View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Carolina Digital Repository

Evaluation of the H_2S Test As An Indicator of Waterborne Fecal Contamination

Lanakila “Ku” McMahan

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Science and Engineering, the Gillings School of Global Public Health.

Chapel Hill
January 2011

Approved by:
Mark Sobsey, Ph.D., advisor

Amy Grunden, Ph.D.

David Weber, M.D.

Louise Ball, Ph.D.

Fred Pfaender, Ph.D.

© 2011
Lanakila “Ku” McMahan
ALL RIGHTS RESERVED

ABSTRACT

LANAKILA MCMAHAN: Evaluation of the H_2S Test As An Indicator of Waterborne Fecal Contamination (Under the direction of Mark D. Sobsey)

A major limitation of the H_2S test for the detection of fecal bacteria is the current uncertainty and variability of its sensitivity and specificity for bacteria of fecal origin based on data from available studies. The purpose of this research was to determine whether a quantitative H_2S test could correctly identify fecally contaminated water samples and determine if there was any relationship between the test results and diarrheal disease risk. Using culture-based biochemical and culture-independent molecular techniques, this research focused on determining the types of microbial community members, including fecal indicator organisms, pathogens, and other microbes present in human sewage samples that are detected in a quantitative H_2S test as microorganisms of concern to human health. Sewage waters, natural waters from the United States, and household drinking water samples from central Vietnam were analyzed for the production of H_2S producing bacteria. The water sources tested in this study were chosen based on the 2002 World Health Organization Guidelines for Drinking Water Quality to be representative of commonly used drinking water sources worldwide and included a rainwater cistern, a protected lake, a well in an agricultural setting, and a well in a forested area. For the culture-based method, samples were analyzed for the presence of fecal bacteria by spread plating the water sample enrichment culture onto selective agar

media. The isolates were then: (1) tested to determine whether they were H_2S producing organisms, and (2) identified to the genus and species level using biochemical methods (primarily Enterotube and API 20E). Terminal Restriction Fragment Length Polymorphisms (TRFLP), a quantitative molecular technique developed for rapid analysis of microbial community diversity, was used to identify microbial community members and understand microbial community differences in water samples. Overall, these experiments showed: (1) when a water sample tests positive for H_2S, there are fecal bacteria in the water sample; (2) greater than 70 percent organisms isolated by the culture based biochemical identification method were also identified using TRFLP analysis; (3) although community composition differed and changed, there was a fairly stable group of identifiable microorganisms in the water samples; and (4) a quantifiable, low-cost version of the H_2S test costing less than 0.75USD had a similar relationship to diarrheal disease as did standard *E. coli* methods for testing drinking water samples in a developing country.

Keywords: H_2S test, microbial water quality, TRFLP, fecal indicator bacteria, diarrheal disease

ACKNOWLEDGEMENTS

First, I would like to thank all of the people from the communities of in Central Vietnam and our EMW and UNC-CH research teams because without their help and patience, none of the field research would have been possible. I also received tremendous support from all of the staff and others that supported the work in Vietnam including our lab manager Douglas Wait. I would also like to thank Hien Vo Thi and Van Ly for all of their support and assistance while in the country. Hien's expertise was a significant source of assistance in making the project a reality.

I would like to acknowledge the support of my doctoral dissertation committee: Mark Sobsey, Amy Grunden, Fred Pfaender, David Weber and Louise Ball. In addition, I have had a great experience working in the Sobsey lab and have to thank all the members for their help; especially my team of undergraduate research assistants. Without Joe Milner, Abhinav Komandur, Karen Roque, Mehrin Islam, Prasant Lokinendi, Naomi Fernando, Shampa Panda, and T Clayton, I would not have been able to get through all of the TRFLP and culture experiments to make the research plan a reality. I also have to give a mention to the members of the EMW team who worked tirelessly day and night to finish the research study.

Finally, I have to say thank you to my friends and family. I know it seems like this has been a never-ending task but your constant support and understanding has made it easier.

Mom, Dad, Barry, and Victor, I never thank you enough for your help over the years.

Mikkos, Namita, Matt, and the others, with your support I’m now Dr. Ku!

TABLE OF CONTENTS

LIST OF TABLES ... xii

LIST OF FIGURES ... xiv

ABBREVIATIONS ... xvi

Chapter 1: Overview and Objectives ... 1

1.1 Introduction and Background ... 1

Global Burden of Disease Attributable to Unsafe Water and Improper Sanitation ... 1

Reasons for Inadequate Water Quality Testing in Developing Countries ... 2

Need for the H_2S Test ... 2

Importance of Improved Water Quality and Opportunities to Document it in Field Studies ... 4

1.2 Objectives ... 6

Chapter 2: Literature Review ... 8

2.1 Diarrheal Disease and Waterborne Illness ... 8

2.2 Hydrogen Sulfide Producing Bacteria ... 9

2.3 H_2S Test Format ... 11

2.4 The H_2S Test as a Fecal Indicator Test ... 13

2.5 Modifications of the H_2S test ... 19

2.6 Currently Available H_2S tests ... 21

Chapter 3: Determination of an appropriate H_2S Test for field application ... 23

3.1 Introduction ... 23

3.2 Materials and Methods **24**

3.3 Results **25**

Comparison of 3 Broth Media 25

Comparison of MPN method at 24 vs. 48 hours 26

Comparison of 125mL Whirl-pak Bags vs. Plastic Bottles 27

Comparison of H_2S and E. coli MPN method 27

Cost of the H_2S Compartment Bag Test 28

Comparison of the H_2S Compartment Bag Test and the IDEXX Quantitray system 32

3.3 Conclusions **35**

Chapter 4: Comparison of Culture-Based and TRFLP Analysis to Identify H_2S Producing Microorganisms in Sewage **36**

4.1 Introduction and Background **36**

4.2 Materials and Methods **38**

Culture-Based Biochemical Detection Methods 38

DNA Extraction 40

PCR Conditions 41

TRFLP 41

Fragment Analysis 42

In Silico© Output 43

4.3 Results **47**

Culture Based Biochemical Detection 47

TRFLP Molecular Analysis 48

Statistical Measure Comparing Culture-Based Method and TRFLP 50

4.4 Discussion **51**

Chapter 5: Comparison of Culture-Based and TRFLP Analysis to Identify H_2S Producing Microorganisms from Multiple Natural Water Sources 54

5.1 Introduction and Background 54

5.2 Materials and Methods 55

Sampling sites 55

Culture-based biochemical detection method 56

DNA extraction 57

PCR conditions 58

TRFLP 58

Fragment analysis 59

Further Phylogenetic Assignment 61

Statistical Measure Comparing Culture-Based Method and TRFLP 62

5.3 Results 63

University Lake Molecular and Cultured Isolate Findings 63

FedEx Global Health Building Rainwater Cistern Molecular and Cultured Isolate Finding. 66

Duke Forest Artesian Well Molecular and Cultured Isolate Findings 67

NCSU Shallow Well Molecular and Cultured Isolate Findings 69

Statistical Measure Comparing Culture-Based Method and TRFLP 70

5.4 Discussion 70

Chapter 6: Hydrogen Sulfide Producing Bacteria and *Escherichia coli* in household drinking water and diarrheal disease risk: evidence from central Vietnam…… 73

6.1 Introduction 73

6.2 Methods 74

Study population and recruitment of households 74

Data collection ... 76

Diarrheal Disease and other Health Related Data ... 76

Water quality data ... 77

Additional Data Collection ... 78

Data Analysis ... 79

6.3 Results ... 82

6.4 Discussion ... 88

Chapter 7: Discussion of the Feasibility of a Simple, Low-cost H_2S Test for Fecal Contamination of Water in Developing Countries and Emergency Situations ... 94

7.1 Summary of Significant Results ... 94

Laboratory evidence ... 94

Field Performance of H_2S CBT Test in determining microbial quality of an improved drinking water supply ... 95

Relationship between the H_2S CBT Results and Human Health Risks ... 96

7.2 Study Results and Relationship to Existing Evidence about the Performance H_2S Test ... 97

Comparison of Laboratory Results ... 97

Comparison of Field Performance Results ... 99

7.3 Health Impact Study and other Evaluations of H_2S Test vs. Human Health ... 102

7.4 Research Limitations ... 102

7.5 Conclusions ... 105

7.6 Further Research on the H_2S Test ... 107

Appendix 1. Microorganisms capable of producing hydrogen sulfide ... 109

Appendix 2. List of Genera Identified in Sewage by Experiment ... 113

Appendix 3. Broth and PBS Recipes ... 118

Appendix 4. Biochemical analysis and TRFLP Protocols 119

Appendix 5. Summary of Fragment Data for Sewage and Natural Water TRFLP Experiments. 126

Literature Cited 128

LIST OF TABLES

Table 2.1 The H2S Test as an Ideal Fecal Indicator 15

Table 3.1 Comparison of H_2S versus *E. coli* tests based on sample positivity or negativity for sewage and seeded water experiments under controlled lab conditions............. 28

Table 3.2 Comparison of H_2S versus *E. coli* tests based on sample positivity or negativity for piped and treated water samples in Central Vietnam. 28

Table 3.3. Criteria for low-cost simple drinking water test for E. coli and H_2S producing bacteria 31

Table 3.4. Comparison of the H_2S Compartment Bag Test and the IDEXX Quantitray system at 27°C, 37°C, and 44°C.. 33

Table 4.1 Kappa values of agreement between results of culture based biochemical identification methods and the TRFLP molecular methods for bacterial isolates from positive H_2S culture tests of sewage samples.. 51

Table 5.1 Kappa Values of Agreement between the Culture Based Identification Method and the TRFLP Molecular Method for the Natural Water Samples.......................... 70

Table 6.1 Household characteristics from individual observations for all study participants over 4 household visits, by exposure status at time of visit.................. 82

Table 6.2 Mean total coliform and E. coli counts (cfu/100ml), H_2S (MPN/100ml) for samples taken from households (untreated and treated water)................................. 83

Table 6.3 Correlation matrix of water samples compared by fecal indicator 83

Table 6.4 Diarrheal disease Odds Ratio estimates fecal indicators in *untreated* drinking water [a,b,c,d] 84

Table 6.5 Diarrheal disease Odds Ratio estimates fecal indicators in *treated* drinking water [a,b,c,d] 85

Table 6.6 Diarrheal disease Odds Ratio estimates of H_2S producing bacteria in *untreated* drinking water by compartment bag category. [a,b,c,d] 86

Table 6.7 Diarrheal disease Odds Ratio estimates of *E. coli* in *untreated* drinking water by WHO category[23]. [a,b,c,d] 86

Table 6.8 Diarrheal disease Odds Ratio estimates of H2S producing bacteria in *treated* drinking water by compartment bag category. [a,b,c,d] 87

Table 6.9 Diarrheal disease Odds Ratio estimates of *E. coli* in *treated* drinking water by WHO category[23]. 87

LIST OF FIGURES

Figure 1.1 A map of the Vietnam water quality study areas 6

Figure 3.1 Concentration of H2S Producing Organisms in various H_2S Formats at 37°C (MPN/100ml) 26

Figure 3.2 Concentration of H2S Producing Organisms in HACH Media at 24 vs. 48 hours at 37°C(MPN/100mL) Note that (A) and (B) were 2 different experimental trials 26

Figure 3.3 Concentration of H2S Producing Organisms in Bags vs. Bottles at 37°C (MPN/100mL) 27

Figure 3.4 Concentration of H2S Producing Organisms (Samonella, Citrobacter, Proteus) at 37°C, 39°C, 41°C, 43°C, and 44°C, (MPN/100mL) 34

Figure 4.1 A workflow diagram describing of the culture based biochemical identification and the TRFLP processes. 40

Figure 4.2 A flow diagram describing the In Silico software (In Silico LLC, Fuquay-Varina, NC) process used to generate TRFLP community profiles (Johnson et. al. 2009). 44

Figure 4.3 Pathogens and H_2S-Producing organisms identified in sewage samples using culture-based biochemical identification methods. 48

Figure 4.4 TRFLP fragment patterns identified at the Genus level in genomic DNA isolated from H_2S-positive liquid cultures of bacteria in sewage-contaminated samples. 49

Figure 5.1 Pathogens and H_2S-Producing organisms identified in natural water samples using culture-based biochemical identification methods. 63

Figure 5.2 TRFLP fragment patterns identified at the Genus level in genomic DNA isolated from H_2S-positive liquid cultures of bacteria in natural water samples. 65

ABBREVIATIONS

ANOVA- Analysis of Variance

CI – Confidence interval

DENR- North Carolina Department of Environment and Natural Resources

dNTP- Deoxynucleotide Triphosphate

EMW-East Meets West Foundation

FC- fecal coliforom

GEE- Generalized Estimating Equations

GBD – Global burden of disease

H_2S- Hydrogen Sulfide

IRB- Institutional Review Board

OR- Odds Ratios

MDG – Millennium Development Goals

MPN- Most Probable Number

NGO – Non-governmental organization

NBCI- National Center for Biotechnology Information

NCSU- North Carolina State University

NW- Natural Water

PBS- Phosphate Buffered Solution

PCR- Polymerase Chain Reaction

OR – OR ratio

OWASA- Orange Water and Sewer Authority

POU – Point of use

RDMA – USAID's Regional Development Mission-Asia

SES – Socioeconomic status

SRB- Sulfate Reducing Bacteria

TC-Total Coliforms

TRFLP- Terminal Restriction Fragment Length Polymorphism

TSA- Tryptic Soy Agar

UNICEF – United Nations International Children's Fund

USD- United States Dollar

USAID- United States Agency for International Development

WHO – World Health Organization

Chapter 1: Overview and Objectives

1.1 Introduction and Background

Global Burden of Disease Attributable to Unsafe Water and Improper Sanitation

It is estimated that 4% of all deaths are a result of the disease burden from inadequate water, sanitation and hygiene and that this accounts for more than 5% of the total disease burden worldwide (Prüss-Üstün and Corvalán 2007). Lack of access to safe water, improper sanitation and poor hygiene contribute to an ongoing global health and crisis resulting in millions of deaths and infectious disease morbidity burdens affecting billions of persons annually. Inadequate water, sanitation and hygiene account for roughly 94 percent of the 4 billion cases of diarrhea that WHO estimates occur globally each year (UNICEF/WHO. 2009).

Drinking and recreational waters, foods and treated fecal wastes such as sewage effluents are routinely tested for fecal indicator bacteria in developed countries. The World Health Organization (WHO), the Food and Agricultural Organization and other international and regional agencies encourage testing of water, waste and food for fecal indicator microbes, and recommend acceptable microbial limits as guidance. However, many developing countries lack the capacity and resources to carry out this type of testing on water, food, waste, and other environmental media. In developed countries, microbiological testing of water and treated human waste streams is ubiquitous, and supported by government regulations, management systems, monitoring specifications,

and sophisticated, costly, well-equipped laboratory facilities. In the developing world especially in remote areas, and after natural disasters, such testing and the infrastructure to support it is rarely available or accessible.

Reasons for Inadequate Water Quality Testing in Developing Countries

In order to know if water is safe to drink and if WHO-recommended Water Safety Plans for hygienic water management are achieving microbially safe water, drinking water and its sources must be tested regularly. One of the greatest challenges in implementing safe water programs in developing nations is the lack of a way for water consumers to identify when their water is and is not microbiologically safe. Outside of areas served by well-equipped centralized water infrastructure, there is a lack of capacity to test water for fecal contamination by even the simplest of methods. In these places, the water people drink is never tested for microbial contamination, and consumers have no way to know if their water is microbiologically safe, if it requires treatment, or if a treatment method they are using is working.

Need for the H_2S Test

Given the lack of access to microbial testing of water in resource-limited settings, there is a need for simple, low cost tests for fecal indicator microbes that can be performed by the water consumer at the point of use. The new and improved tests must not require the usual laboratory equipment, infrastructure, and other standard (but often inaccessible) materials to monitor microbial water quality. Instead, these tests must have the potential to be performed by people and institutions lacking specialized training in environmental microbiology or water quality analysis. Consumer-accessible tests for

fecal microbes in water can potentially reduce pathogen exposures and waterborne disease. The access of water providers, public health authorities, emergency/disaster relief agencies, and water consumers to such tests empowers, educates, builds capacity and enables people and institutions to make critical health-related management decisions about water quality.

To develop such tests, the appropriate fecal indicators must be chosen as targets. Hydrogen sulfide-producing (H_2S) bacteria have been proposed as an alternative fecal indicator. Methods for detection of H_2S bacteria were developed in the early 1980s to fill this need for a simple, reliable field test for use by village public health workers to detect fecal contamination in drinking water (Manja et al. 1982; Sobsey and Pfaender, 2002). The H_2S test detects the presence of microbial hydrogen sulfide production as a black iron sulfide precipitate in suspension. The H_2S method to detect fecal contamination has been compared to more traditional fecal indicator bacteria and their detection methods and has demonstrated relatively good correlation with conventional bacterial indicators of fecal contamination (Sobsey and Pfaender, 2002).

Advantages of this test include the ease of isolating, identifying, and enumerating H_2S producing organisms, and the ease and low cost of the quantitative MPN format of the H_2S test. In addition, the H_2S test may be more applicable than typical fecal indicator bacteria such as fecal coliforms, *E. coli* and enterococci because it can indicate the risks from the more resistant pathogens such as human protozoan parasites, including Giardia cysts and Cryptosporidium oocysts because the test detects spores of *Clostridium perfringens* and related sulphite-reducing clostridia, a better indicator of protozoan parasites.

However, prior laboratory and field research from other studies have made it clear that there currently is inadequate data to advocate for the H_2S test as an indicator because it fails to meet many of the criteria for an ideal or desirable fecal indicator. An ideal fecal indicator should be absent in unpolluted water and present when the source of pathogenic microorganisms of concern (fecal contamination) is present. As described by Kaspar et al., (1992), this may not be true of H_2S producing organisms. H_2S producing organisms may themselves be pathogenic depending on the concentration present in the water of interest. In addition it is presently unclear whether or not H_2S producing organisms are normally present in greater numbers than the pathogenic microorganisms of interest, and that they respond to natural environmental conditions and water treatment processes in a manner similar to the pathogens of concern. This research will examine some of these areas in greater detail and examine sewage and natural water sources to determine if the quantitative version of the H_2S test meets the requirements of an ideal fecal indicator in both laboratory and field settings.

Importance of Improved Water Quality and Opportunities to Document it in Field Studies

There have been no studies that directly relate quantitative H_2S test results to diarrheal disease risks. This information is key because currently WHO and EPA will not accept the H_2S test for water quality purposes because their own preliminary testing and their review of available literature question the sensitivity and specificity of the test. However, if more readily available data show that not only are H_2S-producing organisms present when other fecal indicators are present in actual drinking water samples, there might be an adjustment of the current recommendations from both organizations. More

importantly, if field studies conclusively show that the quantitative H_2S test works as well or better than current fecal indicator tests, there would be further evidence to recommend its adoption as a simple, low-cost alternative to currently available testing. As the National Research Council noted (2006), "The most important biological attribute is a strong quantitative relationship between indicator concentration and the degree of public health risk." However, field studies examining microbiological testing, especially water sampling, are often extremely expensive and time-consuming, further limiting their implementation.

Fortunately, this research benefitted from an already ongoing collaboration between UNC and USAID in Southeast Asia known as WaterSHED that was conducting a post-implementation assessment of household microbial water quality examining *E. coli* as an indicator of fecal contamination in rural areas of central Vietnam with East Meets West (EMW) Foundation. WaterSHED focuses on a collaboration of NGO, government, and increasingly, private sector led programs to increase access to water and sanitation improvements in the rapidly developing economies of Southeast Asia. The goal of EMW is the proper functioning and financial sustainability of clean water and sanitation systems in underserved communities in Central Vietnam. East Meets West (EMW) has now installed over 8,000 piped water connections and hundreds of pour-flush latrines in central Vietnam.

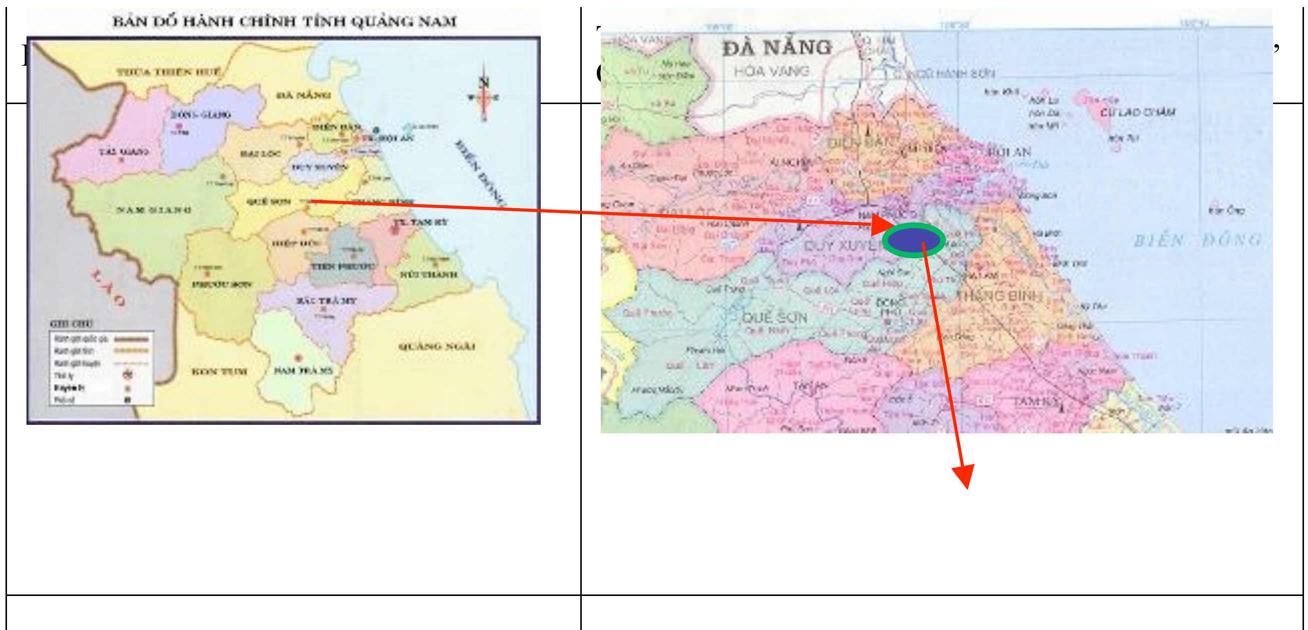

Figure 1.1 A map of the Vietnam water quality study areas

For a quantitative version of the H_2S test to be an acceptable indicator, data is necessary on both its field application, it relationship with an accepted fecal indicator, and its relationship with human illness. The post-implementation study presents an opportunity to compare *E. coli* and H_2S levels in diverse household drinking water samples and then to compare both indicators separately to diarrheal disease.

1.2 Objectives

1. Evaluate different formats of the H_2S test and determine the most effective format for field use by:

a. Evaluating the application of the H_2S test through an estimation of the concentration of H_2S bacteria from the most probable number method using multiple dilutions and sample volumes.

b. Comparing lab made and commercially-made H_2S media for their performance for detection and quantification in wastewater and fecally contaminated water.

c. Comparing fecal contamination of water using an optimized H_2S test versus an *E. coli* test when applied to samples of fecally contaminated water and wastewater.

2. Using biochemical and molecular methods, determine whether there are similarities or differences in the bacterial communities of waters and wastewaters that produce positive H_2S test results.

a. Examine bacterial communities in samples with known fecal contamination-associated fecal microorganisms and determine if the H_2S test can correctly identify these samples.

b. Examine bacterial communities in natural water samples with unknown compositions and concentrations of fecal organisms and determine if the H_2S test can correctly identify fecal contamination in these samples.

c. Examine bacterial communities in household drinking water samples in a group of small Vietnamese communities with unknown levels of fecal organisms and determine if the H_2S test can correctly identify the levels of fecal contamination in these samples compared to a standard test for *E. coli.*

3. Determine the relationship between the presence and numbers of H_2S producing bacteria in drinking water and diarrheal disease risks in a longitudinal study compare it to the presence and levels of the widely accepted *E. coli* fecal indicator.

a. Determine H_2S levels in household drinking water samples over time. Compare these levels to *E. coli* levels.

b. Determine diarrheal disease levels and compare those estimates to the occurrence and level of microbial drinking water contamination as measured by H_2S testing and *E. coli* testing.

Chapter 2: Literature Review

2.1 Diarrheal Disease and Waterborne Illness

Diarrheal disease is a major cause of morbidity and mortality in young children. Recent estimates suggest that diarrhea accounts for more than 1.6 million deaths annually (WHO, 2006). Mortality from diarrheal disease has decreased over the past four decades yet a recent study on the global burden of the disease suggests that there has not be an accompanying decrease in morbidity (Kosek et al., 2003). The average child in the developing world experiences 3 or more diarrheal disease episodes per year; accounting for more than 4 billion cases of diarrhea annually.

Pathogens transmitted through the fecal-oral route often cause diarrheal disease. They are typically considered enteric pathogens because they can infect the gastrointestinal tract and once shed into the environment via excreta they are capable of being transmitted in a variety of ways including through contact with contaminated water and person-to-person. Disease transmission by water can be classified into four categories: waterborne, water-washed, water-based and water- related (White, Bradley, & White, 2002). Ingesting fecally contaminated water transmits waterborne pathogens. Lack of adequate quantity of water for washing and bathing transmits water-washed pathogens. Water-related pathogens are transmitted via an insect vector that breeds in water. Interventions in drinking water quality to reduce diarrheal disease target primarily

waterborne pathogens. Waterborne pathogens comprise a broad range of microorganisms ranging from viruses to bacteria to parasites. For example, a recent case-control study in Ecuador documented cases of diarrhea as a result of all three classes of pathogens: *E. coli*, Rotavirus and *Giardia* (Eisenberg et al., 2006).

