological principles support the growth of fecal bacteria in soil environment?

III. Materials and Methods Used

A. Collection of soil samples to determine the prevalence of fecal indicator bacteria in soils on Oahu, Hawaii

1. Selection of sampling sites

To determine the prevalence of fecal indicator bacteria in the soils of Hawaii, we decided to sample soil from various sites representing major soil affiliations (groups) on the island of Oahu, Hawaii. For this purpose, we consulted Dr. Haruyoshi Ikawa, Soil Scientist (now retired), University of Hawaii and with his help we selected the sampling sites. A map of Oahu showing the sampling locations is presented in **Figure 2.1**.

2. Collection and processing of soil samples

Samples of surface soil (0-6 cm depth) were collected from various locations as per standard protocols and placed into sterile bottles/plastic bags and transported back to the laboratory in a cooled ice chest. The samples were analyzed within 24 hours after sampling.

3. Microbiological analysis

The soil samples collected from various locations were analyzed for concentrations of fecal coliform, *E. coli* and enterococci using two different methods. First, by eluting the soil with a buffer and analyzing the soil elute for concentrations of fecal indicator bacteria using the membrane filtration method (Roll and Fujioka, 1993); second, by the most probable number (MPN) technique as described in the Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WEF 1992). The methodology used in this study to recover fecal bacteria from the soil is summarized in **Table 2.1**.

B. Cobalt irradiation of natural Waimanalo soil

Large quantities of sub-surface (5-30 cm depth) soil were collected at the Waimanalo Experimental Research Station of the University of Hawaii for various experiments in this study. A sample of the soil was sent to Agricultural Diagnostic Service Center, University of Hawaii to determine its chemical properties (**Table 2.2**). A portion of the natural soil was stored in plastic bags and refrigerated for later use. The remaining soil was air-dried under laboratory conditions for two days. The air-dried soil was passed through a 4 mm sized sieve to remove big chunks of soil. The sieved soil was transferred to double plastic bags and cobalt irradiated (dose, 1750 kilo rad) for 33 h at the Hawaii Research Irradiator, University of Hawaii, under the supervision of Dr. James H. Moy. The irradiated soil (in plastic bags) was stored under refrigeration for later use. The irradiated was confirmed to be free of any viable microflora.

C. Isolation, identification and characterization of the soil-borne fecal indicator bacteria using standard cultural and biochemical methods

1. E. coli

The isolates of coliform bacteria from soil were speciated using the API 20E (bioMerieux Vitek, Inc., Hazelwood, MO) identification scheme. The API 20E is a traditional identification scheme used for identifying bacteria belonging to the family enterobacteriaceae. About 40 API 20E-confirmed *E. coli* strains were inoculated into Biolog's gram negative (GN) microplates (**Table 2.3**) to reconfirm the API 20 E identification (of soil *E. coli* isolates) and also to examine whether the Biolog identification system (which is based on carbon source utilization pattern), would facilitate in further characterization of the soil *E. coli* strains. *E. coli* (ATCC 25922) was used as a reference strain in this study. The test was repeated at least twice for each isolate. To determine whether *E. coli* strains from soil tend to cluster into distinct groups, cluster analysis was performed using the Microlog's software *mlclust*.

2. Enterococci

The enterococcal isolates (presumptive) from soil were initially identified by standard tests such as gram reaction, esculin hydrolysis, growth at elevated temperature (45°C), growth in 6.5% NaCl at 35°C and the type antigen on the cell surface (APHA/AWWA/WEF, 1992). The enterococci are recognized as gram positive cocci, have the ability to grow at 45°C and in 6.5% NaCl at 35°C, hydrolyze esculin and carry group D antigen on their cell surface. About 40 enterococcal isolates were identified to the species level using the API 20 Strep Kit (bioMerieux Vitek, Inc., Hazelwood, MO).

The Biolog identification scheme was used to determine the metabolic diversity of soil enterococci. In this study, 28 strains belonging to 6 different species of enterococcus (*Enterococcus avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium* and *E. gallinarum*) from soil were inoculated into Biolog's gram-positive (GP) microplates as per procedure outlined in the Biolog Manual. *E. faecalis* (ATCC 29212) and *E. faecalis* (ATCC 19433) were used as reference strains. Each strain was tested at least twice to make sure that the ID pattern was reproducible. Cluster analysis was performed to group enterococcal strains.

D. Evaluation of soil as a medium for the growth of fecal bacteria

1. Preparation of soil extract

The soil used to prepare soil extract was collected from the Waimanalo Experimental Research Station of the University of Hawaii. To prepare soil extract, 1000g of fresh soil in 1 l of tap water was autoclaved at 121°C for 30 minutes. After autoclaving, 0.5 g of calcium carbonate ($CaCO_3$) was added to the suspension to flocculate the clay

fraction and let stand for 30 minutes. The suspension was filtered repeatedly until a clear liquid was obtained. After filtration, the volume was restored to 1000 ml by adding water. The soil extract, thus prepared, was dispensed into smaller volumes, sterilized and stored for later use.

2. Recovery efficiency of E. coli and E. faecalis on soil extract agar and other traditional media

To find out whether nutrients in soil are adequate to support the growth of *E. coli*, a pure culture of an *E. coli* strain (ATCC 25922) was plated on plain soil extract agar (PSEA, soil extract + agar), standard soil extract agar {designated as SEA , soil extract + minimal levels of carbon (glucose), nitrogen and phosphorus + agar} and other traditional media for *E. coli* such as nutrient agar (NA), fecal coliform agar (mFC), eosine methylene blue (EMB) agar and tryptic soy agar (TSA). Similarly, *E. faecalis* (ATCC 29912) was plated on SEA, NA, TSA, brain heart infusion (BHI) agar and m enterococcus (m ENT) agar. At least three plates were used for each growth medium tested. The plates were incubated at 30°C for 24-96 h, and at the end of the incubation time, the colony forming units (CFU) were enumerated and expressed as mean CFU/ml.

3. Growth of E. coli in soil extract broth

To determine whether nutrients are adequate to support the growth of *E. coli*, a second experiment was designed as follows. Three different broth media namely, minimal medium {MM, containing only carbon (glucose, 1 g/l), P (K_2HPO_4, 0.5 g/l) and N (KNO_3, 0.10 g/l)}, soil extract broth (1 part of soil extract + 1 part of deionized water + C, N and P at concentrations as above) and tryptic soy broth (TSB) as a reference medium were used to inoculate *E. coli*. Three hundred ml each of sterile minimal medium, soil extract broth and tryptic soy broth were aseptically transferred to three separate sterile conical flasks. The broths in the three flasks were inoculated with an overnight culture of *E. coli* (ATCC 25922) at the rate of 7.25×10^6 cells/ml. After inoculation, the flasks were placed on a rotating shaker set to 32°C. At regular intervals, samples were drawn to measure both optical density as well as cell concentration. Cell density (CFU/ml) was determined by plating several dilutions on EMB agar.

E. Assessment of environmental variables (soil moisture, nutrients) on the population dynamics of E. coli and enterococci

1. Effect of soil moisture

The growth of microorganisms in natural habitat such as soil is influenced by various physical (available water, temperature, partial pressure, atmospheric composition, electromagnetic radiation and solid surfaces), chemical (carbon and energy sources, mineral nutrients, growth factors, ionic composition, pH and oxidation reduction potential) and biological (microorganisms and interaction between microorganisms) factors (Stotzky,

1989). Available water is perhaps the most important physicochemical factor that affects microbial growth in the soil environment. To determine the effect of soil desiccation on the viability of fecal indicator bacteria two experiments were conducted under laboratory conditions.

a). Experiment one: Sensitivity of laboratory cultures of *E. coli* and *E. faecalis* to soil moisture stress. Pure cultures of *E. coli* (ATCC 25922) and *E. faecalis* (ATCC 29212) were grown overnight in tryptic soy broth (TSB) and brain heart infusion (BHI) broth, respectively. The cells were washed twice in sterile phosphate buffer to prevent carry over of any nutrients from the broth media to the soil following inoculation. Both *E. coli* and *E. faecalis* were seeded into cobalt irradiated Waimanalo soil at the concentrations: *E. coli*, 2.95×10^6 and *E. faecalis*, 2.15×10^6 Viable cells per g soil. There were two treatments: (i) control, wherein the inoculated soil was maintained at a constant moisture (about 60% of the water holding capacity) throughout the experimental period and (ii) desiccated soil, the inoculated soil (initially at 60% of water holding capacity) was dried under laboratory conditions for several days. The counts of *E. coli* and *E. faecalis* were determined over time from both the control and the desiccated soil treatments by the plate count method (**Table 2.1**). Loss of recovery of *E. coli* and *E. faecalis* that may be attributable to stress (lack of moisture) was examined using a more sensitive detection method such as MPN. Whether addition of moisture alone or both moisture and nutrients were required in order to resuscitate the stressed bacteria was also investigated.

b). Experiment two: Sensitivity of indigenous *E. coli* and enterococci to soil desiccation. Samples of surface soil (0-5 cm depth) from the banks of the Manoa Stream which flows adjacent to the campus were collected and mixed thoroughly. A portion of the fresh soil was immediately assayed for moisture content as well as concentrations of *E. coli* and enterococci by the MPN method. The remaining soil was spread in a thin layer on a sheet of paper and the soil was left to dry at room temperature (25°C). During the next five days, samples were assayed for moisture content and counts of *E. coli* and enterococci by the MPN method.

F. Evaluation of soil as a medium for multiplication of fecal indicator bacteria

To obtain a definitive answer to the question whether fecal indicator bacteria are able to multiply or not in the soil environment of Hawaii, various experiments were conducted under laboratory, simulated field and actual field conditions. The experimental approach (methodology) used in these studies is as follows.

1. Experimental design one: laboratory-based experiments

The rationale for conducting the laboratory-based studies prior to conducting simulated field or in situ studies is that microbial growth under natural conditions is influenced by numerous factors (temperature, available moisture, pH, soil type, nutrient availability, competition for nutrients) and therefore, often it is difficult to interpret the

results from such studies. On the other hand, under laboratory conditions, it is possible to control and measure (determine) the effect of known environmental variables on microbial growth and reproduction. Since most of the variables can be controlled, definitive conclusions can be made from laboratory-based experiments. These kinds of data are required before complex field experiments are designed and conducted.

a). Metabolic status of *E. coli* in soil as determined by the dehydrogenase assay. The oxidation of organic compounds is commonly a dehydrogenation process mediated by enzymes referred to as dehydrogenases. The overall process for dehydrogenation can be summarized as below:

$$XH_2 + A \rightarrow X + AH_2$$

where, XH_2 is a hydrogen donor and A is a hydrogen acceptor (SSSA, 1994). The dehydrogenase enzyme systems play a significant role in the oxidation of soil organic matter and these enzyme systems are an essential part of the microorganisms. As to the correlation between the dehydrogenase assay and the metabolic status of microorganisms, Skujins (1976) suggested that dehydrogenase activity would indicate the average activity of the active population.

To demonstrate whether *E. coli* can remain metabolically active or not in soil, the dehydrogenase assay was used in this study. A pure culture of *E. coli* strain (ATCC 25922) was grown overnight in tryptic soy broth (TSB). The cells were washed twice with sterile phosphate buffer. To 200 g of cobalt irradiated Waimanalo soil, 2 g of calcium carbonate ($CaCO_3$) was added and the soil was thoroughly mixed. A six g portion of this mixture was transferred to each of 10 test tubes (16 by 150 mm) representing various treatments (inoculated or uninoculated and with or without amendments). The soil in 6 test tubes was inoculated with *E. coli* at a concentration of about 1.48×10^8 cells /g dry soil. For comparison, a natural Waimanalo soil amended or unamended with peptone was placed in 4 tubes. To each tube 1 ml of 3% aqueous solution of 2,3,5-Triphenyltetrazolium chloride (TTC) and 2.5 ml of distilled water was added and the contents in each tube was mixed thoroughly and stoppered with a cap. The tubes were transferred to an anaerobic jar and incubated at 37°C for 48 h. The extraction of triphenyl formazan (TPF) from the soil and measurement of its concentration, an indicator of dehydrogenase activity, were done according to the protocols outlined in methods of soil analysis (SSSA, 1994).

b). Successful colonization of fecal indicator bacteria added to soil. An experiment was designed to determine whether *E. coli* and enterococci from a sewage contaminated soil could colonize and increase in counts following the mixing of the sewage contaminated soil (1 part) with a sterile soil (9 parts) not containing any fecal bacteria. In this experiment, 25 g of sewage contaminated soil containing *E. coli* and enterococci (1.03×10^3 and 6.78×10^4, respectively, per g of dry soil) was mixed thoroughly with 225 g of sterile (autoclaved) soil. Following mixing, the soil was brought to about 65% of maximum water holding capacity (MWHC) and maintained at that level through out the experiment. The soil was held under laboratory conditions and as a function of time, counts of *E. coli* and enterococci were determined by the MPN method (**Table 2.1**).

c). Fate of *E. coli* and enterococci from sewage introduced into autoclaved soil. Samples of surface soil (0-3 cm depth) were collected from an area of about 10 square meters on the banks of the Manoa Stream. Big chunks of soil, leaf debris and plant roots were removed and the remaining soil (~ 4 mm sized particles) was spread on clean paper in a thin layer and autoclaved at 121°C for 15 minutes. The autoclaved soil was transferred to a sterile glass jar and refrigerated for later use. The autoclaved soil was free of any indigenous *E. coli* and enterococci.

A raw sewage sample was obtained from the waste water treatment plant located in Hawaii Kai, on the island of Oahu, Hawaii. The concentrations of *E. coli* and enterococci in the raw sewage were 2.08×10^7 and 1.12×10^6, respectively, per 100 ml. A small volume of the diluted sewage (20 ml of 10^{-1} dilution) was added to 500 g (309 g on dry weight basis) of autoclaved soil and mixed thoroughly well. After the soil was inoculated with sewage it was transferred to a sterile glass jar and incubated under laboratory conditions (temperature 23.1°C) for several days. During this period, the soil was maintained at approximately 65-70% of MWHC. Counts of *E. coli* and enterococci were determined on a regular basis by the MPN method (**Table 2.1**).

d). Growth of fecal bacteria from dog feces in cobalt sterilized soil. To determine whether fecal bacteria from animal feces can establish in soil and increase in number over time, an experiment was designed and conducted as follows. A sterile (cobalt irradiated) Waimanalo soil was used in this study. A three hundred g portion (on dry weight basis) of the soil was weighed and transferred to a sterile plastic jar (1000 ml capacity). Dog feces from Animal Services Laboratory (University of Hawaii) was aseptically collected and analyzed for concentrations of fecal coliform (total), *E. coli* and enterococci. The initial concentrations of these bacteria were 5.71×10^7, 4.28×10^7 and 1.57×10^7, respectively, per g dry weight of feces. The feces was diluted 10-fold and 21.4 ml of the 10-fold dilution was used to inoculate the soil. The soil moisture was adjusted to about 65% of MWHC and maintained at that level throughout the experiment. The soil was incubated at room temperature (23-25°C). The counts of fecal coliform, *E. coli* and enterococci were determined on a daily basis over the next seven days by the membrane filtration technique.

e). Fate of fecal coliform and *E. coli* from sewage introduced into cobalt irradiated and natural Waimanalo soil. In this study, diluted primary-treated sewage was added to both the cobalt irradiated and the natural Waimanalo soil to determine the growth characteristics of these bacteria in the two soils. After soil inoculation, the samples were transferred to separate sterile glass jars and incubated under room temperature (23-25°C) for several days. The soil moisture was maintained at about 65% of MWHC throughout the experimental period. Only to the natural Waimanalo soil, simple nutrients (carbon, nitrogen and phosphorus) at minimal levels were added at day 5. These nutrients were added as glucose, ammonium nitrate and monobasic potassium phosphate at the rate of 1 g, 114.30 mg and 17.54 mg, respectively, per 100 g of soil. Sub-samples were analyzed for fecal coliform and *E. coli* on a daily basis for nine days by the membrane filtration (MF) technique.

f). Fate of *E. coli* population with specific gene marker in sterile soil. A recombinant strain of *E. coli* containing the lux plasmid (lux AB) was constructed using the kit obtained from Modern Biology, Inc., West Lafayette, IN. In brief, a strain *E. coli* (DH 5) was transformed with the lux plasmid (plasmid containing lux AB genes and an ampicillin resistance marker) according to the protocols supplied with the kit. Initially, several media were evaluated to grow the lux *E. coli* strain and after several trials, we found that the strain grew better on mFC agar containing 0.1% glucose and ampicillin (250 μg/ml). The strain produced light when grown in the dark.

An increase in population of *E. coli* with lux gene in soil is a direct evidence that *E. coli* can multiply in soil. To determine whether the lux *E. coli* strain can persist and multiply in sterile soil, a laboratory experiment was designed. The experimental procedure is briefly described below.

The soil used in this experiment was a cobalt irradiated Waimanalo soil. Two hundred g (~ 175 g on dry weight basis) portions of the soil were transferred to separate dark brown colored glass jars. The soil in one of the glass jars was designated as control (unamended) and to the soil in the other glass jar, glucose was added at the rate of 1 g /100 g of soil.

The lux *E. coli* strain was grown overnight in TSB containing ampicillin (250 μg/ml). The cells were washed twice with sterile phosphate buffer solution (PBS). The pellet was suspended in 20 ml PSB and mixed well. The inoculum was diluted 100-fold and 15 ml of the 100-fold dilution was used to inoculate the soil to establish a concentration of 3.61×10^5 cells/g dry of soil. The glass jars were transferred to a Styrofoam box and the box was placed in a secured place under laboratory conditions (temperature, 23-25°C). Sub-samples of soil were analyzed for concentrations of *E. coli* on a regular basis by the dilution plate technique. Appropriate dilutions were plated on mFC containing glucose (0.1%) and ampicillin (250μg/ml). The plates were incubated in the dark for 48 hours. Colony forming units (CFU) as well as light production were recorded. The experiment was terminated after 68 days.

g). Growth of indigenous enterococci in soil under laboratory conditions. Enterococci have been previously reported to require more complex nutrients for their multiplication than *E. coli*. To determine whether enterococci indigenous to soil similarly require complex nutrients for their multiplication, a laboratory study was conducted. The particulars of this study are as follows. The soil for this study was obtained from the banks of the Manoa Stream. Samples of surface soil (0-5 cm depth) containing enterococci were collected from the stream bank. The sub-samples were pooled together and mixed thoroughly. Immediately following sampling, the soil assayed for initial concentrations of enterococci. The initial counts (MPNs) of enterococci were 4.47×10^3/g dry soil. One hundred fifty g portions of the soil (on dry weight basis) were transferred to three separate sterile glass jars. The soil in one of the three glass jars was designated as control (unamended). The soil in the remaining two glass jars, was amended with either sodium

azide (0.02 g/100 g of soil) alone or both sodium azide (0.02 g/100 g soil) plus peptone (1 g/100 g of soil). The soil moisture was adjusted to about 65% of MWHC and maintained at that level until the experiment was terminated. Sub-samples of soil were drawn as a function of time to determine the counts of enterococci by the MPN method (**Table 2.1**).

2. Experimental design two: simulated field studies

The rationale for conducting experiments under simulated field conditions is that under these settings, some environmental conditions can still be controlled while allowing certain natural environmental conditions (variables) to take place. As a result, these studies provide a better understanding of the response of fecal bacteria to variable environmental conditions such as daily changes in temperature and light.

a). Construction of growth chamber. For simulated field studies, a wooden, rectangular growth chamber (a wooden frame measuring 36" wide, 26" deep and 18.5" high and completely screened with a nylon cloth except the bottom side, **Figure 2.2**) was constructed to house the potted soils containing indigenous or introduced fecal bacteria. The growth chamber was placed on the outer walkway on the second floor of Holmes Hall. This is a secured area as no one outside of our laboratory uses this external walkway and this area is exposed natural conditions such as fluctuating temperature and sunlight. The nylon cloth prevented external contamination by such things such as birds or insects. It was recognized that the small soil samples in this growth chamber would dry out much faster than soil found naturally on the ground. As a result, in all simulated field studies, the soil was maintained at optimum moisture level (between 65-70% of MWHC) throughout the experimental period by adding water as and when required.

b). Experiment one: Growth of indigenous and introduced fecal indicator bacteria in soil under simulated field conditions. Five hundred g (on dry weight basis) each of natural Waimanalo soil containing fecal coliform, *E. coli* and enterococci (1.26×10^3, 1.26×10^3 and 3.50×10^2, respectively, per g of dry soil) and cobalt irradiated Waimanalo soil which was deliberately contaminated with a primary-treated sewage from the Sand Island Sewage treatment Plant on Oahu (fecal coliform, 2.00×10^3, *E. coli*, 1.81×10^3, and enterococci, 1.29×10^2 per g of dry soil) were placed in separate two plastic pots. The pots containing the soil were transferred to the growth chamber and remained there until the experiment was terminated. Basal nutrients such as carbon (glucose), nitrogen (NH_4NO_3) and phosphorus (KH_2PO_4) were added at minimal levels to both the soil treatments 4 days after incubation. These nutrients were added at the following concentrations: glucose, 1 g, NH_4NO_3, 114.30 mg, and KH_2PO_4, 17.54 mg per 100 g of soil. The average day time (7.00 AM to 6.00 PM) air temperature was 32.1°C while the soil temperature during this period was 33.8°C. Sub-samples of soil were analyzed for fecal coliform, *E. coli* and enterococci on a daily basis for 6 days. When the treatments were replicated, sub-samples were pooled together and analyzed.

c). Experiment two: Biological control of soil *E. coli* by other indigenous microorganisms. The soil used in this study was natural Waimanalo soil containing

indigenous populations of *E. coli*. The initial counts of *E. coli* in this soil were 4.7 X 10^1/g dry soil. The study design consisted of various soil treatments as follows: (i) unamended (control) soil, (ii) glucose (1 g/100 g of dry soil) amended soil, (iii) bile salts (0.15 g/100 g of dry soil) treated soil and (iv) both glucose and bile salts amended (at concentrations as above) soil. The bile salts are used in commercial media (for instance, mFC) as a selective inhibitor of non-fecal bacteria. The purpose of adding bile salts (at small concentrations) to the soil in this study was to test our hypothesis that indigenous soil microorganisms control the growth of fecal bacteria through competition for available nutrients.

Fresh Waimanalo soil was weighed into four plastic pots (250 g on dry weight basis/pot). The soil amendments were added to the designated pots. The pots containing soil were transferred to the growth chamber and left there until the experiment was finished. The average day time (7.30 AM to 5.30 PM) air and soil temperature in the growth chamber during the experimental period were 35.5°C and 36.4°C., respectively. Sub-samples were analyzed over time to monitor changes in *E. coli* population.