The H_2S test has existed since 1982 and has been suggested as a test for the presence of fecal indicator bacteria in water. As described earlier, data exists showing a fairly strong correlation between the results produced by the H_2S test and those produced by *E. coli* and other fecal indicator tests. However, to date there are no published studies showing a relationship between levels of H_2S producing organisms in drinking water and human illness. One of the goals of this research is to determine if there is a relationship between H_2S producing organisms in drinking water sources and diarrheal disease.

2.2 Hydrogen Sulfide Producing Bacteria

There are several genera and species of bacteria that can produce hydrogen sulfide. Hydrogen sulfide is a key compound in the sulfur cycle, and many microbes mineralize or decompose organic sulfur (from living cells or of synthetic origin), oxidize elemental sulfur and inorganic compounds such as sulfides and thiosulfate, or reduce sulfate and other anions to sulfide. H_2S is produced when bacteria consume sulfate oxygen for organic processes. This often occurs in anerobic situations when oxygen is not available in its elemental form nor as a part of a nitrate. In the H_2S test, organisms reduce the SO_4 available in the media to bisulfide, which combines with the Fe to form Ferrous sulphide (FeS) forming a black deposit that denotes a positive reaction (Madigan and Martinko 2008).

A major group of environmental bacteria producing H_2S are referred to as sulfate reducing bacteria (SRB), and are key players in the global sulfur cycle. These bacteria are ubiquitous and occur in a variety of habitats, including marine and freshwaters and their sediments, soils, biofilms, microbial mats, intestinal contents, termite guts, walls of "black smokers" (hydrothermal sea vents) and in association with marine worms (Sobsey and Pfaender 2002).

Based on 16S rRNA sequencing the SRB can be phylogenetically divided into five distinct lineages: (1) Gram-negative mesophilic SRB (delta-Proteobacteria), (2) Gram-positive spore forming SRB (Low G+C Gram-positive Bacteria), (3) thermophilic bacterial SRB (Nitrospira phylum), (4) thermophilic bacterial SRB (Thermodesulfobacterium group), and (5) thermophilic archaeal SRB (Euryarchaeota).

In surface and subsurface geohydrothermal environments (e.g., hot springs, subsurface and submarine hydrothermal vents, etc), H_2S is produced by sulfur respiration with molecular hydrogen.

In ground waters, particularly those contaminated with human or animal wastes or those containing reduced sulfur from natural or anthropogenic sources, there is a high potential for anaerobiasis in the aquifer and the resulting formation of sulfides by bacteria that are not of human or animal origin. In many rural areas small-scale industry, animal husbandry, and human dwellings are all contiguous, which creates the potential for sulfide formation from sediment-derived degradation of organic wastes from these sources, only some of which are fecal sources (Sobsey and Pfaender 2002). In addition, H_2S producers may also be present in Iron-containing waters that contain other Iron-metabolizing and related corrosion bacteria. These bacteria may not have any relationship

with fecal contamination. Moreover, rapid reaction of the iron with sulfide already present in a water sample could produce a darkening in an H2S test almost immediately upon addition of the sample.

The relationship of sulfate-reducing bacteria (SRB) and microbial mats is another recently discovered and described phenomenon, and may affect the results and interpretation of the H_2S test. Many recent studies have shown that the presence of oxygen is not necessarily toxic to SRB and that their habitat range goes beyond anoxic environment. As Minz *et. al* (1999) noted, "the highest rates of sulfate reduction yet documented in a natural system were observed in the highly oxic near-surface region of a cyanobacterial microbial mat. Thus, the contribution of SRB to biogeochemical cycling may be significantly greater than is now appreciated." In addition, other studies have shown that sulfate reduction occurred consistently within the well-oxygenated photosynthetic zone of the mats during both high and low light conditions (Dillon et al. 2007). These studies suggest that SRB may be found in more environments than previously thought and may increase the number of microenvironments in which H_2S producing bacteria that are not necessarily of fecal origin may be found.

2.3 H_2S Test Format

Manja et al. (1982) observed that the presence of coliform bacteria in drinking water was consistently associated with the presence of organisms that produce hydrogen sulfide (H_2S), and developed a test based on the formation of an iron sulfide precipitate on a paper strip or in the sample container. This precipitate is formed by the reaction of H_2S with iron. The test is intended to detect bacteria of fecal origin, some of which are

able to reduce organic sulfur to sulfide (as H_2S gas), which then reacts rapidly with iron to form a black precipitate.

Over the last two decades, various investigators have tested this method and modifications of it in tropical and temperate regions, including Indonesia, Peru, Paraguay, and Chile, Nepal, Bangladesh, and South Africa (Ratto et. al., 1989; Kromoredjo and Fujioka, 1991, Kaspar et al., 1992; Castillo et. al., 1994; Venkobachar et al., 1994; Rijal and Fujioka, 2001; Genthe and Franck, 1999; Pant et al., 2002; Anwar et al., 2004; Oates et al., 2003; Tewari et al., 2003; Pathak and Gopal, 2005; Roser et al., 2005; Gupta et al., 2008), and compared it to traditional bacterial indicators of fecal contamination in water.

The H_2S method does not consistently measure the presence of total coliforms, fecal coliforms or *E. coli*. However, many members of the fecal coliform family are known H_2S producers including: *Klebsiella pneumoniae, K. oxytoca*, *Enterobacter cloacae, and Citrobacter freundii* (LeClerc et. al 2001). Both enteric and non-enteric bacteria from a variety of habitats can release sulfide from proteins, amino acids and other reduced sulfur compounds by reduction reactions, including some coliforms (e.g., *Citrobacter* spp. and *Budvicia aquatica*) and other enteric bacteria (e.g., *Clostridium perfringens*) (Sobsey and Pfaender 2002). Therefore, there are many bacteria that may produce a positive result in the H_2S test.

Wetzel (2001) noted that there would be little sulfate for bacteria to use if the concentration of the substrate is low in freshwater. However, where sulfate concentrations in water are high, such as in geothermal environments, sulfate-reducing bacteria could give positive results in H_2S tests. Sulfate reducers do not metabolize

complex organic compounds such as those included as substrates in H_2S test medium, instead requiring short chain organic acids and other products of fermentation. It is possible that they would not grow and give positive results in H_2S tests. However, in mixed communities of microorganisms SRB could give a positive result because other bacteria (heterotrophs) would ferment sugars and provide the organic acids used by the SRB to give a positive result (Widdel, 1988). For a positive reaction to occur, the test sample would need to become anaerobic, allowing the fermentative bacteria to produce the required short-chain organic acids and other preferred SRB substrates and leading to the growth of SRB in the test sample. These conditions are not as likely to be achieved in the incubation times typically used in H_2S tests (1-2 days), though they are possible.

2.4 The H_2S Test as a Fecal Indicator Test

Microbial water quality indicators are used in hazard identifications, exposure assessments, and to evaluate the effectiveness of risk reduction actions (Committee on Indicators for Waterborne Pathogens 2004). Currently *E. coli*, *enterococci*, and the fecal coliform group are considered the "gold standards" of microbial water quality testing. WHO and the EPA do not consider the H_2S test to be in this category. To be considered an ideal fecal indicator, a test must have the following attributes:

- Correlates to health risk
- Similar (or greater) survival to pathogens
- Similar (or greater) transport to pathogens
- Present in greater numbers than pathogens
- Specific to a fecal source or identifiable as to source of origin
- Specificity to desired target organism

- Broad applicability
- Precision
- Adequate sensitivity
- Rapidity of results
- Quantifiable
- Measures viability or infectivity
- Logistical feasibility (Training and personnel requirements, Utility in field, Cost, Volume requirements)

Table 2.1 The H2S Test as an Ideal Fecal Indicator*

Ideal Indicator	**H_2S Test**	Comments	Reference
Correlated to health risk	**Unknown.**	No published health studies to date	
Similar (or greater) survival and transport to pathogens	**Yes**	Survival similar to *Salmonella* and *Clostridium* spp. Given that they are H_2S producing organisms	Nagaraju and Sastri (1999), Martins et al. (1997), Castillo et al. (1994),
Present in greater numbers than pathogens	**Yes**	Multiple non-pathogenic species produced H_2S including: *Citrobacter freundii*, *Salmonella, Proteus mirabilis, Klebsiella spp., Enterobacter spp., Acinetobacter spp., Morganella spp.*	Nagaraju and Sastri (1999), Castillo et al. (1994), Manja et al. (1982)
Specific to a fecal source or identifiable as to source of origin	**Yes**	Tested by comparison with other fecal indicators	Manja et al. (2001 & 1982), Nagaraju and Sastri (1999), Venkobachar et al. (1994), Nair et al. (2001) Ratto et al. (1989), Kaspar et al. (1992) Castillo et al. (1994), Martins et al. (1997), Kromoredjo and Fujioka (1991) Genthe and Franck (1999) Sivaborvorn (1988)
Specificity to desired target organism	**No**	Multiple Organisms Produce H_2S	Ratto et al. (1989), Kaspar et al. (1992), Venkobachar et al. (1994), Sivaborvorn (1988), Martins et al. (1997),
Broad applicability	**Yes**	1. Test has been applied to groundwater, surface water, bore wells, dug wells, rainwater cistern, and municipal water supplies 2. Test has been conducted on waters from: a. India b. Peru c. Chile d. Indonesia e. South Africa f. Thailand	See below. Ratto et al. (1989), Castillo et al. (1994), Martins et al. (1997), Kromoredjo and Fujioka (1991) Genthe and Franck (1999) Sivaborvorn (1988)
Precision	**Yes among samples, No between labs**	1. Test 100% matched TC in raw water. 81% match for treated waters. 2. 100% Agreement with *E. coli*	Martins et al. (1997) Rijal et al. (2000)
Adequate sensitivity	**Yes in most cases**	1. 82% and 86% agreement with FC test 2. Similar to *E. coli* test 3. 95% Agreement with FC Tests	Genthe and Franck (1999) Rijal et al. (2000) Ratto et al. (1989)
Rapidity of results	**Yes**	24 h heavy/moderate contamination. 48 h for light contamination.	Manja et al. (2001 & 1982), Nagaraju and Sastri (1999), Venkobachar et al. (1994), Nikaeen et al. (2010) Castillo et al. (1994), Martins et al. (1997), Genthe and Franck (1999), Rijal et al. (2000)

Quantifiable	**Yes with MPN method**	1. Similar detection by the MPN version of the H_2S test and *E. coli* 2. 90% agreement with MPN results	Rijal et al. (2000) Manja et al. (2001)
Measures viability or infectivity	**Yes**		Genthe and Franck (1999)
Logistical feasibility (Training and personnel requirements; Utility in field; Cost; Volume requirements)	**Yes**	Cheap and easy-to-use; Minor Training Needed; Moderate Volume; Demonstrated Field Use	Nikaeen et al. (2010), Genthe and Franck (1999), Nagaraju and Sastri (1999), Venkobachar (1994), Kaspar et al. (1992), Kromoredjo and Fujioka (1991), Ratto et al. (1989), Manja et al. (1982),

* Parts of Table Adopted from Sobsey and Pfaender 2002

As has already been noted, though the most important biological attribute is a strong quantitative relationship between indicator concentration and the degree of public health risk, no studies have been published comparing H_2S-producing bacteria to human illness. The Committee on Indicators for Waterborne Pathogens (2004) does say that correlating prospective indicator concentrations and pathogen levels is an alternative means of demonstrating the relationship to health risk, and this is what most research on H_2S-producing bacteria has done.

As Table 2.1 shows, many investigators have attempted to speciate the bacteria that produce positive results in the H_2S test. Castillo et al. (1994) found a large variety of bacteria in samples giving positive reactions in the H_2S test, primarily *Clostridium perfringens* and members of the *Enterobacteriaceae* (including *Enterobacter,* Clostridia*, Klebsiella*, *Escherichia, Salmonella, Morganella*) and other organisms known to cause illness in humans *(Acinetobacter, Aeromonas)*. Ratto et al. (1989) found *Citrobacter* was a common organism in positive H_2S tests.

Sobsey and Pfaender (2002) suggest that while the organisms producing a positive H_2S result many not be all coliforms, they are organisms typically associated with the intestinal tracts of warm-blooded animals, which are not necessarily of human

origin. Moreover, the H_2S test may detect bacteria that are naturally occurring in water and not of fecal origin. One of the major weaknesses of H_2S test for the detection of fecal bacteria is the variation in sensitivity and specificity for bacteria of fecal origin obtained across studies. Nikaeen et al. (2010) found that a P/A H2S test had only 60.9% agreement with standard MPN technique for fecal coliforms.

Previous studies applying the H_2S test to groundwater samples have demonstrated false positive results, where H_2S-positive samples contained no fecal coliforms or *E. coli* (Kaspar et al., 1992; Pant et al., 2002). False negative results, where H_2S-negative samples were found to contain *E. coli*, have been shown in other studies (Desmarchelier et al. 1992,Tewari et al., 2003). In study of groundwater contaminated by septic seepage, Roser *et al.*, (2005) found that an assessment based on a single (p/a) result was unable to distinguish unambiguously between heavily contaminated and mildly contaminated waters. However, multiple test sets, especially the ten by 10 mL arrays, provided a clear distinction between the most and least contaminated locations. While H_2S producers showed this pattern, average H_2S producer counts were greater than *E. coli* or *Enterococci* and therefore the test probably detected bacteria other than these indicators. On the other hand, the test appeared much more sensitive than measurements of somatic and F-specific coliphages and protozoan pathogens which were detected in septic supernatant samples only. Overall, the H_2S test seems to have fairly high sensitivity, specificity and precision when comparing the results across studies.

These investigators observed that many samples in which no *E. coli* were detected gave positive results using the H_2S test. Many of these samples positive for H_2S bacteria and negative for *E. coli* were positive for *Enterococci* and/or sulphite reducing *Clostridia*

(SRC), both of which are accepted microbial indicators of fecal contamination (Roser et al., 2005). In addition, Gawthorne et al. (1996), found that when FC and *E. coli* testing failed to indicate the presence of *Salmonella*, the H_2S test was successful in indicating the presence of 4 different *Salmonella* species. These studies indicate a need for investigation of the microbial community present in samples that give positive results for the H_2S test.

If there is sulfide already present in a water sample, the rapid reaction with the iron in the test media could produce a darkening in an H_2S test almost immediately upon addition of the sample. For this reason, it is very important that the test procedure include visual checking for a rapid positive reaction, after a few minutes to one hour of incubation (Sobsey and Pfaender 2002). A rapid positive result is an indication that the sample already contains sulfides. Such a result cannot be interpreted as an indicator of fecal contamination. Rather, a minimum of 18 hours is required for an adequate interpretation from a highly contaminated sample, with more time required for samples suspected to have low to moderate contamination.

There are other important aspects to microbiological water quality test beyond the biology alone. Precision (which includes not only repeatability with a laboratory, but variability across laboratories) is of particular importance, because decisions must sometimes be made on a limited number of samples. As Table 2.1 shows, though many studies demonstrate consistent agreement between H_2S producing bacteria and TC, FC, *or E. coli*, there is not yet a systematic, standardized method for the H_2S test (with many tests using P/A and others using 20 or 50mL volumes), hindering inter-laboratory agreement of results. The next section describes in detail the differences in H_2S testing methods.

2.5 Modifications of the H_2S test

A variety of H_2S test formats have been previously evaluated. Variations include:

Media composition

Media preparation procedures,

Test formats, such as paper strip, powder, and agar media

Test endpoints {presence-absence, Most Probable Number (MPN), and membrane filter},

Sample volumes

Incubation times

Incubation temperatures.

Over the last two decades, several investigators have evaluated the H_2S test with various modifications such as medium composition, incubation period and temperature, in different tropic and temperate regions including Indonesia, Peru, Chile, Nepal and South Africa. They have also compared it to conventional water bacteriological methods listed below. Ratto et al. (1989) evaluated the H_2S test at 22°C and 35°C and compared it to Most probable number (MPN) and fecal coliform tests for 20 potable water samples in Peru technique for coliforms and fecal coliforms using laurel tryptose broth and brilliant green lactose bile broth with fecal coliform confirmation in EC broth. These investigators concluded that the H_2S test was at least as sensitive test as total coliform (TC) and fecal coliform (FC) tests. Castillo et al. (1994) concluded that the simplicity and low cost of the H_2S test makes it very applicable to tropical and subtropical potable waters. Genthe and Frank (1999) evaluated the specificity of the H_2S test using seeded samples and reported that the test produced positive results in all seeded samples. Pillai et al. (1999) concluded that positive H_2S results were generally obtained in 18 to 48 hours of

incubation at 25°C-44°C. Gupta et al. found that the incubation period had significant effect on the effectiveness of the H_2S test was in comparison to *E. coli* MPN testing, and as incubation period increased from 24 to 48 h, the effectiveness also increased from 47% to 95 at room temperature and 63% to 96% at 37°C.

Manja et al. (1982) also conducted a comparative assessment of H_2S tests at various incubation temperatures and periods with standard tests and found that H_2S producing organisms are consistently associated with the presence of coliforms in water. Recently, Tambekar et. al (2010) analyzed 1050 water samples from various sources (open and tube wells, restaurant and hotel water samples) and compared H_2S –producing bacteria to *E. coli* at both 27ºC and 37ºC after 18, 24, and 48 h of incubation. They found that the number of samples testing positive for both H_2S and *E. coli* using MPN methodology was only 50% at 24 hours and rose to 81% after 48 hours. There was a 62% and 89% correlation of H_2S and *E. coli* at 37ºC after 24 and 48 hours respectively.

These studies suggest that the adding cystine or cystiene to the media with longer incubation times (24-48 hours) and incubation temperatures in the range of 25-35°C can increase the ability of the test to detect low levels (5 CFU per sample) of H_2S producing bacteria (Sobsey and Pfaender 2002).

Though much of the research done on the H_2S test has compared its results to standard *E. coli*, TC, and FC measures, there are only a few studies that used an MPN format. The MPN format would provide more information than the standard presence/absence form of the test, because it would give a semi-quantitative measure of the numbers of H_2S producing organisms in a given water sample. Having quantified or semi-quantified levels of fecal contamination is important for efforts to relate the levels

of bacterial contamination in water to waterborne disease risks. However, there has been no systematic comparison of the various types of H_2S tests used by different investigators, and no effort to standardize the test procedure.

2.6 Currently Available H_2S tests

Until recently, test media or materials were not readily available from commercial sources, and all of the H_2S tests required the use of media formulated in the laboratory and applied to paper strips manually. However, several commercial H_2S tests are now available, providing a basis for uniformity and standardization of testing. In India, there have been efforts to have the medium made commercially and to implement performance criteria for the commercially prepared medium. In the United States the HACH Company has marketed an H_2S test kit (PathoScreen) for use by small labs and consumers. However, in its current form this commercial test is probably too complex and costly to be used for water quality testing in the developing world.

Research and development studies have been done in India by UNICEF (United Nations Children's Fund)-India and its partners (Rajiv Gandhi National Drinking Water Mission, Department of Drinking Water Supply, Ministry of Rural Development, Government of India) to develop, evaluate and disseminate the specifications for a H_2S test and field kit for use in drinking water (Manja et al., 2001). The test is recommended for use by community workers to monitor water supply sources, and is not advocated as a replacement for conventional coliform and other bacteriological testing. In addition researchers in Bangladesh, India, and other parts of south and southeast Asia are currently using a presence/absence form of the test as an indicator of fecal contamination and as an education tool for improved water, sanitation, and hygiene (Tambekar et. al

2010). One such example is the use of the test in post-flooding situations in Pakistan when a presence/absence version of the H2S test was used to assess microbial water quality (WASH Cluster meeting notes 2010).

As the Committee on Indicators for Waterborne Pathogens (2004) notes, logistical feasibility often governs the choice of indicator methods. Those concerns might include: labor; materials; capital; training costs (especially when large numbers of samples are needed for screening purposes); and simplicity (simpler methods with proven field utility and small volume requirements are generally preferred). Given these concerns, a simple, low-cost, quantitative version of the H_2S test would be preferable because it could be standardized for use around the globe. The proceeding chapter describes an effort to create such a test.

Chapter 3: Determination of an appropriate H_2S Test for field application

3.1 Introduction

Hydrogen sulfide-producing (H_2S) bacteria have also been proposed as alternative fecal indicators. Methods for detection of H_2S bacteria were developed to fill a need for a simple, reliable field test for use by village public health workers to detect fecal contamination in drinking water (Manja et al. 1982; Sobsey and Pfaender, 2002). The H_2S test detects the presence of microbial hydrogen sulfide production as a black iron sulfide precipitate in solution. The H_2S method to detect fecal contamination has been compared to more traditional fecal indicator bacteria and their detection methods and has demonstrated relatively good correlation with conventional bacterial indicators of fecal contamination (Sobsey and Pfaender, 2002).

The ultimate goal of the work reported here was to establish and validate a low-cost test to detect and quantify fecal contamination of water. Once developed, the test can be made accessible and affordable to people and institutions who now lack access to tests to determine the microbial safety of their water. The objectives were to: 1) validate existing H_2S bacterial detection methods at ambient incubation temperatures; 2) develop and evaluate simple, inexpensive test formats to detect *E. coli* and H_2S-producing bacteria in water, and 3) compare these newly developed methods to standard methods for microbial water quality testing.

3.2 Materials and Methods

This test was evaluated for accuracy, precision, sensitivity and specificity using three different formulations of H_2S media: commercially available media (Hach, Loveland, CO), autoclaved lab-made broth, and tyndalized lab-made broth. The lab-made broths were a modification of the recipe of Manja et al. (2001). The tyndalized media was made by boiling for 5 minutes, cooling for 6 hours, re-boiling for 10 minutes, cooling for 24 hours, and a third boil for 10 minutes. All of these formulations were compared at three different incubation times (24, 48, 72 hours), and two temperatures (25°C and 35°C). Tests were run using both plastic Whirl-pak bags and 125-mL polypropylene bottles as test containers.

For all broth samples, 450mL of PBS was placed into 5L glass bottles and 50mL of spiked sewage water (0.5mL of sewage into 499.5mL of University Lake water) was added and then serially ten-fold diluted to 10^{-6} in 5L bottles. 3-100mL samples of each broth were aliquoted into plastic 125mL polypropylene bottles. 5mL of both Tyndalized and Autoclaved broths and 1 HACH PathoScreen reagent packet were added to the 100mL samples. 10mL and 1mL out of each 100mL sample were then aliquoted into 16x150mm glass test tubes and 13x100 glass test tubes, which were then capped and incubated at 37°C for 24 and then 48 hours. After 24 and 48 hours, each sample was examined for the production of the black precipitate that is the byproduct of the H_2S reaction. The number of positive samples was then scored and a MPN value was computed from those measurements.

In addition, the MPN format of the H_2S test was compared to three other bacteriological tests: *E. coli* spread plating method on Bio-Rad Rapid *E. coli* 2 Agar,

Salmonella by membrane filtration on Bismuth Sulfite Agar, and detection of H_2S producing facultative anaerobes by membrane filtration on H_2S agar (a formulation consisting of the Hach PathoScreen H_2S media and Bacto-Agar). The mpn Compartment Bag Test (CBT) was developed after confirmation of the success of the H_2S media and equipment comparisons were complete. The CBT was then tested and compared to standard microbial indicator testing at 27°C, 37°C, and 44°C using the molecular and biochemical method described in Appendix 4.

Statistical comparisons of the different candidate fecal indicator microbes (*E. coli*, H_2S-producing bacteria) were made with side-by-side with standard fecal indicator tests and standard incubation conditions by both parametric and non-parametric methods, such as t-tests, ANOVAs, and correlation analyses. All correlation analyses will include a significance test of completion and a 95% Confidence Interval of R^2.

3.3 Results

Comparison of 3 Broth Media

As Figure 1 shows, there was no significant difference in levels of H_2S producing bacteria for the commercially available HACH media vs. the lab-made H_2S broths ($p= 0.49$). This was true in both protected source University Lake Natural water, natural water spiked with sewage, and undiluted sewage.

MPN values ranged from <0.71 (the lower detection limit) to 260 per 100 ml and generally decreased as dilution of untreated sewage increased. The numbers of H_2S organisms in a sample as measured by HACH and Autoclaved broth did not differ significantly at incubation temperatures at 24 or 48 hours.

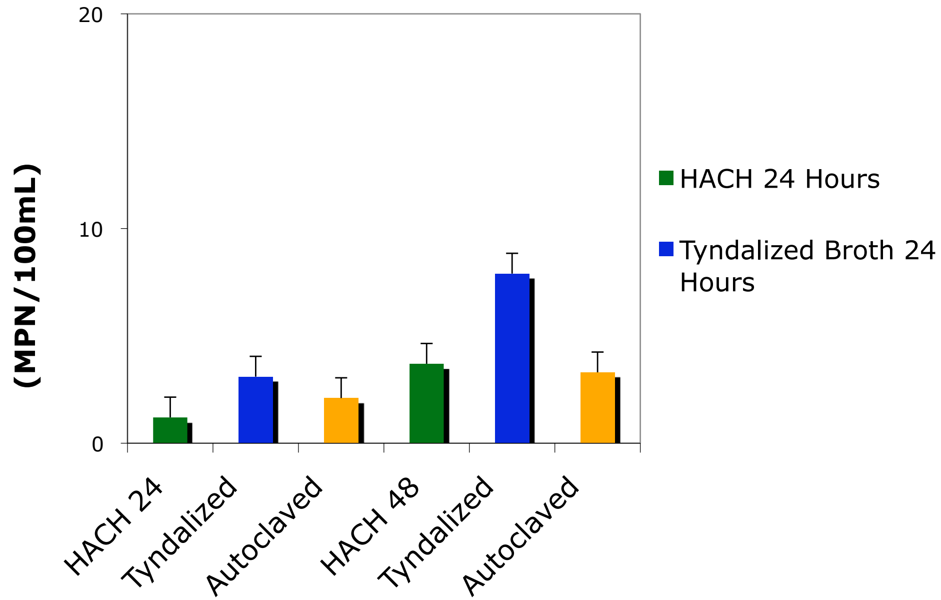

Figure 3.1 Concentration of H_2S Producing Organisms in various H_2S Formats at 37°C over 5 experiments (MPN/100ml)

Comparison of MPN method at 24 vs. 48 hours

As Figure 2 shows, there was no significant difference in the levels of H_2S producing bacteria in the HACH media after 24 hours of incubation vs. 48 hours ($p= 0.45$).