3. Experimental design three: study under natural field conditions

The rationale for conducting experiments under actual field conditions was to asses the fate of fecal bacteria under natural field conditions which encompasses all the factors and variables. As a result, based on data collected from field studies, definitive conclusions can be made whether fecal bacteria are multiplying in the soil environment of Hawaii. Moreover, to demonstrate whether fecal bacteria can multiply in the soil environment, studies were conducted in a sequential order starting with laboratory-based experiments before finally culminating with a field study. It is therefore perceived that the data collected from all these studies not only answer the basic question whether fecal bacteria are able to multiply in soil, but also provide ample opportunity to understand the ecology of fecal bacteria in the soil environment.

a). Development of an antibiotic resistant strain of *E. coli* for soil inoculation. To determine whether a specific population of *E. coli* can multiply in soil under natural conditions, we decided to use an *E. coli* strain with a specific marker (antibiotic resistance) for soil inoculation. Initially, several *E. coli* strains from the American Type Culture Collection (ATCC) carrying antibiotic resistance markers were screened to select a suitable *E. coli* strain for subsequent field studies. However, the ATCC strains were found to be unsuitable for in situ soil experiments because these strains not only failed to grow at higher antibiotic concentrations, but also grew very poorly on traditional media used for *E. coli* such as mFC, EMB which can easily distinguish *E. coli* from other background organisms. Moreover, whenever these strains were recovered from soil following inoculation, there was too much interference by the background microorganisms that were naturally resistant to the antibiotics used. As a result, we decided to develop our own *E. coli* strain with a higher antibiotic resistance to overcome the problems such as background interference and difficulty in monitoring the seeded organism.

Previously we had recovered over 200 strains of *E. coli* from soil sampled at different locations on the island of Oahu, Hawaii. For this study, over 20 strains of *E. coli* were tested for their inherent resistance to various antibiotics (chloramphenicol, streptomycin, and tetracycline). After several screenings, we found an *E. coli* strain, (designated as **2X**) which was highly resistant to the antibiotic streptomycin. This strain could grow on all traditional media (mFC, EMB) for *E. coli* containing streptomycin as high as **400mg/ml**. The advantage of using the 2X strain in the field study was that the strain was indigenous to soil, and therefore, it was expected to grow better in the soil under natural conditions.

b). Experimental design. A natural Waimanalo soil was used in this study. The soil was thoroughly mixed and 774 g (600 g dry weight basis) of fresh soil was weighed into each of four plastic pots measuring 15.2 cm (diameter) by 15.2 cm (height). The soil in the pots was unamended (control) or amended with either bile salts (0.15 g/100 g of soil) or bile salts plus glucose at the rate of 0.15 g and 1 g, respectively, per 100 g of soil. There were two controls treatments: (1) unamended natural soil containing indigenous *E. coli* and (2) unamended natural soil inoculated with *E. coli* strain 2X.

The *E. coli* strain 2X was grown overnight in TSB containing 1000 µl of streptomycin. The cells were washed twice with sterile buffer and after the second wash, the pellet was suspended in 20 ml buffer and the inoculum was serially diluted. Twenty five ml from 10^{-1} dilution was used to inoculate the soil in three pots to establish an inoculum density of 7.5 X 10^{6} cells/g dry soil. After soil inoculation, the pots were transferred to open area (open court yard) on the ground floor of Holmes Hall. The pots were buried in the ground up to their brim (**Figure 2.3**). The area was marked and protected to prevent any intrusion. Moisture was added to the soil when required. Sub-samples of soil were drawn over time to determine the counts of *E. coli* by the spread plate technique. Soil dilutions were plated on mFC agar containing 1000 µg/ml of streptomycin. To recover indigenous *E. coli* (uninoculated control treatment) plain mFC was used.

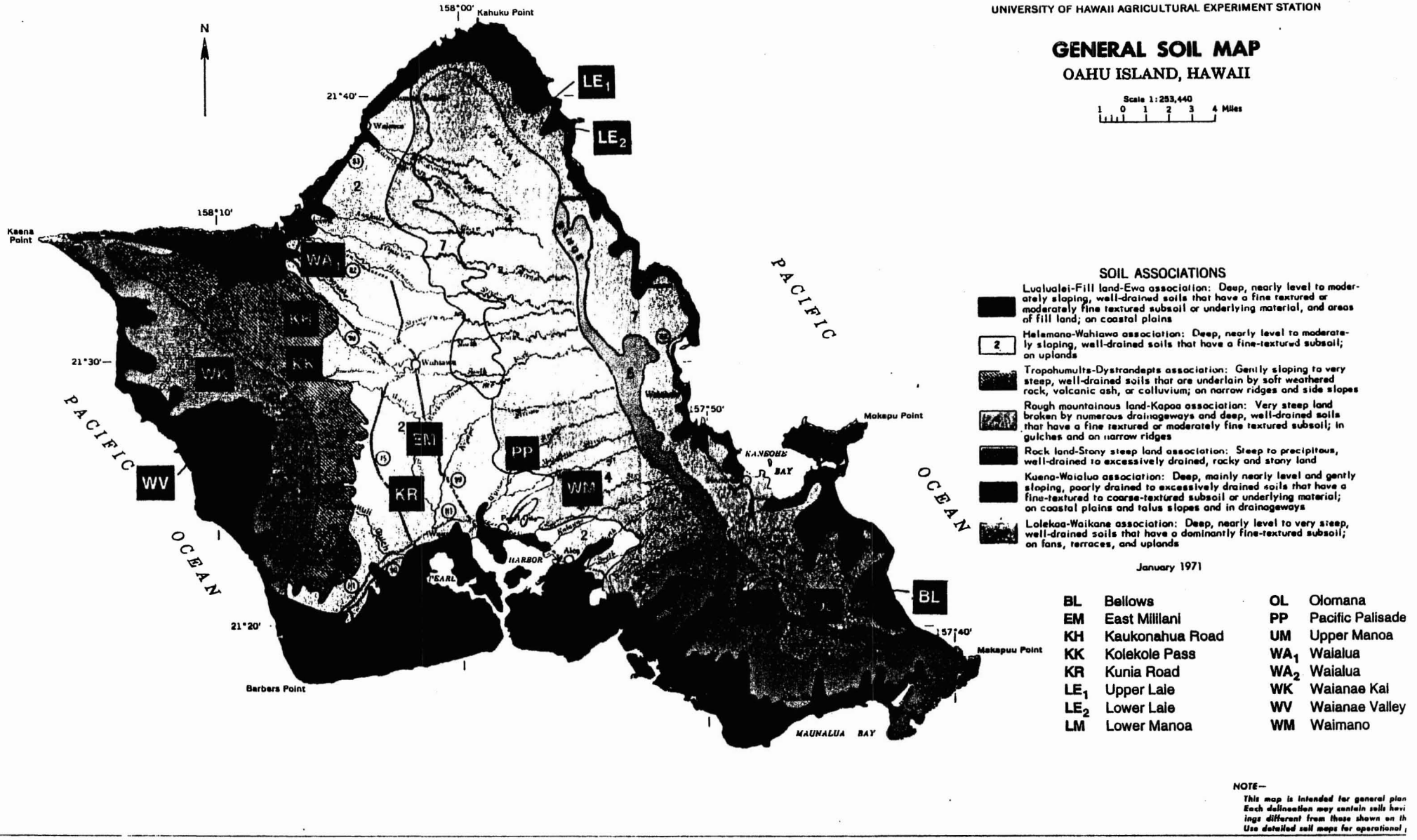


Figure 2.1. Map of Oahu Showing Soil Sampling Locations Representing Major Soil Groups (Affiliations) Found on the Island.

Table 2.1. Experimental Methodology, Media, Presumptive and Confirmatory Tests Used for Isolation and Initial Identification of Fecal Indicator Bacteria from Soil and Water.

Recovery Method	Fecal coliform	*E. coli*	Enterococci
Most Probable Number (MPN) technique	EC broth (44.5°C)	• Presumptive EC broth (44.5°C) • Confirmation 1. Green metallic sheen on EMB agar 2. β-glucuronidase activity (nutrient agar + 4-methylumbelliferyl-β-D-glucuronide (MUG): production of blue fluorescence following exposure to long-wavelength UV lamp	• Presumptive Azide dextrose broth (ADB, 35°C) • Confirmation 1. Pfizer selective enterococcus (PSE) agar 2. Growth in brain heart infusion (BHI) broth at 45°C and BHI + 6.5% NaCl at 35°C
Membrane Filtration (MF)	mFC agar (44.5°C)	• Presumptive mTEC agar (44.5°C) • Confirmation Urease activity	• Presumptive mE agar (41°C) • Confirmation Esculin hydrolysis (substrate test on EIA agar)
Dilution Plate Technique (to recover *E. coli* and *E. faecalis* from soil following inoculation)		• Presumptive mFC agar (44.5°C) • Confirmation 1. Growth on EMB agar (green metallic sheen) 2. β-glucuronidase activity	m Enterococcus agar (35°C)

Reference: Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WEF 1992).

Table 2.2. Chemical Characteristics* of the Waimanalo Soil Used in This Study.

pH			Nutrients			
	OC (%)	N (%)	P	K	Ca	Mg
				ppm		
6.2	1.75	0.17	66	320	3700	980

OC = organic carbon
N = Nitrogen
P = phosphorus
K = potassium
Ca = calcium
Mg = magnesium

* Chemical analysis was performed by the Agricultural Diagnostic Service Center, University of Hawaii.

Table 2.3. Biochemical Tests Used to Confirm the Existence of *E. coli* and Enterococci in Hawaii's Soil Environment.

Organism	Preliminary/Initial Identification	Additional Tests (To reconfirm the initial ID and to characterize soil-borne *E. coli* and enterococci)
Escherichia coli	Test kit: API 20E identification system for enterobacteriaceae. Source: bioMérieux Vitek, Inc. Identification: Based on 20 biochemical tests.	Test: Biolog identification system Source: Biolog, Inc. Identification: Based on carbon source utilization profile (metabolic fingerprinting). Test panel: Gram-negative (GN) microplate containing 95 different carbon sources.
Enterococci	Test kit: API 20 Strep identification system for streptococci. Source: bioMérieux Vitek, Inc. Identification: Based on 20 biochemical tests.	Test: Biolog identification system Source: Biolog, Inc. Identification: Based on carbon source utilization profile (metabolic fingerprinting). Test panel: Gram-positive (GP) microplate containing 95 different carbon sources.

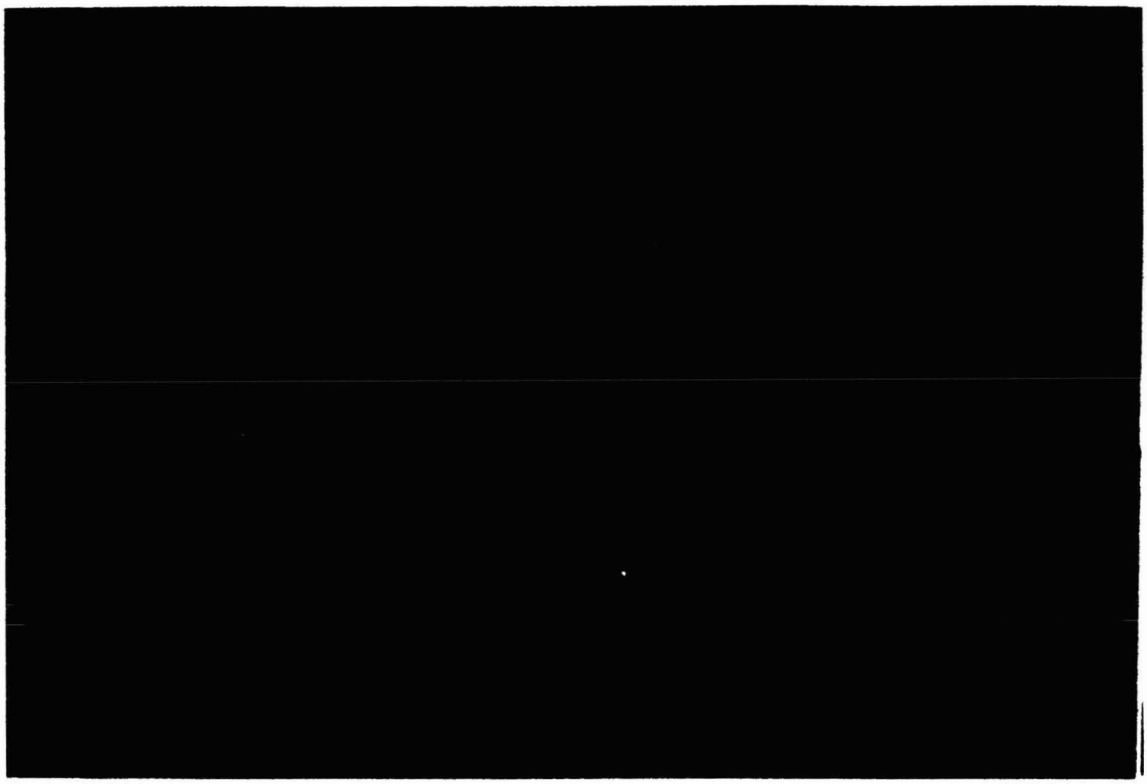


Figure 2.2. A Model of the Growth Chamber Used to Incubate Soil in Simulated Field Studies.

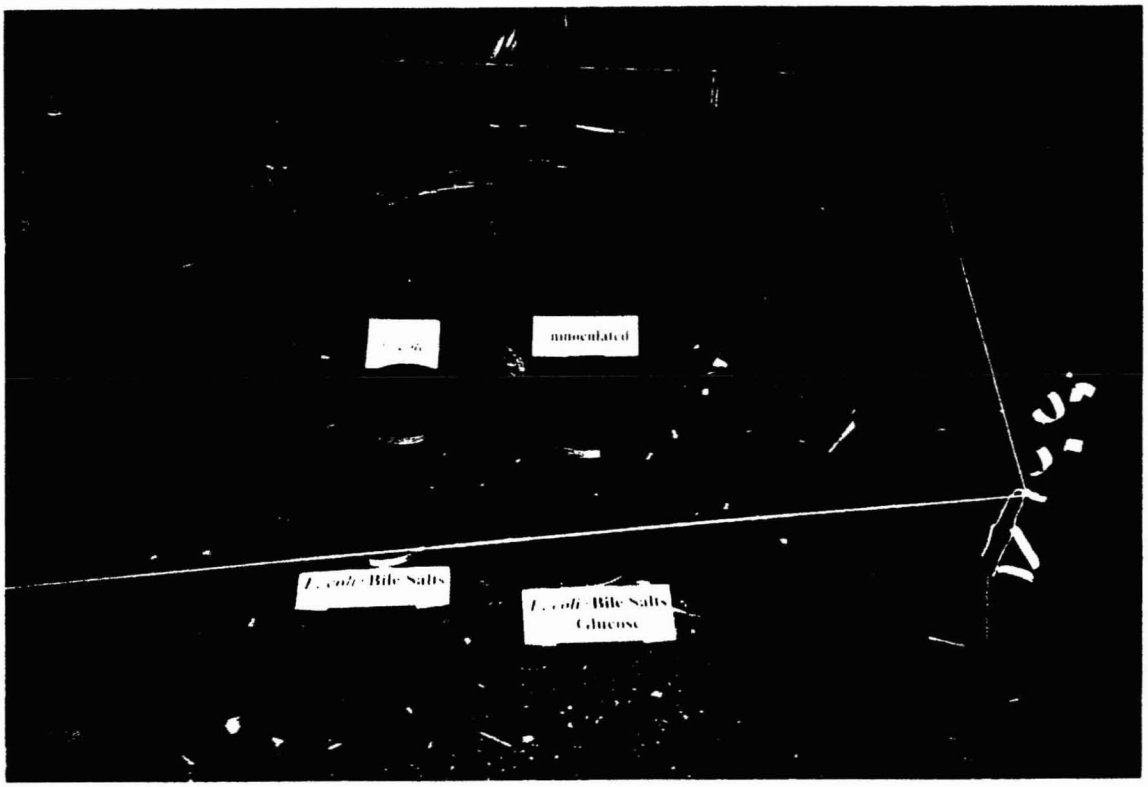


Figure 2.3. A View of the Field Experiment Conducted to Determine Whether *E. coli* Can Multiply in Soil Under Natural Field Conditions.

CHAPTER 3

EVIDENCE FOR PERSISTENCE OF FECAL INDICATOR BACTERIA IN HAWAII'S SOIL ENVIRONMENT

I. Objectives

Preliminary studies conducted earlier had indicated that fecal indicator bacteria could be readily recovered from soil samples collected from the banks of the Manoa Stream as well as grassy areas on campus. In order to determine the extent of prevalence of these bacteria in Hawaii's soil environment, a detailed study was conducted. The objectives for this phase of the study were as follows:

- To determine the prevalence and relative abundance of fecal indicator bacteria (fecal coliform, *E. coli* and enterococci) in the different soil groups on the island of Oahu, Hawaii.
- To identify and to confirm the existence of *E. coli* and enterococci in Hawaii's soils using traditional biochemical techniques.
- To determine the metabolic diversity of soil *E. coli* and enterococci on the basis of their carbon source utilization profiles.

II. Experimental Design

To determine the prevalence and abundance of fecal indicator bacteria in Hawaii soil environment, samples of surface soil representing major soil groups were collected from various sites on the island of Oahu, Hawaii (**Figure 2.1**). The samples were analyzed for concentrations of fecal coliform, *E. coli* and enterococci by the most probable number (MPN) technique. The identification of soil *E. coli* and enterococci was accomplished using specific biochemical tests (reactions) for these bacteria. The metabolic diversity of soil *E. coli* and enterococci was determined by analyzing their carbon source utilization profiles. The experimental methodology is discussed in detail in **Chapter 2.**

III. Results and Discussion

A. Occurrence of fecal bacteria in all major soil groups on Oahu

The microbiological analysis of the soil samples (representing 7 major groups of soil, **Figure 2.1**) collected from various locations on the island of Oahu indicated that fecal bacteria such as fecal coliform, *E. coli* and enterococci could be readily recovered from all the major soil groups. The geometric mean concentrations of fecal coliform, *E. coli* and enterococci ranged from 26-1711, <1-172, and 452-3831 (**Table 3.1**). The concentrations of these bacteria in the soil samples ranged from 0 to 8.00 X 10^4 (fecal coliform), 0 to 1.20 X 10^4 (*E. coli*), and 0 to 1.77 X 10^4 (enterococci), respectively, per g of soil. Variations in their abundance within a soil group or among soil groups probably reflect important environmental variables such as soil moisture and available nutrients.

One of the uniqueness of the Hawaiian islands is the occurrence of all the soil orders (the highest level of classification) described according to the present soil classification scheme. For instance, 8 of the 10 soil orders can be found on the island of Oahu (refer map in **Figure 1.1**). This study not only demonstrates a wide-spread occurrence of fecal bacteria in Hawaii's soil environment, but also indicates that there no limitation for fecal bacterial adaptability to diverse soil groups.

A wide-spread occurrence of fecal bacteria in the soil environment has not been previously reported. Therefore, the persistence of fecal indicator bacteria in the soil in Hawaii, Guam and Puerto Rico clearly indicates that these bacteria have adopted to the soil conditions in these tropical locations and are part of natural soil biota. Thus, high concentrations of fecal bacteria in natural stream waters in Hawaii have been reported to be attributable to rainfall washing and transferring of these soil-borne fecal bacteria into stream waters (Hardina and Fujioka, 1991).

B. Confirming the identity of E. coli and enterococci recovered from soil samples

The fecal coliform bacteria recovered from soil were identified and speciated on the basis of biochemical reactions using the API 20E strips. For isolates that were identified as *E. coli*, the degree of identification varied from *E. coli* likelihood to *E. coli* excellent ID.

Various tests such as gram reaction, catalase reaction, esculin hydrolysis, growth at elevated temperature (45°C), growth in 6.5% NaCl at 35°C and the type of antigen on the cell surface (Standards Methods for the Examination of Water and Wastewater, 1992) were used to ascertain whether the isolates recovered from soil were indeed belonging to the genus enterococcus. Moreover, these tests were felt necessary before screening a number of isolates for further characterization (speciation).

To find out what species of enterococcus were prevalent in the soils on Oahu, additional biochemical tests were run using the API 20 Strep identification scheme. Of the 47 isolates screened, a positive species identification was obtained for 40 isolates. The predominant isolates (35/40, 87.5%) were found to be belonging to the genus enterococcus. No identification was obtained for 7 isolates because of doubtful biochemical profiles. However, based on additional information gathered on motility, hippurate hydrolysis and presence of yellow pigment, 4 of the 7 isolates with doubtful profiles were assigned to the species *Enterococcus casseliflavus*.

The API 20 Strep identification scheme used in this study revealed that at least six species of enterococcus namely, *Enterococcus avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium* and *E. gallinarum* were found to be present in the soil on Oahu. Based on the available data, *E. casseliflavus* appears to be more numerous in soil in contrast to other species of enterococcus (**Figure 3.1**). *E. faecalis* is generally as abundant as *E. gallinarum* while the remaining species (*E. avium*, *E. durans* and *E. faecium*) are less numerous in the soil.

In summary, the biochemical tests employed in the identification of fecal bacteria from soil confirm the existence of both *E. coli* and enterococci in Hawaii's soil environment.

C. Metabolic diversity of soil E. coli and enterococci

The objective of this study was to determine whether Biolog's carbon source utilization profiles could be used not only as an additional tool to reconfirm the API 20E and API 20 Strep identification of soil *E. coli* and enterococci, but also to determine the extent of metabolic diversity of these bacteria. The results are summarized below.

All the 37 API 20E-confirmed soil *E. coli* strains (isolates) were typed as *E. coli* by the Biolog identification scheme with similarity index (SIM) ranging from 0.576-0.982 (**Figure 3.2**). Using the Microlog's software, *mlclust*, a cluster analysis was performed on the 37 isolates and the results of the analysis (*Dendrogram Distances and the 2-D Distances*) are depicted in **Figure 3.3** and **Figure 3.4**. In brief, 34 of the 37 strains of *E. coli* clustered into three major groups, with group C being the largest one consisting of 17 strains (**Figure 3.3**). The other two groups namely, group B and group D, contained 12 and 5 strains, respectively. A two dimensional plot showing the interrelationship between groups containing strains of *E. coli* is shown in **Figure 3.4**.

All the API 20 Strep-confirmed *E. faecalis* strains were typed as *E. faecalis* by the Biolog identification scheme with a similarity index ranging from 0.872-0.959 (data not shown). Two strains, one each belonging to *E. avium* (strain 7 AC°) and *E. faecium* (strain 7 AQ°), initially identified by the API 20 Strep scheme, came out with similar species identification by the Biolog scheme.

Results of the cluster analysis for *E. faecalis* strains are shown in **Figure 3.5** (*Dendrogram Distances*) and **Figure 3.6** (*2-D Distances*). In general, the soil *E. faecalis* strains clustered into four distinct groups (A to D). Soil strains from group C were found to be more closer to the ATCC strain than other strains from group A, B or D.