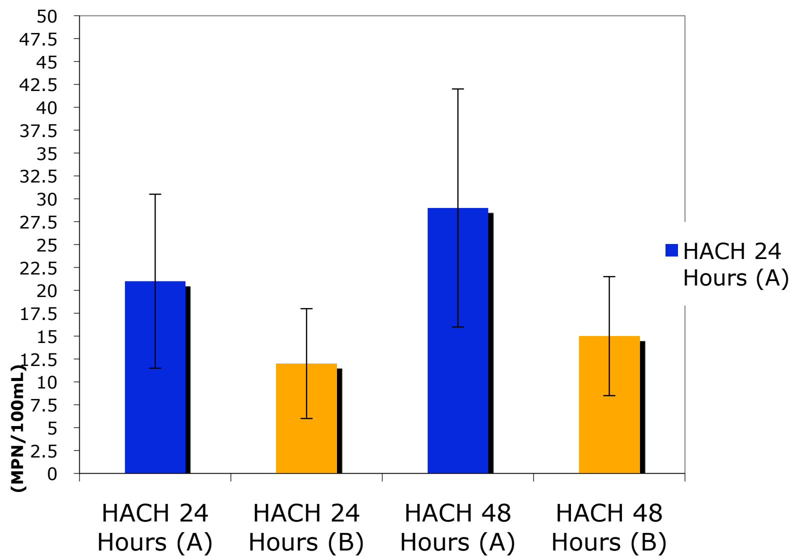

Figure 3.2 Concentration of H_2S Producing Organisms in HACH Media at 24 vs. 48 hours at 37°C(MPN/100mL) Note that (A) and (B) were 2 different experimental trials

Comparison of 125mL Whirl-pak Bags vs. Plastic Bottles

As can be seen in Figure 3.3, the bacterial numbers detected by an MPN H_2S test incubated in plastic bags were slightly higher than numbers from tests incubated in bottles, but the difference was not significant after 48 hours ($p= 0.31$).

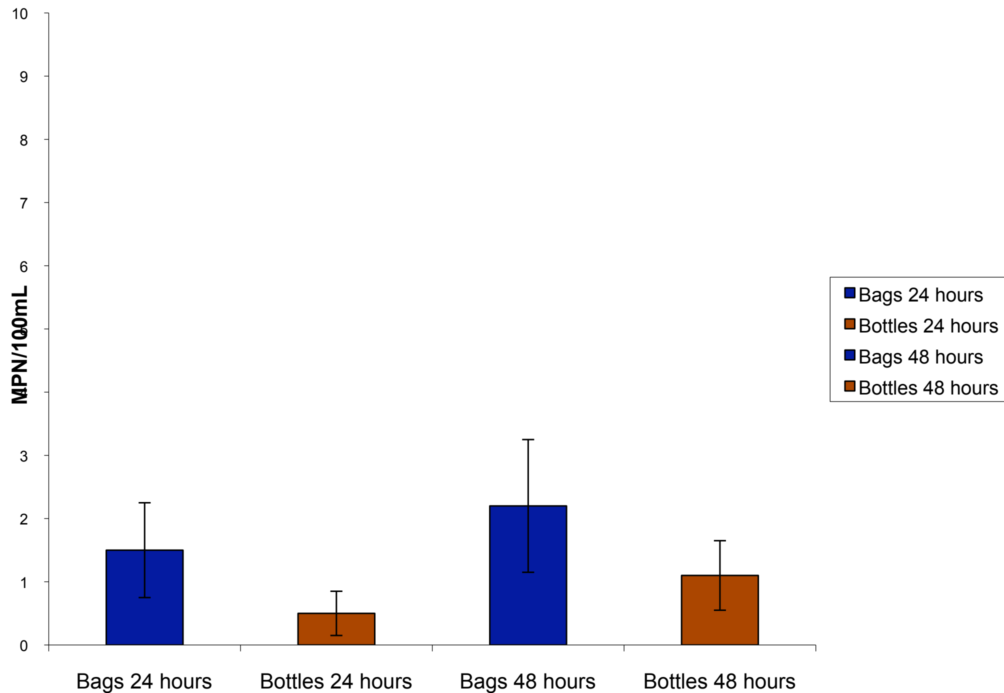

Figure 3.3 Concentration of H2S Producing Organisms in Bags vs. Bottles at 37°C (MPN/100mL)

Comparison of H_2S and E. coli MPN method

The ability of the H_2S test to detect fecal contamination was also compared to the detection of *E. coli* using the Colilert Quantitray MPN method. This analysis was done for natural water samples spiked with primary effluent (Table 3.1) and drinking water samples from Vietnam (Table 3.2). As shown in Table 3.1 and Table 3.2, comparison of presence or absence of H_2S producing bacteria and *E. coli* in primary effluent spiked natural water gave similar results for both fecal indicators ($p=0.0000$).

Table 3.1 Comparison of H_2S versus *E. coli* tests based on sample positivity or negativity for sewage and seeded water experiments under controlled lab conditions.[a]

	Test Outcomes	+	-	Total
E. coli	+	8	0	8
	-	1	5	6
Total		9	5	14
Sensitivity	0.889	PPV	1	
Specificity	1	NPV	0.833	
Kappa	0.408			

[a] (SE) =Sensitivity, (Sp) =Specificity, (PPV) =Positive Predictive Value, & (NPV)= Negative Predictive Value

In the indicator and methods comparison for water supplies in the Central region of Vietnam, given a positive H_2S test, there was a 76% chance that there was also *E. coli* present in the water. Given a negative H_2S test, there was a 65% chance that *E. coli* was not present in the water. If *E. coli* were absent from the water samples, there was a 82% chance that the H_2S test was negative for the same volume of water.

Table 3.2 Comparison of H_2S versus *E. coli* tests based on sample positivity or negativity for piped and treated water samples in Central Vietnam.[a]

			H_2S	
	Test Outcomes	+	-	Total
E. coli (from Colilert)	+	467	145	612
	-	359	653	1012
Total		826	798	1,624
Sensitivity	0.565	PPV	0.763	
Specificity	0.818	NPV	0.645	
Kappa	0.382			

[a](SE) =Sensitivity, (Sp) =Specificity, (PPV) =Positive Predictive Value, & (NPV)= Negative Predictive Value

Cost of the H_2S Compartment Bag Test

As described earlier, developing countries and areas impacted by natural disasters often lack resources necessary to do microbial water quality testing. As

Table 3.3 shows, the availability of electricity, amount of space to run the test, amount of sample water available, the availability of trained personnel as well as the amount of money available for testing are just a few factors that must be considered when choosing a test. Other factors include:

- Volume of sample the test analyzes
- Quantification method
- Need for electricity
- Need for supplemental equipment
- 1 step vs. multiple step
- Perishability
- Portability/compactness
- Convenience of application
- Presence/absence
- Readily detectable endpoint

- Easily visualize *E. coli* on point (range of countable CFUs)
- Sensitivity
- Specificity
- Precision
- Unit cost
- Performance of method at non-conventional temperatures

(National Research Council of the National Academies, 2004)

These factors are outlined in the table below and described according to method. The table is followed by detailed comparisons of the methods across criteria. The following abbreviations are used: Membrane filtration with MI agar (MI), membrane filtration with Bio-Rad agar (BR), Colilert method (COL), Petrifilm (PET), Easygel (EZ), and the H_2S CBT (CBT). The unit cost of the test of particular importance, because it is only useful in developing country and emergency settings if it is affordable. None of the currently available fecal indicator tests are available at an affordable level for widespread use in low-resource settings. MTF and MF tests require expensive supplemental equipment and materials such as membrane filter funnels, Petri dishes, and test tubes. The QT test costs $5.25 per 100 mL sample, which is still too expensive for widespread use in low-resource settings, and this cost does not include the $4000 QT sealer required to perform the test. While the EZ method does not require any expensive supplemental equipment, the unit cost per test is $15. In many low-resource areas of the world, a large portion of the population lives on less than $1-$2 a day. Thus, in order for a test to be affordable it would have to cost significantly less than those currently available.

	MI Agar	BioRad	Colilert	Petrifilm	EasyGel	H_2S CBT	E. Coli CBT
Applied sample volume for test	1 mL or 10 mL	1 mL or 10 mL	100 mL	1 or 5 mL	1 mL or 5 mL	100ml	100ml
Convenience of application	NO	NO	NO	YES	YES	YES	YES
Portability/compactness	NO	NO	NO	YES	YES	YES	YES
Need for electricity	YES	YES	YES	YES	YES	NO	NO
Need for supplemental equipment	YES	YES	YES	NO	NO	NO	NO
Single step vs. multiple step	Multiple	Multiple	Multiple	3 steps	4 steps	3 steps	3 steps
Perishable	YES	YES	YES	YES	YES	YES	YES
Readily detectable endpoint	YES	YES	YES	YES	YES	YES	YES
Easily visualize fecal organism at endpoint (countable range of CFUs	Adequate	Adequate	Adequate	Can be limited with small sample volume.	Can be limited with small sample volume.	Limited to less than 100MPN /mL	Limited to less than 100MPN /mL
Broth vs. agar vs. pectin	Agar	agar	Broth	gelling agent	Pectin	Broth	Broth
Sensitivity	YES	YES	YES	NO	NO	YES	YES
Specificity	YES	YES	YES	YES	NO	YES	YES
Precision	YES	YES	YES	MAYBE	MAYBE`	YES	YES

Table 3.3. Criteria for low-cost simple drinking water test for E. coli and H_2S producing bacteria

*(Micrology Laboratory, 2007)

** (IDEXX Colilert Customer Service Representative)

Unit cost (not including cost of incubator)	$3.18 per gram; Expensive supplemental equipment required***	HIGH	$5.25 per sample plus tax; Sealer required = $4,000 **	LOW	$15 per sample*	$0.40 per sample	$1.70 per sample

*** (Fisher Scientific, 2008)

As can be seen above, the H_2S CBT is among the simplest tests to perform, and is the lowest cost of all currently available fecal indicator tests. In addition, it doesn't require incubation in tropical environments, and requires no other supplemental equipment.

Comparison of the H_2S Compartment Bag Test and the IDEXX Quantitray system

An additional 9-week analysis was done to examine the effects of temperature (27°C, 37°C, and 44°C) on the H_2S test performed in the compartment bag versus the IDEXX Quantitray system. In addition, molecular and cultivation microbial analysis was done to determine what organisms were found in positive H_2S tests at different temperatures and how that compared with the initial water sample before the H_2S test was performed.

Table 3.4 shows that concentrations of H_2S producing bacteria in the CBT compared to the QT were not significantly different at incubation temperatures of 25°C, or 37°C, or

44^oC. However, results at 44^oC were significantly different between from results at 25^oC, 37^oC in both the CBT and the QT.

Table 3.4. Comparison of the H_2S Compartment Bag Test and the IDEXX Quantitray system at 27°C, 37°C, and 44°C

Dunn's Multiple Comparison Test	Significant @ $P < 0.05$?
25 Tray vs 25 Bag	No
25 Tray vs 37 Tray	No
25 Tray vs 37 Bag	No
25 Tray vs 44 Tray	Yes
25 Tray vs 44 Bag	Yes
25 Bag vs 37 Tray	No
25 Bag vs 37 Bag	No
25 Bag vs 44 Tray	Yes
25 Bag vs 44 Bag	Yes
37 Tray vs 37 Bag	No
37 Tray vs 44 Tray	Yes
37 Tray vs 44 Bag	Yes
37 Bag vs 44 Tray	Yes
37 Bag vs 44 Bag	Yes
44 Tray vs 44 Bag	No

A subsequent analysis more closely examined the temperature range between or 37°C and 44°C to provide a maximum temperature value at which the H_2S method can be guaranteed effective since ambient temperatures in some environments can sometimes surpass 44°C. It is important to determine if the CBT test cannot function in these environments and prompted a questioning of the maximum temperature at which this method could be effectively used. In an effort to determine this upper limit, we evaluated the growth of three different coliform bacteria (*Salmonella, Citrobacter, Proteus*) at incremental temperatures approaching 44ºC and estimated the concentration of the bacteria using the most probable number (MPN) method.

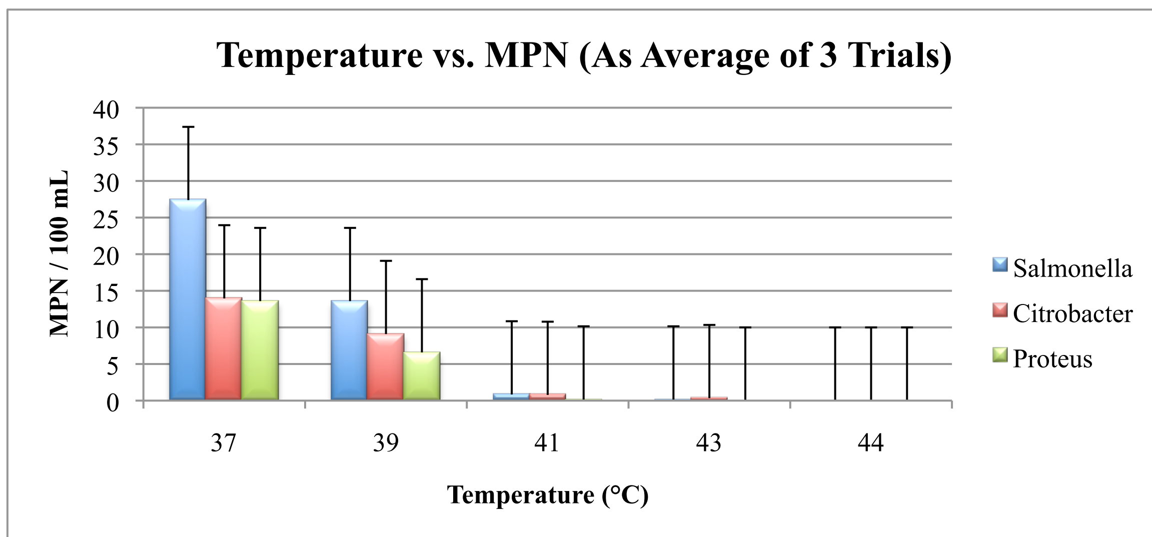

Figure 3.4 Concentration of H2S Producing Organisms (Salmonella, Citrobacter, Proteus) at 37°C, 39°C, 41°C, 43°C, and 44°C, (MPN/100mL)

As shown in Figure 3.4, none of the organisms grew substantially beyond 39ºC. Based on this information, and the results from the CBT versus Quantitray experiments, it is inadvisable to perform the H_2S CBT above 39ºC.

3.3 Conclusions

The H_2S MPN compartment bag is easy-to-use, economical, and fairly robust as a test that could also be used in low-resource settings. Laboratory testing has shown that there is no significant difference between the levels of H_2S producing bacteria given 3 different broth recipes, at 24 and 48 hours, and in different sample containers. In addition, lab testing has shown that there is no significant difference between the levels of H_2S producing bacteria in the standard methods and those determined by the compartment bag. Both laboratory and field-testing with potable and non-potable water samples showed that the presence of H_2S-producing bacteria was strongly associated with the presence of *E. coli*.

Given that *E. coli* is currently considered the gold standard fecal indicator for microbiological water quality testing, these results provide evidence that the quantitative H_2S test in a compartmentalized plastic bag has some promise as a low-cost, easy to use alternative to current expensive, labor intensive and lab-based microbial water quality testing practices for *E. coli*.

Chapter 4: Comparison of Culture-Based and TRFLP Analysis to Identify H_2S Producing Microorganisms in Sewage

4.1 Introduction and Background

One of the major weaknesses of the H_2S test for the detection of fecal bacteria is the variation in sensitivity and specificity for bacteria of fecal origin obtained across different studies. Previous studies applying the H_2S test to groundwater samples have demonstrated false positive results, where H_2S-positive samples contained no fecal coliforms or *Escherichia coli* (Kaspar et. al 2009, Pant et. al 2002). False negative results, where H_2S-negative samples were found to contain *E. coli*, have been reported in other studies (Tewari et. al 2003). The observed lack of uniformity, reported inconsistencies and the unavailability of the test in a ready-to-use, quantitative form in many locations are barriers to the widespread adoption and use of the H_2S tests. For the H_2S bacteria test to be an acceptable tool to evaluate water quality for the presence and magnitude of fecal contamination, data are needed indicating which microorganisms produce positive results in the test, under what conditions test results indicate actual fecal contamination of water, and ultimately a quantitative version of the test is needed to estimate the magnitude of fecal contamination.

The purpose of this research is to determine if the H_2S test is effective in detecting and quantifying bacteria of fecal origin. Although we are primarily concerned with

testing water samples, for the test to be effective it must first be capable of detecting fecal bacteria when applied to sewage samples, as waters contaminated with human sewage are used as drinking water sources in many impoverished areas worldwide. Using culture-based biochemical and culture-independent molecular techniques, this research is focused on determining the types of microbial community members, including fecal indicator organisms, pathogens, and other microbes present in human sewage samples that are detected in a quantitative H_2S test as microorganisms of concern to human health.

Molecular genetics techniques utilizing extracted nucleic acids now allow microbial community analysis to be coupled with a phylogenetic framework. Terminal Restriction Fragment Length Polymorphism (TRFLP) was the nucleic acid based method employed in this study because it provides a way to determine the presence of common species in a sample with or without culturing the organisms, facilitates finding major differences between communities, and allows for testing hypotheses based on a comparison of samples (Kent et. al 2003). By using TRFLP, Liu et al. (1997) were able to distinguish all bacterial species in a model bacterial community, and the pattern was consistent with the predicted outcome. TRFLP analysis of complex bacterial communities revealed high species diversity in activated sludge, bioreactor sludge, aquifer sand, and termite intestines (Blackwood et. al 2007, Kim and Marsh 2004). Others have also compared the results of TRFLP and the traditional culture-based approach and found that TRFLP often provides a more detailed analysis than the traditional approach (Morales et. al 2006, Pidiyar et. al 2004). In this study the results of TRFLP and traditional culture-based isolation and biochemical characterization methods were compared to determine what microorganisms are growing in positive H_2S samples

from municipal sewage. If both techniques found that this new H_2S test was effective with samples that should have organisms of concern, future analysis can focus on the effectiveness of the test with waters that may or may not contain organisms normally found in sewage and other fecal waste sources that are a concern to human health.

4.2 Materials and Methods

Culture-Based Biochemical Detection Methods

To determine the genera and relative numbers of bacteria present in sewage-spiked phosphate buffered saline (PBS) samples, grab samples of 120 ml volumes of raw untreated sewage were obtained on 3 separate occasions from the Orange Water and Sewer Authority (OWASA) wastewater treatment plant (Chapel Hill, NC). The OWASA system serves a university community having no major sources of industrial wastes. Each sample of collected sewage was considered a separate experiment, since samples were collected every 2 weeks over a six-week interval. From the 120-ml raw, untreated sewage sample, duplicate 10 ml aliquots were removed and pelleted at 3,500 rpm for 20 min, and the pellets were overlaid with 300 µl of the original sample and archived at -80°C for future DNA analysis (see Figure 1). PathoScreen reagent for H_2S bacteria testing of a 100 ml water sample (Hach Company, Loveland, CO) was added to the remaining 100 ml of raw sewage in a 5-compartment MPN bag and incubated at 37°C for 24 h. The MPN bag is a clear polyethylene bag, 15 cm wide x 23 cm long (Whirl-Pak®, Nasco, Fort Atkinson, WI) in which there are 5 internal vertical compartments in the lower two-thirds of the bag, each with a volume of 1, 3, 10, 30 and 56 ml, respectively. For biochemical analysis of bacteria from the culture-based detection method, a 0.5 ml sample from each H_2S-positive compartment of an H_2S MPN bag sample was diluted serially 10-fold in

PBS to a dilution of 10^{-5}. Then, 100 µl volumes of each dilution were spread onto duplicate 13 x 150 mm diameter plates of the following agar media to isolate colonies: Bio-Rad RAPID' E. coli 2 agar, Salmonella-Shigella agar, Phenyl ethanol agar, m-Aeromonas Selective agar, and H_2S agar (22). All plates were incubated aerobically at 37°C for 24 h. Isolates from spread plates were obtained by streak plating characteristic colonies onto Tryptic Soy Agar on three successive days. These colony isolates were archived in 0.8 ml of Tryptic Soy Broth at -80°C. The isolates were then tested to determine if they produce H_2S by culturing in H_2S medium and were further identified at the genus and species level using standard biochemical identification test kits, specifically BBL Enterotube II (BD Diagnostic Systems, Sparks, MD) and the API 20E System (bioMerieux, Inc., Hazelwood, MO).

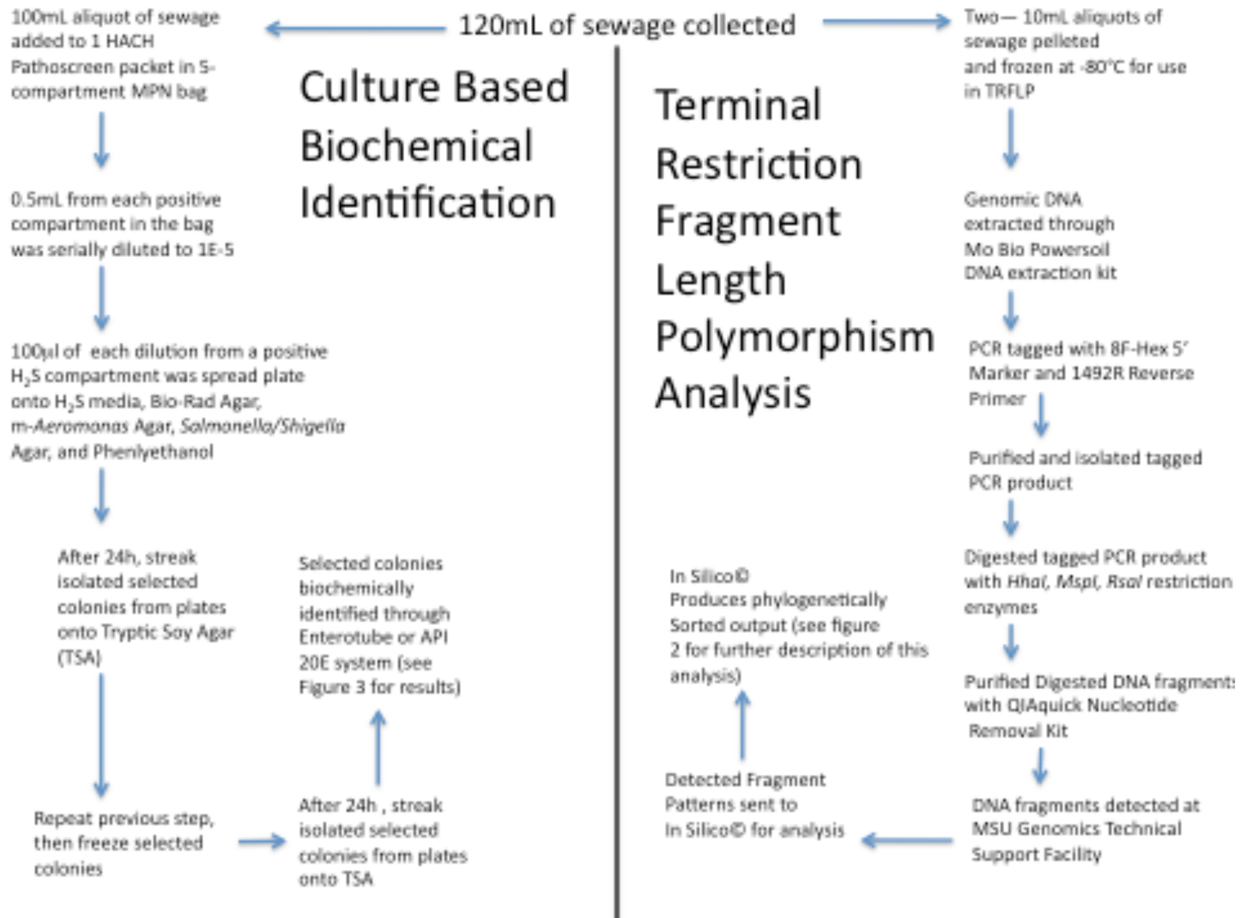

Figure 4.1 A workflow diagram describing of the culture based biochemical identification and the TRFLP processes.

DNA Extraction

Two compartment bags of positive H_2S media were pelleted and the resulting pellets were overlaid with 1 ml of the H_2S positive sample spent medium and archived at -80°C for future DNA isolation for TRFLP molecular community analysis (Figure 1). Genomic DNA (gDNA) was extracted using the Mo Bio Powersoil^{TM} DNA extraction kit (Mo Bio Laboratories Inc, Solana Beach, CA), according to the manufacturer's protocol. Agarose gel electrophoresis was used to visualize whether sufficient quality gDNA was isolated from each sample. A 3 µl volume of each gDNA sample was electrophoresed

through a 1% TAE Agarose gel containing 15 µl of ethidium bromide per 100 ml of Agarose gel. Isolated gDNA was stored at -80ºC until it was used for PCR reactions.

PCR Conditions

3 µl volumes of each DNA sample were added to 97 µl of Master Mix (per sample: 10 µl 10X Reaction Buffer; 0.8 µl dNTP [Deoxynucleotide Triphosphate]; 83.7 µl PCR grade water; 0.5 µl each of the bacterial-specific 16S rDNA primers 8F-Hex 5'-AGA GTT TGA TC(A/C) TGG CTC AG and reverse primer 1492R 5'-GGT TAC CTT GTT ACG ACT T; 0.5 µl of Qiagen HotstarTaq DNA polymerase (Qiagen, Valencia, California). Each DNA sample was amplified in triplicate. The forward primer for the PCR reaction was labeled on the 5' end with a hexamide fluorescent marker to allow the terminal fragment to be tracked. PCR was performed in a Perkin-Elmer 9600 thermocycler by using an initial denaturation step of 15-min at 95°C, followed by 35 cycles consisting of denaturation (1 min at 94°C), annealing (1 min at 50°C), and extension (2 min at 72°C) and a final extension at 72°C for 7 min. PCR replicates of each sample were then pooled and purified by using the UltraClean™ PCR Clean-up Kit (MoBio Laboratories Inc, Solana Beach, CA) according to the manufacturer's protocol.