The Biolog identification scheme could not distinguish *E. casseliflavus* from *E. gallinarum*. Enterococcus strains that were initially identified as *E. casseliflavus* (on the basis of the biochemical reactions from API 20 Strep identification scheme and other additional tests), were all typed as *E. gallinarum* by the Biolog scheme. The inability of the Biolog identification scheme to distinguish these two species is understandable because of the fact that both *E. casseliflavus* and *E. gallinarum* are very closely related. The phylogenetic analysis based on sequence homology of 16S rRNA indicates that these two species share 99.8% sequence similarity (Devriese et al., 1993).

In the current study it was found that the carbon source utilization profiles alone were insufficient to distinguish strains of *E. casseliflavus* from strains belonging to *E. gallinarum*. Therefore, it was realized that a cluster analysis of the data would probably differentiate the strains of these two species and group them into distinct groups. The results of the cluster analysis depicted graphically in **Figure 3.7** and **Figure 3.8**, not only support our initial API 20 E identification and assignment of enterococcal strains from soil into *E. casseliflavus* and *E. gallinarum*, but also show distinct branching patterns without any overlaps. A combined cluster analysis including strains belonging to different species of enterococcus is shown in **Figure 3.9** (*Dendrogram Distances*) and **Figure 3.10** (*2-D Distances*). The data presented in **Figures 3.9** and **Figure 3.10** represent a summary of the results of the cluster analyses performed earlier (refer **Figure 3.5** through **Figure 3.8**).

IV. Summary and Conclusion

In this phase of the study, three lines of evidence were obtained to show that fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) are naturally present in high concentrations in the soil environment of Hawaii. First, soil appears to be the principal environmental source of fecal bacteria in Hawaii as these bacteria could be recovered from major soil groups indicating their ability to colonize soil and adapt to this (soil) environment. Second, various biochemical tests used in this study confirm the persistence in soil of *E. coli* and enterococci. Third, strains of soil *E. coli* and enterococci exhibit metabolic diversity and this supports the hypothesis that many strains of *E. coli* and enterococci are able to colonize soil rather than a few strains.

Taken together, these results provide definitive data that there is an environmental source of fecal bacteria in Hawaii. Therefore, the assumption used in interpretation of recreational water quality standards that ***there should not be an environmental source of fecal bacteria*** is not valid in Hawaii.

Table 3.1. Concentrations of Fecal Indicator Bacteria in the Major Soil Groups (Affiliations) Found on the Island of Oahu.

Soil Group	Location	N		Concentration of Indicator Bacteria (MPN/g Soil)		
				Fecal Coliform	*E. coli*	Enterococci
1	Lower Manoa	07	GM	270	<1	1255
			Range	0-80000		200-17750
2	East Mililani	03	GM	65	23	985
			Range	0-1200	0-1200	35-8000
3	Kolekole Pass	03	GM	67	<1	3236
			Range	0-920		1150-12000
4	Pacific Palisades	04	GM	50	1	452
			Range	0-5500	0-10	40-5500
5	Upper Manoa	07	GM	26	26	793
			Range	0-400	0-400	0-14000
6	Bellows	03	GM	1711	143	3831
			Range	250-12000	0-12000	1350-12000
7	Olomana	04	GM	851	172	1297
			Range	115-12000	0-12000	130-12000

N = Number of Samples; GM = Geometric Mean

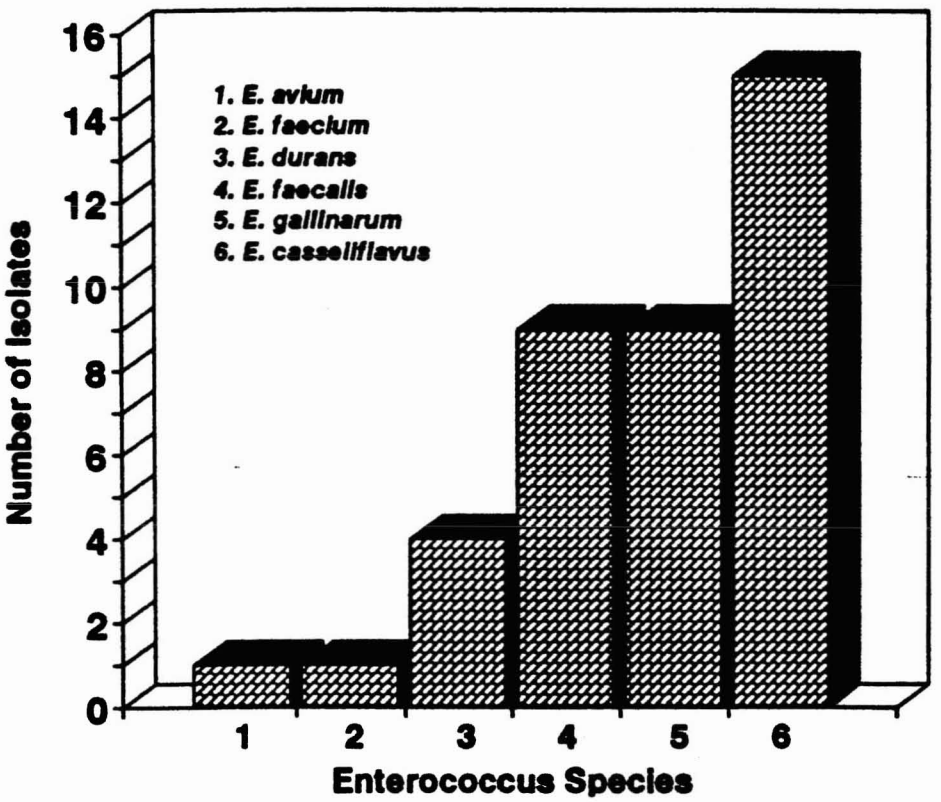


Figure 3.1. Relative Abundance in Soil of Various Species Belonging to the Genus Enterococcus.

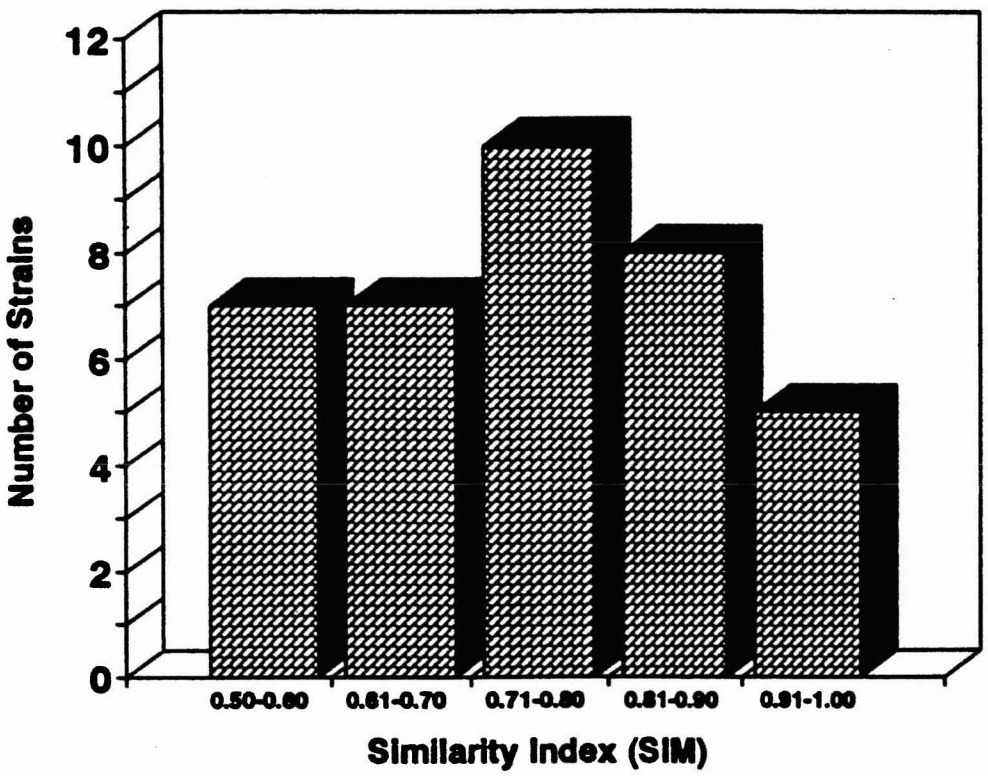


Figure 3.2. Grouping of Soil *E. coli* Strains on the Basis of Their Similarity to Biolog's *E. coli* strains in the Data Base.

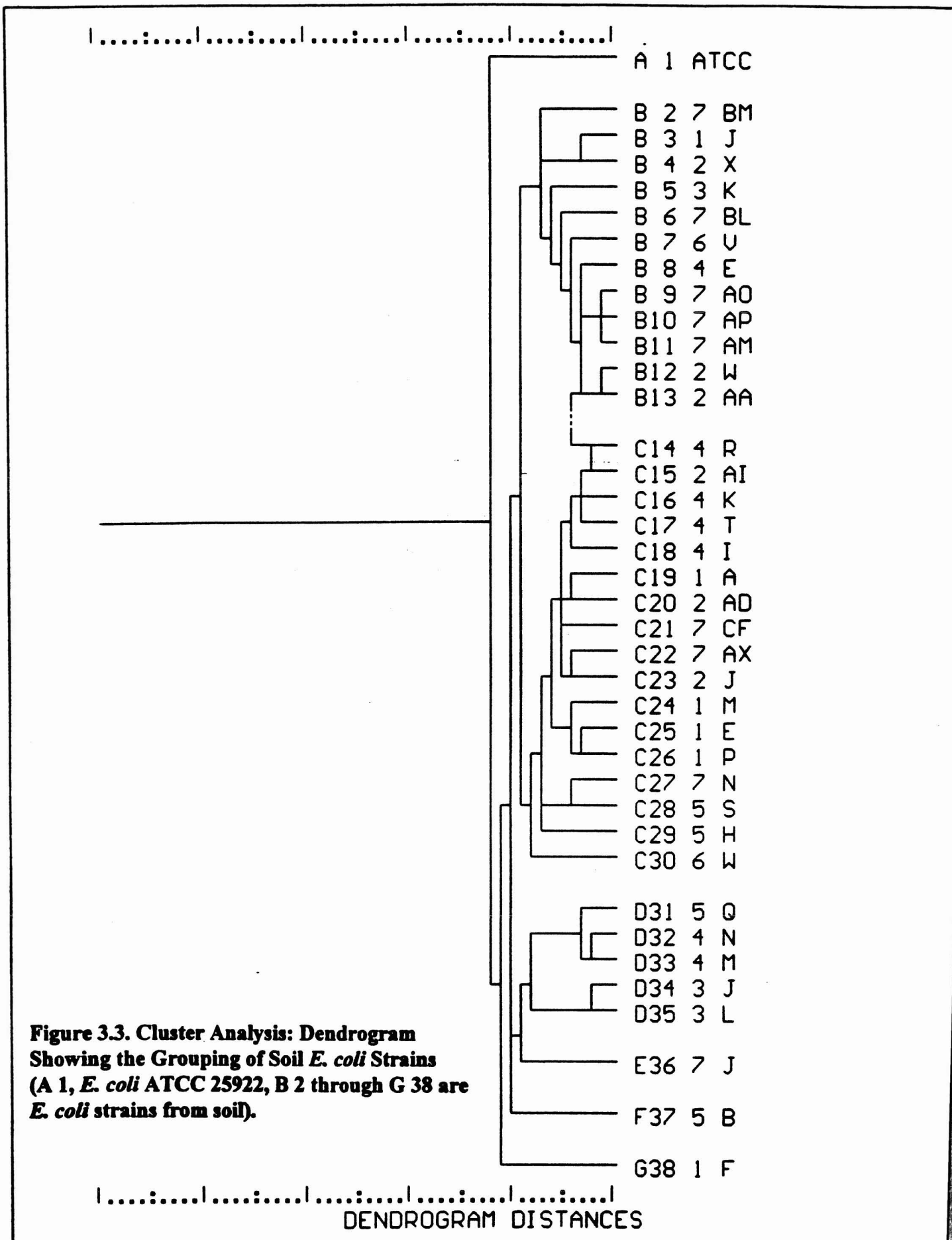
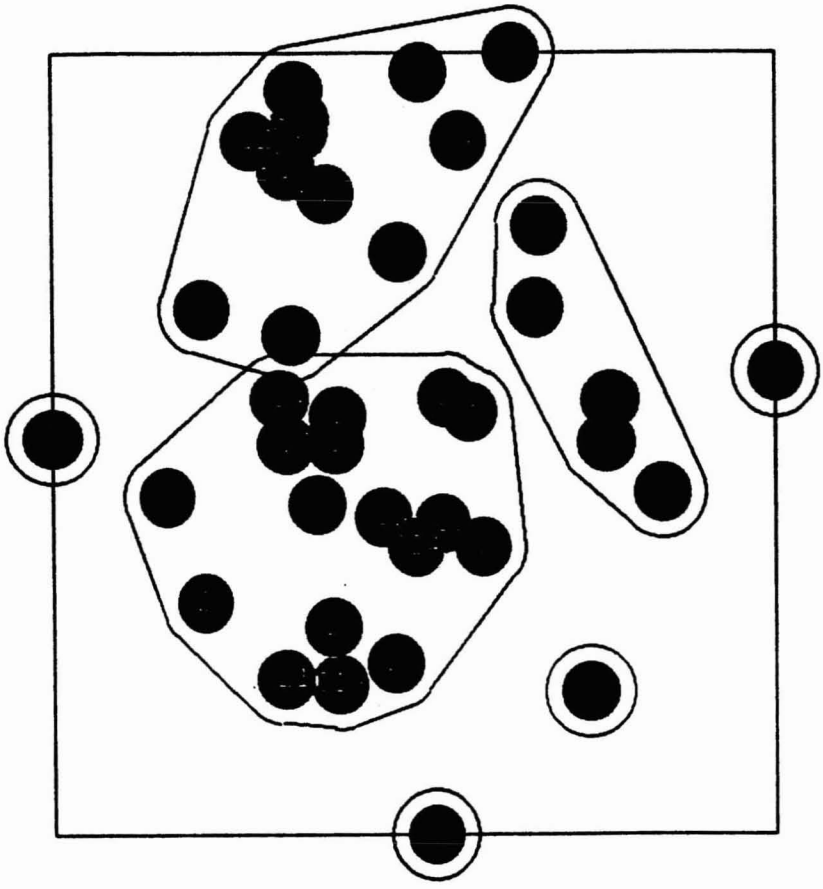


Figure 3.3. Cluster Analysis: Dendrogram Showing the Grouping of Soil *E. coli* Strains (A 1, *E. coli* ATCC 25922, B 2 through G 38 are *E. coli* strains from soil).



A1 ATCC
B2 7 BM
B3 1 J
B4 2 X
B5 3 K
B6 7 BL
B7 6 V
B8 4 E
B9 7 AO
B10 7 AP
B11 7 AM
B12 2 W
B13 2 AA
C14 4 R
C15 2 AI
C16 4 K
C17 4 T
C18 4 I
C19 1 A
C20 2 AD
C21 7 CF
C22 7 AX
C23 2 J
C24 1 M
C25 1 E
C26 1 P
C27 7 N
C28 5 S
C29 5 H
C30 6 W
D31 5 Q
D32 4 N
D33 4 M
D34 3 J
D35 3 L
E36 7 J
F37 5 B
G38 1 F

2-D DISTANCES

Figure 3.4. Cluster Analysis: The 2-D Plot Showing the the Grouping of Soil *E. coli* Strains (A 1, *E. coli* ATCC 25922, B 2 through G 38 are *E. coli* strains from soil).

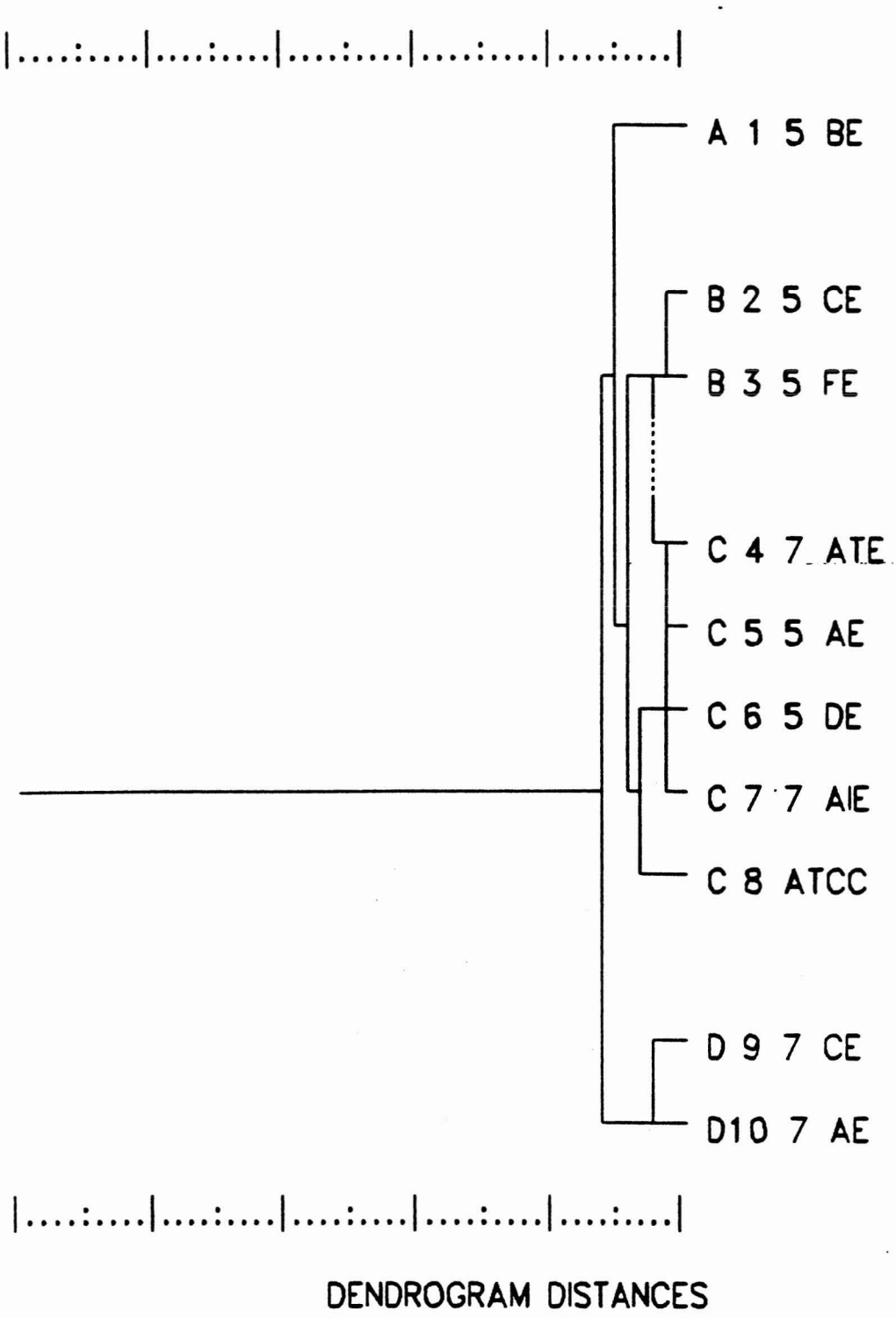


Figure 3.5. Cluster Analysis: Dendrogram Showing the Grouping of Soil *E. faecalis* Strains (C 8, *E. faecalis* ATCC 29212 and other strains in group A, B, C and D are from soil).

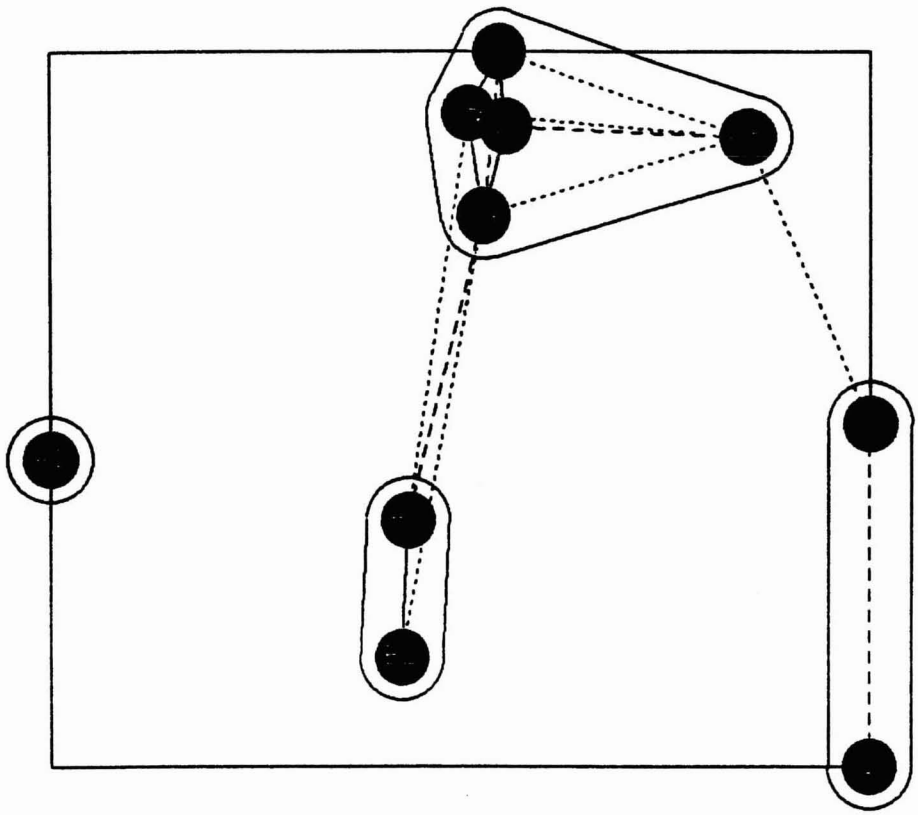


Figure 3.6. Cluster Analysis: The 2-D Plot Showing the Interrelationship Between Groups Containing Strains of *E. faecalis* (C 8, *E. faecalis* ATCC 29212 and other strains in group A, B, C and D are from soil).