TRFLP

For the TRFLP analysis of the amplified bacterial 16S rDNAs, three restriction enzymes, *Rsa*I, *Hha*I, and *Msp*I (New England Biolabs, Inc., Ipswitch, MA) were used. For the *Rsa*I digest, 30 µl of purified PCR product (approximately 30 µg) was mixed with 10 µl of Reaction Buffer#1, 59 µl PCR grade water, and 1µl of restriction enzyme. For the *Hha*I digest, 30 µl of purified PCR product (approximately 30 µg) was mixed with 10

µl of Reaction Buffer#4, 1µl BSA, 58µl PCR grade water, and 1µl of restriction enzyme. For *Msp*I, 30 µl of purified PCR product (approximately 30 µg) was mixed with 10 µl of Reaction Buffer#4, 1µl BSA, 58µl PCR grade water, and 1µl of restriction enzyme. Restriction digests were incubated overnight at 37°C. For clean-up, restriction digests were heat treated at 60°C for 20 min to heat inactivate the restriction enzymes. The QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany) was then used to purify the digested DNA according to the manufacturer's protocol, except 50 µl of warmed (60°C) PCR grade water was added instead of kit elution buffer, and the water was allowed to incubate on the column for 5 min prior to elution of the DNA. DNA samples were then frozen at -20º C. Fragment detection was carried out at the MSU Genomics Technical Support Facility according to their detection protocols (http://gtsf.msu.edu/dna-fingerprinting-and-genotyping).

Fragment Analysis

Data tables containing fragment size and abundance data for each digest of the DNA of sewage samples was exported from GeneScan, and the resulting text files were sent for pattern detection by the *In Silico*© database. Each file contained all the detected fragments for a given restriction digest (e.g., data obtained from one of the *Hha*I digests for a sample would be contained in one file, *Msp*I fragments would be contained in another file, and *Rsa*I fragment data would be contained in a third file). Each entry in these data files contained fragment length size, retention time on gel, peak height, fragment identification number, and a peak area found in the sample. For calculation of the diversity indices, the TRFLP analysis peak area was used as the amount measurement, and its relative abundance was measured by dividing individual peaks by

the total fluorescence of the sample. The results for each diversity measure are representative of the number of fragments in each experimental sample.

In Silico© Output

Pattern detection and pattern identification were carried out using the *In Silico*©, software package (*In Silico*©, RTP, NC). The *In Silico*© output is a comprehensive dataset which includes the following diversity analysis values: Simpson Index of diversity, a measure of the richness (the number of different species per sample) and evenness; Reciprocal Simpson, an inverse of the Simpson's index (lowest value is 1; the higher the value the greater the diversity); Species richness (the number of species within a community); and the Shannon Weaver diversity index, which is one of several diversity indices used to measure diversity in categorical data and takes into account the number of species and the evenness of the species. The index is increased either by having additional unique species, or by having greater species evenness (Blackwood et. al 2007, Kim and Marsh 2004). *In Silico*© also provides information on the fragment parameters, including: total fragment utilization, which is the proportion of the fragments used in the analysis compared to the total number of fragments available; the total number of fragment patterns detected; and evenness, which is a measure describing how much each individual fragment contributes to the whole (on a 0-1 scale; closer to 1 the better) (Kim and Marsh 2004).

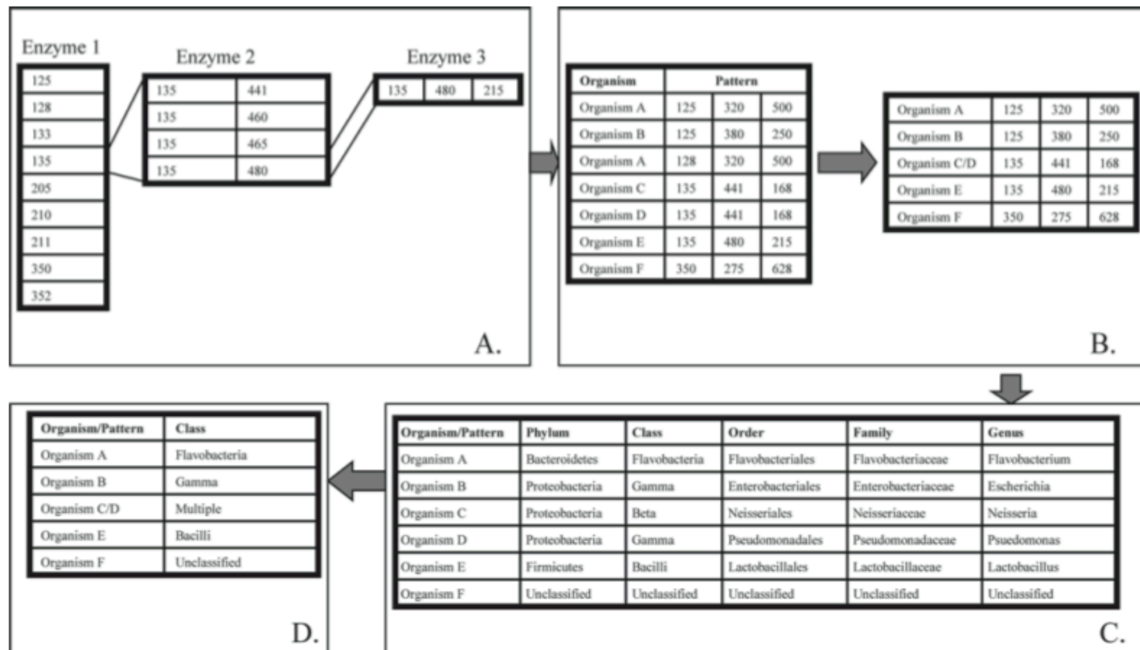

Figure 4.2 A flow diagram describing the In Silico software (In Silico LLC, Fuquay-Varina, NC) process used to generate TRFLP community profiles (Johnson et. al. 2009).

A) Fragments generated from separate restriction digests are sequentially matched to patterns found in the In Silico database. B) Matched patterns are compressed by removing extraneous patterns that belong to a single organism, as in organism A, or by combining multiple organisms that have the same pattern, as in organism C and organism D. C) Patterns are then matched to phylogenetic information in the In Silico software and reported at 5 different levels. D) In the final output of the In Silico software package, organisms are identified by genera. Note that the pattern belonging to organisms C and D is identified as "multiple" because the 2 organisms belong to different classes. Organism F is defined as unclassified because no phylogenetic information is available for the pattern.

As described in Johnson et al. (2009), fragments were sequentially matched to patterns as described previously (Kent et. al 2003, Figure 2A). A compression utility was then used to remove multiple matches to the same organism and to combine patterns that matched multiple organisms (Figure 2B). A phylogenetic sorting algorithm matching the 16S rDNA genes in the National Center for Biotechnology Information (NCBI) sequence database was then applied to the final pattern set (Figure 2C). Although the identified patterns can be reported by *In Silico*© at 5 different phylogenetic levels (phylum, order, class, family, and genus; Figure 2), the data in this study are presented at the genus level because this gives information on the hydrogen sulfide production capabilities of each

type of microorganism.

The “unclassified” group of patterns differs from unmatched fragments because little or no phylogenetic information is deposited with their respective sequence. Unclassified patterns make up a large percentage of the data since sequences are often deposited without sufficient phylogenetic identification. Sequences in the “multiple classification” category fit into more than one distinct phylogenetic group and consist of all the unique species from phylogenetically different groups that match a single fragment pattern.

Further Phylogenetic Assignment

Given the relatively high percentage of fragment patterns from the *In Silico*© output that were either in the “Multiples” or “Unclassified” categories, further analysis was conducted. Any fragment pattern that was assigned to either the “Multiples” or “Unclassified” category was then re-analyzed by entering it into the BLAST tool in the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nucleotide/), and the retrieved nucleic acid sequence listed in NCBI was then entered into the Michigan State University Ribosomal Database Project classifier website (http://rdp.cme.msu.edu/classifier/classifier.jsp) using a 95% confidence threshold for phylogenetic assignment (Johnson et. al 2009, Wang et. al 2007). For example, the first unclassified pattern in Sewage experiment 1 was reported by the *In Silico*© database as “Uncultured bacterium clone ICBTF7 16S ribosomal RNA gene, partial sequence”. That identity was entered into the NCBI database and produced a 522bp sequence. That 16S rRNA gene sequence was then submitted to the RDP Classifier website, which generates a genus level identification of the organism with 95% confidence.

The classifier takes a sequence and assigns it to the lowest taxonomic level possible within a certain degree of confidence. If that organism was identified with 95% confidence or higher, it was removed from the “Unclassified” category and was reclassified. In some cases, all of the fragment patterns from a “multiple” categorization could be analyzed using BLAST and the RDP Classifier and were identified as the same organism. When this occurred, and the organism was identified with 95% confidence or higher, it was removed from the “Multiples” category and was reclassified. Some fragment patterns that were originally labeled as “Multiples” contained only organisms that have been identified as fecal coliforms and Gram-negative enteric pathogens (Committee on Indicators for Waterborne Pathogens 2004). Therefore, a separate “Gram negative enteric bacteria” category was created for those “Multiples”, which included the following genera: *Escherichia*, *Klebsiella*, *Proteus*, *Salmonella*, and/or *Shigella* (LeClerc et. al 2001).

Statistical Methods to Compare Culture-Based and TRFLP Results.

Kappa Tests of agreement were performed comparing the genera found in the culture-based method to those found in the TRFLP output. Kappa Tests of agreement are a measure of association (correlation or reliability) between two measurements of the same item when the measurements are categorical. Values closer to zero indicate slight to little agreement, while values closer to one indicate strong agreement (Landis and Koch 1977).

4.3 Results

Culture Based Biochemical Detection

A diverse group of microorganisms was isolated and cultured from the H_2S-positive sample volumes cultured from sewage samples, with 24 different species isolated. *Citrobacter freundii, E. coli,* and *Enteric Group 60* represented more than 50% of the total isolates as identified by either the Enterotube or the API20E system. In addition, known enteric pathogens such as *Shigella* were identified. Importantly, a number of different possible H_2S-producing enteric microorganisms were also isolated including: *Acinetobacter wolffii, Aeromonas hydrophila*, *Citrobacter freundii, Klebsiella ozonae, K. pneumoniae, Salmonella* sp., and *Yersinia enterocolitica.* Figure 3 shows the extent of detection of H_2S-producing microorganisms by the H_2S test using culture-based biochemical identification. Although there are more non-H_2S producing pathogens/fecal indicator organisms than any other group, each sample had multiple H_2S-producing organisms that were also pathogens. As was described earlier, after the isolates were identified by the Enterotube system, they were re-analyzed for their ability to produce hydrogen sulfide. Overall, 96 of the 216 isolates (44.4%) from all sewage experiments were positive for H_2S production when further tested for this ability.

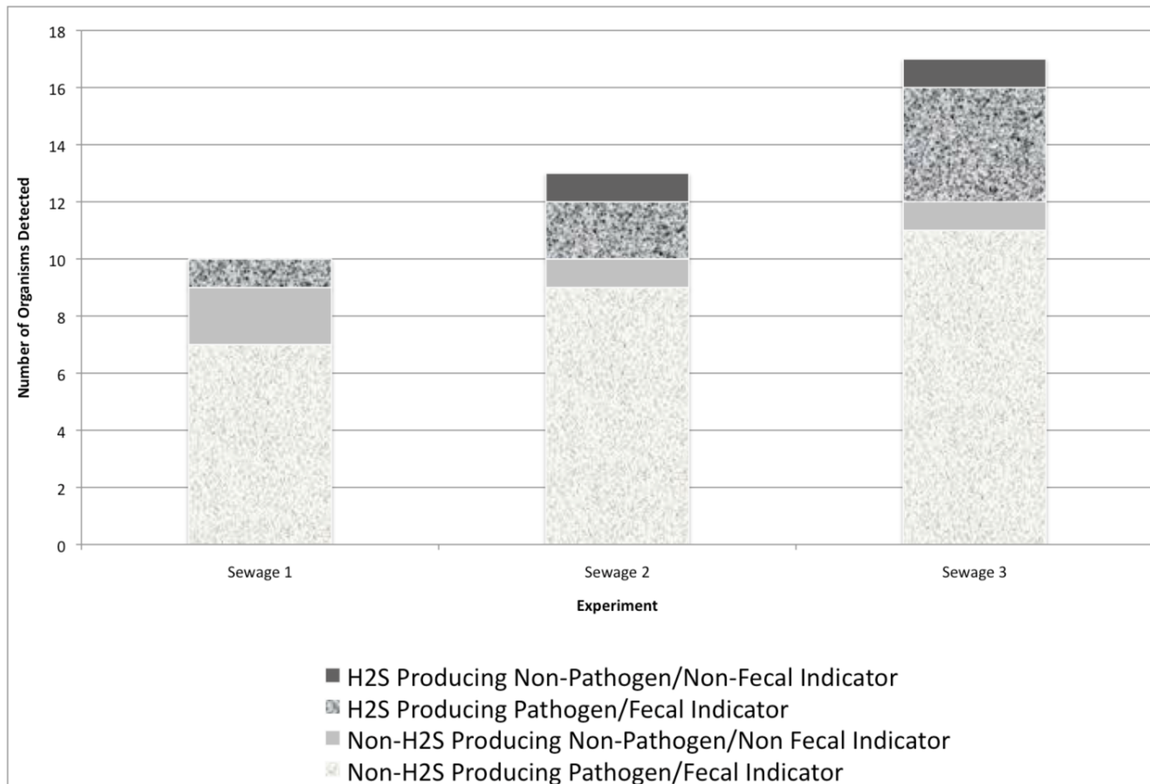

Figure 4.3 Pathogens and H_2S-Producing organisms identified in sewage samples using culture-based biochemical identification methods.

The following categories divide the identified organisms based on whether they are a likely Pathogen/ Fecal Indicator Organism and whether or not they produce Hydrogen Sulfide: H_2S Producing Pathogen/Fecal Indicator (bottom of bar) (Committee on Indicators for Waterborne Pathogens 2004); H_2S Producing Non-Pathogen/Non-Fecal Indicator (Reis et al. 2002) Non-H_2S Producing Pathogen/Fecal Indicator (Widdel 1988); Non-H_2S Producing Non-Pathogen/Non-Fecal Indicator (bottom of bar)

TRFLP Molecular Analysis

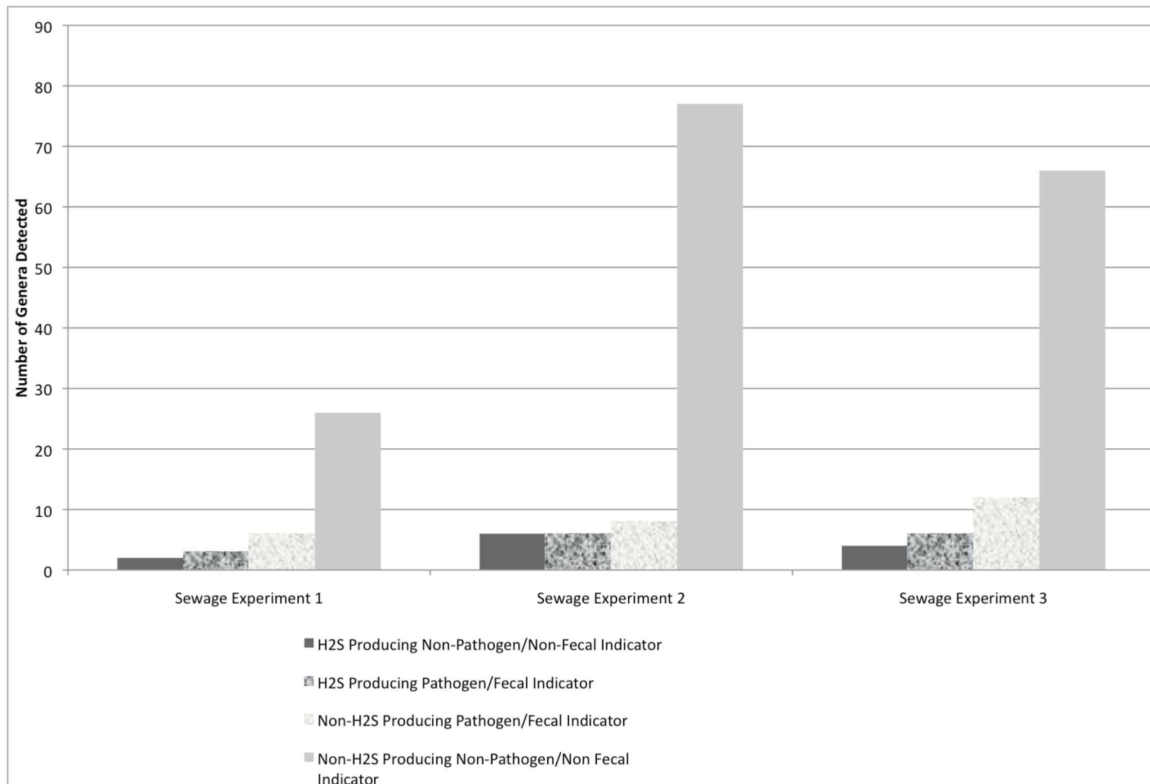

Figure 4.4 TRFLP fragment patterns identified at the Genus level in genomic DNA isolated from H_2S-positive liquid cultures of bacteria in sewage-contaminated samples.

The following categories divide the genera based on whether they are a likely pathogen/ Fecal Indicator Organism and whether or not they produce Hydrogen Sulfide: H_2S Producing Pathogen/Fecal Indicator (Committee on Indicators for Waterborne Pathogens 2004); H_2S Producing Non-Pathogen/Non-Fecal Indicator (Reis et al. 2002); Non-H_2S Producing Pathogen/Fecal Indicator (Widdel 1988); Non-H_2S Producing Non-Pathogen/Non Fecal Indicator

A total of 162 genera, were identified in the TRFLP molecular analysis. Of the 162 genera identified by the molecular analysis, 145 were not identified through cultivation and biochemical identification processes. However, as shown in Table 1, 33 of 40 genera (82.5%) that were isolated and identified from all sewage experiments through the culture-based biochemical techniques were also identified by TRFLP, with *Hafnia* and *Yersinia* being the only exceptions. Given the large number of bacteria detected, it is possible that there was not enough DNA from these two organisms to molecularly detect them. Moreover, the two organisms could have been misclassified by the biochemical classification methods. Seven percent of the 162 genera identified were detected in all

three experiments, and 31% were detected in at least two of the experiments. Results of agreement in genera identification were more similar in Sewage experiments 2 and 3 than in Sewage experiment 1, with 80% of the matched genera identified from experiments 2 and 3. Sewage experiment 2 yielded the most genera identifications (192); followed by Sewage experiment 3 (180); with Sewage experiment 1 having the fewest genera identified (59). Gram-negative enterics, the classification of "Multiples" that are all known gram-negative organisms in the *Enterobacteriaceae* family, represented the largest percentage of classified organisms (13.6%, 4.8%, and 3.9% for experiments 1, 2, and 3, respectively). As Figure 4 shows, there were a considerable number of potentially pathogenic H_2S producing genera found by the TRFLP analysis, with 20% of classified organisms in sewage experiment 2 and 19% from sewage experiments 1 and 3 considered potential pathogens (Committee on Indicators for Waterborne Pathogens 2004). Of the genera identified by TRFLP analysis, 25% in sewage experiment 1 and 16% in sewage experiments 2 and 3 were known H_2S producers according to a previous taxonomic listing (Sobsey & Pfaender 2002). Of the total fragment patterns obtained in experiments 1, 2 and 3, 27%, 13%, and 14%, respectively, were classified as "Unclassified", and 13%, 11%, and 16%, respectively in experiments 1, 2 and 3, matched more than one known microorganism and were classified as "Multiples". These percentages were obtained after the results were re-analyzed through the NCBI database and the RDP Classifier website.

Statistical Measure Comparing Culture-Based Method and TRFLP

A Kappa Test of agreement that compared the families identified in the culture-based method to those found using TRFLP is shown in Table 1. The Kappa value of

0.737 suggests strong agreement in the genera identified by the two identification methods.

Table 4.1 Kappa values of agreement between results of culture based biochemical identification methods and the TRFLP molecular methods for bacterial isolates from positive H_2S culture tests of sewage samples

	Number of Organisms Identified by TRFLP (by Genera)[a]			
		+	-	Total
Number of Organisms Identified by Culture (by Genera)	+	33	7	40
	-	212	0	212
	Total	245	7	252
SE	0.135	PPV	0.825	
Sp	0	NPV	0	
Kappa	0.737			

[a] (+) indicates that the organism was found in the identification process; (-)indicates that the organism was not found in the identification process
[b] (SE) =Sensitivity, (Sp) =Specificity, (PPV) =Positive Predictive Value, & (NPV)= Negative Predictive Value

4.4 Discussion

The compartmentalized plastic bag, liquid culture MPN method employed to detect and quantify H_2S-producing bacteria in this study is easy to use and economical for low-resource settings. However, it is well-known that H_2S-producing bacteria are diverse based on their taxonomy, ecology and metabolism, and there are concerns that not all H_2S-producing bacteria detected by the H_2S test are of fecal origin and indicative of fecally contaminated water (Rijal et. al 2000). The purpose of this research was to determine if a sample that we presumed would be positive for H_2S-producing fecal bacteria, sewage, was culture-positive for H_2S-producing bacteria, and would also contain organisms of concern as a benchmark for future natural water based studies. To

address this concern, the H_2S-producing bacteria in sewage samples analyzed by a quantal (MPN) H_2S culture test were characterized and identified by both biochemical and nucleic acid-based molecular methods. Results of both the culture-based biochemical and the TRFLP molecular method indicated that this H_2S test correctly identified both known enteric pathogens and fecal indicator bacteria in sewage.

In these experiments applied to sewage, H_2S-positive sample volumes contained many enteric microorganisms of public health concern, including: *Salmonella, Shigella, Yersinia,* and *Klebsiella*. In addition, known H_2S producing organisms were identified including: *Acinetobacter wolffii, Aeromonas hydrophila*, *Citrobacter freundii, Klebsiella ozonae, K. pneumoniae, Salmonella* sp., and *Yersinia enterocolitica.* TRFLP was able to identify other microbes that are known water-borne human pathogens or fecal indicators including: *Bacteroides, Burkholderiaceae, Erwinia, Escherichia, Enterococcus, Helicobacter, Klebsiella, Pseudomonas, Ruminococcus, Salmonella, Serratia, Shewanella, Staphylococcus, Streptococcus, Streptomycetes,* and *Vibrio* (UNICEF 2009). In fact, results from the TRFLP analysis demonstrate that at least 16% of the organisms classified were known H_2S producers, and the majority of those organisms were also potentially pathogenic or likely of fecal origin.

It is not surprising that the TRFLP and biochemical results do not match exactly, given the inherent bias and limitations of culture methods. Specifically, the culture-based isolation techniques used in this study focused on culturing and identifying members of the *Enterobacteriaceae* family, to the exclusion of most others, and it is well documented that culture methods generally underestimate both the diversity and concentrations of bacteria in environmental samples for a variety of reasons. Molecular methods also have

implicit biases due to the sampling method, nucleic acid extraction methods, the PCR reaction conditions, and human error (Blackwood et. al 2007, Kim and Marsh 2004).

However, TRFLP analysis was not only able to detect the H_2S producers that were detected via cultivation, but was also able to detect other H_2S producing organisms that were not identified by the cultivation methods used. While this research provides taxonomic evidence for a likely link between sewage-contaminated water samples having known H_2S-producing enteric microorganisms and their ability to be quantified and identified in positive H_2S test results, future work will examine whether or not positive H_2S samples from diverse fecally contaminated natural water sources have the same or similar relationships. In addition, future work will also determine whether or not negative H_2S test results from such water samples contain known waterborne pathogens or fecal indicator bacteria, an indication of false negative results. If these questions can be adequately answered, a quantitative version of the H_2S-producing bacteria test as described here may be recommended for use in developing countries and other resource-limited settings such as emergency situations world-wide.

Chapter 5: Comparison of Culture-Based and TRFLP Analysis to Identify H_2S Producing Microorganisms from Multiple Natural Water Sources

5.1 Introduction and Background

Many investigators have attempted to identify the bacteria at the species level that produce positive results in the H_2S test (Sobsey and Pfaender 2002). Castillo et al. (1994) found a large variety of bacteria in samples giving positive reactions in the H_2S test, primarily *Clostridium perfringens* and members of the *Enterobacteriaceae* (including *Enterobacter, Clostridium, Klebsiella*, *Escherichia, Salmonella, Morganella*) and other organisms known to cause illness in humans *(Acinetobacter, Aeromonas)*. Ratto et al. (1989) found *Citrobacter* was a common organism in positive H_2S tests.

However, it is currently unclear if a positive H_2S test is actually indicative of fecal organisms that are of concern to human health. Sobsey and Pfaender (2002) suggest that while the organisms producing a positive H_2S result many not be all coliforms, they are organisms typically associated with the intestinal tracts of warm-blooded animals, which are not necessarily of human origin. Moreover, the H_2S test may detect bacteria that are naturally occurring in water and not of fecal origin.

Culture-based and molecular methods could be used to address this problem. The purpose of this study was to examine natural water samples and determine, through a comparison of culture-based biochemical and molecular methods, if water samples that

produce positive H_2S tests do contain organisms of concern to human health and whether those that produce negative H_2S tests lack organisms of concern.