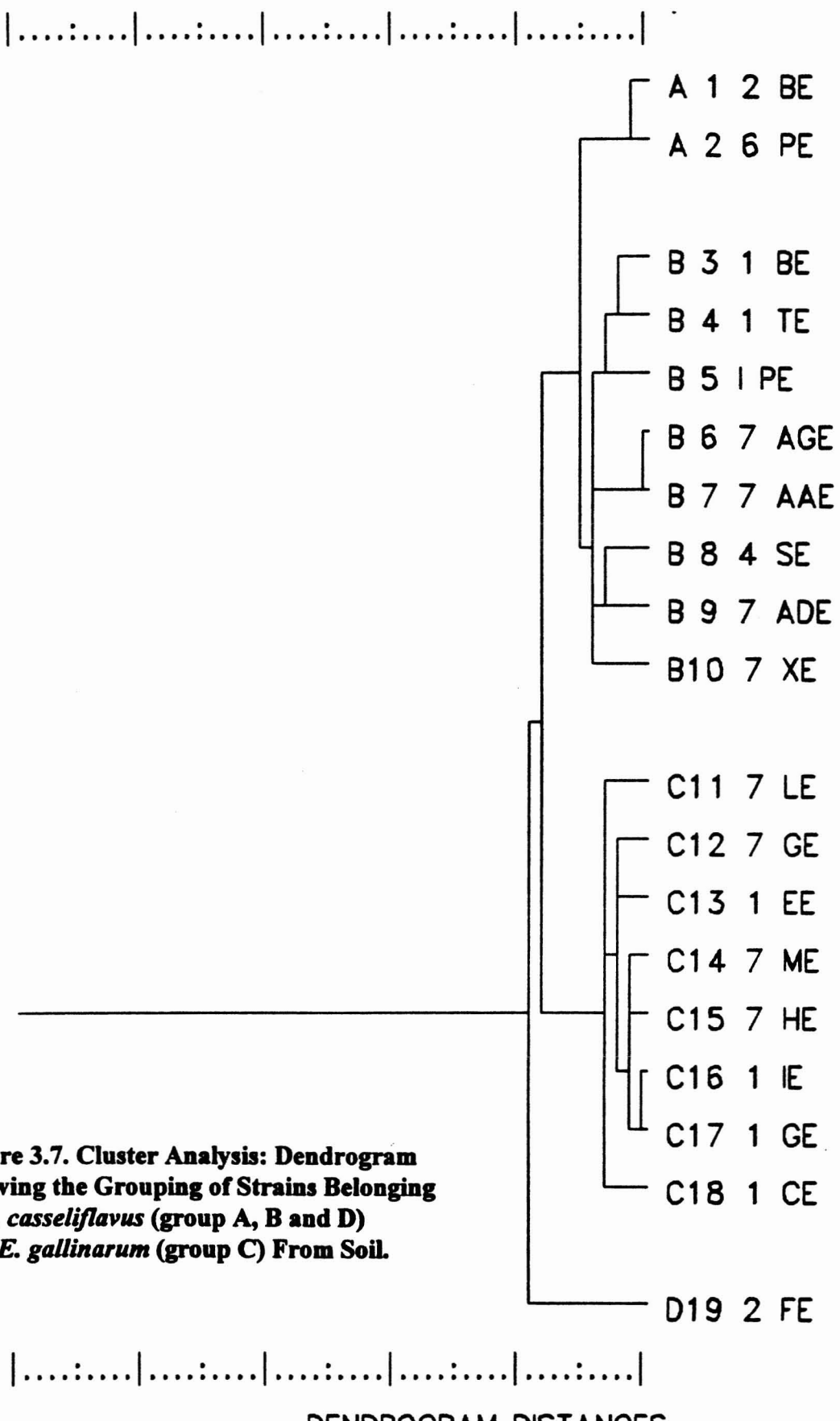
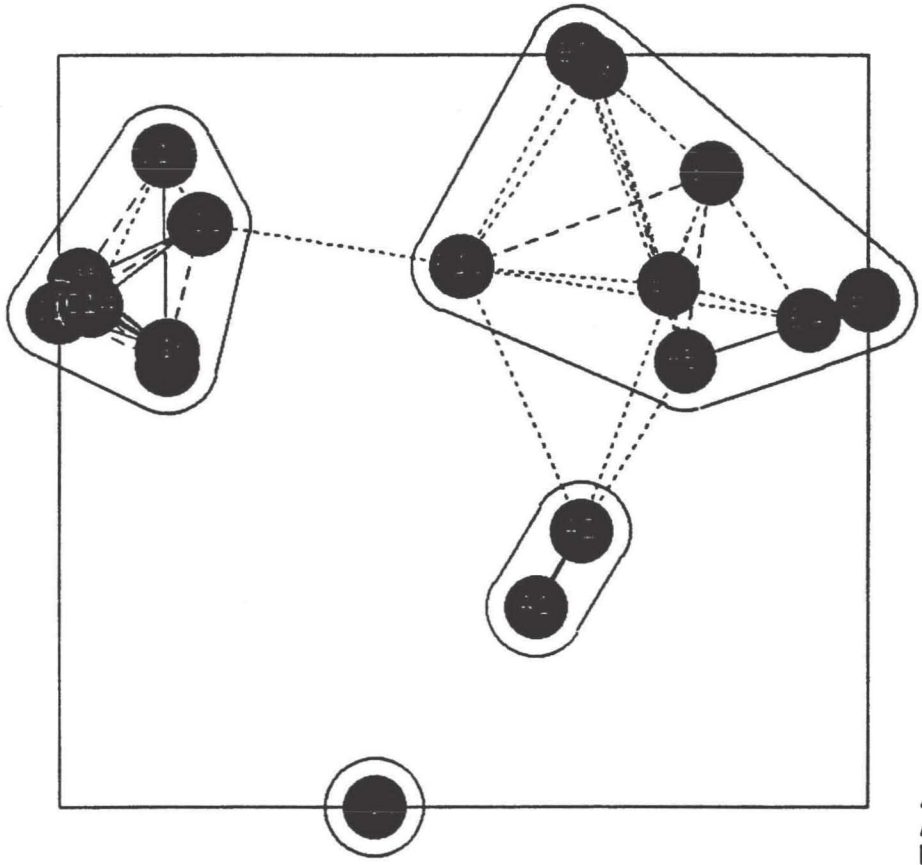


Figure 3.7. Cluster Analysis: Dendrogram Showing the Grouping of Strains Belonging to *E. casseliflavus* (group A, B and D) and *E. gallinarum* (group C) From Soil.



KEY

<= 1.0
<= 2.0
<= 3.0
<= 4.0

A1 2 BE
A2 6 PE
B3 1 BE
B4 1 TE
B5 I PE
B6 7 AGE
B7 7 AAE
B8 4 SE
B9 7 ADE
B10 7 XE
C11 7 LE
C12 7 GE
C13 1 EE
C14 7 ME
C15 7 HE
C16 1 IE
C17 1 GE
C18 1 CE
D19 2 FE

2-D DISTANCES

Figure 3.8. Cluster Analysis: The 2-D Plot Showing the Interrelationship Between Groups Containing Strains of *E. casseliflavus* (group A, B and D) and *E. gallinarum* (group C) From Soil

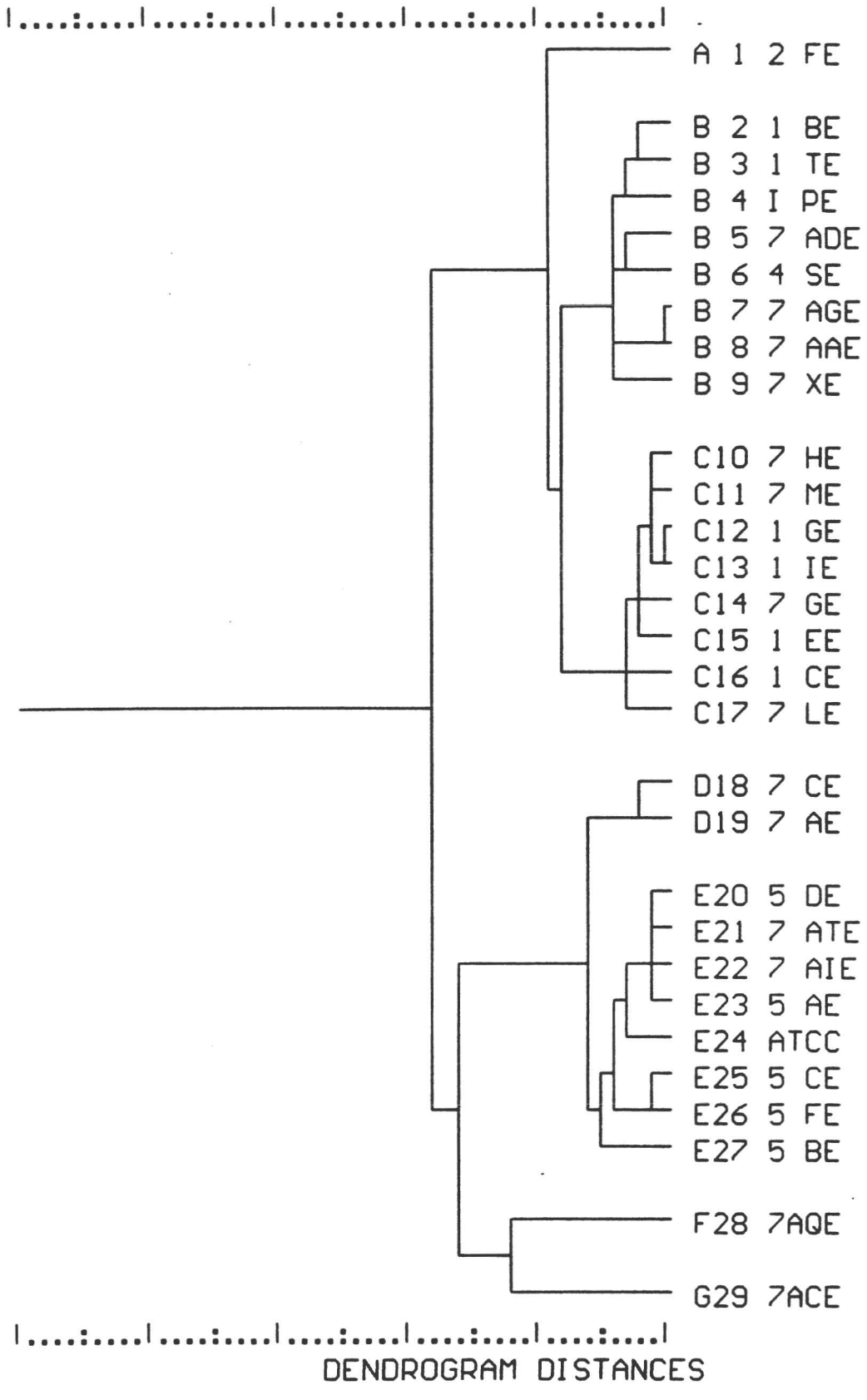
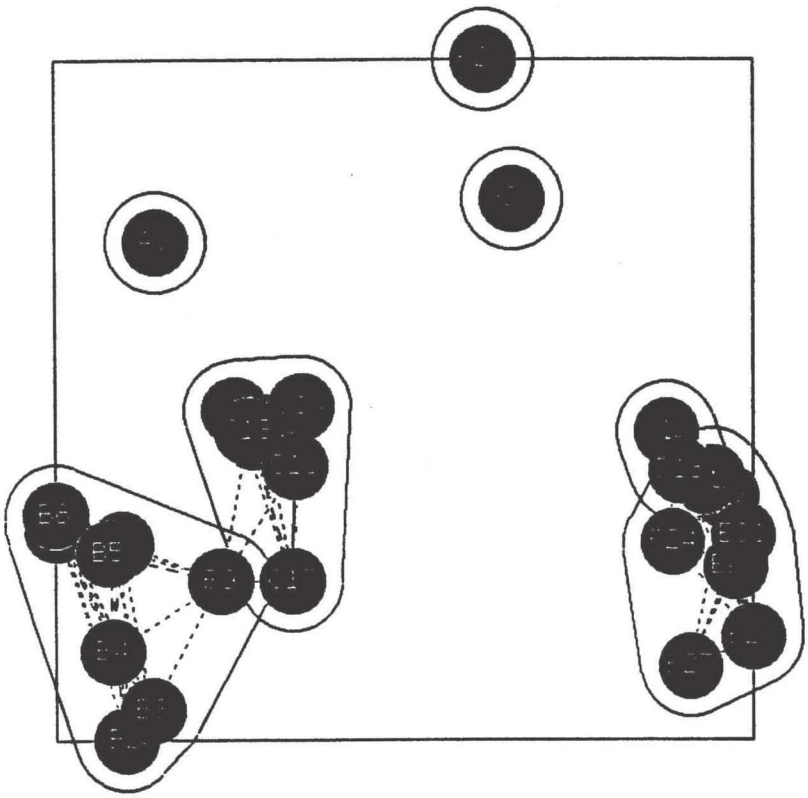


Figure 3.9. Cluster Analysis: Dendrogram Showing the Grouping of Strains Belonging to Different Species of *Enterococcus* (group G, *E. avium*, group A, & B, *E. casseliflavus*, group D & E, *E. faecalis*, group F, *E. faecium* and group C, *E. gallinarum*) Recovered From Soil.



KEY

<= 1.3
<= 2.5
<= 3.8
<= 5.0

A1 2 FE
B2 1 BE
B3 1 TE
B4 I PE
B5 7 ADE
B6 4 SE
B7 7 AGE
B8 7 AAE
B9 7 XE
C10 7 HE
C11 7 ME
C12 1 GE
C13 1 IE
C14 7 GE
C15 1 EE
C16 1 CE
C17 7 LE
D18 7 CE
D19 7 AE
E20 5 DE
E21 7 ATE
E22 7 AIE
E23 5 AE
E24 ATCC
E25 5 CE
E26 5 FE
E27 5 BE
F28 7AQE
G29 7ACE

2-D DISTANCES

Figure 3.10. Cluster Analysis: 2-D Plot Showing the Interrelationship Between Groups Containing Strains Belonging to Various Species of Enterococcus From Soil (for explanation of groups refer figure 3.9).

CHAPTER 4

EVALUATION OF SOIL AS A MEDIUM FOR THE GROWTH OF FECAL INDICATOR BACTERIA

I. Objectives

We previously reported that fecal indicator bacteria such as fecal coliform, *E. coli* and enterococci naturally occur in the soil environment of Hawaii. Having established the fact that these bacteria are part of natural soil biota under tropical conditions of Hawaii, it was felt necessary to evaluate soil as a medium for the growth of fecal indicator bacteria before answering the question whether these bacteria can multiply under soil conditions. Consequently, the objectives of this phase of the study were as follows:

- To determine whether soil contains enough nutrients to support the growth of fecal bacteria such as *E. coli* and enterococci.
- To establish a growth curve for *E. coli* using soil extract as a medium as compared to growth in standard bacteriological medium.
- To determine the effect of soil desiccation on the population dynamics of *E. coli* and enterococci.

II. Experimental Design

To find out whether soil contains adequate nutrients to support the growth of fecal bacteria, nutrients were extracted from soil and a sterile soil extract growth medium (soil extract agar) was prepared. Pure cultures of known *E. coli* and *E. faecalis* were grown on the soil extract agar as well as other traditional media generally used to culture these bacteria. To determine relative suitability of soil as a potential growth-promoting medium for fecal bacteria, an experiment was designed in which a growth curve for *E. coli* was established using soil extract as a medium. Finally, the effect of soil desiccation on the activity fecal bacteria was determined under laboratory conditions using pure cultures of *E. coli* and *E. faecalis* as well as populations of fecal bacteria that are naturally present in soil. The experimental methodology for each of these experiments is discussed in detail in **Chapter 2**.

III. Results and Discussion

A. Growth and recovery efficiency of E. coli and E. faecalis on soil extract agar and other traditional bacterial growth media

For *E. coli* and enterococci to grow in soil environment, the soil must provide minimal levels of nutrients. Soil extract agar (SEA), is a medium which has been extensively used to recover soil bacteria based on providing nutrients from soil. The concept of soil extract as a component of bacteriological medium (to recover bacteria from soil) was originally developed by James (1958). Since then, researchers (Olsen and Bakken, 1987; Parkinson et al., 1971; Wollum, 1982) have extensively used soil extract as an essential ingredient of soil extract agar with slight modifications from what James (1958) developed earlier. For most soil microbiologists, the SEA has been a medium of choice to study soil bacteria.

In the initial experiment to determine whether nutrients in soil are adequate to promote the growth of *E. coli* and *E. faecalis*, pure cultures of these bacteria were plated on soil extract agar and other traditional media (BHI, EMB, mENT, mFC, NA, TSA) which are routinely used to culture these bacteria in the laboratory. After inoculation, the plates were incubated at 30°C. The results of this experiment are summarized below.

The recoveries of *E. coli* on all media including the soil extract agar were generally similar and the counts ranged from 8.45 to 8.56 log CFU/ml (**Figure 4.1**). Visible colonies of *E. coli* on plain SEA (containing only soil extract plus agar) appeared 96 h after incubation. On the other hand, if the soil extract was supplemented with minimal levels of carbon (glucose) and salts (the medium designated as standard SEA), distinct colonies of *E. coli* appeared after 24 h. Similarly, the recoveries of *E. faecalis* on SEA and other standard media after 48 h of incubation were similar, and the counts ranged from 8.27 to 8.38 log CFU/ml (**Figure 4.2**).

These results indicate that soils in Hawaii contain adequate nutrients to support the growth of fecal bacteria such as *E. coli* and *E. faecalis*. However, it should be noted that under natural soil conditions, the growth of fecal bacteria may not be as dramatic as that observed on synthetic media under laboratory conditions because the nutrient concentrations may be lower and other soil microorganisms would be competing for available nutrients.

B. Growth curve for E. coli using soil extract as a medium

The objective of this experiment was to determine the extent by which nutrients in soil can support the growth of fecal bacteria. In this study, a pure culture of *E. coli* was inoculated into the following three different broth media: (1) minimal medium [containing only a simple source of carbon (glucose) and salts], (2) soil extract broth (soil extract and

minimal levels of glucose and salts) and (3) tryptic soy broth (TSB). As TSB is commonly used in the laboratory to grow most bacteria, it was used as a reference medium in this study. The growth of *E. coli* in the three broth media at 32°C was monitored both by recording the absorbance readings as well as determining the colony forming units (by plating on EMB agar) as a function of time.

The growth of *E. coli* in the reference medium (TSB) was as expected. There was a short lag phase followed by a rapid increase in the population and reaching the stationary phase in about 10 h (**Figure 4.4**). The growth of *E. coli* in the minimal medium as well as in the soil extract broth was not as dramatic as observed in TSB. In the minimal medium, there was a marginal increase in the absorbance during the first three hours and thereafter, the absorbance remained relatively constant until the experiment was terminated (**Figure 4.3**). On the other hand, *E. coli* seeded into soil extract broth exhibited a shorter lag phase and a prolonged exponential phase, clearly showing that the soil extract broth was a better growth medium than the minimal medium. The data on the viable counts of *E. coli* (**Figure 4.4**) as a function of time were consistent with the absorbance data (**Figure 4.3**).

In the interpretation of the data it should be noted that the tryptic soy broth was used in this experiment only as a reference medium (positive control) and this medium contains maximum concentrations of all required nutrients. The minimal medium was used in this experiment as a negative control. Thus, the soil extract broth was found to be a better growth medium for *E. coli* than the minimal medium. Although both soil extract broth and minimal medium contained glucose and salts, these nutrients alone appeared to be insufficient for rapid growth of *E.coli* as evidenced by a marginal increase in *E. coli* counts in the minimal medium. On the other hand, the rate and extent of *E. coli* growth in the soil extract broth was much higher, indicating that the better growth of *E. coli* in this medium was attributable to nutrients in the soil extract.

C. Sensitivity of E. coli and enterococci to soil desiccation

Available water is perhaps the most important physicochemical factor that affects microbial growth in the soil environment. The amount of water actually available for microbial use is expressed as *water activity* which is abbreviated as a_w (Brown, 1976; Reid, 1980). The water activity of free distilled water is 1.0 and most microorganisms need a_w values above 0.96 in order to be metabolically active (Atlas and Bartha, 1993). However, some microorganisms such as filamentous fungi and lichens are capable of growing at low water activity (for instance, a_w as low 0.60).

As far as the soil environment is concerned, water availability in soils is usually determined in relation to water holding capacity (WHC), which is the amount of water retained by soil after excess water is allowed to drain. Optimum growth of aerobic microorganisms in soil occurs between 50 and 70% of WHC, which is analogous to 0.98-0.99 a_w (Atlas and Bartha, 1993). Since available water fluctuates in the soil environment, the effect of soil moisture loss on fecal bacteria needs to be documented.

1. Experimental situation one: E. coli and E. faecalis seeded into cobalt irradiated soil maintained at optimum moisture (control) or left for desiccation

To determine the fate of *E. coli* and *E. faecalis* in soil containing optimum moisture (35% soil moisture), pure cultures of these two bacteria were introduced into cobalt irradiated soil and the seeded soil was incubated under laboratory conditions for several days while maintaining soil moisture at 35%. The counts of *E. coli* in the control soil increased by about 1 log unit during the first 2 days and thereafter, the population was relatively stable during the next 6 days (**Figure 4.5**). On the other hand, the counts of *E. faecalis* remained unchanged throughout the experimental period. These results indicate that fecal indicator bacteria such as *E. coli* and enterococci can maintain a stable population in sterile soil containing adequate moisture.

To determine the effect of soil moisture loss on *E. coli* and *E. faecalis*, an experiment was designed in which a cobalt irradiated soil seeded with these bacteria and initially maintained at optimum moisture was allowed to lose moisture over time. The effect of soil desiccation on these bacteria was monitored and the results of this study are as follows.

The loss of soil moisture as a result of desiccation, had a pronounced effect on *E. coli*, but not *E. faecalis* (**Figure 4.6**). The counts of *E. coli* dropped by 3 log units in 2 days, and by day 4, no viable counts of *E. coli* could be recovered from the desiccated soil. At this time the soil contained about 14% moisture (a decline of about 60% of the optimum level). In contrast, a soil moisture level of as low as 10% seemed to have very little effect on the viability of *E. faecalis*. These results indicate that *E. coli* is more sensitive to soil desiccation than *E. faecalis*.

Realizing that the recovery method (mFC incubated at 44.5°C) used for *E. coli* was not ideal for stressed bacteria, a more sensitive technique such as MPN was used to determine whether there were any viable counts of *E. coli* in the desiccated soil that could not be recovered by the platting technique. The MPN recovery method indicated that a few viable cells (5-6 cells/g of dry soil) of *E. coli* were present in the desiccated soil indicating that most but not all cells had been killed (data not shown). When the soil was replenished with lost moisture, *E. coli* counts approximating that at time zero (0) could be recovered in 2 days following the addition of moisture (**Table 4.1**). These results indicate that *E. coli* population undergo various stages of stress depending up on moisture content and recovery of viable concentrations of *E. coli* from soil depends on method used which can resuscitate the stressed bacteria .

2. Experimental situation two: effect of soil desiccation on E. coli and enterococci indigenous to soil

In this experiment, soil samples collected from the banks of the Manoa Stream containing indigenous populations of *E. coli* and enterococci were used to determine the sensitivity of these bacteria to soil desiccation under laboratory conditions. The results are

summarized in **Figure 4.7**. The data indicate that recovery of viable counts of *E. coli* was very sensitive to drop in soil moisture while enterococci was more resistant to changes in soil moisture. These patterns of relative sensitivities of native *E. coli* and enterococci to soil desiccation as a function of time were very similar to that observed for pure cultures of these bacteria seeded into cobalt irradiated soil and dried under similar conditions.