At present, obstacles remain that limit the widespread use of H_2S tests because of their lack of uniformity and unavailability in a ready-to-use form. For the H_2S test to be an acceptable tool for water quality evaluation, data are needed on which organisms produce positive results in the test, under what conditions test results indicate fecal contamination of water, the sensitivity and specificity of the test, and the relationship between water quality as measured by the H_2S test and the incidence of diarrheal disease.

5.2 Materials and Methods

Sampling sites

This research focused on four drinking water source types. The sources were chosen based on the 2006 WHO Guidelines for Drinking Water Quality to be representative of the most often used drinking water sources worldwide. University Lake (Chapel Hill, North Carolina) was chosen as a representative of surface water. The University of North Carolina built the lake in 1932. University Lake holds 450 million gallons of water and is home to varied and abundant terrestrial and aquatic wildlife. Its tributaries include Morgan Creek, Phil's Creek, Neville Creek, Price Creek and Pritchard's Mill Creek. The lake has a 213-acre surface area, and is one of two protected primary drinking water sources for the Orange County Water and Sewer Authority. The North Carolina Department of Environment and Natural Resources (DENR) has access to an unprotected artesian well that has been chosen as the representative of an unprotected well. In addition, DENR has access to a set of wells on the North Carolina State University (NCSU) agricultural campus that are surrounded by several farming and

livestock operations. One of the shallow wells (25ft) was chosen as a representative of a groundwater/Aquifer source that is high in iron and sulfide. Lastly the FedEx Global Health building (Chapel Hill, NC) has a water catchment system that accumulates water from the roof in a rainwater cistern that was used as a representative of a rainwater collection system. The water from this system is being used for irrigation and toilet flushing in the FedEx building. For each of these drinking water sources, three separate experiments were run over a three-month period. In addition, given the already identified problems with geothermal and other water sources that are high in H_2S or Iron, the NCSU well was chosen because it has high sulfide and iron levels. This water sample collection plan was designed to help experimentally determine how the H_2S test performs under different microbiological and chemical conditions.

Culture-based biochemical detection method

To determine the genera and relative numbers of bacteria present in the natural water (NW) samples, 160 mL of water was obtained from each water source (Duke Forest Artesian Well; NC State University Shallow Well; University Lake in Chapel Hill, NC; Rainwater cistern at UNC-Chapel Hill) on 3 separate occasions. Each water collection was treated as a separate experiment, since they were collected every 2 weeks over a six-week interval. 160 mL of each natural water (NW) source was obtained and two 50 mL aliquots were removed and pelleted at 3,500 rpm for 20 min, and the pellet was overlaid with 300 µL of the original sample and archived at -80°C for future DNA analysis. A MPN experiment was performed by diluting 50 mL of the natural water sample into 450 mL of Phosphate Buffer Solution. The natural water mixture was then serially diluted out to 1×10^{-5}. Four 100 mL samples of each dilution were then aliquoted

into plastic bags. One PathoScreen (Hach Company, Loveland, CO) reagent packet was added to the 100 mL samples. Then 9 mL and 1 mL aliquots from each 100 mL sample were put into 16x150 mm glass test tubes and 13x100 mm glass test tubes, respectively. The test tubes were then capped and incubated at 37°C for 24 h.

Organisms were isolated from positive H_2S MPN test tube and bottle samples through the following process (Figure 1): A dilution series was performed out to $1x10^{-5}$ by taking 1 mL of the positive H_2S test (from the undiluted 100 mL of NW) and placing it in 9 mL of phosphate buffered saline (PBS). Then, 100 µL volumes of each dilution were spread onto duplicate 13 x 150 mm diameter plates of the following agar media to isolate colonies: Bio-Rad RAPID E. coli 2 agar, Salmonella-Shigella agar, Phenyl ethanol agar, m-Aeromonas Selective agar, and H_2S agar. All plates were incubated aerobically at 37°C for 24 h. Isolates from spread plates were obtained by streak plating characteristic colonies onto Tryptic Soy Agar on three successive days. These colony isolates were archived in 0.8 mL of Tryptic Soy Broth at -80°C. The isolates were then tested to determine if they produce H_2S by culturing in H_2S medium and were then identified at the genus and species level using standard biochemical test kits (BBL Enterotube II, BD Diagnostic Systems, Sparks, MD and API 20E System, (bioMerieux, Inc., Hazelwood, MO).

DNA extraction

As can be seen in Figure 1, two compartment bags of positive H_2S media were pelleted and the resulting pellet was overlaid with 1 ml of the H_2S positive sample spent medium and archived at -80°C for future DNA isolation for TRFLP molecular community analysis. Genomic DNA (gDNA) was extracted using the Mo Bio Powersoil

DNA extraction kit (Mo Bio Laboratories Inc, Solana Beach CA), according to the manufacturer's protocol. Agarose gel electrophoresis was used to visualize whether sufficient quality gDNA was isolated from each sample. A 3 µl volume of each gDNA sample was electrophoresed through a 1% TAE Agarose gel containing 15 µl of ethidium bromide per 100 ml of Agarose gel. Isolated gDNA was stored at -80ºC until it was used for PCR reactions.

PCR conditions

3 µl volumes of each DNA sample were added to 97 µl volumes of Master Mix (per sample: 10 µl 10X Reaction Buffer; 0.8 µl dntp [Deoxynucleotide Triphosphate]; 83.7 µl PCR grade water; 0.5 µl each of the bacterial-specific 16S rDNA primers 8F-Hex 5'-AGA GTT TGA TC(A/C) TGG CTC AG and reverse primer 1492R 5'-GGT TAC CTT GTT ACG ACT T; 0.5 µl of Qiagen HotstarTaq DNA polymerase(Qiagen, Hilden, Germany). Each PCR sample was performed in triplicate. The forward primer for the PCR reaction was labeled on the 5' end with a hexamide fluorescent marker to allow the terminal fragment to be tracked. PCR was performed in a Perkin-Elmer 9600 thermocycler by using an initial denaturation step of 15-min at 95°C, followed by 35 cycles consisting of denaturation (60 s at 94°C), annealing (60 s at 50°C), and extension (120 s at 72°C) and a final extension at 72°C for 7 min. PCR replicates of each sample were then pooled and purified by using the UltraClean™ PCR Clean-up Kit (MoBio Laboratories Inc, Solana Beach CA) according to the manufacturer's protocol.

TRFLP

For the TRFLP analysis of the amplified bacterial 16S rDNAs, three restriction enzymes, *Rsa*I, *Hha*I, and *Msp*I (New England Biolabs, Inc., Ipswitch, MA) were used.

For the *Rsa*I digest, 30 µl of purified PCR product (approximately 30 µg) was mixed with 10 µl of Reaction Buffer#1, 59 µl PCR grade water, and 1µl of restriction enzyme. For the *Hha*I digest, 30 µl of purified PCR product (approximately 30 µg) was mixed with 10 µl of Reaction Buffer#4, 1µl BSA, 58µl PCR grade water, and 1µl of restriction enzyme. For *Msp*I, 30 µl of purified PCR product (approximately 30 µg) was mixed with 10 µl of Reaction Buffer#4, 1µl BSA, 58µl PCR grade water, and 1µl of restriction enzyme. Restriction digests were incubated overnight at 37°C. For clean-up, restriction digests were heat treated at 60°C for 20 min to heat inactivate the restriction enzymes. The QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany) was then used to purify the digested DNA according to the manufacturer's protocol, except 50 µl of warmed (60°C) PCR grade water was added instead of kit elution buffer, and the water was allowed to incubate on the column for 5 min prior to elution of the DNA. DNA samples were then frozen at -20º C. Fragment detection was carried out at the MSU Genomics Technical Support Facility according to their detection protocols (http://gtsf.msu.edu/dna-fingerprinting-and-genotyping).

Fragment analysis

Data tables containing fragment size and abundance data for each digest of the DNA of sewage samples was exported from GeneScan, and the resulting text files were sent for pattern detection by the *In Silico*© database. Each file contained all the detected fragments for a given restriction digest (e.g., data obtained from one of the *Hha*I digests for a sample would be contained in one file, *Msp*I fragments would be contained in another file, and *Rsa*I fragment data would be contained in a third file). Each entry in these data files contained fragment length size, retention time on gel, peak height,

fragment identification number, and a peak area found in the sample. For calculation of the diversity indices, the TRFLP analysis peak area was used as the amount measurement and its relative abundance was measured by dividing individual peaks by the total fluorescence of the sample. The results for each diversity measure are representative of the number of fragments in each experimental sample.

Pattern detection and pattern identification were carried out using the *In Silico*©, software package (*In Silico*©, RTP, NC). The *In Silico*© output is a comprehensive dataset which includes the following diversity analysis values: Simpson Index of diversity, a measure of the richness (the number of different species per sample) and evenness; Reciprocal Simpson, an inverse of the Simpson's index (lowest value is 1; the higher the value the greater the diversity); Species richness (the number of species within a community); and the Shannon Weaver diversity index, which is one of several diversity indices used to measure diversity in categorical data and takes into account the number of species and the evenness of the species. The index is increased either by having additional unique species, or by having greater species evenness. *In Silico*© also provides information on the fragment parameters, including: total fragment utilization, which is the proportion of the fragments used in the analysis compared to the total number of fragments available; the total number of fragment patterns detected; and evenness, which is a measure describing how much each individual fragment contributes to the whole (on a 0-1 scale; closer to 1 the better) (Blackwood et. al 2003, Kim et. al 2004, Blackwood et. al 2007).

As described in Johnson et al. (2009), fragments were sequentially matched to patterns as described previously (10, Figure 2A). A compression utility was then used to

remove multiple matches to the same organism and to combine patterns that matched multiple organisms (Figure 2B). A phylogenetic sorting algorithm matching the 16S rDNA genes in the National Center for Biotechnology Information (NCBI) sequence database was then applied to the final pattern set in the (Figure 2C). Although the identified patterns can be reported by *In Silico*©, at 5 different phylogenetic levels (phylum, order, class, family, and genus; Figure 2), the data in this study are presented at the genus level because this gives information about the different types of organisms' capacity of the microorganisms to produce hydrogen sulfide.

The "unclassified" group of sequences differs from unmatched fragments because little or no phylogenetic information is deposited with their respective sequence. Unclassified sequences make up a large percentage of the data since sequences are often deposited without sufficient phylogenetic identification. Sequences in the "multiple classification" fit into more than one distinct phylogenetic group and consist of all the unique species from phylogenetically different groups that match a single fragment pattern.

Further Phylogenetic Assignment

Given the relatively high percentage of fragment patterns from the *In Silico*© output that were either in the "Multiples" or "Unidentified" categories, further analysis was conducted. Any fragment pattern that was assigned to either the "Multiples" or "Unidentified" category was then re-analyzed by entering it into the BLAST tool in the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nucleotide/), and the retrieved nucleic acid sequence listed in NCBI was then entered into the Michigan State University Ribosomal Database Project classifier website

(http://rdp.cme.msu.edu/classifier/classifier.jsp) using a 95% confidence threshold for phylogenetic assignment (Johnson et al 2009, Wang et. al 2007). For example, the first unidentified pattern in Sewage experiment 1 was reported by the In Silico database as "Uncultured bacterium clone ICBTF7 16S ribosomal RNA gene, partial sequence". That identity was entered into the NCBI database and produced a 522bp sequence. That 16S rRNA gene sequence was then submitted to the RDP Classifier website, which generates a genus level identification of the organism with 95% confidence.

The classifier takes a sequence and assigns it to the lowest taxonomic level possible within a certain degree of confidence. If that organism was identified with 95% confidence or higher, it was removed from the "Unidentified" category and was reclassified. In some cases, all of the fragment patterns from a "multiple" categorization could be analyzed using BLAST and the RDP Classifier and were identified as the same organism. When this occurred, and the organism was identified with 95% confidence or higher, it was removed from the "Multiples" category and was reclassified. Some fragment patterns that were originally labeled as "Multiples" contained only organisms that have been identified as fecal coliforms (Committee on Indicators for Waterborne Pathogens 2004). Therefore, a separate "Gram negative enteric bacteria" category was created for those "Multiples", which included the following genera: *Escherichia*, *Klebsiella*, *Proteus*, *Salmonella*, and/or *Shigella* (LeClerc et. al 2001).

Statistical Measure Comparing Culture-Based Method and TRFLP

Kappa Tests of agreement were performed comparing the genera found in the culture-based method to those found in the TRFLP output. Kappa Tests of agreement are a measure of association (correlation or reliability) between two measurements of the

same item when the measurements are categorical. Values closer to zero indicate slight to little agreement, while values closer to one indicate strong agreement (Landis and Koch 1977).

5.3 Results

University Lake Molecular and Cultured Isolate Findings

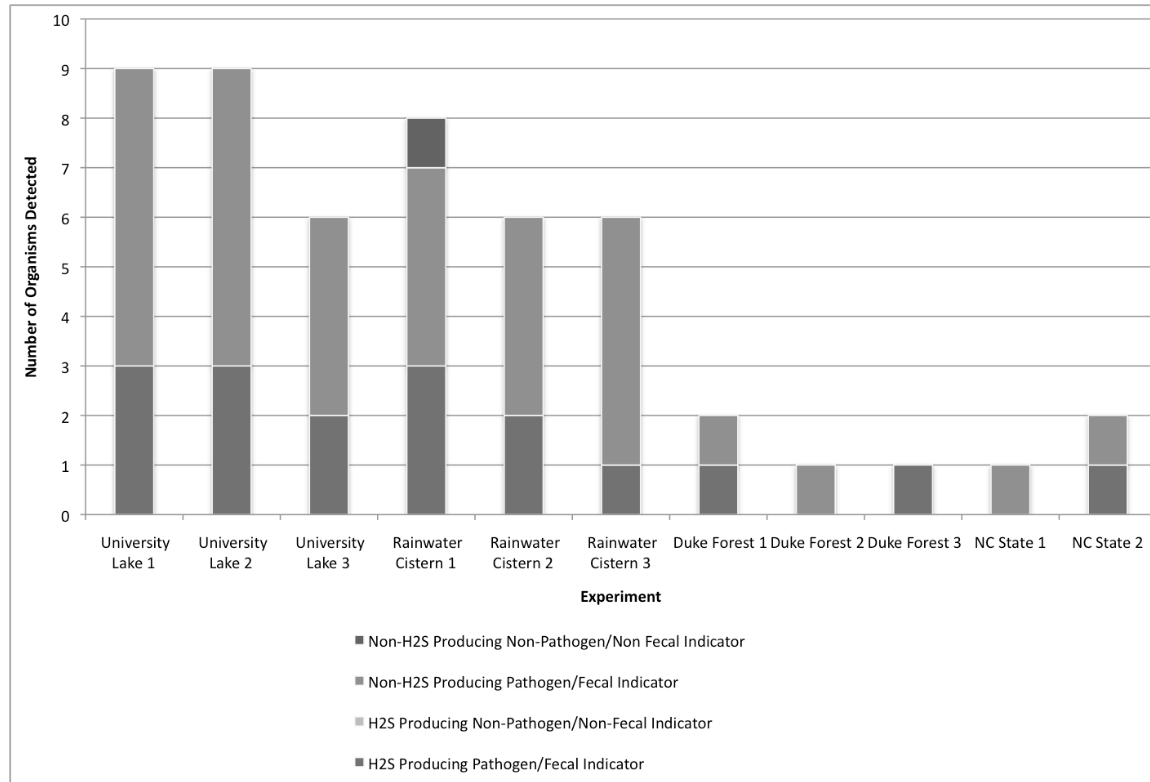

Figure 5.1 Pathogens and H_2S-Producing organisms identified in natural water samples using culture-based biochemical identification methods.

The following categories divide the genera based on whether they are a likely pathogen/ Fecal Indicator Organism and whether or not they produce Hydrogen Sulfide: H_2S Producing Pathogen/Fecal Indicator (Committee on Indicators for Waterborne Pathogens 2004); H_2S Producing Non-Pathogen/Non-Fecal Indicator (Reis et al. 2002); Non-H_2S Producing Pathogen/Fecal Indicator (Widdel 1988); Non-H_2S Producing Non-Pathogen/Non Fecal Indicator

Cultured Isolates

As can be seen in Figure 5.1, University Lake had the most species richness and diversity of the water sources examined in both the cultured and molecular experiments. *Escherichia coli, Citrobacter freundii, Klebsiella pneumoniae, Hafnia alvei, and*

Aeromonas hydrophila represented at a minimum 67% of all organisms cultured and isolated from the three experiments. Figure 3 shows that 10 different organisms were isolated. *C. freundii, E. coli,* and *Enteric Group 60* represented more than 50% of the total isolates as identified by either the Enterotube or the API20E system. Known pathogens such as *Proteus, Salmonella* and *Klebsiella* were also identified. Most importantly, possible H_2S producing organisms were also isolated, including: *Klebsiella ozonae*, *Proteus mirabilis, K. pneumoniae* and *C. freundii.* The detection of H_2S-producing organisms by the H_2S test was confirmed by the culture-based biochemical detection findings in which 54 of 282 isolates were positive for H_2S production. However, Figure 5.1 shows that there are differences in both the number of organisms in the sample and the overall community composition from these H_2S enriched samples.

While experiment 1 isolates were composed of 30% *C. freundii* and 22% *E. coli*, Experiment 2 isolates were shown to consist of 19% *C. freundii* and 51% *E. coli*, and Experiment 3 University Lake samples contained no *C. freundii* isolates but 41% of the isolates were identified as *E. coli*. There were also fewer organisms isolated in Experiment 3 than in Experiments 1 or 2.

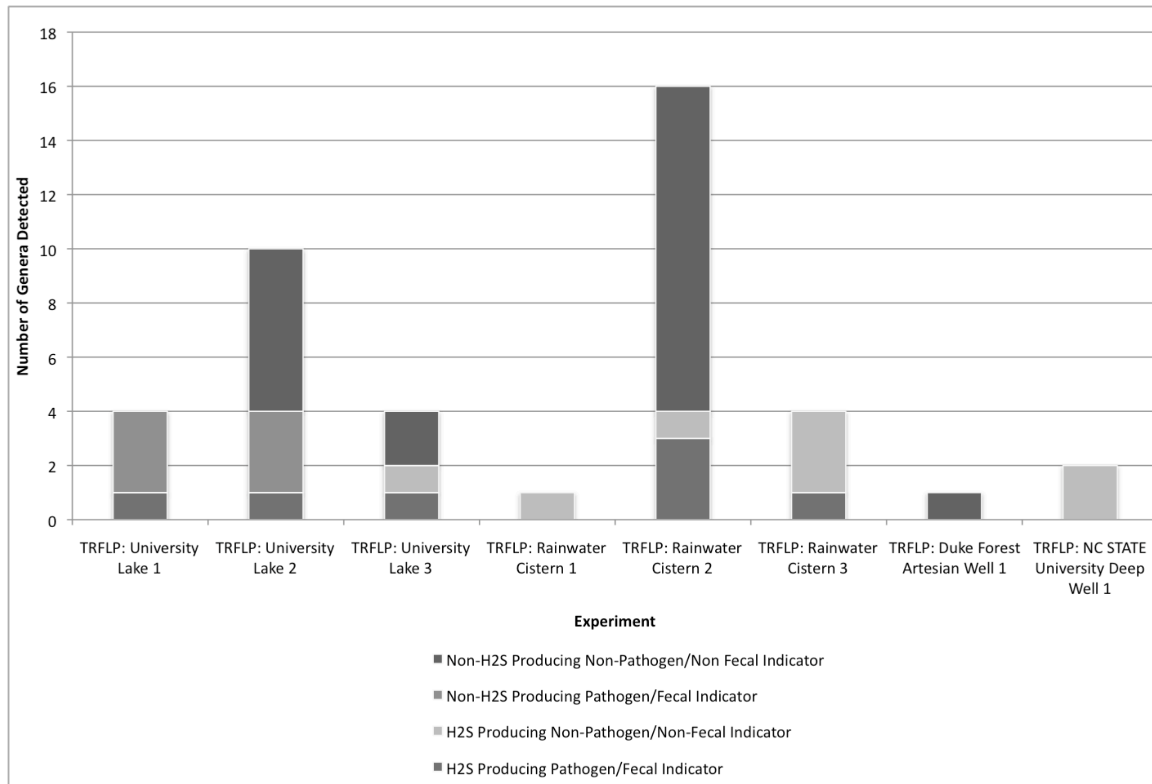

Figure 5.2 TRFLP fragment patterns identified at the Genus level in genomic DNA isolated from H_2S-positive liquid cultures of bacteria in natural water samples.

The following categories divide the genera based on whether they are a likely pathogen/ Fecal Indicator Organism and whether or not they produce Hydrogen Sulfide: H_2S Producing Pathogen/Fecal Indicator (Committee on Indicators for Waterborne Pathogens 2004); H_2S Producing Non-Pathogen/Non-Fecal Indicator (Reis et al. 2002); Non-H_2S Producing Pathogen/Fecal Indicator (Widdel 1988); Non-H_2S Producing Non-Pathogen/Non Fecal Indicator

TRFLP

In addition, microbial community identifications were carried out via TRFLP analysis of University Lake Experiment 1, 2, and 3 samples, and fragments for all three restriction enzymes were detected. Figure 5.2 shows 12 different genera identified between the three University Lake experiments. As the figure shows, there were only 2 genera that were identified that were not isolated through the cultivation process. These are *Rhodothermus* and *Shewanella.* However, every genus isolated through the biochemical cultivation and isolation process was also identified in the TRFLP analysis. University Lake experiment 2 was the most diverse, with 10 different genera identified.

These results indicate the presence of potential pathogens, including: *Shewanella*, *Serratia*, *Nitrosomonas*, *Enterobacter*, and Gram negative enterics. Results from University Lake Experiment 3 were unlike Experiments 1 and 2 in that *Methylobacter*, *Cryptomonad*, *Comomonas*, and *Caldithrix* were identified as present in Experiment 3 samples but not Experiment 1 or 2 samples. The number of unclassified patterns was 20%, 15%, and 0% for the three experiments, respectively. In addition, 13%, 21%, and 29% of the fragment patterns were classified as "multiples" since these fragment patterns were identical for a set of bacteria, and as such the fragment cannot be positively identified as having come from a particular single bacterium.

FedEx Global Health Building Rainwater Cistern Molecular and Cultured Isolate Findings

Cultured Isolates

The FedEx cistern was second in species richness amongst water samples. As Figure 5.1 indicates, there was considerable variability in the community structure of the isolates. Known pathogens such as *Serratia marcescens, Salmonella* sp*., Shigella* sp*.,* and *Yersinia enterocolitica* were identified in Experiment 1. Though *E. coli* represented 28% and 25% of the Experiment 2 and 3 isolates, respectively, it was not isolated in samples from Experiment 1. Cistern Experiment 2 was not similar to Experiment 1 in the types of organisms identified. *C. freundii, E. coli, and Enteric Group 60* were all isolated in high percentages. Pathogens and H_2S producing organisms were isolated from Cistern Experiment 2. The known pathogen *Klebsiella* was identified. In addition, possible H_2S producing organisms *Aeromonas hydrophila and Citrobacter freundii* were isolated. As

was expected, based on previous experiments, 75 of 264 isolates tested positive for H_2S production.

TRFLP

The TRFLP results for the three Cistern Experiments vary greatly. While Experiment 1 only identified an "unclassified Lachnospiraceae", Experiment 2 identified 16 different genera, and Experiment 3 identified 4 genera. Known H_2S producing genera were identified in all samples except those from Experiment 1. With the exception of Rainwater Cistern Experiment 3, the TRFLP analyses for the Cistern experiments differ greatly with respect to the biochemical analysis. The number of unclassified patterns was 50%, 28%, and 6.3% for the three experiments, respectively. In addition, 0%, 30%, and 70% of the fragment patterns were "multiples" and could have been more than one organism.

Duke Forest Artesian Well Molecular and Cultured Isolate Findings

Cultured Isolates

The Duke Forest well samples were more affected than any other by rainfall changes, probably due to the fact that it is a shallow artesian well. Figure 5.1 shows those differences. In periods with high rainfall, such as in experiment 1, there is an increase in H_2S production and in the number of organisms found. On the other hand, low rainfall periods produced very little organism diversity. Overall, there was very little diversity in the well. In fact, only Experiment 1 had more than one organism isolated. It was the only instance where the H_2S test was positive as well. The only H_2S producing organism identified in any of the experiments was *C. freundii* in Experiment 1. In addition, there were a few isolates of *Enterobacter aerogenes.* The presence of H_2S-producing

organisms was confirmed with 71 of 73 isolates being positive for H_2S production in Experiment 1. Only *Enterobacter cloacae* were isolated in Experiment 2. There were no pathogens or H_2S producing organisms isolated from this experiment with 0 of 71 isolates testing positive for H_2S production. Duke Forest Experiment 3 was not similar to Experiment 1 or 2 in the types of organisms identified. Only *Pseudomonas aeruginosa,* a potential opportunistic pathogen, was isolated. Figure 5.1 shows that there were H_2S producing organisms isolated from Duke Forest Experiment 3 with 0 of 47 isolates testing positive for H_2S production.

TRFLP

The TRFLP analysis results for Duke Forest Experiment 1 was somewhat similar to the data obtained using biochemical methods. Figure 5.2 shows the genera identified by this analysis. Every genus isolated through the biochemical cultivation and isolation process was also identified in the TRFLP analysis. The number of unclassified patterns was 17% for Experiment 1. In addition 50% of the fragment patterns were "multiples" and could have been more than one organism. The quantity of DNA isolated from bacteria collected in Duke Forest Experiments 2 and 3 was not adequate to run TRFLP. There was a second and a third attempt to recover DNA and conduct the TRFLP analysis; however, neither effort provided any DNA. The difficulty in recovering DNA from the Artesian Well samples is likely due to a low number of organisms that would grow in the culture based methods.

NCSU Shallow Well Molecular and Cultured Isolate Findings

Cultured Isolates

The NC State agricultural campus has a set of wells surrounded by agriculture and livestock production. Like the Duke Forest well samples, the biochemical tests for the NCSU well samples showed that there was little diversity in the organisms isolated from this well. In fact there was no growth on the NCSU experiment 3 plates (also no DNA was recovered), and much less growth in experiments 1 and 2 than in other water sources tested. Figure 5.1 shows the results from experiment 1 and 2. Unlike any of the other water sources, no H_2S samples were positive for the well experiments even though organisms were isolated. In addition, though this was the water source that was high in iron and sulfides, none of the cultured samples from the wells produced a positive H_2S test.

Only *Enterobacter aerogenes* were isolated from Experiment 1. There were no pathogens or H_2S producing organisms isolated from this experiment. None of the 65 isolates tested positive for H_2S production. NCSU Experiment 2 water isolates did not match those from NCSU Experiment 1 in that *Pseudomonas cepecia* and *Enteric Group 60* were isolated from Experiment 2 and had not been identified in Experiment 1. As was expected based on previous experiments, 0 of 55 isolates tested positive for H_2S production. There were no culture based isolates from NCSU Experiment 3.

TRFLP

There was adequate DNA to run a TRFLP for NCSU Experiment 1. As Figure 5.2 shows, *Enterobacter* and *Comamonas* were identified by TRFLP. This closely matched the culture-based experiments. As was the case with other previously described

experiments, the quantity of the DNA recovered from NCSU Experiment 2 was deemed to be inadequate based on DNA gel analysis. There was a second and a third attempt to recover DNA and run the TRFLP. However, neither effort provided isolated DNA.

Statistical Measure Comparing Culture-Based Method and TRFLP

A Kappa Test of agreement that compared the families found in the culture-based method to those found in the TRFLP output is shown in Table 5.1. The Kappa value of 0.664 suggests fairly strong agreement on the families identified by the two methods.

Table 5.1 Kappa Values of Agreement between the Culture Based Identification Method and the TRFLP Molecular Method for the Natural Water Samples

	No. Organisms Identified by TRFLP (by Genera)[a]			
		+	-	Total
No. Organisms Identified by Culture (by Genera)	+	17	8	25
	-	29	0	29
	Total	46	8	54
SE	0.369565217	PPV	0.68	
Sp	0	NPV	0	
Kappa	0.664254703			

[a] (+) indicates that the organism was found in the identification process; (-)indicates that the organism was not found in the identification process

5.4 Discussion

Overall, these experiments have shown that when a water sample tests positive for H_2S there are fecal bacteria in the water sample, and when they test negative, there are no bacteria of concern detected either by biochemical or TRFLP methods. In the NCSU experiments, there were organisms isolated, but the number that grew in the H_2S media was much lower than in any of the other experiments. The H_2S MPN method is easy-to-

use and economical for low-resource settings. However, H_2S-producing bacteria are diverse based on their taxonomy, ecology and metabolism, and there are concerns that not all H_2S-producing bacteria in the H_2S bacteria test are of fecal origin and associated with fecally contaminated water (Sobsey and Pfaender 2002). There is a possibility that some may be of non-fecal origin and thereby cause false positive results in the H_2S test.

To address this concern, the H_2S-producing bacteria in representative water samples or cultured from water samples in the H_2S test were characterized and identified by both biochemical and molecular methods. The goal of these analyses was to determine whether H_2S bacteria detected were likely of fecal origin rather than non-fecal environmental H_2S-producers. In addition, laboratory testing indicated that compartment bags with positive H_2S tests often contain pathogens, *E. coli*, and other fecal coliforms.

In these experiments, positive H_2S samples contained many organisms of concern, including: *Aeromonas hydrophila, Escherichia coli, Enterobacter aerogenes, Enterobacter agglomerans, Enterobacter cloacae, Hafnia alvei, Morganella morganii, Plesiomonas shigelloides, Providencia alcalifaciens, Providencia rettegeri, Pseudomonas aeruginosa, Pseudomonas cepecia, Serratia liquifaciens, Serratia marcescens, Serratia plymuthica, and Shigella* sp.

In addition, known H_2S producing organisms were identified including: *Acinetobacter wolffii, Aeromonas hydrophila*, and *Citrobacter freundii, Klebsiella ozonae, K. pneumoniae, Proteus mirabilis, Salmonella* sp.*, and Yersinia enterocolitica.* TRFLP also identified other genera that are known human water-borne pathogens including: *Cryptomonadaceae* (WHO 2006).

Unlike previous work done with sewage samples, the molecular and biochemical results were very similar when there was enough DNA to analyze. In addition, there is clear evidence that this version of the H_2S test is not impacted by high sulfur and high iron water sources, with little to no culturable growth and no positive H_2S tests in those samples. Given the strong relationship between positive H_2S tests and organisms of concern, these results provide evidence that the quantitative H_2S test in a compartmentalized plastic bag has promise as a low-cost, easy to use alternative to current expensive, labor intensive and lab-based microbial water quality testing practices.

In addition, waters with negative H_2S tests contained very few pathogens. These results were determined for a number of different water source types, locations, and compositions, and suggest that the H_2S test has great potential for low-cost microbial water quality testing in resource poor environments. Future experimentation should look at varied water sources and their H_2S levels and organisms found therein.

Chapter 6: Hydrogen Sulfide Producing Bacteria and *Escherichia coli* in household drinking water and diarrheal disease risk: evidence from central Vietnam

6.1 Introduction

At present, obstacles remain that limit the widespread use of H_2S tests because of their lack of uniformity and unavailability in a ready-to-use form. For the H_2S test to be an acceptable tool for water quality evaluation, data are needed to determine the relationship, if any, between water quality as measured by the H_2S test and the incidence of diarrheal disease. Our previous culture-based biochemical and molecular work has provided taxonomic evidence for a likely link between sewage-contaminated water samples having known H_2S-producing enteric microorganisms and their ability to be quantified and identified in positive H_2S test results from sewage samples. Moreover, our findings show that there is clear evidence that this version of the H_2S test is not impacted by high sulfur and high iron water sources, and that there is a strong relationship between positive H_2S tests and organisms of concern in natural water samples of different water source types, locations, and compositions. In addition, waters with negative H_2S tests contained very few pathogens.

Numerous studies examining the relationship between measured microbial indicators in drinking water and health outcomes reveal only a limited association (e.g., Moe et al. 1991) or no association (Gundry et al. 2004; Jensen et al. 2004). To date,

published results have only examined the relationship between H_2S-producing bacteria and other fecal indicators. However, this research is the first to directly examine possible relationships between human illness (in this case diarrheal disease) and hydrogen sulfide-producing bacteria in drinking water samples in a low-resource setting in a developing country where the test is most needed. Moreover, this study examines the relationship between *E. coli* and H_2S-producing bacteria and between *E. coli* and diarrheal disease from household drinking water samples in rural central Vietnam. In addition, this research describes the utility of a new simple, low-cost form of the H_2S test in comparison with standard membrane filtration and IDEXX Colilert MPN quantification of *E. coli*.

6.2 Methods

Study population and recruitment of households

Binh Dao Commune in Thang Binh District, Quang Nam Province in central Vietnam is a poor community along the Truong Giang River, 56km south of Da Nang City. Tra Doa Hamlet has 7,592 people living in 650 households, with 95% of the population earning their living by farming and the remaining 5% running small businesses. The local people have difficult circumstances, which makes contributing financially to the construction of a clean water system challenging.

The main sources of drinking water in the area are wells and irrigation systems. Most of the sources have high levels of salinity and alum. The presence of four fish processing units and many graves in the area has seriously affected the present water sources. In order to improve water quality, local residents must currently filter water themselves. Furthermore, most wells are out of water in the dry season. In September

2005, East Meets West (EMW) constructed a clean water system for 600 households in the commune, and the system is still operating under the management of the local authorities.

In this work, we report on a 20-week study in rural Vietnam near DeNang that included a monthly collection of household drinking water quality (*E. coli* and H_2S-producing bacteria) and diarrheal disease data in 300 households. Regression analysis was applied to measure the association between the levels of *E. coli* in household drinking water and the outcomes of diarrheal disease (all types) experienced by household members in the previous seven days.

The cohort enrolled 300 households (141 households connected to a piped water supply system, 83 connected to a piped system and a pour-flush latrine, and 76 households who had received the East Meets West Foundation (EMW) program offer but declined to invest in either). All households were located in central Vietnam's Da Nang province. In collaboration with EMW, an initial map of all households within seven villages in the area was constructed and households were approached in randomized order within each village. Households were recruited until the a priori sample size criterion of 300 households was met. All households with piped water were connected to an EMW-sponsored system, however.

Eligibility criteria were that the households stored drinking water at the household level, had one or more children under five years of age (up to 48 months at the time of enrollment; infants who were not yet drinking water were excluded from the study), that the household was located within the study area, and that the head of household and the primary caregiver (if a different person) agreed to voluntarily participate. If the

household was eligible, we presented the primary caregiver (senior female, charged with caring for children and collecting water) with the informed consent form. If the primary caregiver and the head of household (if different) consented to participate, the household would be enrolled in the study. All survey data was collected from the primary caregiver, who acted as the study's point of contact with the household.

Data collection

The study had an initial cross-sectional sampling and recruitment phase (1 month) and a longitudinal data collection phase (4 months). Data on diarrheal disease, water quality, and other household factors were collected as part of a broader study of household-level environmental health, including the effectiveness of water quality interventions in reducing diarrheal disease outcomes. Each household was visited once upon recruitment and then approximately three more times at the rate of once per month, with sampling ending in August 2009. The first interview was extensive (one hour) and with subsequent brief follow-up interviews (15 minutes) to monitor changing covariates. Pre-structured, pre-tested (by back-translation and use in focus groups and pilot interviews) questionnaires were prepared in Vietnamese prior to use in the study. Each interview was administered in Vietnamese.

Diarrheal Disease and other Health Related Data

A longitudinal diarrheal disease surveillance and household water quality monitoring survey was performed. The head of the household was asked to provide a one-week recall of diarrheal disease for herself and all members of her household. Diarrhea was defined for participants as three or more loose or watery stools in a 24-hour

period and dysentery was defined as stool with the presence of blood, based on World Health Organization definitions (WHO 2009).

Data on water use and handling practices, sanitation and hygiene, and other potentially important covariates were gathered at each visit as well, including the following characteristics and water use practices that have been shown to be associated with diarrhea disease: socioeconomic status, sanitation services (including human excreta management such as latrines or sewerage, water services and sources, water quantity use, hygiene behavior, and solid waste services), the presence of domestic animals in the home, and type and construction of housing as wealth indicators. Data to ascertain discrete cases or case duration were not collected.

Water quality data

Two water samples of 500 ml volume were taken from each household by study staff at the initial visit and each follow up visit to determine the effectiveness of the piped water system in reducing the concentrations of microbes present in drinking water sources. The first water sample was untreated drinking water, which was defined as water taken directly from the household before any household treatment was performed. This sample could have been from a surface water source, a tube well or borehole, a dug well, a spring, rainwater, or a piped water system. The second sample was treated drinking water, which was defined as water treated in the household by either boiling (widely practiced in most study households), filtration, or chemical disinfection (usually chlorine). Sample water pH, turbidity, and free and total chlorine were analyzed, with Chlorine measures taken as soon as the water samples were taken, using the following methods: pH (Sension1 meter, Hach Company, Loveland, CO)) and turbidity with a

turbidimeter (Hach Pocket 2100p, Hach Company, Loveland, CO Turbidity of water samples was measured in triplicate using a turbidimeter and arithmetic mean values reported as NTU).

Additional samples were taken for analysis if households used multiple storage containers, multiple sources, or multiple treatment methods. Samples were kept cool (25°C) and transported as quickly as possible to the laboratory in Ho Chi Minh, Vietnam, where analysis was performed as soon as possible, in all cases within 24 hours.

Hydrogen sulfide (H_2S)-producing bacteria, Total coliforms (TC) and *E. coli* were the microbial indicators used in this study. Membrane filtration techniques on Bio-Rad (Hercules, CA) selective agar and IDEXX Colilert® (Westbrook, Maine) consistent with methods described in *Standard Methods for the Examination of Water and Wastewater* (2005) were used to determine TC and *E. coli* levels. All samples were processed in duplicate using a minimum of two dilutions, three replicates each, with positive and negative controls. To test for H_2S-producing bacteria, PathoScreen reagent for testing of a 100 ml water sample (Hach Company, Loveland, CO) was added to the remaining 100 ml of each water sample in a 5-compartment MPN bag and incubated at 37°C for 24 h. The MPN bag is a clear polyethylene bag, 15 cm wide x 23 cm long (Whirl-Pak®, Nasco, Fort Atkinson, WI) in which there are 5 internal vertical compartments in the lower third of the bag, each with a volume of 1, 3, 10, 30 and 56 ml, respectively. Arithmetic mean values are reported.

Additional Data Collection

In addition to measures of health, questions were asked to determine usage of the water and/or sanitation intervention, water acquisition, treatment, storage and use

practices and to document sanitation and hygiene conditions and practices – all possible covariates to use in the analysis of diarrheal disease data. Observational data, such as presence of soap in the home, data on types and numbers of water storage containers, details on family water treatment practices, and presence of animals or animal waste in the home were used to supplement survey data collected in interviews to help determine whether or not there were other covariates that would affect the relationship between or bacterial measures of interest and diarrheal disease.

Data Analysis

Interviews initially collected data on paper surveys. Survey and water quality data were then transferred into a Microsoft Excel spreadsheet and copied into Stata 10.1 (StataCorp, College Station, Texas, USA) for analysis, excluding direct personal identifiers of the study participants. All data were entered twice by separate data entry staff and compared to minimize data entry error. In addition, these data without direct identifiers were copied onto USB memory keys monthly (as back-up) and were sent to study staff as additional back-up copies.

Data collected for water quality and from household surveys was analyzed using stratified or tabular analysis to assess for trends (microbial concentrations and turbidity in water and diarrheal disease incidence rates) and in the longitudinal phase of the study for differences between the three groups. Risk ratios were computed via a Poisson extension of generalized estimating Equations (GEE), which allows for the adjustment of estimates within households and within individuals over time due to clustering (Liang & Zeger 1986; Zeger & Liang 1986). To examine possible associations between water quality and health, microbial counts for H_2S producing bacteria, total coliform, and *E. coli* were used

as categorical variables based on standard log-levels of contamination (<1, 1-10, 11-100, 101-1000, 1001+ organisms per 100 ml) [23] in GEE regression with diarrheal disease as the dependent variable. Because the H_2S MPN compartment bag is based on a Poisson distribution, a different categorization was performed to take into account the non-continuous nature of the data. Based on the likelihood function of the Poisson distribution of the MPN method in the 5-compartment bag, there were only 32 possible outcomes, 8 of which were much more likely than all other outcomes. Those 8 outcomes were used to determine the 5 different categories used (<5, 5-20, 21-50, 51-200, 201+ organisms).

Diggle et al. (2002) describe the application of the GEE model and its application to binary longitudinal data. The model uses the marginal expectation (average response for observations with the same covariates) as a function of covariates in the analysis; correlation between individual observations is computed via a variance estimation term. The GEE model assumed that missing observations are Missing Completely at Random (MCAR) as described by Little & Rubin (2002): that the probability of an observation being missing is not related to measured or unmeasured cofactors that may be related to the exposure (water quality) or the outcome (diarrhea). Covariates were considered in a backward elimination procedure and were identified based on an a priori change-in-effect criterion of 10%. Adjusted estimates are reported. All potential measured confounders, including presence of a water quality intervention (piped water, boiling), socio-economic status, age, water quality and quantity, and sanitation and hygiene-related factors, were assessed in the analytical model. In addition, the H_2S levels were compared to the "gold standard" *E. coli* measure through Spearman Rank correlations and ANOVA. Observations made by the survey team showed that individuals drank both untreated

water and treated (through filtration, boiling, and/or chemical disinfection), and estimates are therefore given for both groups.

Protection of Human Subjects

This study was reviewed and approved by UNC Institutional Review Board (IRB) and the relevant local ministries and local authorities.

6.3 Results

Diarrheal disease and water quality

Characteristics of study participants are presented by water source in Table 6.1.

	All	<1	1-10	11-100	101-1000	1000+
Sex						
Male	2900	900	742	735	416	103
Female	3212	1001	751	871	462	124
Age						
0-5	1324	424	324	328	192	56
6+	4636	1441	1131	1229	662	166
Cases diarrheal disease, all ages	**153**	**38**	**29**	**43**	**32**	**11**
Observational periods, all ages	**6116**	**1902**	**1494**	**1608**	**878**	**227**
Cases diarrheal disease, under 5	**63**	**17**	**14**	**12**	**14**	**6**
Observational periods, under 5	**1324**	**424**	**324**	**328**	**192**	**56**
Odds of diarrheal disease, all ages	**2.5%**	**2.0%**	**1.9%**	**2.7%**	**3.65%**	**4.8%**
Odds of diarrheal disease, under 5	**4.8%**	**4.0%**	**4.3%**	**3.65**	**7.29%**	**10.7%**
Education of primary caregiver:						
No school		21	23	5	20	15
Primary school		312	212	321	148	31
Some secondary school		1267	1109	1062	587	160
Some university/technical or higher		171	95	94	67	9
House construction wealth indicators						
Sheet metal roof	32.7%	32.0%	30.3%	30.4%	38.4 %	46.9%
Cement floors	57.6%	55.8 %	61.9 %	56.7 %	60.1 %	42.3 %
Cement walls	87.6%	89.1%	88.2%	84.7%	90.0 %	83.7%
Water Source (Dry Season)						
piped	72.5%	70.1%	86.0%	72.7%	59.3%	53.2%
tube well or borehole	20.3%	25.9%	12.0%	20.3%	24.3%	12.6%
protected dug well	2.5%	1.6%	1.2%	3.2%	3.5%	9.0%
unprotected dug well	4.7%	2.4%	0.8%	3.9%	12.9%	25.2%
Soap Available:						
No	19.2%	18.9%	14.9%	22.3%	22.2%	18.1%
Yes	80.7%	81.1%	85.1%	77.6%	77.8%	81.9%
After restroom, do people wash hands:						
Yes (everyone all the time)	37.1%	35.3%	35.3%	39.5%	39.4%	39.2%
Sometimes (not everyone or not all the time)	62.9%	64.7%	64.7%	60.5%	60.6%	60.8%
Wash hands with:						
Water only	38.4%	39.9%	34.5%	41.3%	37.5%	35.2%
Water and soap	61.6%	60.1%	65.7%	58.7%	62.5%	64.8%

Table 6.1 Household characteristics from individual observations for all study participants over 4 household visits, by exposure status at time of visit

Water quality data, (mean, median, 95% interval) Untreated Water			Water quality data, (mean, median, 95% interval) Treated water		
TC/ 100ml (N=6108)	*E. coli*/ 100ml (N=6109)	H2S/ 100ml (N=4086)	TC/ 100ml (N=6066)	*E. coli*/ 100ml (N=6059)	H2S/ 100ml (N=4068)
5019, 414, (0-14000)	248, 6.3, (0-687)	94, 14, (0.1-480)	1594, 1.36, (0-14000)	165, 0, (0-`164)	10.3, 0, (0-48)

Table 6.2 Mean total coliform and E. coli counts (cfu/100ml), H_2S (MPN/100ml) for samples taken from households (untreated and treated water).

Table 6.2 lists the fecal indicator measurements by exposure status. One-way ANOVA showed that there is a clear and significant difference between the means of the untreated and treated water quality across groups ($p< 0.0001$). There is a 10-fold difference in the Total Coliform (TC) and H_2S measurements, and at least a 2-fold difference in the *E. coli* measurements.

Table 6.3 Correlation matrix of water samples compared by fecal indicator

Categorization Method	***Water Source***					
	Untreated Drinking Water			**Treated Drinking Water**		
As a Continuous Measure		*E. coli*	Total Coliforms		*E. coli*	*Total Coliforms*
	Total Coliforms	**0.0985**		Total Coliforms	**0.1146**	
	H_2S	**0.1039**	**0.3235**	*H_2S*	**0.1352**	**0.0667**
By WHO Category		*E. coli*	Total Coliforms		*E. coli*	Total Coliforms
	Total Coliforms	**0.5003**		Total Coliforms	**0.3389**	
	H_2S	**0.3166**	**0.4198**	*H_2S*	**0.2035**	**0.3344**
By Compartment Bag Category		*E. coli*	Total Coliforms		*E. coli*	Total Coliforms
	Total Coliforms	**0.4308**		Total Coliforms	**0.2773**	
	H_2S	**0.3123**	**0.3841**	*H_2S*	**0.1513**	**0.2649**

Table 6.3 shows that there was a significant relationship between the levels of H_2S bacteria, TC, and *E. coli* in untreated and treated drinking water samples as a continuous measure, when categorized according to the WHO scale, and when categorized by the compartment bag scale. The relationship between *E. coli* and H_2S bacteria in untreated drinking water was only 0.10 as a continuous variable, but increased to 0.31 in both categorizations. There was a stronger relationship between TC and H_2S bacteria and *E. coli* in untreated drinking water, with a correlation of 0.50 and 0.41, respectively based on the WHO categorization. Though the relationships in treated water samples were significant (at p=0.05), they were overall much lower than the relationships in the untreated water samples. The relationship between *E. coli* and H_2S bacteria in treated drinking water was only 0.13 as a continuous variable, but increased to 0.20 in both categorizations. There was a stronger relationship between TC and H_2S bacteria and *E. coli* in untreated drinking water, with each having a correlation of 0.33 based on the WHO categorization.

McNemar's test, which determines whether or not there is a relationship between diarrheal disease and the fecal indicator measure of interest, showed that there was a significant relationship between *E. coli*, and H_2S- producing bacteria, and diarrheal disease in untreated drinking water samples. Results of GEE analysis are presented in Tables 6.4-6.9.

Table 6.4 Diarrheal disease Odds Ratio estimates fecal indicators in *untreated* drinking water [a,b,c,d]

Variable	OR	P-value	95%LL	95%UL
H_2S-producing bacteria as a continuous measure	**1.000881**	0.023	1.00012	1.0016
E. coli as a continuous measure	**1.000085**	0.012	1.000017	1.000153
Total Coliforms as a continuous measure	**1.000012**	< 0.0001	1.000008	1.000015
H_2S-producing bacteria categorized by Compartment Bag classification [b]	**1.21**	.005	1.06	1.37
E. coli categorized by Compartment Bag classification [b]	**1.22**	< 0.0001	1.11	1.34

Total Coliforms categorized by Compartment Bag classification [b]	1.07	0.241	0.95	1.22
H_2S-producing bacteria categorized by WHO classification [c]	**1.24**	0.028	1.02	1.51
E. coli categorized by WHO classification [c]	**1.28**	< 0.0001	1.13	1.47
TC categorized by WHO classification [c]	**1.21**	0.026	1.02	1.42

[a] Bolded Odds Ratios indicate a significant relationship
[b] Based on 10% A priori change in estimate criterion no adjustments were necessary
[c] Compartment Bag Categorization was as follows: (<5, 5-20, 21-50, 51-200, 201+ organisms)
[d] WHO Categorization was as follows: (<1, 1-10, 11-100, 101-1000, 1001+ organisms)

Table 6.4 shows the Odds Ratios (OR) of the relationship between diarrheal disease and the bacterial measures in all untreated drinking water samples as a continuous measure, when categorized according to the WHO scale, and when categorized by the compartment bag scale. Note that there were significant relationships between diarrheal disease and both *E. coli* and H_2S-producing bacteria, but not for TC.

Table 6.5 Diarrheal disease Odds Ratio estimates fecal indicators in *treated* drinking water [a,b,c,d]

Variable	OR	P-value	95%LL	95%UL
H_2S-producing bacteria as a continuous measure	0.99	0.216	0.98	1.01
E. coli as a continuous measure	0.9999	0.644	0.9999	1.00
Total Coliforms as a continuous measure	0.99999	0.0542	0.376	1.00
H_2S-producing bacteria categorized by Compartment Bag classification [b]	0.62	0.124	0.55	1.009
E. coli categorized by Compartment Bag classification [b]	0.90	0.191	0.77	1.05
Total Coliforms categorized by Compartment Bag classification [b]	1.07	0.175	0.97	1.19
H_2S-producing bacteria categorized by WHO classification [c]	0.93	0.491	0.74	1.15
E. coli categorized by WHO classification [c]	0.90	0.233	0.76	1.07
TC categorized by WHO classification [c]	1.02	0.763	0.91	1.14

[a] Bolded Odds Ratios indicate a significant relationship at p=0.05
[b] Based on 10% A priori change in estimate criterion no adjustments were necessary
[c] Compartment Bag Classification was as follows: (<5, 5-20, 21-50, 51-200, 201+ organisms)
[d] WHO Categorization was as follows: (<1, 1-10, 11-100, 101-1000, 1001+ organisms)

Table 6.5 shows the Odds Ratios of the relationship between diarrheal disease and the bacterial measures in all treated drinking water samples as a continuous measure, when categorized according to the WHO scale, and when categorized by the

compartment bag scale. Note that there were no significant relationships between diarrheal disease and any of the bacterial measures.

Table 6.6 Diarrheal disease Odds Ratio estimates of H_2S producing bacteria in *untreated* drinking water by compartment bag category. [a,b,c,d]

H_2S-producing bacteria/ 100mL in untreated household drinking water	OR	P-value	95%LL	95%UL
<5	1(referent)			
5-20	1.14	0.658	0.64	2.01
21-50	**2.29**	0.001	1.40	3.75
50-200	1.41	0.242	0.79	2.51
200+	**2.30**	0.009	1.23	4.32

[a] Bolded Odds Ratios indicate a significant relationship at p=0.05
[b] Samples were stored household drinking water. Households were asked to provide a sample of the water that the family was drinking at the time of visit.
[c] Odds Ratios of those reporting diarrhea within the previous 7 days. Diarrhea was defined as 3 or more loose or watery stools within 24 hours.
[d] Computed by Poisson extension of generalized estimating Equations (GEE), adjusted for clustering within households and in individuals over time. No other confounding variables were identified based on a 10% a priori change-in-estimate criterion, including presence of a water quality intervention (ceramic filter or boiling).