IV. Summary and Conclusions

In this phase of the study, two lines of evidence were obtained to show that the soil environment of Hawaii provides sufficient means to promote the growth of fecal bacteria such as *E. coli* and enterococci. First, soil contains adequate nutrients to support growth and to maintain a stable population of *E. coli* and enterococci. This was ascertained by relative recovery efficiency of both *E. coli* and *E. faecalis* on soil extract agar and other traditional media for these bacteria. Second, additional supporting information on soil's ability to promote the growth of *E. coli* was collected by establishing a growth curve for *E. coli* using soil extract broth as a growth medium.

The effect of soil moisture, an important environmental variable, on the population dynamics of *E. coli* and enterococci was established. *E. coli* was found to be more susceptible to soil desiccation than enterococci. Drop in soil moisture (for instance, from 35% to 13% for *E. coli*) results in bacterial populations to become stressed, but not completely die out. Up on return of moisture, viable concentrations of fecal bacteria increase again. This study indicates that viable counts of *E. coli* in soil under natural conditions can vary significantly depending up on available moisture.

Taken together, these results provide definitive data that since soil contains sufficient nutrients and moisture to support the growth of *E. coli* and enterococci, the basic requirements for the multiplication of the fecal indicator bacteria under natural environmental conditions are available.

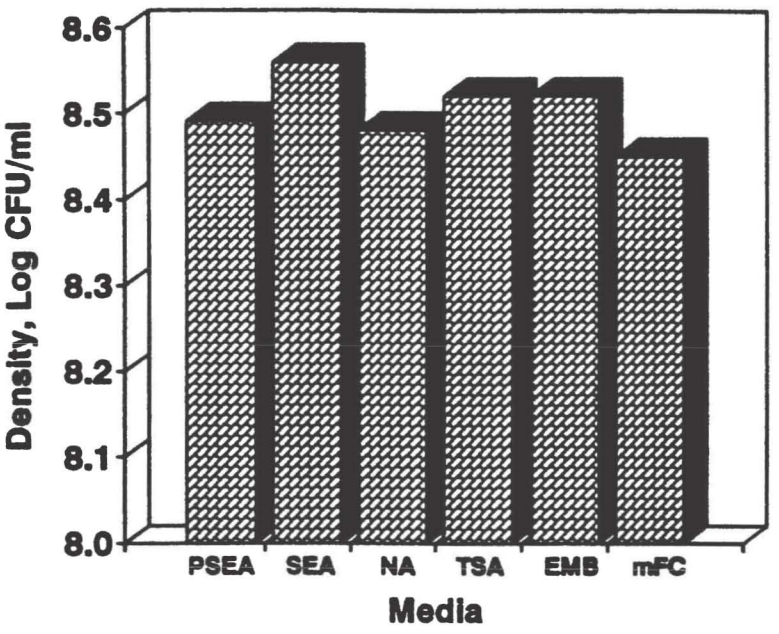


Figure 4.1. Growth and Recovery Efficiency of *E. coli* on Soil Extract Agar and Other Traditional Media After 24-96 Hours at 30°C.

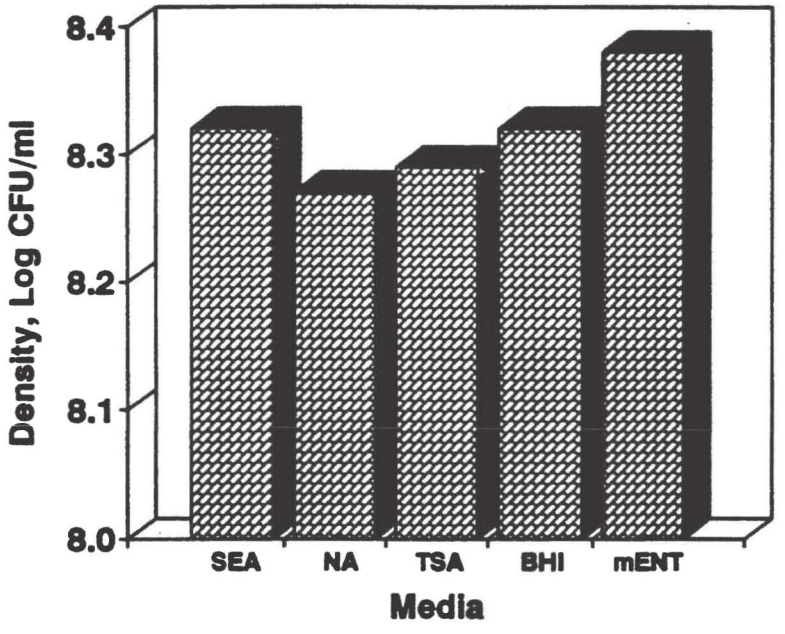


Figure 4.2. Growth and Recovery Efficiency of *E. faecalis* on Soil Extract Agar and Other Traditional Media After 48 Hours at 30°C .

PSEA = Plain Soil Extract Agar, SEA = Soil Extract Agar (Standard), NA = Nutrient Agar, TSA = Tryptic Soy Agar, EMB = Eosine Methylene Blue Agar, mFC = Fecal Coliform Agar, BHI = Brain Heart Infusion Agar, mENT = m Enterococcus Agar

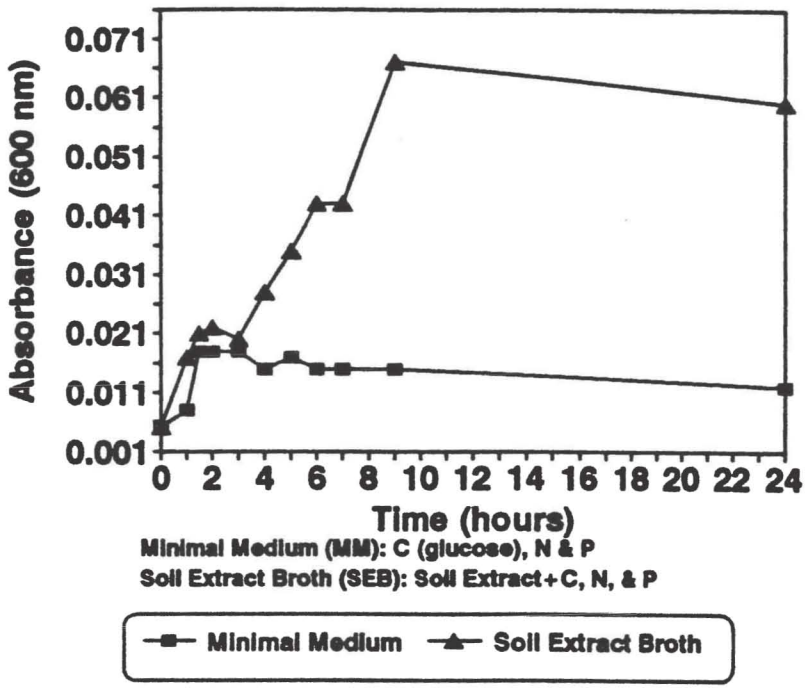


Figure 4.3. Growth Curves for *E. coli* Grown in Soil Extract Broth (SEB) and Minimal Medium (MM) at 32°C.

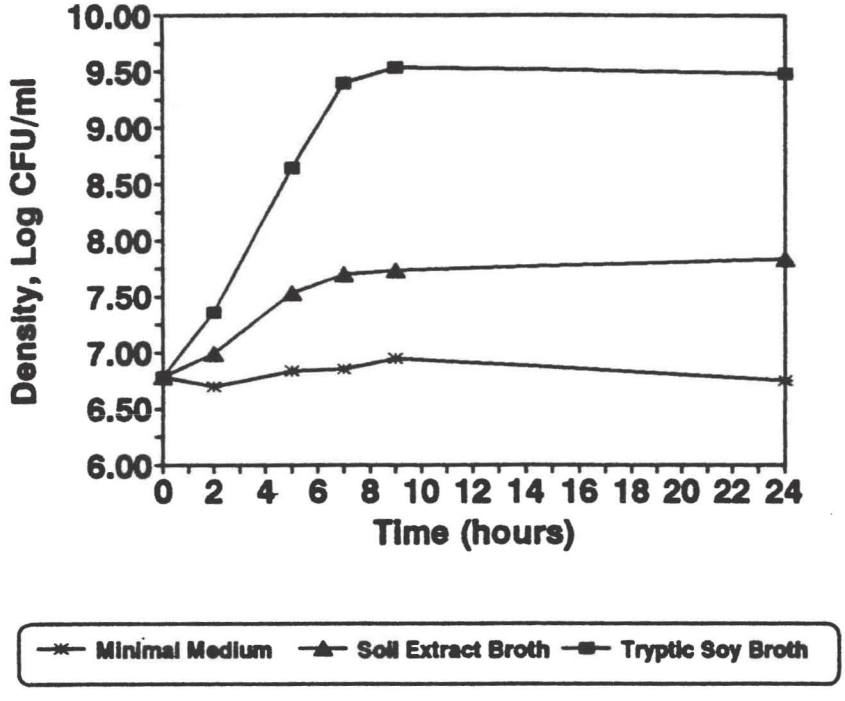


Figure 4.4. Colony Forming Units (CFU) of *E. coli* Grown in Three Different Media at 32°C As a Function of Time.

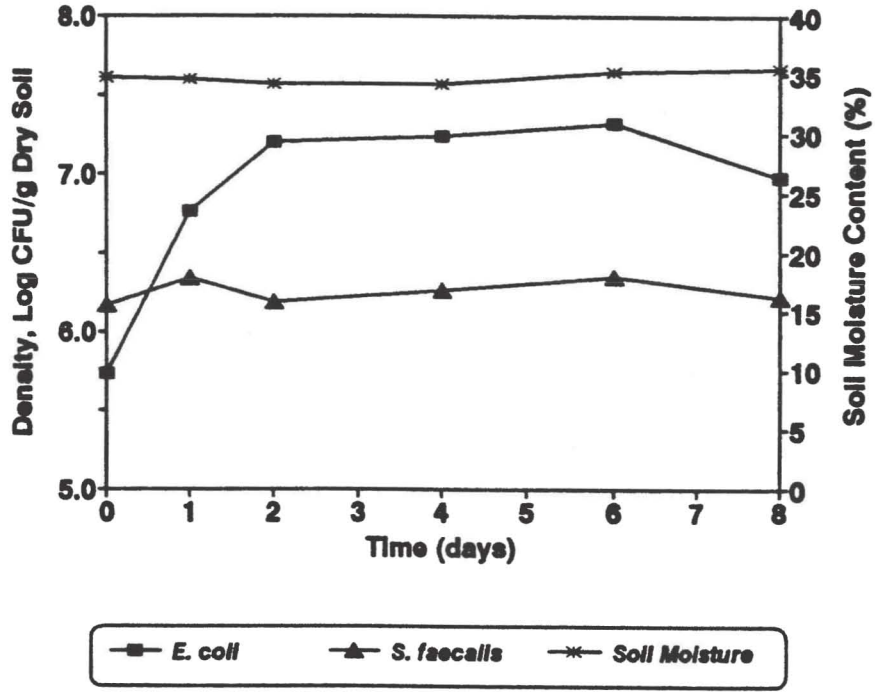


Figure 4.5. Survival of *E. coli* and *E. faecalis* Introduced into Cobalt Irradiated Soil Held at Optimum Soil Moisture (35%) for Microbial Growth at 25 ±1 °C.

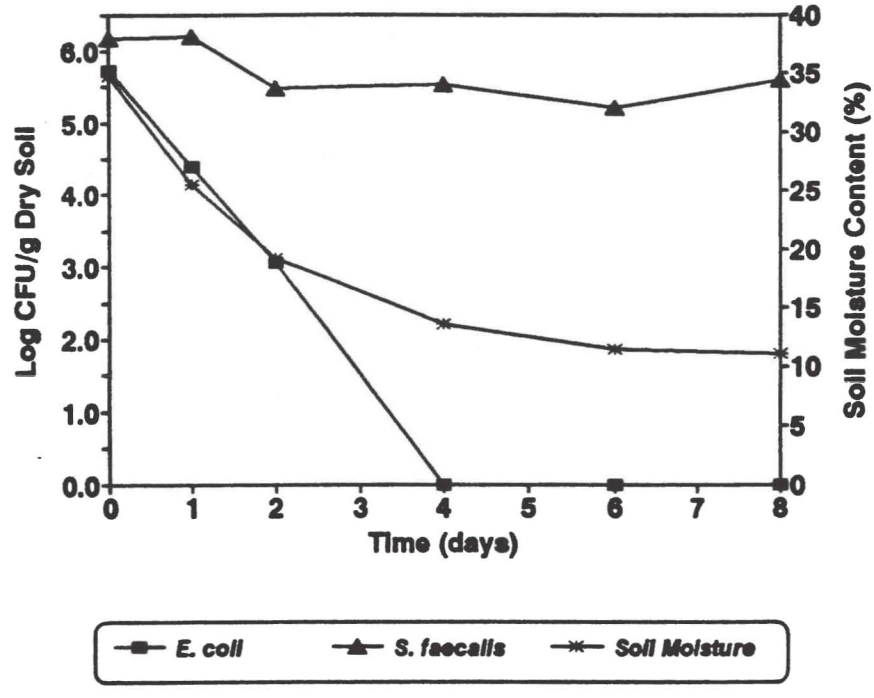


Figure 4.6. Effect of Soil Desiccation As a Function of Time on the Viability of *E. coli* and *E. faecalis* Introduced into Cobalt Irradited Soil Held at 25 ±1 °C.

Table 4.1. Sensitivity of *E. coli* and Enterococci to Soil Desiccation: Recovery of Stressed Bacteria.

Days After Desiccation	Counts in Desiccated Soil (Log CFU/g Dry Soil)		Counts After Replacing Lost Moisture *(Log CFU/g Dry Soil)	
	E. coli	*E. faecalis*	*E. coli*	*E. faecalis*
0	5.73	6.17	ND	ND
1	4.40	6.20	ND	ND
2	3.05	5.48	ND	ND
4	000	5.53	ND	ND
6	000	5.21	5.86	5.77
8	000	5.60	6.55	5.79

Pure cultures of *E. coli* and *E. faecalis* were inoculated into cobalt irradiated soil containing optimum soil moisture (~35%) for microbial growth at time zero. The soil was allowed to lose moisture under laboratory conditions (25 ± 1°C), and as a function of time, viable counts of both *E. coli* and *E. faecalis* were determined by plating soil dilution on mFC and m Enterococcus agar, respectively. The loss of recovery of *E. coli* counts at day 4 and after was believed to be due to lack of adequate moisture in the soil for *E. coli* to remain in a viable state. *To determine whether addition of moisture to the dry soil would rejuvenate the stressed *E. coli*, moisture was added to a portion of the desiccated soil on day 4 and *E. coli* counts were determined on day 6 and day 8 of the experiment.

ND = Not Determined

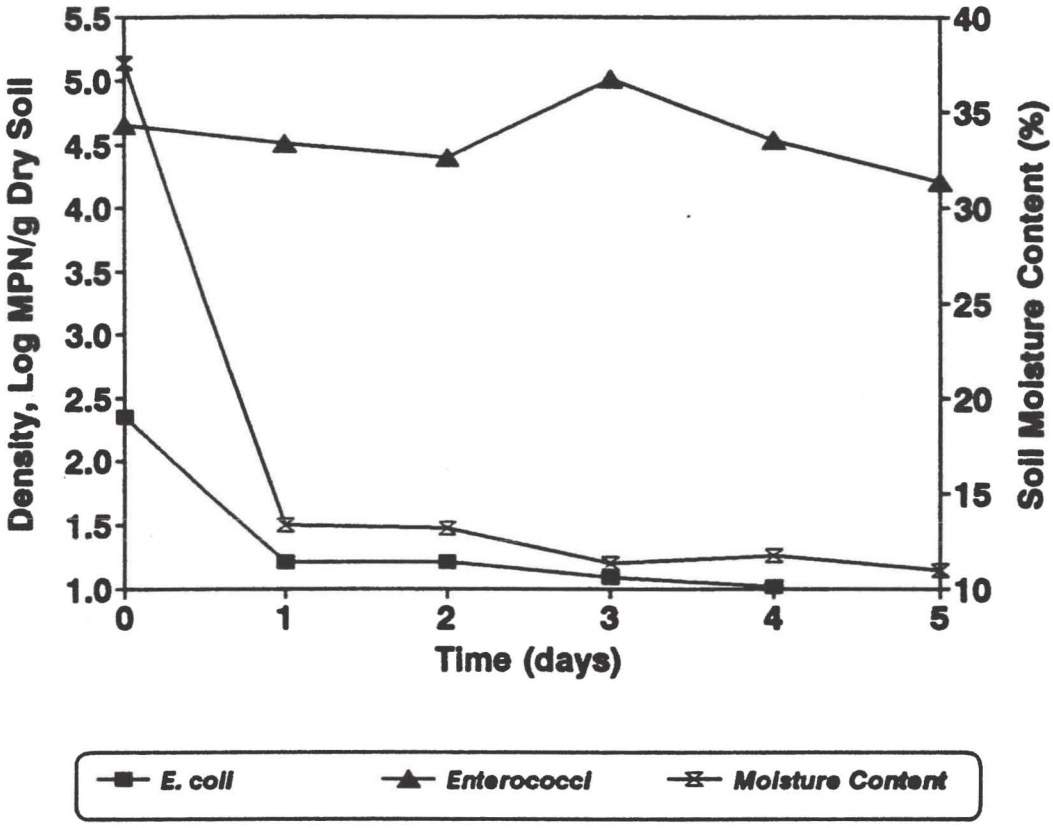


Figure 4.7. Sensitivity of Indigenous Populations of *E. coli* and Enterococci in Soil to Desiccation Under Laboratory Conditions (25 ± 1°C).

CHAPTER 5

EVIDENCE FOR MULTIPLICATION OF FECAL INDICATOR BACTERIA IN THE SOIL ENVIRONMENT OF HAWAII

I. Objectives

Having determined from earlier studies that the soil environment under tropical conditions of Hawaii provides sufficient nutrients and moisture for fecal indicator bacteria to exist and to maintain a stable population in the soil, the next phase of the study was to obtain definitive data whether fecal indicator bacteria such as fecal coliform, *E. coli* and enterococci can multiply in the soil environment under natural conditions. Accordingly, the objectives of this phase of the study were as follows.

- To determine whether *E. coli* can remain metabolically active in soil.
- To determine whether fecal indicator bacteria such as fecal coliform, *E. coli* and enterococci can multiply in soil under (1) laboratory, (2) simulated field and (3) actual field conditions.

II. Experimental Design

Experiments to show that fecal indicator bacteria can multiply in soil are difficult because soil content in terms of physical, chemical and biological composition is complex and varies from one sample to next. Moreover, the natural soil environments undergo many changes such as temperature, sunlight, moisture, additional impacts by plants, animals and other sources of contamination each day. As a result, experiments conducted directly under natural field conditions are difficult to interpret. To predict events occurring in soil environments, the experimental approach was to initially conduct experiments under laboratory controlled conditions so that most of the variables could be controlled and the effect of one variable such as moisture could be measured and interpreted confidently. In some of these experiments, soil samples had to be sterilized so the variable impacts of the microbial population in the soil could be negated. In this regard, for two of the initial experiments, soil samples were sterilized by autoclaving. For all subsequent experiments soil samples were sterilized by cobalt irradiation since irradiation causes less of change in the structure and content of soil. After most of the data under laboratory controlled conditions were completed, some experiments were conducted under simulated field conditions so some of the variables under natural field conditions could still be controlled. For simulated field conditions, the soil samples were incubated in a chamber to protect

against outside contamination while allowing the samples to undergo changes in temperature and sunlight. The final experimental design was to conduct field experiments in which the samples were placed into a soil environment exposed to all the variables naturally found under field conditions. Details of all of the experiments are described in **Chapter 2.**

III. Results and Discussion

A. Laboratory-based experiments

1. Colonization of sewage-borne fecal bacteria from 1 part of contaminated soil to 9 parts of uncontaminated and autoclave-sterilized soil

The objective of this experiment was to provide experimental evidence that fecal bacteria (*E. coli* and enterococci) from sewage can establish (colonize) and maintain a stable population when they are released into a new soil environment. In this experiment, 1 part of soil which was contaminated with sewage was mixed with 9 parts of sterile soil and incubated for up to 4 days at 25°C. The results summarized in **Table 5.1** show a steady increase in the counts of *E. coli* over a period of 4 days. The initial concentration of *E. coli* at time zero was 0.92×10^1/g of dry soil, and after 4 days, the counts had increased about 2 logs to 1.31×10^3/g of dry soil. The initial concentration of enterococci at time zero was 6.08×10^2 cells/g of dry soil and the population had increased almost two logs to 2.95×10^4 and 3.85×10^4 per g of dry soil by day 3 and day 4. The significance of this experiment is that sewage-borne fecal bacteria from the contaminated soil was able to colonize the 9 parts of soil which had not been contaminated. These results provide evidence that fecal indicator can readily multiply in sterilized soil.

2. Multiplication and persistence of sewage-borne E. coli and enterococci added directly to autoclave-sterilized soil

The objective of this experiment was to determine the relative rate by which the fecal indicator bacteria multiply in sterile soil and how long they can persist. In this experiment, sewage containing *E. coli* and enterococci was added to sterile soil and the concentrations of these fecal indicator determined over 18 days at 25°C. The results as plotted in **Figure 5.1** show that the counts of *E. coli* from sewage increased by approximately 4 log units during the first 2 days, followed by a marginal increase (~ 1 log unit) between day 3 and day 4. Thereafter, the population remained relatively stable during the next 14 days. Similarly, the counts of enterococci increased by about 2 log units after day 1 followed by nearly another 2 log units during the next 2 days. Thereafter, the population of enterococci was more or less stable in the next 14 days. These results provide direct evidence that both *E. coli* and enterococci populations in sewage can readily multiply in sterile soil and persist for up to two weeks in the soil. These results indicate

that in the absence of other soil microorganisms, soil is a favorable environment for the multiplication and survival of fecal indicator bacteria

3. Purified E. coli added to cobalt-sterilized soil are metabolically active as measured by the dehydrogenase assay

The objective of this experiment was to measure the metabolic status of *E. coli* in soil using an enzyme assay commonly used by soil microbiologists. In this regard, only metabolically active cells can multiply. As a result, increase in metabolic activity is also indicative of cell multiplication. In this experiment a pure culture of *E. coli* was added to cobalt sterilized soil without added nutrients and with minimal and twice the minimal concentrations of peptone. After 48 hours at 37°C under anaerobic conditions, the soil was measured for triphenyl formazan (TPF) as indicator of dehydrogenase activity. Positive and negative controls were included. Details of this experiments are described in Chapter 2.

The results of this experiment as summarized in **Figure 5.2** show that negative controls consisting of uninoculated cobalt sterilized soil with and without added peptone revealed no dehydrogenase activity indicating that soil and peptone did not contribute to dehydrogenase activity. As positive control, we analyzed natural Waimanalo soil with all its indigenous soil microflora plus added peptone. Maximum dehydrogenase activity was observed in this soil sample as measured by the amount of farmazan produced (1032 μg/g dry soil). This high value was not surprising since the dehydrogenase activity in this soil is attributable to the metabolic activity of the total indigenous microflora which are present in natural soil. In comparison, there was a steady increase in metabolic activity in soil samples inoculated with only *E. coli* (92.7 μg farmazan/g dry soil), *E. coli* plus 1X peptone (243.2 μg farmazan/g dry soil) and *E. coli* plus 2X peptone (295.4 μg farmazan/g dry soil). These results provide direct enzymatic evidence that *E. coli* in soil remain metabolically active and will multiply when nutrients are present.