Table 6.6 gives a more detailed analysis of the relationship between the compartment bag categorization of H_2S-producing bacteria in untreated water and reported diarrheal disease. Though there are some strata that did not have significant Odds Ratios (5-20 organisms per 100mL & 50-200 organisms per 100mL), there is an overall increasing trend of increasing Odds Ratios as bacterial levels rose.

Table 6.7 Diarrheal disease Odds Ratio estimates of *E. coli* in *untreated* drinking water by WHO category[23]. [a,b,c,d]

E. coli/ 100mL in untreated household drinking water	OR	P-value	95%LL	95%UL
<1	1 (referent)			
1-9	.91	.720	0.54	1.52
10-99	1.35	.177	0.87	2.09
100-999	**1.89**	0.005	1.22	2.94
1000+	**2.83**	0.001	1.50	5.35

[a] Bolded Odds Ratios indicate a significant relationship at p=0.05
[b] Samples were stored household drinking water. Households were asked to provide a sample of the water that the family was drinking at the time of visit.

[c] Odds of those reporting diarrhea within the previous 7 days. Diarrhea was defined as 3 or more loose or watery stools within 24 hours.
[d] Computed by Poisson extension of generalized estimating Equations (GEE), adjusted for clustering within households and in individuals over time. No other confounding variables were identified based on a 10% a priori change-in-estimate criterion, including presence of a water quality intervention (ceramic filter or boiling).

Table 6.7 shows that the same relationship between reported diarrheal disease and increasing concentrations of *E. coli* in untreated drinking water occurs, although the relationship is significant only at extremely high levels (101-1000, 1001+ organisms per 100 ml).

Table 6.8 Diarrheal disease Odds Ratio estimates of H2S producing bacteria in *treated* drinking water by compartment bag category. [a,b,c,d]

H_2S-producing bacteria / 100mL in treated household drinking water	OR	P-value	95%LL	95%UL
<5	1(referent)			
5-20	**.0006**	< 0.0001	.00049	.00068
21-50	**.0004**	< 0.0001	.00026	.0005
50-200	**.0002**	< 0.0001	.00016	.00037

[a] Bolded Odds Ratios indicate a significant relationship at p=0.05
[b] Samples were stored household drinking water. Households were asked to provide a sample of the water that the family was drinking at the time of visit.
[c] Odds of those reporting diarrhea within the previous 7 days. Diarrhea was defined as 3 or more loose or watery stools within 24 hours.
[d] Computed by Poisson extension of generalized estimating Equations (GEE), adjusted for clustering within households and in individuals over time. No other confounding variables were identified based on a 10% a priori change-in-estimate criterion, including presence of a water quality intervention (ceramic filter or boiling).

Table 6.8 shows that there is a statistically significant but negligible relationship between H_2S-producing bacteria and diarrheal disease in treated drinking water.

Table 6.9 Diarrheal disease Odds Ratio estimates of *E. coli* in *treated* drinking water by WHO category[23].

E. coli / 100mL in treated household drinking water	OR	P-value	95%LL	95%UL
<1	1(referent)			
1-9	1.18	0.541	0.70	2.00
10-99	0.63	0.213	0.30	1.31
100-999	0.81	0.632	0.33	1.95
1000+	0.71	0.428	0.30	1.65

[a] Bolded Odds Ratios indicate a significant relationship at p=0.05
[b] Samples were stored household drinking water. Households were asked to provide a sample of the water that the family was drinking at the time of visit.

[c] Odds of those reporting diarrhea within the previous 7 days. Diarrhea was defined as 3 or more loose or watery stools within 24 hours.
[d] Computed by Poisson extension of generalized estimating Equations (GEE), adjusted for clustering within households and in individuals over time. No other confounding variables were identified based on a 10% a priori change-in-estimate criterion, including presence of a water quality intervention (ceramic filter or boiling).

McNemar's test showed that there was not a significant relationship between any of the fecal indicator measures (*E. coli*, total coliforms, and H_2S- producing bacteria) and diarrheal disease in treated drinking water samples (see Table 6.9). Type II errors were low for both tests, with the probability that both the H_2S test and *E. coli* were both in the high risk WHO category given that the individual did not have diarrhea (weighted by the number of individuals in a HH) at 11.5%. The probability that the *E. coli* test was in the high risk WHO category given that the individual did not have diarrhea (weighted by the number of individuals in a HH) was 18.6%. However, the probability that the H_2S test was in the high risk WHO category given that the individual did not have diarrhea (weighted by the number of individuals in a HH) was 49.3%. No confounding variables were identified based on the a priori 10% change-in-effect criterion for adjustment, including presence of a water quality intervention (piped water or boiling).

6.4 Discussion

There are clear limitations to the quality of the data available for analysis in this study. Most importantly, even after an exhaustive survey of households, there was no way to say with certainty which of the water samples collected (treated, untreated, stored, etc) was actually being used by the household, or if the grab samples are representative of the average daily levels microbiological contamination. If the households were drinking mostly the untreated water before boiling, we would have expected much higher rates of illness. If they were drinking only treated water after boiling we would have expected little to no illness. Given that we observed both occurrences, it was necessary to analyze

both situations. In addition, household members may be drink from water sources outside of the home that were not available for analysis. Moreover, given that we only sampled households monthly, there is a question of how representative the water samples were of the actual water quality of the household. However, the water samples may be representative given that our interviewers came to the household unannounced and collected water samples, preventing the household from skewing the water quality as more or less contaminated. In most households sampled, there was often very little difference between the bacterial levels in the different samples.

There are also problems with self-reported disease data (Fewtrell and Colford, 2005). In the current survey, because respondents are primarily reporting on their own illness and that of their household it is quite possible that the validity of our data is compromised by response bias. Moreover, if this is true then the results of any statistical tests conducted on these data may be biased because these results are wholly dependent on how well the variables have been measured. However, repeated household visits and asking multiple questions related to diarrheal disease in different forms hopefully allowed the participants to give a more accurate recall of household illness within the week prior to data collection.

Though the H_2S test has been around for almost 30 years, there has never been a detailed analysis to determine if the fecal indicator test has any relationship with human diseases attributable to drinking water. Though this has not prevented some organizations from prescribing the H_2S test as a viable fecal indicator, others, namely WHO, CDC, and EPA refuse to approve the H_2S test as a viable fecal indicator test. Instead, all previous analyses have compared the test to other fecal indicators. In this study, 69% of samples

were either both positive or both negative for E. coli and H_2S-producing bacteria. It was necessary to examine both untreated and treated water samples separately because our interviewers noticed that people were drinking both the treated drinking water, and the untreated water directly from the source. In addition, though Type II errors for both tests (at levels considered high risk by WHO) compared to diarrheal disease were low, each test individually had much higher type II error. This may justify using a low-cost version of both tests to monitor fecal indicator levels.

It was necessary to examine both untreated and treated water samples separately because our interviewers noticed that people were drinking both the treated drinking water, and the untreated water directly from the source. Table 6.4 shows that though there are significant relationships between TC, *E. coli*, and H_2S-producing bacteria in both untreated and treated drinking water samples, those relationships are not very strong. This could be due to a variety of reasons. Previous work by Castillo et al. (1994) and Ratto et al. (1989) have shown that there are a number of fecal organisms that are often found in positive H_2S tests. However, Table 6.2 and other unpublished work has shown that *E. coli* levels are sometimes still higher than H_2S levels in both temperate and tropical water samples. This is not surprising, considering that Fujioka & Unutoa (2006) & Winfield and Groisman (2003) found that *E. coli* sometimes proliferates under certain conditions in tropical waters.

Consistent with Moe et al (1991) and Brown et al (2008), Table 6.6 and Table 6.7 show that regardless of the categorization scheme used for the analysis there is a weak non-monotonic, relationship between both *E. coli* and H_2S-producing bacteria and diarrheal disease in untreated water samples. This is an important fact because no

previous work has examined a relationship between H_2S and diarrheal disease, and this work shows that the relationship is equivalent between both bacterial measures and diarrheal disease. Moe et al. (1991) found no relationship between diarrheal illness rates and good quality (1 *E.coli*/100 mL) versus moderately contaminated drinking water (2 – 100 *E. coli*/100 mL) in a field study from the Philippines. It was only when *E. coli* levels in water were 100 cfu/100 ml that increasing concentrations were associated with increasing risks of diarrheal disease. Similarly, we conclude that overall there is not a strong linear dose-relationship between diarrheal disease and either *E. coli* or H_2S-producing bacteria, but that there is an association at extremely high levels of both groups of organisms. When using the compartment bag categorization, we found an interesting drop in the OR at levels between 50-200 H_2S organisms/100mL. Multiple re-analyses could not explain that drop, but no previous work has found a linear relationship between fecal indicator bacteria and diarrhea. This anomaly could also be due in part to the low levels of diarrhea found in the study. This might also be partially attributed to multiple other causes for diarrheal disease, including: viral illness, parasitic infection, foodborne illness, and other non-waterborne or water-related sources (UNICEF/WHO 2009). Future studies with a higher diarrheal disease rates may have more success in analyzing this relationship.

Table 6.5 shows that with all of the categorization schemes used for the analysis there is never a significant association between both *E. coli* and H_2S-producing bacteria and diarrheal disease in treated water samples. This holds true for *E. coli* even when the fecal indicator was categorized (see Table 6.9). This most likely due to the fact that because there are so few bacteria in most of the treated water samples (as shown in Table

6.2), there are just not enough to determine any relationship. McNemar's test, which determines whether or not there is a relationship between diarrheal disease and the fecal indicator measure of interest, showed that there was a significant relationship between each of the fecal indicator measures (*E. coli*, total coliforms, and H_2S- producing bacteria) and diarrheal disease in untreated drinking water samples. The same relationship was not found for *E. coli* and total coliforms in treated drinking water samples. However, Table 6.8 shows that there was a significant relationship between H_2S producing bacteria and diarrheal disease in treated drinking water.

The data we present suggests that a quantifiable, low-cost version of the H_2S test that costs less than 0.45USD has a similar relationship to diarrheal disease as standard *E. coli* methods (usually costing more than 4USD/test) have in water quality testing of drinking water samples in a developing country. Given its much lower cost, we may now be able to get a more expansive view of fecal bacteria at the household level in many more communities globally by doing 10 tests instead of 1.

Since there are many organisms that can produce a positive result to an H_2S test and *E. coli* levels can fluctuate in tropical waters, it is not surprising that the tests do not correlate well with each other in numbers of organisms. However, in comparison with diarrheal disease, both tests perform similarly. One advantage of this study is that the tested drinking water samples come from a multitude of sources and disparate areas, making it slightly more feasible to assume that the H_2S test can work with a variety of drinking water types. Further comparisons of both tests to each other and diarrheal disease need to be performed in other locations to determine if this select set of

household samples in Central Vietnam is an isolated occurrence, or if these tests will continue to produce similar relationships with diarrheal disease.

Chapter 7: Discussion of the Feasibility of a Simple, Low-cost H_2S Test for Fecal Contamination of Water in Developing Countries and Emergency Situations

7.1 Summary of Significant Results

The field and lab research provided in the previous chapters makes a significant contribution to the body of evidence on the H_2S tests and its relationship with waterborne human health risks. The most important findings from the research can be grouped into three categories: laboratory evidence for the effectiveness of the H_2S test, field performance of the H_2S test, and the relationship of the H_2S test results to human health risks.

Laboratory evidence

1) This was the first study to examine multiple testing formats and media formulations in a laboratory setting. Overall, there was no significant difference in levels of H_2S producing bacteria for the commercially available HACH media vs. the lab-made autoclaved and thrice-boiled H_2S broths ($p= 0.49$). This was true in analysis of both a protected natural surface water source ((University Lake), natural surface water spiked with sewage, and undiluted sewage. This is a key finding because it shows that in low-resource settings where autoclaves and ready access to commercial products are not available, a basic low-cost H_2S medium made from scratch can perform as well as a more expensive commercial H_2S medium. In addition, the numbers of H_2S

organisms in a sample as measured by HACH and autoclaved lab-made broth did not differ significantly at incubation temperatures at 24 or 48 hours. However, there was a significant difference in the bacterial levels between the 24 and 48-hour results for the thrice-boiled medium.

2) Overall, whether in plastic or glass tubes, plastic or glass bottles, or in non-compartmentalized or compartmentalized clear polyethylene bags, there was no significant difference between the levels of H_2S producing bacteria detected in the samples analyzed. In addition, there was no significant difference between the MPN estimates for H_2S producing bacteria given by the CBT and Quanti-tray overall or at the separate test temperatures of 27°C, 37°C, and 44°C. This is a key finding because it shows that when properly performed, the simple, low-cost, 5-chambered CBT can be used in laboratory settings and provide quantitative results similar to more involved, expensive and time-consuming standard tests.
3) Given that current water microbiology detection and decision-making frameworks of WHO, US EPA and CDC use *E. coli* as the target fecal indicator organism, it is noteworthy that the comparison of presence or absence of H_2S producing bacteria and *E. coli* in primary effluent spiked natural water gave similar results for both fecal indicators ($p=0.0000$).

Field Performance of H_2S CBT Test in determining microbial quality of an improved drinking water supply

4) This was the first study to use both molecular and traditional culture and biochemical analysis methods to examine the bacterial composition of H_2S-positive and H_2S negative natural water samples. These sources were of varied

environments and were chosen to represent typically used drinking water sources as determined by WHO. The Kappa value of 0.664 suggests strong agreement in the bacteria genera identified by the two analysis methods. Overall, these experiments have shown that when a water sample tests positive for H_2S producing bacteria there are fecal bacteria in the water sample, and when they test negative, there are no bacteria of human health concern detected either by biochemical or TRFLP methods, **providing further evidence that the H_2S test should be considered among the candidate fecal indicator bacteria.**

5) The H_2S CBT was performed in over 1600 household drinking water samples in Vietnam. Although performed by Master's level trained microbiologists, this was the first work done using a simple, low-cost quantitative MPN form of the H_2S test in a setting with minimal laboratory facilities and equipment.
6) Molecular results from a select sub-sample of household drinking water samples taken during the Vietnam study revealed that when a water sample tests positive for H_2S bacteria, there are fecal bacteria in the water sample and there was at least 1 pathogen detected in a small subset of samples that were subjected to molecular analysis.

Relationship between the H_2S CBT Results and Human Health Risks

7) This was the first work ever done to examine the relationships among a quantitative test of H_2S-producing bacteria, diarrheal disease and other fecal indicators. In the longitudinal Vietnamese household drinking water study, the generalized estimating equation of binary regression showed that there were

significant relationships between diarrheal disease risks and levels of both *E. coli* and H_2S-producing bacteria, but not for levels of TC bacteria.

8) There was a significant relationship between the levels of H_2S bacteria, TC, and *E. coli* in untreated and treated drinking water samples as a continuous measure, when categorized according to the WHO microbial water quality decimal scale, and when categorized by the compartment bag water quality scale in the longitudinal Vietnamese household drinking water study.

7.2 Study Results and Relationship to Existing Evidence about the Performance H_2S Test

Comparison of Laboratory Results

Though recent quantitative work by Tambekar et. al (2010) and Gupta et. al (2008) have been published, there are only a few studies that used an MPN format of the H_2S test. This present research has shown that there is no significant difference between the levels of H_2S producing bacteria detected by MPN assay using 3 different medium formulations differing in either broth recipe or method of sterilization, at 24 and 48 hours of incubation, and different sample containers. In addition, lab testing has shown that there is no significant difference between the levels of H_2S producing bacteria found in water and sewage using standard sampling containers (EPA approved IDEXX Quanti-tray System) and those determined by the compartment bag at standard incubation temperatures (27°C, 37°C, and 44°C).

A number of investigators have attempted to speciate the bacteria that produce positive results in the H_2S test. However, this was the first study to use both molecular and traditional culture and biochemical analysis methods to examine the bacterial

composition of H_2S-positive sewage samples. Although it was expected that gut pathogens and indicator organisms would be found, this work also determined that H_2S positive samples did indeed have fecal indicators and a range of pathogens, and would also contain organisms of concern as a benchmark for subsequent studies in natural waters.

A diverse group of microorganisms was isolated and cultured from the H_2S-positive sample volumes cultured from sewage samples, with 24 different species isolated. Like Castillo et al. (1994) and Ratto et al. (1989), both the molecular and cultured isolations steps in this work found a large variety of bacteria in samples giving positive reactions in the H_2S test, primarily *Clostridium perfringens* and members of the *Enterobacteriaceae* (including *Enterobacter,* Clostridia*, Klebsiella*, *Escherichia, Salmonella, Morganella*), Citrobacter, and other organisms known to cause illness in humans *(Acinetobacter, Aeromonas)*. The numbers of H_2S organisms in a sample as measured by HACH and autoclaved scratch broth did not differ significantly at 37°C at 24 or 48 hours.

Similar to the work of Rijal et. al (2000) there are more non-H_2S producing pathogens/fecal indicator organisms identified in H_2S-positive samples than in any other group. However, each sample had multiple H_2S-producing organisms that were also pathogens. As was described earlier, after the isolates were biochemically identified by the Enterotube system, they were re-analyzed for their ability to produce hydrogen sulfide. Overall, 96 of the 216 isolates (44.4%) from all sewage experiments were positive for H_2S production when further tested for this ability. The Kappa value of 0.737 suggests strong agreement in the genera identified by the two independent identification

methods, TRFLP and culture-biochemical analysis. Indeed, 33 of 40 genera (82.5%) that were isolated and identified from all sewage experiments through the culture-based biochemical techniques were also identified by TRFLP, with *Hafnia* and *Yersinia* being the only exceptions. As shown in Appendix 2, a total of 162 genera were identified in the TRFLP molecular analysis, with 145 not isolated through cultivation and biochemical identification processes. Though many of the genera not isolated by cultivation were not pathogens, approximately 15% of the non-cultured genera were potentially pathogenic. In addition, another 5% of the non-cultured genera have been previously associated with fecal contamination. This evidence seems to suggest that the TRFLP molecular method can identify more genera of bacteria than cultivation and biochemical identification, which is not surprising considering that many other researchers that have compared culturing bacteria to molecular methods have identified many more organisms through molecular techniques (Blackwood et al. 2007, Broderick et al. 2004).

Comparison of Field Performance Results

In the work that followed, molecular and the combination of cultivation and biochemical isolation were applied to H_2S positive and H_2S negative samples in natural waters that could be used as possible sources for drinking water as described by the World Health Organization's Guidelines for Drinking Water Quality including: a protected deep well in an agricultural setting, an forested unprotected artesian well, a protected reservoir, and a rainwater catchment system. Like Ratto et al. (1989) and Pillai et al. (1999), we evaluated the H_2S test at different temperatures (27°C, 35°C, and 44°C) using the most probable number (MPN) method, and found that H_2S producing organisms were consistently associated with the presence of coliforms in water.

This work agrees with previous work done by others (Kromoredjo and Fujioka, 1991, Kaspar et al., 1992; Venkobachar et al., 1994; Rijal and Fujioka, 2001; Genthe and Franck, 1999; Pant et al., 2002; Anwar et al., 2004; Oates et al., 2003) that cultured bacteria out of positive H_2S tests and found fecal indicators and pathogens. However, this was the first work to use molecular methods in addition to traditional cultivation and biochemical identification techniques and found that compartment bags with positive H_2S tests often contain *E. coli*, other fecal coliforms, and many organisms of concern, including: *Aeromonas hydrophila, Escherichia coli, Enterobacter cloacae, Hafnia alvei, Morganella morganii, Plesiomonas shigelloides, Providencia alcalifaciens, Providencia rettegeri, Pseudomonas aeruginosa, Pseudomonas cepecia, Serratia marcescens, and Shigella* sp. Unlike previous researchers (Kaspar et al., 1992; Pant et al., 2002) that applied the H_2S test to groundwater samples and demonstrated false positive results, there is clear evidence that this version of the H_2S test is not adversely impacted by high sulfur and high iron water sources as samples from varied NC sources and VN drinking water samples, with little to no culturable growth and no positive H_2S tests in those samples. Moreover, unlike the Tewari et al. (2003) study, there were few false negative results, with 92% of NC water samples and 76% of 1600 Vietnamese household drinking water samples having both *E. coli* and H_2S-producing bacteria. The results of the H_2S test in comparison with the *E. coli* results provides even more evidence that the H_2S test should be treated as a fecal indicator.

Cost should also be a factor in determining which microbial test to choose, and it is clear that the H_2S version of the CBT (0.45USD) is much less expensive than either the *E. coli* version of the CBT (1.75USD) or other E. coli testing (average 4USD). This is

mostly due to the expense of the chromogenic and flourogenic components of the *E. coli* media, which is much more expensive than the ingredients for the H_2S media. This is a crucial difference between the two tests because we can now do almost 10 H_2S tests for every on standard *E. coli* test on the market now. Moreover, as was shown in the Paskistan Post-Flooding situation, there are many instances where local organizations in South and Southeast Asia are using the qualitative forms of the H_2S test without WHO approval, and this version of the test gives quantitative information and is probably of similar cost.

Though EPA and WHO are slow to recognize the H_2S test and view *E. coli* as the gold standard, it is important to remember that just 25 years ago both organizations viewed TC as the gold standard fecal indicator, and there was slow acceptance of *E. coli* and *enterococci*. Though there seems to be resistance in these organizations to new evidence that challenges their "gold standards", they nevertheless will update and modify their recommendations to meet the current needs. As more evidence on a standardized, quantitative, simple, low-cost version of the H_2S test emerges, there will probably be a reduction in the resistance to its use. Now, many organizations (especially those in disaster relief) are looking for more cost-effective ways to get a basic measurement of water quality, and *E. coli* is currently cost prohibitive for their needs. It would be better to use the H_2S test in these situations, even if there are some instances with high Type II errors, to get a baseline measure of household water quality in areas where E. coli testing is too expensive.

7.3 Health Impact Study and other Evaluations of H_2S Test vs. Human Health

This was the first work ever reported that looked at the relationship between diarrheal disease and quantitative H_2S test results in drinking water samples in a low-resource setting in a developing country, as an example of a setting where the test is most needed. Numerous studies examining the relationship between measured microbial indicators in drinking water and health outcomes reveal only a limited and quantitatively inconsistent association (e.g., Moe et al. 1991, Brown et. al. 2008) or no association (Gundry et al. 2004; Jensen et al. 2004). To date, published results have only examined the relationship between H_2S-producing bacteria and other fecal indicators. McNemar's test, which determines whether or not there is a relationship between diarrheal disease and the fecal indicator measure of interest, showed that there was a significant relationship between *E. coli*, H_2S- producing bacteria, and diarrheal disease in untreated drinking water samples from this study done in Vietnam. In addition, this was the first work to show that regardless of the categorization scheme used for the quantitative results of the H_2S bacteria analysis, there is a weak positive but non-monotonic relationship between both *E. coli* and H_2S-producing bacteria levels in untreated drinking water source samples and household diarrheal disease in a central VN community.

7.4 Research Limitations

This research has begun to fill significant gaps in the knowledge about the H_2S test for microbial water quality detection and its relationship to waterborne disease risk; both under controlled laboratory conditions and in the field. However, there are some

limitations to the research. The laboratory studies were done with a beta version of the CBT and it is not yet clear if the final were will perform in the same way. More research on the commercialized version of the CBT using the H_2S test in the laboratory could help to determine if they perform the same and help us better understand and interpret the results from field studies of the CBT. It is also possible that the laboratory results are not representative of conditions in the field with untrained personnel using the CBT. There are also limitations to the molecular analysis.

It is not surprising that the TRFLP and biochemical results do not match exactly, given the inherent bias and limitations of culture methods. Specifically, the culture-based isolation techniques used in this study focused on culturing and identifying members of the *Enterobacteriacae* family, to the exclusion of most others, and it is well documented that culture methods generally underestimate both the diversity and concentrations of bacteria in environmental samples for a variety of reasons (Kim et al. 2004). Molecular methods also have implicit biases due to the sampling method, nucleic acid extraction methods, the PCR reaction conditions, and human error. In addition, given that there are many organisms that can produce a positive result to an H_2S test, and that *E. coli* levels can fluctuate in tropical waters, it is not surprising that the tests do not correlate well with each other in numbers of organisms. There were also some issues with the household drinking water study.

The field research on the relationship between the H_2S CBT and health impacts suffer from lack of generalizability. The participants who were part of the study represent a specific set of conditions that are unique to the Central Vietnam and do not encompass the range of conditions under which people collect, treat, and use household water. For

example, there may have been an underestimation of health impact because there were so few cases of diarrheal disease. However, this limitation is not restricted to this study alone but is a limitation of all studies that take place in only one location at one point in time.

There are also clear limitations to the quality of the data available for analysis in this study. Most importantly, even after an exhaustive survey of households, there was no way to say with certainty which of the water samples collected (treated, untreated, stored, etc) was actually being used by the household, or if the grab samples are representative of the average daily levels microbiological contamination. If the households were drinking mostly the untreated water before boiling, we would have expected much higher rates of illness. If they were drinking only treated water after boiling we would have expected little to no illness. Given that we observed both occurrences, it was necessary to analyze both situations. In addition, household members may be drink from water sources outside of the home that were not available for analysis. Moreover, given that we only sampled households monthly, there is a question of how representative the water samples were of the actual water quality of the household. Most importantly, the diarrheal disease we observed may be due to a variety of factors that we didn't measure (viruses, parasites, foodborne illness). There are also problems with self-reported disease data (Fewtrell and Colford, 2005). In the current survey, because respondents are primarily reporting on their own illness and that of their household it is quite possible that the validity of our data is compromised by response bias. Moreover, if this is true then the results of any statistical tests conducted on these data may be biased because these results are wholly dependent on how well the variables have been measured.