4. Multiplication of fecal bacteria from animal feces added to cobalt-sterilized soil

The objective of this experiment was to determine whether fecal bacteria from animal feces will grow in sterile soil because feces of animals (rats, birds, dogs) are often deposited on the soil and are common sources of fecal bacteria added to soil. In this experiment, feces of dog was diluted in water, added to sterile soil and incubated under laboratory conditions (25°C) for seven days. The results summarized in **Figure 5.3** show that fecal coliform and *E. coli* bacteria from dog feces readily multiplied in the cobalt sterilized soil. The counts of these bacteria increased by approximately 2 log units after day 1, and thereafter the counts were relatively stable during the next five days. In contrast, the enterococci increased by only 0.8 log unit and maintained this level for 5 days. These results are similar to those in which the source of fecal bacteria was human sewage. Based on these results, we conclude that fecal bacteria from animal feces can multiply in sterile soil and therefore the source of the indigenous populations of fecal indicator recovered from soil, especially in remote areas, is probably animal feces.

5. Comparative fate of sewage-borne fecal coliform and E. coli added to cobalt-sterilized versus natural Waimanalo soil

Previous experiments showed that fecal bacteria multiplied when added to sterile soil by utilizing available nutrients in the soil. However, in the natural environment, soil is comprised of many different kinds of indigenous microflora and it is well known that these different groups of microorganisms compete for available nutrients. Moreover, soil microorganisms are especially suited to obtaining nutrients under environmental conditions. The objective of this experiment was to compare the fate of sewage-borne fecal coliform and *E. coli* added to sterile soil and to natural soil after incubation at 25°C for 9 days.

The results of this experiment are summarized in **Figure 5.4** and show that fecal coliform and *E. coli* added to sterile Waimanalo soil increased 4-5 log units within the first two days of incubation and continued to increase slowly over the 9 day period. These results confirm our previous observation that *E. coli* and fecal coliform bacteria can readily multiply in sterile soil. However, when the same sewage borne fecal bacteria were added to the same Waimanalo soil which had not been sterilized, multiplication of the fecal bacteria in the soil sample was not observed over the first 5 days (**Figure 5.5**) clearly indicating that in the presence of indigenous soil microorganisms, fecal indicator bacteria are not able to multiply. On day 5, nutrients in the form of glucose was added to the soil and this resulted in the immediate multiplication of fecal coliform and *E. coli* to approximately 3 log units within 1-2 days and maintained that concentration for the next three days. Based on these results we conclude that indigenous soil microorganisms are much more efficient in obtaining nutrients from soil under ambient conditions (25°C) and multiply at the expense of other microorganisms such as fecal coliform which cannot successfully obtain sufficient nutrients and therefore cannot multiply. However, when nutrients in excess are available, even the fecal bacteria can multiply in the presence of indigenous soil microorganisms. These results also show that although fecal bacteria were initially suppressed from multiplying, they were poised to multiply when conditions became favorable.

6. Multiplication of E. coli population with genetic marker in cobalt-sterilized soil

In soil experiments, it is desirable to show that the increase in population of *E. coli* represents the increase in numbers of a specific population of *E. coli*. To accomplish this, there is a need for a strain of *E. coli* with a specific gene marker. This was accomplished by creating a special strain of *E. coli* carrying the lux plasmid (see Materials and Methods section for details). Tracking this strain of *E. coli* was easy since the colonies fluoresced as a result of the lux genes. To determine whether this particular strain of *E. coli* can multiply in soil under laboratory conditions, the recombinant *E. coli* was inoculated into sterile soil with or without nutrients. During the next 68 days, sub-samples of soil were analyzed at regular intervals for concentrations of *E. coli* by plating soil dilution on mFC agar containing 0.1% glucose and ampicillin (250μg/ml).

The results summarized in **Figure 5.6** show that in the soil without added glucose, *E. coli* population rapidly increased by more than 2 log units in four days and increased slightly over the next 8 days and then remained more or less stable during the next 44 days. At day 68, the soil still contained a residual level which was 1.5 log units higher than the inoculated level. In contrast, the counts of *E. coli* in the soil with added glucose increased by approximately 4 logs (from 4.06 to 7.86 log CFU/g dry soil) in 8 days and slowly dropped over the 68 days of incubation to a residual level which was 1.5 log units higher than the inoculated level. The results of this study show that a specific population of *E. coli* did multiply in sterile soil under laboratory conditions. These results provide direct genetic evidence for the multiplication of *E. coli* in the soil environment.

7. Enterococci require complex nutrients for multiplication in soil

In our earlier experiments it was observed that in sterile soil, fecal coliform bacteria generally grew and multiplied at a much faster rate than enterococci. Moreover, enterococci have been previously reported to require more complex nutrients for their multiplication than *E. coli*. Therefore, it was realized that in soil, non-availability of such complex nutrients would certainly restrict enterococci growth and multiplication. To obtain direct evidence as to whether enterococci require complex nutrients for multiplication in the soil environment, an experiment was designed in which the growth of indigenous enterococci was determined in natural soil after the addition of various combinations of complex nutrients and a selective inhibitor of non-enterococci.

The results summarized in **Figure 5.7** show that indigenous enterococci did not multiply in the unamended natural soil and growth was only marginal when sodium azide was added to natural soil to inhibit the growth of other soil microorganisms and thereby reduce competition for available nutrients. However, in the presence of both sodium azide and peptone, enterococci increased to a significant level from day 5 onwards. The results of this experiment provide evidence that unlike fecal coliform bacteria, enterococci require complex nutrients such as peptone to multiply in soil.

B. Simulated field experiments

Before natural field experiments were conducted, some additional experiments were conducted under simulated field conditions so that some of the environmental variables could be controlled. In this experimental design, soil samples were placed in plastic pots and left in a screened chamber outdoors to expose these soil samples to varying temperature and light conditions while preventing any external contamination and maintaining adequate soil moisture (see Materials and Methods in **Chapter 2**).

1.Predictable fate of indigenous populations of fecal bacteria in soil

The same experiment completed under laboratory conditions which traced the fate of indigenous populations of fecal coliform, *E. coli* and enterococci in natural soil was

carried out under simulated field conditions. In this experiment, natural soil containing indigenous populations of fecal bacteria was placed in plastic pots and exposed to simulated field conditions for several days. Sub-samples of soil were analyzed at regular intervals for concentrations of fecal coliform, *E. coli* and enterococci by the MPN technique. The results are summarized in **Figure 5.8** and show that in natural soil, the viable counts of indigenous fecal coliform and *E. coli* fluctuated during the three day exposure to changing temperature and sunlight. By day 3, the concentrations of fecal coliform and *E. coli* were reduced by two log units. On day 4, minimal concentrations of simple nutrients (glucose and salts) were added to this soil and this resulted in the three to four log units increase in concentrations of both fecal coliform and *E. coli* over the next two days. These results are consistent with that obtained from experiments conducted under controlled laboratory conditions. Thus, even under simulated field conditions indigenous soil microorganisms suppress the growth of fecal bacteria by competitively using available nutrients. When glucose was added to the soil on day four, the populations of fecal coliform and *E. coli* immediately responded to the added amendments and multiplied in significant numbers indicating that their nutritional requirements were simple.

In contrast, the measured concentrations of enterococci remained fairly constant throughout the experiment and did not respond to the addition of simple nutrients such as glucose. These results support our earlier conclusions from a study carried out under laboratory conditions (see **Figure 5.7**) that enterococci require complex nutrients for multiplication in soil.

2. Predictable fate of sewage-borne fecal bacteria added to cobalt-sterilized soil

The same experiment conducted under laboratory conditions which showed that fecal bacteria from sewage was able to multiply when added to cobalt sterilized soil was completed under simulated field conditions. The results shown in **Figure 5.9** indicate that in cobalt irradiated soil, the concentrations of fecal coliform and *E. coli* increased steadily over the first four days of incubation and increased at a faster rate after the addition of glucose on day 4. The fate of enterococci in the cobalt irradiated soil was quite different. Concentrations of enterococci remained constant over the six days and did not respond to the addition of glucose on day four. In summary the results obtained under simulated field were predicted based on the results obtained under laboratory controlled conditions. Thus, the same conclusion which were obtained under laboratory controlled conditions applies to the results obtained under simulated field conditions. First, that fecal colifrom and *E. coli* are able to extract nutrients from soil in the absence of competing indigenous populations of soil microorganisms and are able to multiply. The addition of glucose enhanced the multiplication of both fecal coliform and *E. coli* but not enterococci because enterococci have more complex nutritional requirements than fecal coliform bacteria.

3. Confirmation that growth of fecal bacteria is suppressed by indigenous soil microflora

Under laboratory conditions indigenous soil microflora was shown to suppress the growth of fecal bacteria. A similar experiment was conducted under simulated field settings. In this study, the growth of indigenous *E. coli* in natural soil was monitored after adding to the soil one of various combinations of simple nutrients and a selective inhibitor (bile salts) of non-fecal bacteria.

The results graphed in **Figure 5.10** show that the addition of moisture alone to the soil (control treatment) did not result in any significant increase in the levels of *E. coli* indicating that other soil microbes were extracting limited nutrients from soil more efficiently than *E. coli*. When the soil was supplemented with glucose, *E. coli* counts increased by about 2 log units during the first 2 days and thereafter the population remained stable. The addition of bile salts alone, allowed the multiplication of *E. coli* in the soil by about 2 log units greater than that recovered in the glucose-treated soil. When the soil was supplemented with both bile salts and glucose, counts of *E. coli* increased by about 5 log units in 48 h.

Bile salts are used as selective agents in media (for instance, mFC medium) to suppress the growth of non-fecal bacteria. Therefore, it was assumed that bile salts may similarly suppress certain bacterial populations in soil and thus reduce competition for available nutrients to facilitate *E. coli* multiplication. Further evidence to the above premise was obtained when soil dilutions were plated on soil extract agar containing or not containing bile salts. Lower recovery of soil bacteria on SEA plus bile salts in contrast to that recovered on SEA not containing bile salts (**Figure 5.11**) supports our hypothesis that bile salts suppress certain bacterial populations in soil and promote *E. coli* multiplication through reduced competition for available nutrients.

C. Natural field experiment

The purpose of this experiment was to determine whether *E. coli* can multiply in soil subjected to natural environmental conditions. In this study, an *E. coli* strain (originally isolated from soil) carrying an antibiotic (streptomycin) resistance marker was inoculated into natural Waimanalo soil placed in several plastic pots. The soil in the plastic pots was either unamended (control) or supplemented with one of three different amendments namely, glucose, bile salts or glucose plus bile salts. The plastic pots containing soil were buried in the ground in an open area and remained there until the experiment was terminated. Sub-samples of soil were analyzed for concentrations of *E. coli* at regular intervals over a period of 34 days by plating soil dilution of mFC agar containing streptomycin (1000µg/ml)..

The results summarized in **Figure 5.12** show that the uninoculated natural soil (overall control) contained a very low concentration of indigenous *E. coli*. The counts of these indigenous *E. coli* were undetectable after day 3 (data not shown). In the seeded soil, but unamended, the counts of *E. coli* declined gradually over a period of 34 days reaching a concentration of 3.56 log CFU/g of dry soil from an initial concentration of 6.70 log CFU/g of dry soil. On the other hand, the addition of bile salts (0.15 g/100 g of soil) as an inhibitor of non-fecal bacteria, resulted in a marginal increase (~ 0.5 log) in *E. coli* counts during the first 3 days and thereafter, the counts were more or less stable over the next 18 days and declined by about 1 log unit in the next 13 days. At day 34, the concentration *E. coli* in the bile salts-treated soil was about 2 log units higher than that recovered from the unamended soil during the same time. In the soil that received both bile salts and glucose, *E. coli* multiplied by approximately by 1.5 log unit during the first 3 days and the counts were relatively stable from day 6 through day 27, and subsequently, the counts declined. The results obtained in this experiment were very similar to that observed under simulated field conditions with comparable soil treatments.

This study again demonstrates that an intense competition for a limited supply of nutrients in natural soil result in suppression of *E. coli* multiplication. In contrast to concentrations of fecal bacteria (in soil), other indigenous microorganisms are much more numerous (**Table 5.2**), and therefore, these organisms always have a competitive advantage over fecal bacteria in extracting nutrients from soil. The addition of both bile salts and glucose to the unamended soil on day 34, resulted in a significant increase (about 3.5 log unit) in *E. coli* population in 5 days (data not shown). These results show that the suppressed population of *E. coli* is still metabolically active and will multiply when nutrients become available.

IV. Summary and Conclusions

In this phase of the study a traditional experiment based on dehydrogenase activity was completed to provide data that *E. coli* populations are metabolically active in soil indicating potential for their multiplication. To obtain direct evidence for multiplication of *E. coli* and enterococci in soil, various experiments had to be designed and conducted under three defined conditions namely, laboratory, simulated filed and actual filed conditions.

In the first experimental study design the objective was to determine whether fecal indicator bacteria such as fecal coliform, *E. coli* and enterococci are able to multiply in soil under laboratory conditions. Various experiments conducted under these conditions revealed that (1) populations of fecal bacteria (fecal coliform, *E. coli*, enterococci) can be established in soil and these fecal bacteria retain their active metabolic status in soil which is a prerequisite for multiplication, (2) populations of fecal coliform and *E. coli* increased significantly in sterile soil while counts of enterococci increased marginally suggesting that fecal coliform bacterial requirements are much simpler than that of enterococci, (3) in

natural soil, the multiplication of fecal bacteria was not evident until some simple nutrients such as glucose and salts or an inhibitor of non-fecal bacteria (bile salts) were added to stimulate the growth of fecal coliform and *E. coli* indicating that other indigenous soil microorganisms were more efficient in extracting a limited supply of energy sources, and (4) enterococci require more complex nutrients for multiplication in soil in contrast to fecal coliform bacteria.

In the second experimental study design, the objective was to extend our laboratory-based studies to simulated filed conditions to determine whether fecal bacteria could multiply in soil under environmental settings that better approximate natural conditions than those under laboratory situations. In brief, the results of the studies conducted under simulated field conditions were consistent with those previously obtained under laboratory settings. The major findings of the studies were: (1) as observed under laboratory conditions before, both fecal coliform and *E. coli* readily multiplied in cobalt irradiated soil however, these bacteria were unable to multiply in natural soil containing indigenous microflora until some simple nutrients (glucose and salts) were added, (2) the counts of enterococci in both sterile and natural soil were relatively stable again indicating that enterococci require more complex nutrients for multiplication, and (3) further evidence was obtained to substantiate our earlier hypothesis that competition for available nutrients restricts *E. coli* multiplication in natural soil.

In the third experimental study design, the objective was to obtain definitive data whether *E. coli* can multiply in soil under natural conditions. In brief, this study also demonstrated that fecal coliform bacteria such as *E. coli* can multiply in natural soil when nutrients are available.

Table 5.1. Evidence for Establishment (Colonization) of Fecal Indicator Bacteria in New Soil Environment.

Time (days)	Concentration of Indicator Bacteria (Log MPN/g Dry Soil)	
	E. coli	Enterococci
0	0.97	2.78
1	2.36	ND
2	2.82	ND
3	2.97	4.47
4	3.12	4.58

One part of sewage-contaminated soil was mixed with nine parts of sterile (autoclaved) soil to determine whether fecal bacteria (*E. coli* and enterococci) would establish in the new soil environment and increase in counts over time.

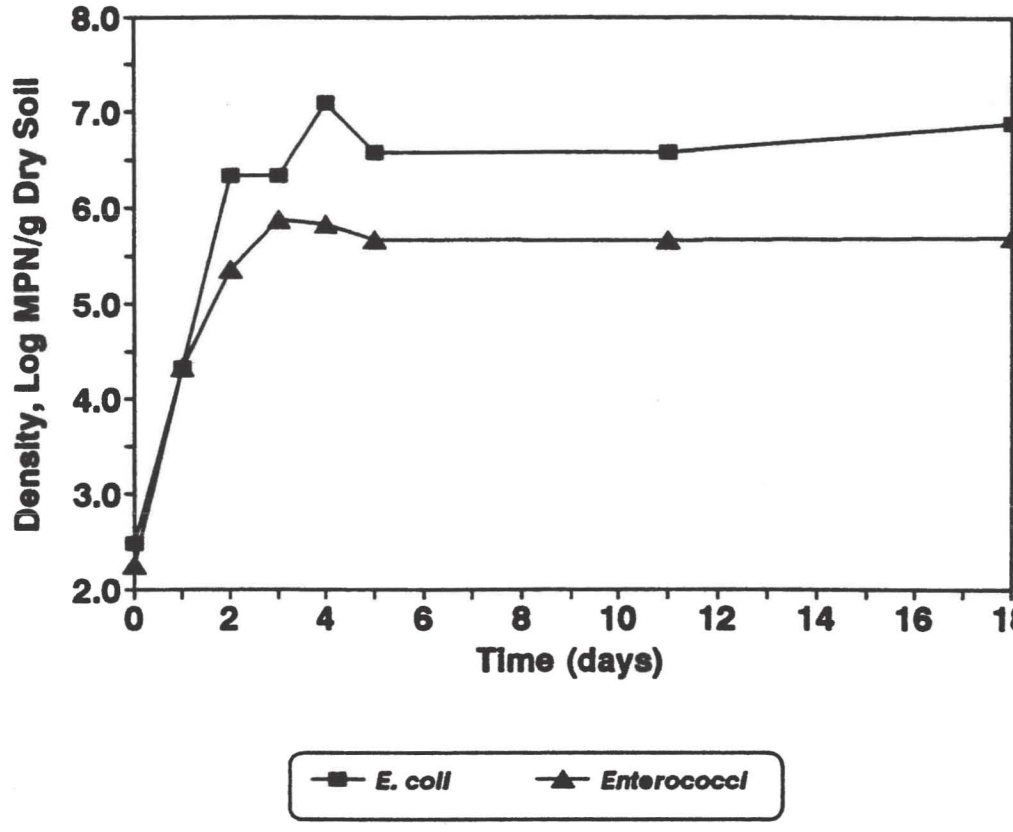


Figure 5.1. Multiplication of *E. coli* and Enterococci From Sewage Introduced into Sterile (autoclaved) Soil Incubated at Room Temperature (25°C).

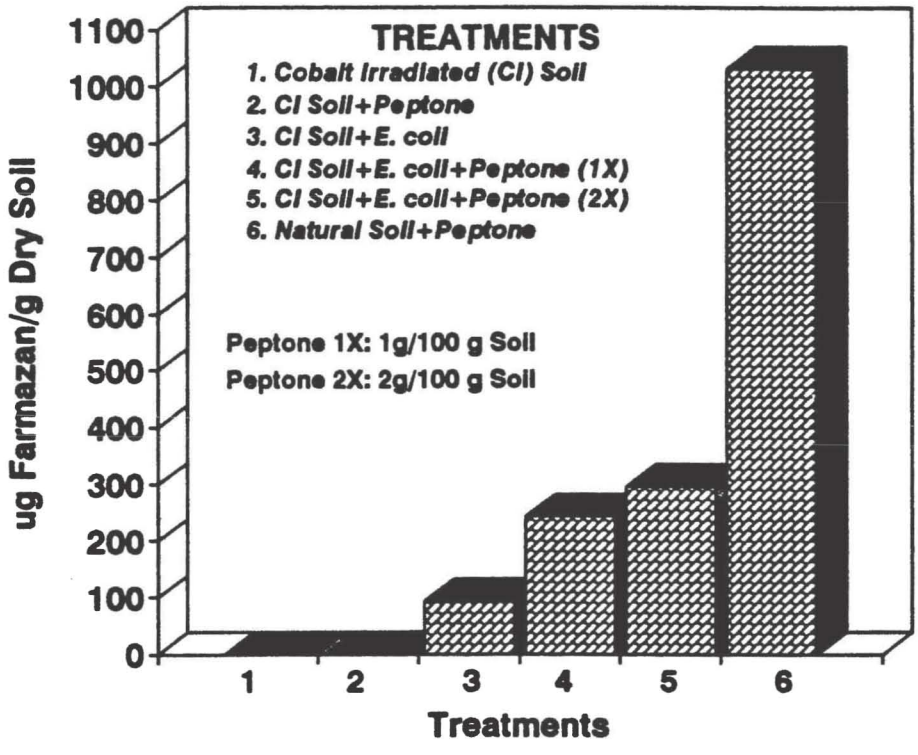


Figure 5.2. Metabolic Status of *E. coli* in Soil As Determined by the Dehydrogenase Assay.

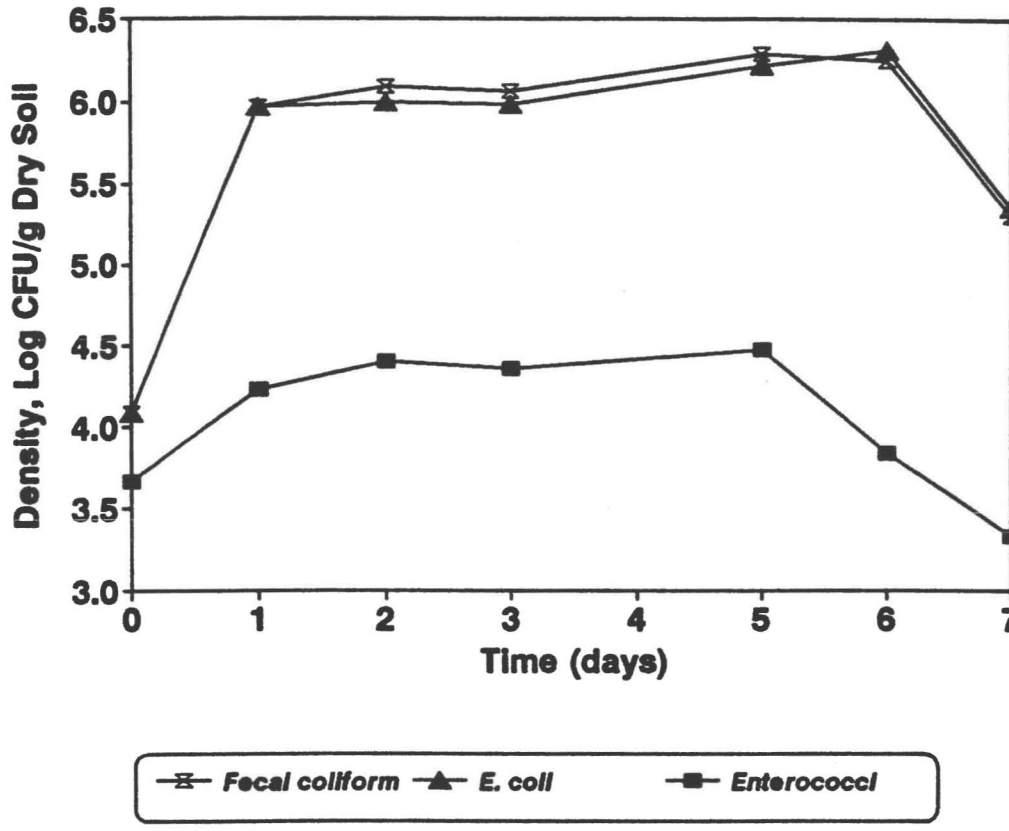


Figure 5.3. Growth of Fecal Bacteria From Animal Feces Introduced into Cobalt-Sterilized Soil Incubated Under Laboratory Conditions (25°C).

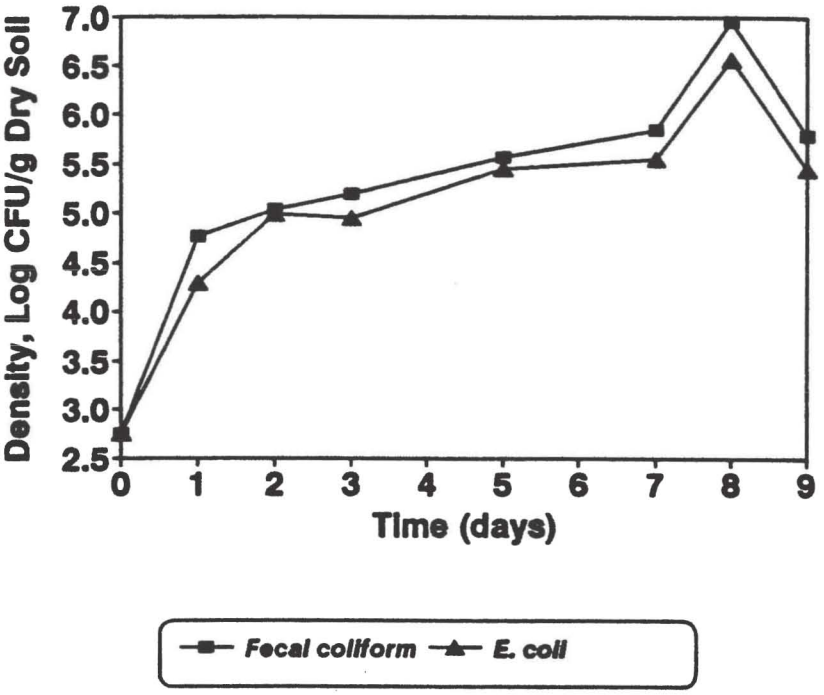


Figure 5.4. Fate of Fecal Indicator Bacteria From Sewage Introduced into Cobalt Irradiated Soil Held Under Laboratory Conditions at 25°C.

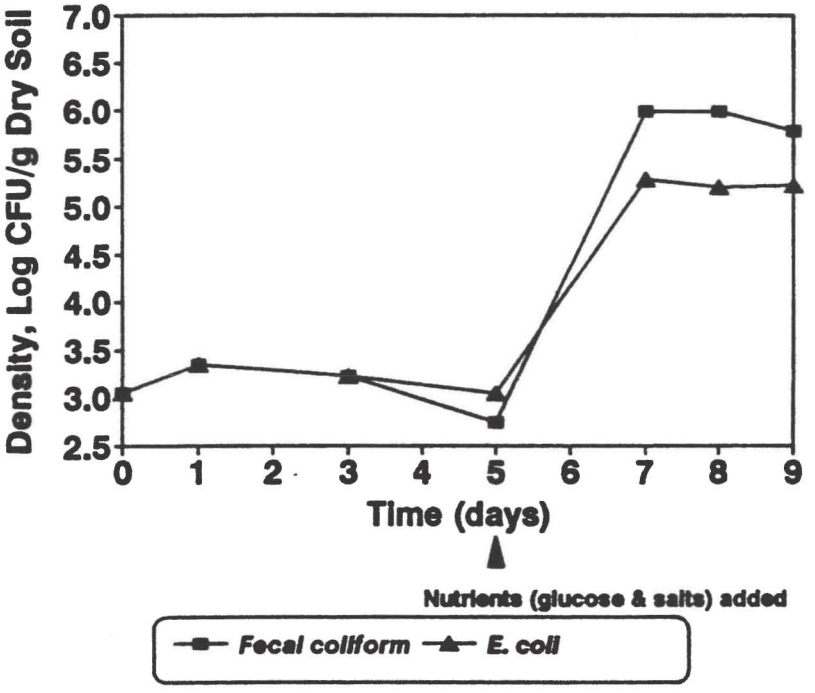


Figure 5.5. Fate of Fecal Indicator Bacteria From Sewage Introduced into Natural Waimanalo Soil Held Under Laboratory Conditions at 25°C.

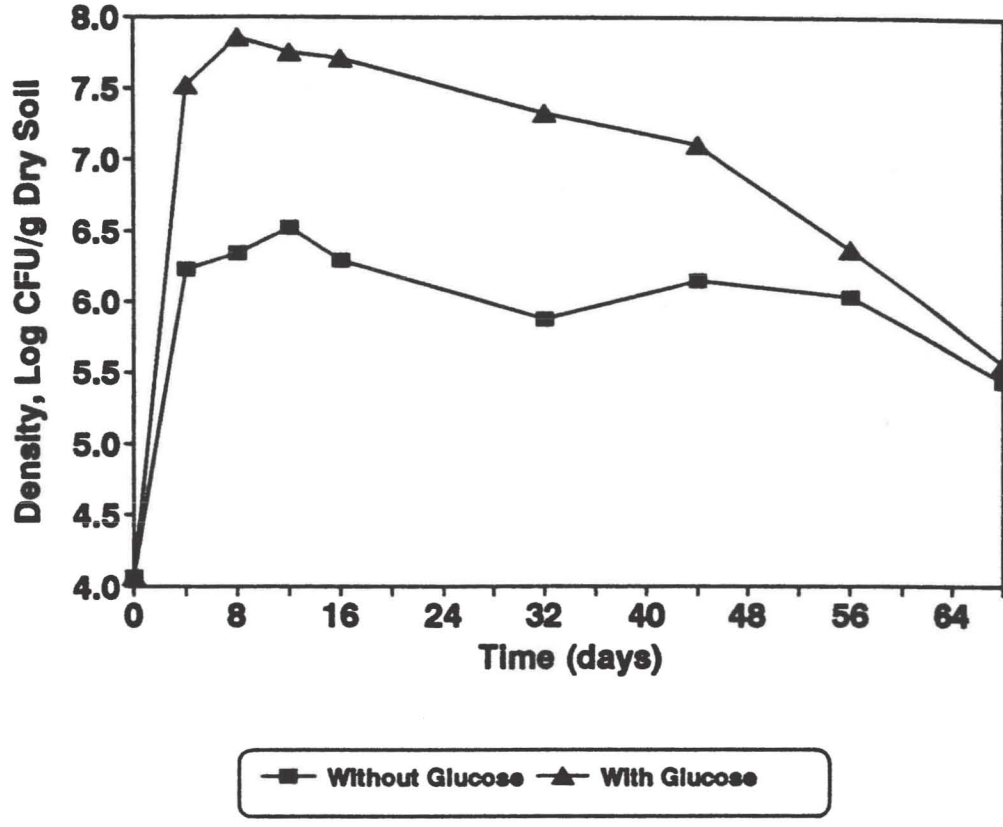


Figure 5.6. Growth of *E. coli* With *Lux* Gene Marker Introduced into Sterile Soil Under Laboratory Conditions (25°C).

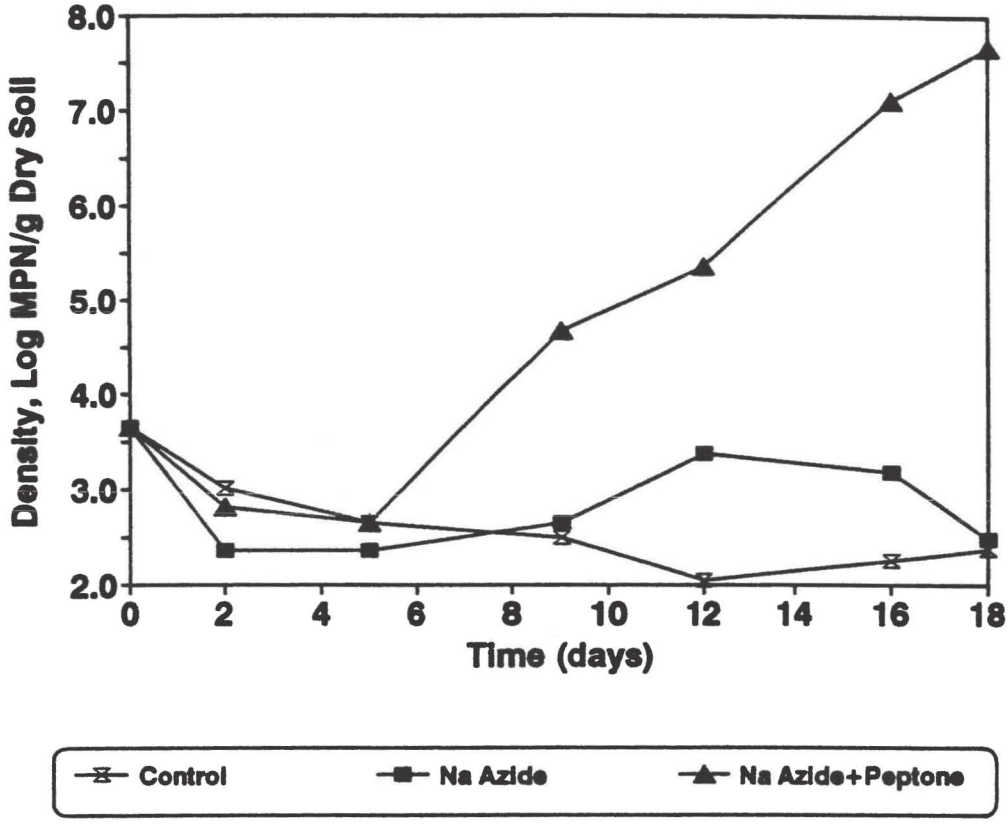


Figure 5.7. Enterococci Require Complex Nutrients for Multiplication in Soil Under Laboratory Conditions (25°C).

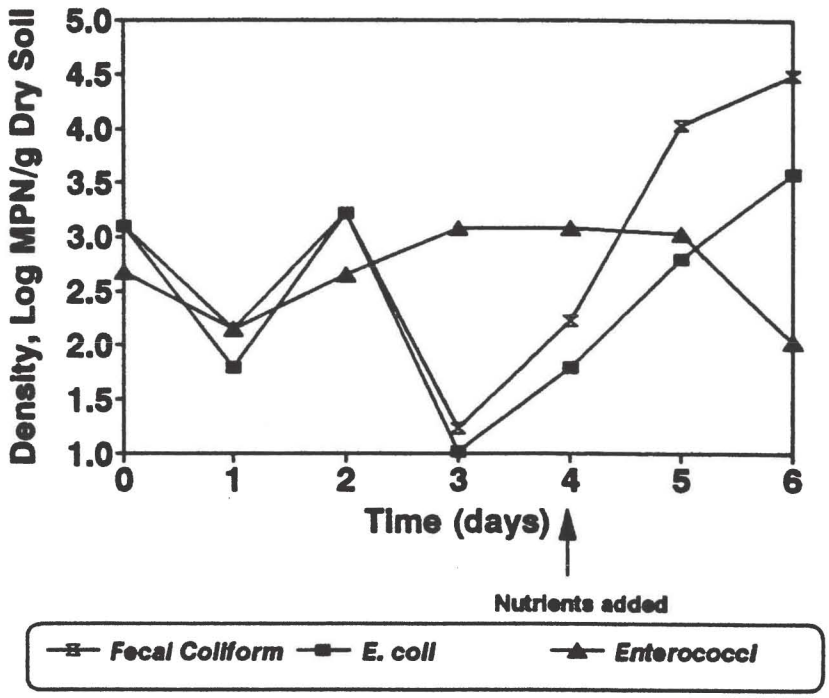


Figure 5.8. Growth of Indigenous Fecal Bacteria in Soil Under Simulated Field Conditions, But Maintaining Adequate Soil Moisture.

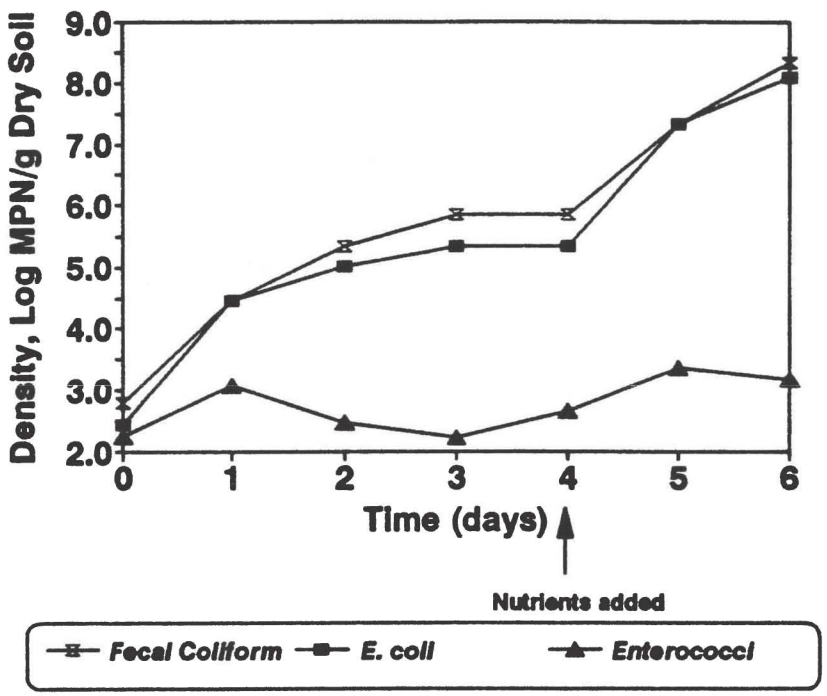


Figure 5.9. Growth of Fecal Bacteria From Sewage Introduced into Cobalt Irradited Soil Held Under Simulated Field Conditions and Maintaining Adequate Soil Moisture.

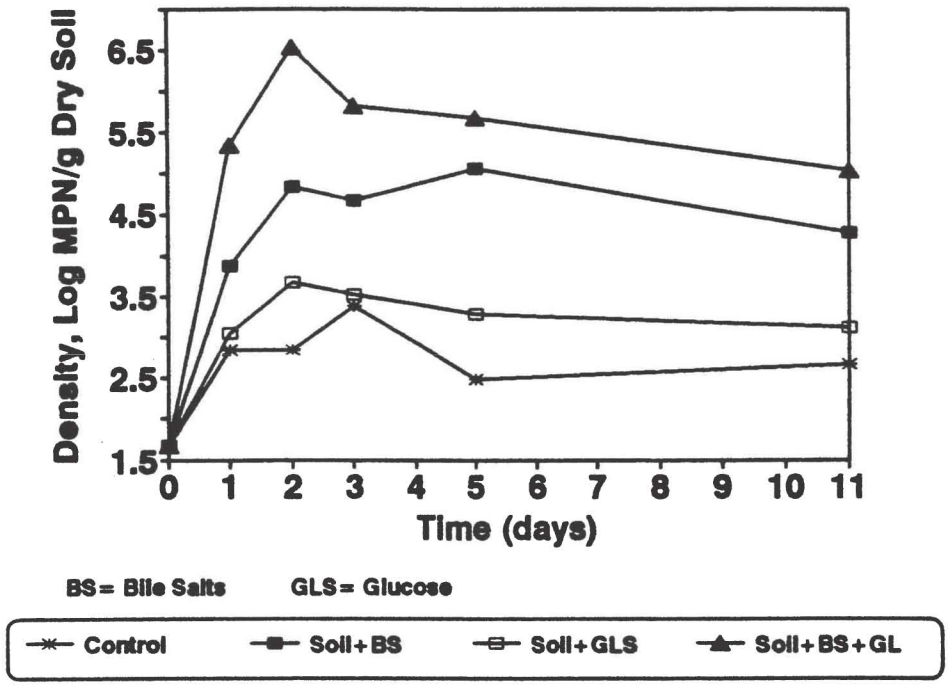


Figure 5.10. Competition for Available Nutrients Limits *E. coli* Multiplication in Natural Soil.

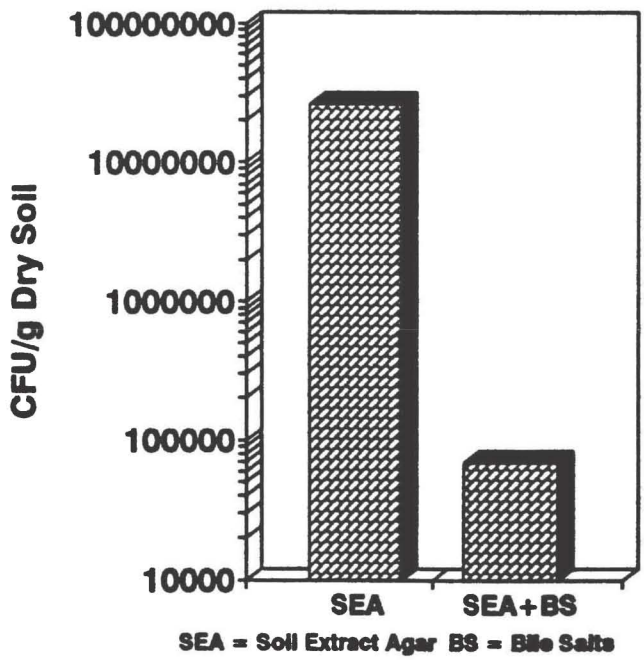


Figure 5.11. Inhibitory Effects of Bile Salts on Soil Bacteria.

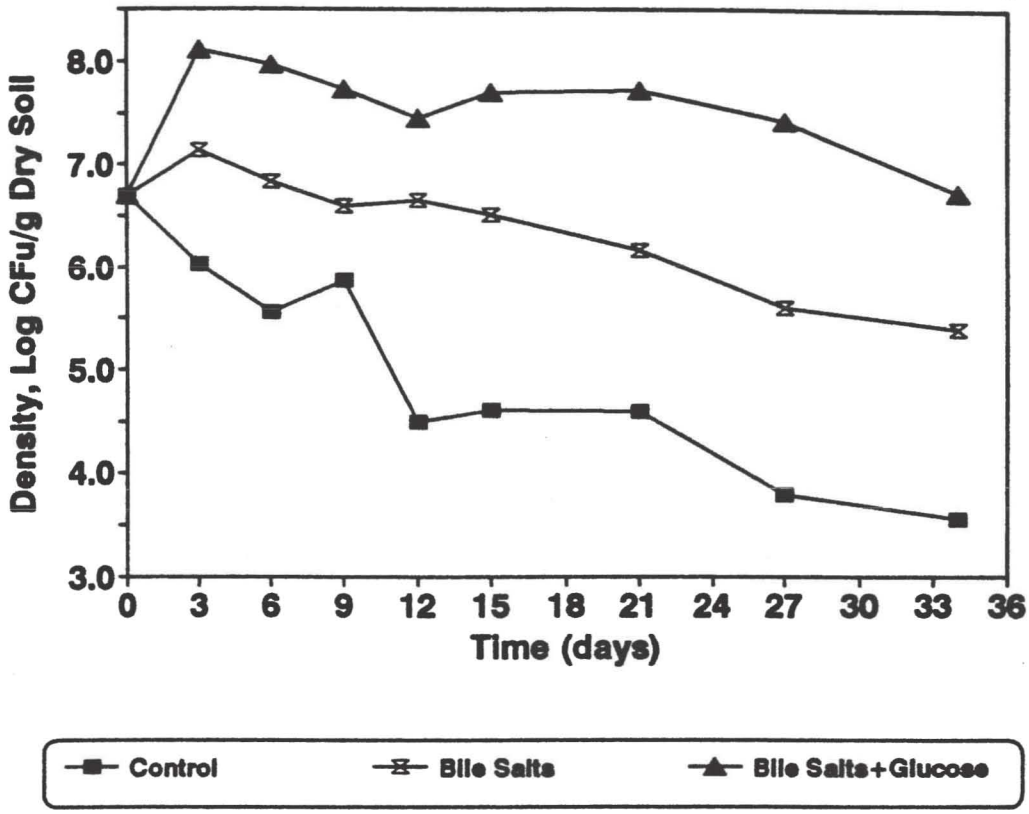


Figure 5.12. Growth Characteristics of Introduced *E. coli* in Natural Soil Under Field Conditions.

Table 5.2. Abundance of Fecal Indicator Bacteria Relative to Other Microorganisms in Soil From Three Different Locations.

Soil/Sample Site	Bacteria (total)	Fungi	Fecal Indicator Bacteria		
			Fecal Coliform	*E. coli*	Enterococci
	Concentration (CFU/g Dry Soil)				
Banks of Manoa Stream					
Site 1	7.96×10^7	ND	1.91×10^2	1.91×10^2	1.28×10^2
Site 2	7.15×10^6	ND	4.86×10^2	2.16×10^2	2.16×10^2
Waimanalo Soil	2.68×10^9	8.32×10^4	9.17×10^2	9.17×10^2	ND

ND = Not Determined

CHAPTER 6

PROJECT SUMMARY AND RECOMMENDATIONS

I. Project Rationale and Project Goal

Historically, concentrations of fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) have been used to determine the hygienic quality of water and to establish water quality standards. In using these fecal bacteria as an index of water quality, two assumptions are made that: (1) *there are no major environmental sources of these bacteria, and* (2) *the fecal bacteria do not multiply in the environment*. However, based on numerous studies conducted by our laboratory in Hawaii and extended to Guam over the past 20 years, we have obtained convincing evidence that freshwater streams in Hawaii contain high concentrations of fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) which consistently exceed the recreational water quality standards as recommended by USEPA. Sanitary surveys indicated that point source contamination such as sewage could not account for the high concentrations of fecal bacteria in all of the streams. However, we detected these same fecal indicator bacteria in high concentrations in the soil environments of Hawaii and concluded that soil was the primary environmental source where these fecal indicator were multiplying. Moreover, since rain is the source of all streams in Hawaii, we concluded that rain was the mechanism by which these soil-bound fecal indicator bacteria was transported to the streams. Based on the results of our studies we concluded that the two assumptions used by USEPA in interpreting recreational water quality standards were not applicable in Hawaii and therefore the USEPA recreational water quality standards are not applicable to Hawaii and other humid tropical islands.

Although we had obtained substantial data and had made reasonable conclusions, our data did not conclusively show that the fecal indicator bacteria were actually multiplying in the soil. Demonstration that fecal indicator actually multiplies in the soil environment of Hawaii is required to show that these fecal indicator bacteria could not be used to establish recreational water quality standards because under these conditions, the numbers of fecal indicator bacteria in soil would not be related to the presence of feces and to the possible presence of sewage borne pathogens. This conclusion is supported by the fact that pathogens such as human viruses and protozoa cannot multiply in the environment. Thus, the primary goal of the present study was to supplement all of the previous studies we have conducted for the purpose of obtaining convincing data that fecal indicator bacteria are able to multiply in the soil environment of Hawaii.

II. Project Experimental Design and Findings

The experimental design of this study was to conduct several different types of experiments to provide broad-based data that fecal indicator bacteria are able to multiply in the soil environment of Hawaii. The findings and conclusions of this study are as follows.

A. Fecal indicator bacteria are naturally found in most of the soil environments of Hawaii

This conclusion was based on the fact that fecal indicator bacteria could be recovered from soil samples representing 7 major soil groups which are found in different parts of the island of Oahu, state of Hawaii. The recovery of these fecal bacteria from the different parts of the island with different types of soil and from different environmental conditions provide evidence that the fecal indicator bacteria are able to colonize or persist in soil environments throughout the island and therefore would have an impact on water quality throughout the islands of Hawaii.

B. The fecal bacteria recovered from soil were speciated using standard identification schemes and determined to represent a metabolically diverse group

This conclusion was based on the use of standardized biochemical tests such as API 20 E, API 20 Strep and the Biolog identification scheme to confirm that the fecal indicator bacteria we recovered from soil were identified as *E. coli* and at least six species of enterococcus namely, *Enterococcus avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium* and *E. gallinarum*. Moreover, using the Biolog identification scheme which tests the ability of isolates to metabolize 95 different carbon sources as nutrients, it was determined that the fecal indicator bacteria recovered from the soil do not represent a few selected strains but represent a metabolically diverse group. These data suggest that the soil environment is not selecting for a mutant strain of fecal indicator with special metabolic pathways to survive in the soil environment. The data suggests that most strains of fecal indicator will be able to colonize or persist in the soil environment of Hawaii.

C. Soil provides sufficient moisture and nutrients to support the growth of fecal indicator bacteria

The conclusion that soil contains sufficient nutrients to support the growth of fecal indicator bacteria was based on two experiments in which nutrients from the soil was extracted and the soil extract made into a growth medium. In the first experiment, the soil extract was made into an agar growth medium and the recovery efficiency of *E. coli* and enterococci was determined on this agar medium as compared to other standard growth medium such as TSA, BHI, EMB and mEnt agar medium. Although the growth rates of

both *E. coli* and enterococci were slower on the soil extract agar as compared to the standard growth medium, the total amount of growth or yield of cells was similar on all growth media after 24-96 hours at 30°C. In the second experiment, the soil extract was prepared as a broth growth medium and the growth rate of *E. coli* at 32°C was shown to undergo a typical bacterial growth curve, although the rate and extent of this growth curve was less as compared to the growth curve in standard broth growth medium such as TSB. These results indicate that the concentrations of nutrients in soil are less than in standard bacterial growth medium but contains enough of all the essential nutrients to support the growth of *E. coli* and enterococci.

The conclusion that moisture in soil controls the viability of fecal indicator bacteria and that soil with naturally high moisture content (between 50-70% of water holding capacity or approximately about 35% moisture in Waimanalo soil in the current study) in the environment allows for the multiplication of fecal indicator bacteria were based on two experimental design. In the first experiment, purified *E. coli* and enterococci were added to cobalt sterilized soil and their concentrations monitored while allowing the soil moisture level in the soil to drop. In the second experiment, the indigenous concentrations of *E. coli* and enterococci in natural soil were determined and the concentrations of these fecal bacteria monitored while allowing the soil moisture to drop. The results showed that when moisture content of soil was reduced to <15%, the concentrations of *E. coli* dropped dramatically while the concentrations of enterococci remained fairly stable. These results are consistent with previous findings that when soil moisture levels drops, bacterial cells become stressed and that gram negative bacteria such as *E. coli* are more easily stressed than gram positive bacteria such as enterococci. The level of soil moisture would account for the variable concentrations of fecal indicator recovered from soil samples.

D. Evidence was obtained to show that fecal indicator bacteria can multiply in soil under laboratory controlled conditions

Initially, most of the experiments were conducted under laboratory controlled conditions because many of the variables of the experiment such as temperature (24 ± 1 °C), light, external contaminating sources and soil moisture could be controlled and the effect of a specific factor could be measured and the results interpreted with confidence. In the first two experiments soil was sterilized by autoclaving. All subsequent experiments were conducted using cobalt sterilized soil since this means of sterilization better maintain the natural state of the soil. Under these controlled conditions, the following findings were found.

1. Colonization of sewage-borne fecal indicator bacteria from 1 part of contaminated soil to 9 parts of uncontaminated and autoclave-sterilized soil

In this experiment one part of sewage contaminated soil was added to 9 parts of sterile soil. After 4 days of incubation at 25°C, both *E. coli* and enterococci populations had increased over 2 logs in the 10 parts of soil. Based on these results, we conclude that populations of *E. coli* and enterococci in sewage had colonized all of the sterilized soil and that soil in the absence of indigenous populations of microorganisms is a suitable environment for the growth of fecal indicator bacteria.

2. Multiplication and persistence of sewage-borne E. coli and enterococci added directly to autoclave-sterilized soil

In this experiment sewage-borne *E. coli* and enterococci were added to sterilized soil and their concentrations monitored over 18 days at 25°C. Both of these fecal indicator bacteria increased rapidly exceeding two log units during day one and up to 4-5 log units over 4 days and retained most of this high level over the next 14 days. These results confirm our previous observation that sewage-borne fecal bacteria can multiply rapidly in sterile soil and moreover, can maintain its population for extended periods.

3. Purified E. coli added to cobalt-sterilized soil are metabolically active as measured by dehydrogenase activity

This enzyme assay is generally used to determine the metabolic status of soil microorganisms. The assay showed that the activity of purified *E. coli* bacteria added to sterilized soil had a low activity and increased when nutrients (peptone) were added to soil. These results provide enzymatic evidence that the metabolic activity of *E. coli* increases *as E. coli* in the soil responds to added nutrients and undergo multiplication. The metabolic activity of *E. coli* in soil was only 20% of the total metabolic activity of all bacteria in natural soil indicating the greater metabolic activity of all the different microflora naturally present in the soil.

4. Multiplication of fecal bacteria from animal feces added to cobalt-sterilized soil

In this experiment diluted animal feces was added to sterile soil and incubated at 25°C. Concentrations of fecal coliform and *E. coli* multiplied to approximately 2 log units in 1 day and maintained that level over the next five days. In contrast enterococci concentrations increased only 0.8 log units in two days and maintained that level for about five days. Based on these results we conclude that fecal indicator bacteria from animal feces can multiply in soil and therefore animal feces may be the source of the indigenous populations of fecal indicator bacteria which can be recovered from soil throughout the island of Oahu.

5. Comparative fate of sewage-borne fecal coliform and E. coli added to cobalt-sterilized versus natural Waimanalo soil

In this experiment the fate of sewage-borne fecal coliform and *E. coli* was measured in sterilized versus natural Waimanalo soil. The results showed that the fecal bacteria immediately multiplied in the sterile Waimanalo soil but did not multiply over a five day period in natural Waimanalo soil. However, when excess nutrients were added to the natural Waimanalo soil on day 5, the suppressed population of fecal coliform and *E. coli* immediately multiplied. Based on these results we concluded that fecal indicator bacteria can utilize the nutrients available in sterile soil and will multiply. However, in natural soil, the indigenous soil microorganisms are much more effective in utilizing the available nutrients and will suppress of the growth of fecal bacteria. When nutrients are supplied and are in excess, even the fecal indicator bacteria can multiply along with the indigenous populations of soil microorganisms.

6. Multiplication of E. coli population with genetic marker in cobalt-sterilized soil

In this experiment, a population of *E. coli* with a genetic marker (lux gene) that enabled the colonies of *E. coli* to produce light was added to sterile soil with and without added nutrients (glucose) at 25°C and monitored over 68 days. In the soil without added nutrients, the *E. coli* population increased more than 2 log units in 4 days and up to 6.5 log units in 12 days. Thereafter, the concentrations remained stable during the next 44 days and then dropped slowly but was still 1.5 log units higher than the inoculated level at day 68. In the soil with added nutrients (glucose), the population of *E. coli* increased approximately 4 log units in 8 days and then dropped slowly but was still 1.5 log units higher than the inoculated level at day 68. These experiments provide genetic data that *E. coli* populations can multiply in soil and persist for up to 68 days. Moreover, that *E. coli* populations can multiply to higher levels when added nutrients are available. Thus, growth of *E. coli* in soil is controlled by available nutrients.

7. Enterococci require complex nutrients for multiplication in soil

Growth of enterococci in soil was less responsive to simple nutrients such as glucose and was always less than that of *E. coli*. In this experiment the growth of indigenous populations of enterococci in natural soil was determined after the addition of a complex nutrient (peptone) in the presence and absence of sodium azide, a known inhibitor of non-enterococci bacteria. Enterococci grew only in the soil sample with peptone and sodium azide. Based on these results, we concluded that enterococci like *E. coli* cannot multiply in the presence of indigenous populations of soil microorganisms but will grow well in soil when the indigenous soil microbial population is suppressed and complex nutrients are available for its growth.

E .Evidence was obtained to show that fecal indicator bacteria multiply in soil under simulated field conditions

Experiments conducted under laboratory conditions are easy to interpret but the data becomes significant if it can predict outcomes under actual field conditions. Initially, experiments under simulated field conditions were designed to allow some control over natural field conditions (external contamination, changing soil moisture) but expose the sample to daily changes in temperature and light.

1. Predictable fate of indigenous populations of fecal bacteria in soil

In this experiment, the indigenous soil populations of fecal indicator bacteria were monitored over 6 days under simulated field conditions. Concentrations of fecal coliform and *E. coli* fluctuated but showed no increase over 3 days in natural soil. On day 4, in response to the addition of simple nutrients (glucose) to the soil, populations of fecal coliform and *E. coli* rapidly increased. In contrast, concentrations of enterococci remained unchanged over the 6 days. Thus, the fate of the indigenous populations of fecal bacteria under simulated field conditions were similar to those conducted under laboratory conditions. Based on these results we conclude that even in field conditions, the indigenous populations of soil microflora are better able to obtain nutrients and thereby suppress the growth of fecal bacteria. However, when excess nutrients are available, even the fecal bacteria can multiply in soil. Fecal coliform and *E. coli* have simple growth requirements as compared to enterococci.

2. Predictable fate of sewage-borne fecal bacteria added to cobalt-sterilized soil

In this experiment sewage-borne fecal bacteria was added to sterile soil and their concentrations monitored over 6 days. The results showed that populations of fecal coliform and *E. coli* are able to multiply in sterile soil and increased to higher levels when additional nutrients (glucose) was added on day 4. In contrast, populations of enterococci remained unchanged over the 6 day experiment. Thus, the fate of the fecal indicator bacteria in sterile soil under simulated field conditions were similar to those conducted under laboratory conditions and support the consistent hypothesis that fecal indicator bacteria can multiply in soil when nutrients are available. Thus, soil, in the absence of indigenous populations of microorganisms, is an environment which is conducive for the growth of fecal indicator bacteria.

3. Confirmation that growth of fecal bacteria is suppressed by indigenous soil microflora

Circumstantial evidence was obtained that indigenous soil microflora suppressed the growth of fecal bacteria in soil. In this experiment, the fate of indigenous populations of *E. coli* was determined in natural soil with and without the addition of bile salt, an inhibitor of non-fecal coliform bacteria. The results clearly showed that the addition of bile salt to natural soil greatly inhibited the multiplication of soil microflora and at the same

time allowed *E. coli* to multiply to high levels. These results provide direct evidence that in soil, growth of indigenous soil microflora is responsible for suppressing the growth of fecal bacteria.

F. Evidence was obtained to show that fecal indicator bacteria can multiply under natural field conditions

Data obtained under natural field conditions are most relevant but are most difficult to obtain and interpret. In this experiment, a population of *E. coli* obtained from soil but determined to be resistant to high concentrations of an antibiotic (streptomycin) was added to soil under natural field conditions and monitored over 34 days. The use of antibiotic resistant *E. coli* was useful because this isolate originated from the soil, could be added at high initial concentration and could be easily recovered using selective medium containing streptomycin. The results showed that in natural soil, the populations of antibiotic resistant *E. coli* dropped slowly over the 34 day period. In contrast, the populations of antibiotic resistant *E. coli* increased slightly in soil samples to which bile salts were added and increased further in soil samples containing bile salts and added nutrients (glucose). Thus, the fate of *E. coli* (in soil) under natural field conditions was similar to that observed under laboratory as well as under simulated field conditions. The increase in counts of the antibiotic resistant strain of *E. coli* provide direct evidence for the multiplication of *E. coli* under natural soil conditions. Based on these results, we conclude that the data and hypothesis we developed based on experiments conducted under laboratory and under simulated field conditions are applicable to natural soil environments.

G. The principle of microbial ecology support the ability of fecal indicator bacteria to multiply in the soil environment of Hawaii

1. Common dogma: fecal bacteria should not multiply in soil environment

The ability of fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) to multiply in soil environments has been a difficult concept to accept by many in the field of water quality because of a common dogma which states that bacteria whose normal habitat is the intestine of warm blooded animals should not be able to multiply and become established in a totally different environment such as the soil. This dogma is plausible when the soil environment represents a temperate climate where the differences in the temperature between the intestines (37°C) and the fluctuating soil temperatures (0-25°C) is drastic. Other important factors includes humidity, lack of available moisture, soil composition and microbial flora. However, this dogma is less plausible when the soil environment represents a humid, tropical climate such as in Hawaii. Under these conditions the soil temperature (15-30°C) remains essentially constant throughout the year. These are temperature range in which the fecal indicator bacteria can readily grow. As a result, the soil environment of Hawaii and other humid tropical countries are characterized

by basic properties such as temperature and moisture which are conducive for the growth of fecal indicator bacteria.

2.Adaptability of bacteria and their drive to survive and multiply

A common principle of microbial ecology is that bacterial populations are genetically adaptable and will use every strategy within its gene pool to survive and to multiply in any environment. Development of various adaptive features by microorganisms permits their survival in diverse and extreme environments (Atlas and Bartha, 1993). In this regard, success for a microbial population is establishment of a population within an environment. The rapidity and extent to which it grows are only degrees of success. Fecal indicator bacteria are highly adaptable. In natural environments such as soils of Hawaii, the major factors which greatly limit the growth of fecal bacteria such as temperature, moisture, and osmotic effects are not limiting. As a result, fecal indicator bacteria are more able to use all of its adaptable strategy to survive and multiply in the humid, tropical soil environment.

3. Microbial adaptability and competition for available nutrients determines the microbial flora of specific soil environments: the feast or famine model

Many environmental niches including soil is comprised of many kinds of microbial flora. All of these soil microbial populations are using all of their survival strategies to become established in that environmental niche. Those better adapted to obtaining nutrients under those conditions will survive well and will predominate by out-competing other microorganisms for the limiting available sources of nutrients. However, growth of every microbial population is controlled by production of its own by-products and disappearance of usable nutrients. Under these conditions, the growth of other microbial populations are favored. In many environments availability of nutrients are sporadic causing great spurts of microbial growth followed by long periods of starvation where the microbial population must wait for another opportunity to obtain nutrients. This is the feast or famine model (Koch, 1971; Morita, 1984) which is the common strategy for most microbial populations in oligotrophic environments such as oceans and some soil. The fate of fecal indicator bacteria in soil fits into this microbial ecological model. Fecal indicator bacteria are not especially suited to grow in soil environments but this environment is within its adaptable range. As a result, the microbial populations whose normal habitat is the soil are much more able to obtain the available nutrients from soil and this prevents the fecal indicator bacteria to obtain sufficient nutrients to multiply dramatically. However, sufficient nutrients are available for slow sporadic multiplication of fecal bacteria or sporadic events when excess nutrients become available allows the fecal bacteria to maintain a population for long periods of time.

4. Impact of rhizosphere environment on growth of soil microflora

The extent of microbial activity in any ecosystem is dependent on the quality and quantity of the available nutrients, particularly energy (Morita, 1997). In this regard, the ability of fecal bacteria to maintain an active metabolic status and to multiply in soil, are also dependent on the availability of energy sources as well as other favorable conditions for growth. Readily usable carbon, a primary source of energy, is limited in soil (Clark, 1965; Lockwood, 1981; Lockwood and Filonow, 1981). As pointed out by Clark (1967), the paucity of food or lack of suitable and accessible energy source is the primary factor limiting bacterial growth. As a consequence, in soil where nutrients are in limited supply, soil-borne fecal bacteria may exhibit a slower growth pattern but enough to maintain an active population as a survival strategy.

In the current study, the experiments designed to determine whether fecal bacteria can multiply in the soil environment were essentially carried out using bare/bulk soil (without vegetation). The microbial activity in bulk soil which is not under the influence of plants roots is generally minimal due to paucity of energy sources. On the other hand, in natural soil, there are areas or pockets that are characterized by intense microbial activity. One such area is the rhizosphere which is defined as the volume of soil adjacent to and influenced by plant root (Hiltner, 1904). The rhizosphere microbial ecology has been extensively reviewed by various researchers (Balandreau and Knowles, 1978; Bolten et al., 1993; Elliott et al., 1984; Foster and Bowen, 1982). In the rhizosphere region, the microbial counts and their activity are several fold greater when compared with that of the bulk soil. An enhanced microbial activity in the rhizosphere has been attributed to a variety of organic compounds released by plant roots. Compounds released by plant roots as exudates include, amino acids, growth-promoting and growth-inhibiting substances, low molecular weight sugars organic acids, polysaccharides and proteins (Hale et al., 1978; Sparling et al., 1982). In fact, the carbohydrates derived from plant roots are the primary sources of carbon and energy for microbial growth and metabolism in the rhizosphere (Foster and Bowen, 1982). The substrates released by plant roots (refer **Table 6.1**) into the surrounding soil are so diverse and complex, it will be able to support the growth of microorganisms such as enterococci that require more complex nutrients. Therefore, the rhizosphere region may provide a special ecosystem in soil to furnish fecal bacteria with nutrients to grow. However, experiments are needed to demonstrate that fecal bacteria are able to grow and multiply in the rhizosphere region under natural conditions. Other indigenous microflora (bacteria) in the rhizosphere are short, gram-negative rods such as Alcaligenes spp., Flavobacterium and Pseudomonas (Alexander, 1977). As fecal coliform bacteria are also gram-negative rods, these bacteria possibly have an active niche in the rhizosphere.

Taken together, these results provide direct evidence that the soil environment of Hawaii can support the growth and multiplication of fecal indicator bacteria. These findings support the hypothesis based on principles of microbial ecology that various adaptive features that are characteristic traits of microorganisms permit their survival in diverse and extreme environments. As a result, fecal indicator bacteria are able to adapt and become established as one of the sub-population of soil microflora. The evidence indicates that these fecal bacteria represent a minor fraction of the diverse populations of bacteria present in soil. Most of the indigenous soil bacteria are well suited to grow in the soil environment and are better able to obtain nutrients and grow to higher concentration than fecal bacteria. However, fecal bacteria have apparently developed a strategy to persist and maintain a stable population in the soil environment. Most soil bacteria grow sporadically because of competition for available nutrients in a fast or famine mode. Undoubtedly the population of fecal bacteria undergo these constraints. Since time for rapid growth and multiplication is not essential for the maintenance of a stable population, these fecal bacteria have evolved a successful strategy to maintain a stable population in the soil environment of Hawaii. Still these fecal bacteria are successful enough to make an impact on existing recreational water quality standards.

H. Need for an alternative and applicable recreational water quality standard for Hawaii

Two of the basic assumptions (that *there are no major environmental sources of these bacteria, and the fecal bacteria do not multiply in the environment*) used by regulatory agencies in monitoring the quality of recreational waters are not applicable in Hawaii. As a result, the current USEPA recommended microbial indicators (fecal coliform, *E. coli*, enterococci) of water quality are not adequate in determining the quality of recreational waters in the island state. Therefore, there is a need to use an alternate microbial indicator to monitor the hygienic quality of recreational waters in Hawaii.

III. Recommendations

Since the existing water quality standards are not applicable for Hawaii, alternative and more reliable recreational water quality standards should be used in Hawaii (. Based on data and methodology considerations, *Clostridium perfringens* is the most reliable and suitable fecal indicator to assess the quality of recreational waters in Hawaii as well as other tropical locations because it meets all the required criteria for fecal indicators (Fujioka and Byappanahalli, 1996; Fujioka et al., 1997).

In brief:

- Regulatory agencies such as USEPA and the State of Hawaii Department of Health should re-evaluate the usefulness of the existing recreational water quality standards as applied to the state of Hawaii and to other tropical islands as well.
- We propose that *C. perfringens* be used to establish the hygienic quality of environmental waters in Hawaii. We propose the following standards based on geometric mean concentrations (CFU/100 ml) of *C. perfringens* using the mCP medium as developed by Bisson and Cabelli (1979):

	Geometric mean (CFU/100ml)
1. Inland waters for recreational use	<50
2. Coastal beaches for recreational use	<5
3. Near-shore marine waters which may become contaminated with sewage from ocean out-fall or waters from ship	<2
4. Pristine, uncontaminated waters	0

Table 6.1. Nutrients Released by Plant Roots into Soil: Organic Compounds Found as Plant Root Exudates.

Class of Organic Compound	Exudate Components
Amino compounds	Asparagine, α-alanine, glutamine, aspartic acid, leucine/isoleucine, serine, aminobutyric acid, glycine, cysteine, /cystine, methionine, phenylalanine, tyrosine, threonine, lysine, proline, tryptophan, β-alanine, arginine, homoserine, cystathionine
Fatty acids and sterols	Palmitic, stearic, oleic, linoleic, and linolenic acids; cholesterol, campesterol, stigmasterol, sitosterol,
Growth factors	Biotin, thiamin, niacin, pantothenate, choline, inositol, pyridoxine, p-amino benzoic acid, n-methyl nicotinic acid
Nucleotides, flavonones, and enzymes	Flavonone, adenine, guanine, uridine/cytidine, phosphatase, invertase, amylase, proteinase, polygalacturonase
Organic acids	tartaric, oxalic, citric, malic, acetic, propionic, butyric, succinic, fumaric, glycolic, valeric, malonic
Sugars	Glucose, fructose, sucrose, galactose, rhamnose, ribose, xylose, arabinose, raffinose, oligosaccharide
Miscellaneous	Auxins, scopolectin, fluorescent substances, hydrocyanic acid, glycosides, saponin, organic phosphorus compounds, nematode cyst or egg hatching factors, nematode attractants, fungal mycelial growth stimulants, mycelium growth inhibitors, zoospore attractants, spore and sclerotium germination stimulants and inhibitors, bacterial stimulants and inhibitors, parasitic weed germination stimulators

Source: Metting (1993)

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