7.5 Conclusions

- **First study to examine multiple testing formats and media formulations in a laboratory setting and to use both molecular and traditional culturing methods to examine the bacterial composition of H_2S-positive and H_2S negative natural water samples.**
- **Overall, there was no significant difference in levels of H_2S producing bacteria for the commercially available HACH media vs. the lab-made Autoclaved and thrice-boiled H_2S broths ($p = 0.49$). This was true in both protected source University Lake Natural water, natural water spiked with sewage, and undiluted sewage.**
- **The numbers of H_2S organisms in a sample as measured by HACH and Autoclaved lab-made broth did not differ significantly at incubation temperatures at 24 or 48 hours.**
- **In low-resource settings where autoclaves aren't available, a basic low-cost media can perform as well as a more expensive commercial media. This is a key finding, since logistical concerns are a key component of the ideal fecal indicator framework.**
- **In plastic or glass tubes, plastic or glass bottles, or in non-compartmentalized or compartmentalized bags, there was no significant difference between the levels of H_2S producing bacteria analyzed.**
- **There was no significant difference between the MPN estimates given by the CBT and Quanti-tray overall or at 27°C, 37°C, and 44°C separately for H_2S Producing bacteria.**

- **When properly performed, the simple low-cost 5-chambered CBT can be used in laboratory settings and gets results similar to more expensive and time-consuming standard tests.**
- **Water samples from natural water sources testing positive for H_2S had fecal bacteria, and those testing negative had few fecal organisms. This provides further evidence that the H_2S test should be considered a candidate fecal indicator.**
- **Comparison of presence or absence of H_2S producing bacteria and *E. coli* in primary effluent spiked natural water gave similar results for both fecal indicators ($p=0.0000$).**
- **The H_2S CBT was performed in over 1600 household drinking water samples in Vietnam. Though performed by Master's level trained microbiologists, this was the first work done on a simple, low-cost quantitative MPN form of the H_2S test.**
- **There were significant relationships between diarrheal disease and both *E. coli* and H_2S-producing bacteria in VN study, but not for TC. This is important because it fulfills on of the key criteria for an ideal fecal indicator.**
- **H_2S version of compartment bag has been lab tested, tested in VN, and possibly will be tested in Peru. Comparisons of the bag to other standard assays have shown that it is effective in undiluted waters up to 400MPN/100ml.**

7.6 Further Research on the H_2S Test

Though much has been learned, the results from this research have lead to many other research questions that should be used to assess the effectiveness and performance of the H_2S CBT test.

1) Laboratory research should continue to investigate the effects on performance of a standardized MPN version of the H_2S test that are like those in the field for frequency of use, water physical and chemical parameters, and variations or changes in water quality and temperature. This will provide better information on how such variable conditions influence H_2S test performance in identifying microbes in water.
2) Field evidence is needed to determine if the H_2S CBT test performs as well in other environmental settings compared to standard fecal indicator testing as it did in the laboratory and in Vietnam. In addition, future testing should use molecular methods with source tracking to determine if the organisms detected are actually pathogenic and not just “potentially” pathogenic.
3) Additional field studies can and should be performed to more clearly identify and better understand the relationship between water quality and diarrheal disease. This should involve re-classifying water quality exposure levels by more specifically assigning water quality to each participant. Furthermore, an attempt should also be made to conduct further health impact analysis of the data in the form of case-control analyses.
4) Additional research on health impact of the H_2S CBT should be performed in different locations to determine and quantify the generalizability H_2S test results

to household diarrheal disease.

5) Additional field research needs to be conducted in low-resource communities to determine if the commercialized form of the H_2S test is user-friendly and easy to understand for household users that want to test their microbial water quality. The pre-packaged version of the test that would be distributed in low-resource settings would include all materials necessary to safely perform and dispose of the test. These components include: the compartmentalized bag, a water collection cup, latex gloves, the dry medium already in the bag, and a chlorine tablet for disinfection of the liquid in the bag prior to disposal. Thus, the three-step procedure would involve: (1) Collecting the proper sample volume in the provided 100 mL collection cup; (2) Pouring the sample into the bag, mixing it with the medium, and distributing it into the compartments; and (3) Folding down the flaps to seal the bag. This is a simple method that could easily be followed using pictorial instructions, which is ideal for low-resource settings where illiteracy may be prevalent. The instructions could be provided in a panel imprinted directly on the exterior of the bag. Training and post-implementation assessments should be done to determine the user friendliness of the H_2S CBT.

Appendix 1. Microorganisms capable of producing hydrogen sulfide

Name	Common Source or Habitat	Pathogens	Capable of Giving Positive H_2S Test[1]
Phototrophic Bacteria G-			
Rhodospirillum	Water- strict anaerobic	None reported	Unlikely- slow growth
Myxobacteria G-			
Flexibacteria	Water, soil	None reported	Possible
Simonsiella	Mammal oral saprophytes	None reported	Possible
Alysiella	Mammal oral saprophytes	None reported	Possible
Budding or Appendaged- G-			
Hypomicrobium	Soil, water	None reported	Unlikely- slow growth
Spirochaetes and Spirals- G-			
Treponema	Animals	Several	Unlikely- fastidious
Spirillum	Fresh and salt water	None reported	Possible
Campylobacter	Man and animals	Several	Possible
G – Rod and Cocci			
Xanthomonas	Soil, water	Plant pathogens	Possible
Agrobacterium	Soil, plant tissues	Plant saprophytes and pathogens	Possible
Halobacterium- Archebacteria	Water	None reported	Not possible - require 12% NaCl
Halococcus- Archebacteria	Water	None reported	Not possible – require 12% NaCl
Brucella	Animal bodies	Many human and animal	Unlikely- require CO_2 to grow
Francisella	Water	Human and animal parasites	Unlikely- fastidious
G- Facultative Rods- Enterics			
Budvicia	Fresh water;	None	Likely +

[1] Fastidious means microbes require blood, serum, sterols, etc; slow growth = not able to produce response in the incubation period of the test; no substrates means the needed nutrients are not in the test medium

	shrew intestines		
Edwardsiella	Water, human & animal intestines	Possible	Likely +
Citrobacter	Water, food, animal feces, urine	None reported	Likely +
Salmonella	Water, food, animals	Many pathogens	Likely +
Proteus	Soil, water, animal feces	Possible, none reported	Likely +
Yersinia	Human and animal intestines	Yes	Likely +
Klebsiella	Widely distributed	Unlikely	Likely +
Erwinia	Plant	Plant pathogens	Unlikely- rarely in water
Aeromonas	Animals	Possible	Likely +
Zymomonas	Beer, fermenting fruit	None reported	Possible
Flavobacterium	Water	Rodent pathogen	Possible- H_2S rare
Pasteurella	Animals	Many animal pathogens	Unlikely- fastidious
Actinobacillus	Animal membranes and tissues	Animal pathogens	Likely +
Cardiobacterium	Human nose and throat	Possible	Unlikely- fastidious
Streptobacillus	Mammal intestines	Animal parasites and pathogens	Unlikely- fastidious
G- Anaerobic			
Bacteroides	Intestinal, oral cavity animals	Rumen bacteria- unlikely	Unlikely- fastidious
Fusobacterium	Animal mucus membranes	Several	Likely + but rare in water
Desulfovibrio	Soil, water, sediment	None reported	Unlikely- no substrates
Desulfomonas	Human intestinal tract	None reported	Unlikely - fastidious
Desulfobacter	Soil, water, sediment		Possible
Desulfococcus	Soil, water, sediment	None reported	Possible
Desulfuromonas	Soil, water, sediment		Possible
Desulfosarcina	Soil, water, sediment		Possible

Butyrivibrio	Rumen	None reported	Unlikely- fastidious
Selenomonas	Animal intestines	None reported	Possible
G- Cocci			
Neisseria	Animals- many tissues	Many	Unlikely- fastidious
Veillonella	Mouth, intestinal track of animals	Possible	Unlikely- slow growth
Megasphaera	Rumen, sheep intestine	None reported	Unlikely- slow growth
G+ Cocci			
Staphylococcus	Human skin, membranes, air, dust	Many	Likely +
Peptococcus	Animals- mostly humans	Possible	Likely +
Peptostreptococcus	Animal respiratory and UG tract	Several	Possible
G+ Endospore Formers			
Clostridium	Soil, water, sediment	Some	Possible
Desulfotomaculum	Soil, water	None reported	Unlikely- no substrates
G+ Non-Spore Formers			
Erysipelothrix	Soil, water, fish, animals	Some	Likely +
Actinomycetes- G+			
Eubacterium	Cavities of man and animals	Some	Possible
Actinomycetes	Soil, water	Both man and animals	Unlikely- slow growth
Arachnia	Soil, humans, animals	Some	Likely +
Rothia	Soil, animals	None reported	Unlikely- slow growth
Actinoplanes	Plants, soil, animal skin	None reported	Unlikely- slow growth
Planobispora	Soil	None reported	Possible
Dactylosporangium	Soil	None reported	Possible
Streptoverticillium	Soil- antibiotic producers	None reported	Unlikely- slow growth
	Soil, animal	None reported	Unlikely- Thermophilic

Thermomonospora	wastes		
Mycoplasmas G-			
Mycoplasma	Parasites of man	Several	Unlikely- fastidious

Appendix 2. List of Genera Identified in Sewage by Experiment

Organism	Sewage Experiment 1	Sewage Experiment 2	Sewage Experiment 3
Achromobacter[d]	--	+	+
Acidobacter[d]	+	+	+
Acidimicrobium[d]	+	+	--
Acidithiobacillus[b]	--	+	+
Acidovorax[d]	--	+	+
***Acinetobacter*[d]**	+	+	+
***Aeromonas*[a]**	--	+	--
Actinobacillus[b]	--	--	+
Afipia[d]	--	+	--
Agromyces[d]	--	+	--
Akkermansia[d]	--	--	+
Alcaligenes[d]	--	+	--
Alishewanella[d]	--	+	+
Alkanindiges[d]	--	+	+
Alteromonas[d]	--	+	+
Aminobacterium[d]	--	+	--
Anaerolinea[d]	--	--	+
Anaeromyxobacter[d]	+	--	+
Anaerovorax[d]	--	+	--
Anaplasma[d]	+	--	--
Aquicella[d]	--	+	+
Aquimonas[d]	--	+	--
Arcobacter[d]	--	+	--
Aricella[d]	--	+	--
Arthrobacter[d]	--	+	--
Azoarcus[d]	--	+	+
Azonexus[d]	--	+	--
Azospira[d]	--	+	--
Azotobacter[d]	--	--	+
Bacillariophyta[d]	--	--	+
Bacillus[a]	--	+	--
***Bacteroides*[a]**	--	+	--
Bangia[d]	--	--	+

Beggiatoa[d]	--	+	--
Bifidobacterium[c]	--	+	--
Bilophila[d]	+	--	--
Bordetella[c]	--	+	+
Bosea[d]	--	--	+
Byssovorax[d]	--	--	+
Butyrivibrio[b]	--	+	--
Caldithrix[d]	+	+	+
Caldilinea[d]	--	--	+
Capnocytophaga[d]	--	+	--
Carnobacterium[d]	+	--	+
Castellaniella[d]	--	+	--
Catellatospora[d]	+	+	+
Catenibacterium[d]	--	+	--
Catenulispora[d]	--	+	--
Comomonas[d]	--	+	+
Conexibacter[d]	--	+	+
Cupriavidus[d]	--	--	+
Curvibacter[d]	--	+	--
Cyanobacteria GpIIa[d]	+	--	+
Cyanobacteria GpVI[d]	+	--	+
Cytophaga[d]	--	+	--
Dechloromonas[d]	--	+	--
Dehalococcoides[d]	--	+	--
Delftia[d]	--	--	+
Desulfocapsa[d]	--	--	+
Desulfomicrobium[b]	--	+	--
Desulfovibrio[b]	+	--	--
Dokdonella[d]	+	--	+
Dyella[d]	--	+	--
Dysgonomonas[d]	--	+	--
Enterobacter[c]	+	+	+
Enterococcus[c]	--	+	--
Entomoplasma[d]	--	+	--
Erwinia[a]	+	--	--
Escherichia[c]	--	--	+
Eubacterium[d]	--	+	--
Gram Negative Enterics[a]	+	+	+
Flavimonas[c]	--	+	--

Fusobacterium[b]	--	+	+
Gallionella[d]	--	+	--
Gemmatimonas[d]	--	+	--
Geobacillus[d]	+	--	--
Haemophilus[c]	--	--	+
Halomonas[d]	--	+	+
Helicobacter[c]	--	--	+
Herbaspirillum[d]	--	+	+
Hydrocarboniphaga[d]	--	+	--
Hydrogenophaga[d]	+	--	--
Hymenobacter[d]	--	+	--
Janthinobacterium[d]	--	+	--
Klebsiella[a]	--	+	+
Kurthia[d]	+	--	+
Lachnospira[d]	--	+	+
Lactococcus[d]	--	+	--
Leptolyngbya[d]	+	--	--
Leptothrix[d]	--	+	+
Levilinea[d]	--	--	+
Limnobacter[d]	--	--	+
Lysobacter[d]	--	+	--
Magnetococcus[d]	--	--	+
Marinomonas[d]	+	--	+
Methylobacter[d]	+	+	--
Methylocaldum[d]	--	+	--
Methylomicrobium[d]	+	--	--
Methylomonas[d]	--	+	--
Methylophilus[d]	--	+	+
Microbacterium[d]	--	+	+
Mitsuaria[d]	--	--	+
Mogibacterium[d]	--	+	--
Moraxella[d]	--	+	--
Moritella[d]	--	--	+
Mycobacterium[c]	--	+	+
Mycoplasma[c]	--	+	--
Myxococcus[d]	+	--	--
Nitrosomonas[d]	--	+	+
Nitrospira[d]	--	--	+
Papillibacter[d]	--	+	--
Patulibacter[d]	--	+	--

Pelomonas[d]	--	+	--
Peredibacter[d]	--	--	+
Planctomyces[d]	--	--	+
Polaromonas[d]	+	+	--
Porphyra[d]	+	--	+
Providencia[c]	**+**	**--**	**+**
Pseudoalteromonas[d]	+	+	+
Pseudomonas[c]	+	+	+
Pseudoxanthomonas[d]	--	+	--
Ralstonia[d]	--	--	+
Raoultella[d]	+	--	+
Reinekea[d]	--	--	+
Rheinheimera[d]	--	+	+
Rhodanobacter[d]	--	+	+
Rhodoferax[d]	--	+	+
Rhodothermus[d]	--	+	+
Rikenella[d]	--	--	+
Rubritalea[d]	--	--	+
Ruminococcus[d]	--	+	--
Salinibacter[d]	--	+	+
Salmonella[a]	--	--	+
Schlegelella[d]	--	+	--
Serratia[c]	+	--	+
Shewanella[c]	+	+	+
Sphingomonas[d]	--	--	+
Spiroplasma[d]	--	+	--
Sporomusa[d]	--	--	+
Staphylococcus[a]	--	--	+
Streptococcus[c]	--	--	+
Streptomyces[a]	+	+	+
Sulfurimonas[d]	--	+	+
Sulfurovum[d]	+	+	+
Sutterella[d]	+	+	+
Syntrophomonas[c]	+	--	+
Tepidiphilus[d]	--	+	--
Teredinibacter[d]	--	--	+
Tetrathiobacter[d]	--	+	--
Thauera[d]	--	+	+
Thiobacter[b]	+	+	+
Thiovirga[d]	--	+	--

Tolumonas[d]	--	+	--
Trichococcus[d]	--	+	+
Verrucomicrobium[d]	+	--	--
Vibrio[a]	--	--	+
Xiphinematobacter[d]	--	--	+
Zobellella[d]	--	+	--
Zoogloea[d]	--	--	+

The following categories divide the identified organisms based on whether they are a likely Pathogen/ Fecal Indicator Organism and whether or not they produce Hydrogen Sulfide: a) H_2S Producing Pathogen/Fecal Indicator (Committee on Indicators for Waterborne Pathogens 2004)2004; b)H_2S Producing Non-Pathogen/Non-Fecal Indicator Reis et al. 2002; c) Non-H_2S Producing Pathogen/Fecal Indicator (Widdel 1988); d) Non-H_2S Producing Non-Pathogen/Non-Fecal Indicator

Appendix 3. Broth and PBS Recipes

H2S broth [20x](per 100 mL):
40 g peptone
3 g K2HPO4
1.5 g Ferric Ammonium Citrate
2.0 g Sodium Thiosulfate
.65 g Sodium Dodecly sulfate
.25 g L-cystine.

PBS (per 5L):
40 g NaCl
1 g KCl
.6g KH2PO4
4.55 g Na2HPO4 (anhydrous).

Appendix 4. Biochemical analysis and TRFLP Protocols

Biochemical Analysis

Preservation of *H2S-producing* Isolates from Natural Water Sources

Monday

1. Obtain 160mL of natural water source.
2. Remove 10 mL and freeze for molecular analysis.
3. Perform an MPN experiment using. 50mL of the sample will be used in an MPN dilution series {Dilute 50mL of natural water into 450mL of PBS. Serially dilute NW mixture out to 10^{-5}. Aliquot 4-100mL samples of each dilution into plastic bags. Prepare negative PBS controls. Add 1 HACH PathoScreen reagent packet to the 100mL samples. Aliquot 9mL and 1mL out of each 100mL sample into 16x150mm glass test tubes and 13x100 glass test tubes. Place caps on the test tubes. Incubate at 37C for 24 hours.}
4. A HACH pathoscreen reagent packet will be added to the remaining undiluted 100mL of NW, and the samples will be incubated at 37C for 24 hours.

Tuesday

Colonies selected from H2S MPN method:

1. Run a dilution series out to $1x10^{-5}$ by taking 1mL of the positive H2S test (from the undiluted 100mL of NW) and placing in 9mL of PBS.
2. Remove 100µLs of each dilution and spread plate directly onto a 100 mm dish containing Bio-Rad agar. Repeat this process more times, giving a total of 10 plates for the agar (2 x 5 dilutions= 10 plates). Repeat this process for Salmonella-Shigella agar, Phenylethanol agar, m-Aeromonas Selective agar, and H2S agar. Repeat steps 1 and 2 for both an H2S positive and an H2S negative sample bag. Incubate Salmonella-Shigella agar, m-Aeromonas Selective agar, and Bio-Rad agar plates aerobically at 37 degrees C for 24 hours. Incubate Phenyl ethanol and H2S agar plates anaerobically at 37 degrees C for 24 hours.
3. Pellet 2-10 ml from the negative control sample and then archive this pellet at -80 deg C for future DNA isolation for your molecular community analysis.

Plates will be labeled as follows:
Medium/Dilution /Isolate Number (1 of ?)/Date/Initials
Ex. BR/-3/2/2.11.08/KM

Wednesday

Colony Differentiation:

For each of the agar plates that were aerobically incubated on Tuesday, select a colony using a sterile wooden stick and streak-plate it onto a 100 mm TSA plate. Do This only for countable dilutions with isolated colonies. Repeat this process for each agar at that dilution. Also repeat this process 3 times for each different morphology found at that dilution. Incubate these TSA plates at 37 degrees C for 24 hours.
For each of the agar plates that were anaerobically incubated on Tuesday, select a colony using a sterile wooden stick and streak-plate it onto a 100 mm of its original agar (Ex: Take a colony from a H2S plate and streak it onto another H2S plate). Incubate these plates anaerobically at 37 degrees C for 24 hours.

Thursday
For each of the agar plates that were aerobically incubated on Wednesday on TSA, select an isolated colony using a sterile wooden stick and streak-plate it onto a 100 mm TSA plate. For each of the agar plates that were anaerobically incubated on Wednesday, select a colony using a sterile wooden stick and streak-plate it onto a 100 mm of its original agar (Ex: Take a colony from a H2S plate and streak it onto another H2S plate). Incubate these plates anaerobically at 37 degrees C for 24 hours.
Friday
In the hood, recover an isolated colony from each TSA agar plate and freeze it (scoop an isolated colony from the plate and place it in 1ml of 80/20 TSB/glycerol). The date and initials will be written on the cap, and the remainder will be written on the body of the tube. The tubes will then be placed in approximately 3 boxes that will be clearly labeled, and then placed in the -80 freezer. Doug will be informed of the position of the boxes and each one's exact contents.
Monday
Take Friday's isolated samples that from the freezer and streak them on TSA again.
Tuesday
Recover an isolated colony from each plate incubated the previous evening. Place the colony in 10mL of H_2S media and incubate at 37C for 24 hours.
Determine which colonies produced a positive H2S test and record the results.
Remove another colony for use in Enterotube analysis.
Enterotube analysis of colonies from TSA plates of the 3 randomly selected isolates:
Using the Enterotube device, select a colony from the appropriate TSA plate and place the device into the Enterotube casing. In all, 50 isolates will be characterized via Enterotube. These will be incubated for 24 hours at 37 degrees C.
The results of each Enterotube will be read and recorded for future reference. For any Enterotube that doesn't give a unambiguous result, API-20E will be performed on that isolate.
Tubes will be labeled as follows:
Medium/Dilution/Isolate Number (X of Y)/Replicate (A or B)/ Date/ Initials
Ex: BR/20/1 of 5/A/2.11.08/KM
T-RFLP Characterization
Day 1
1. Following the FDA 2500 protocol, 50mL of either the drinking water or wastewater sample will be spun down by centrifuge for 20 min at 9000 rpm. Remove Supernatant (except for 300 μLs and resuspend pellet.
2. DNA extraction (Following the Powersoil DNA extraction kit protocol listed below)
a.To the PowerBead Tubes provided, add 0.25 gm of soil sample.
b. Gently vortex to mix.
c. Add 60ml of Solution C1 and invert several times or vortex briefly.
d. Secure PowerBead Tubes horizontally on a flat-bed vortex pad with tape & Vortex at maximum speed for 10 minutes.
e. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature.
f. Transfer the supernatant to a clean 2 ml Collection Tube (provided).
g. Add 250ml of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.

h. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
i. Avoiding the pellet, transfer up to, but no more than, 600ml of supernatant to a clean 2mL Collection Tube (provided).
j. Add 200ml of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.
k. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
l. Avoiding the pellet, transfer up to, but no more than, 750ml of supernatant into a clean 2 ml Collection Tube (provided).
m. Add 1200ml of Solution C4 to the supernatant and vortex for 5 seconds.
n. Load approximately 675ml onto a Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675ml of supernatant to the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. **Note**: A total of three loads for each sample processed are required.
o. Add 500ml of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
p. Discard the flow through.
q. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
r. Carefully place Spin Filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.
s. Add 100ml of Solution C6 to the center of the white filter membrane.
t. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
u. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required. Store DNA frozen at (-20° to -80°C).
3. Run 3µL of each DNA sample with 1µL of 6X loading dye dots on 1% Agarose gel for 40min at 110V(2g Agarose, 200mL TAE, 7µL Ethidium bromide. Microwave for 1min and then swirl. Microwave for and additional minute and then swirl. Microwave for 30s and then swirl. Pour gel and let cool until solidified). {confirmation of DNA smear=good!}
4. Observe gel under UV conditions and print picture.

Day 2

PCR conditions. This experiment calls for 100µL PCR reactions. Make sure to have a negative control (PCR reaction with no sample DNA). Also have a dedicated PCR that's always clean and keep reaction solutions separate in the -20°C freezer.
1. Clean Station with detergent buffer.
2. Obtain reagents (Per sample: 10µL 10X Reaction Buffer; .8µL DNTP [deoxynucleoside Triphosphate]; 83.7µL PCR grade water; .5µL of 5' and 3' Primers; .5µL of enzymes; 3µL of DNA sample), Materials (10, 100, 1000µL pipetter and aerosol barrier tips, PCR tubes and centrifuge tubes), and let DNA thaw.
3. Make Master mix (For 4 DNA samples there will be 4 tubes of DNA/sample for a total of 16 samples. There will also be a negative control and 1 extra run for pipetting error for a total of 18 PCR reactions) Master Mix for 18 reactions consists of: 180µL 10X Reaction Buffer; 14.4µL DNTP; 1560.0µL PCR-grade water; 9µL of 5'-AGA GTT TGA TC(AC) TGG CTC AG; 9µL of 1492 reverse primer (or 3' primer) 5'-GGT TAC CTT GTT ACG ACT T; 9µL of Qiagen HotstarTaq DNA polymerase).

4. Add 3µL of DNA sample to 97µL of Master Mix for a total reaction volume of 100µL. Place samples into thermocycler and run under the following conditions: 15-min hot start at 95°C using, followed by 35 cycles consisting of denaturation (60 s at 94°C), annealing (60 s at 50°C), and extension (120s at 72°C) and a final extension at 72°C for 7 min.
5. Verify amplified DNA by electrophoresis of aliquots of PCR under the following conditions:
a. Make a 20 lane gel for 17 samples by adding .6g Agarose to 60mL of TAE. Microwave for 30s and then swirl. Microwave for and additional 30s and then swirl. Microwave for 10s and then swirl. Pour gel and let cool until solidified (~15 minutes). Add enough TAE to pour over the gel up to the fill line.
b. Place .5µL dots of each PCR sample on filter paper.
c. Add .5µL of loading dye to each dot (~10µL total) and then add each dye/sample combination to the wells in the gel. Make sure to place the marker first (3µL of the loading dye) on both the top and the bottom rows of the gel.
d. Connect (+) and (-) connections {Red to red, black to black}.
e. Set meter to 110 V for 45 minutes.
f. Remove gel and place in Ethidium Bromide solution (50µL EtBr in 200mL of water) for 5 minutes.
g. Detain in water solution for 10 minutes.
h. Image products and obtain printout of picture.
6. Place PCR products in 4°C freezer.

Day 3
1. Obtain 8 spin-bind columns{1 for 2 samples. Ex: 1 column for samples #1 and #2).
2. Remove PCR products from 4°C freezer. Label all spin columns and micro-centrifuge tubes correctly.
3. Transfer total volume of PCR product into each PCR tube.
4. Place 1mL (or 5 times the amount of the combined 2 PCR products) of Spin Bind Buffer in the 2 combined PCR products.
5. Place ½ of the total volume (~600µL) in the spin column. Repeat for all combined PCR products.
6. Centrifuge for 1min @ 10,000 RPM.
7. Pour off supernatant.
8. Repeat steps 5 & 6 for the other ½ volume of combined PCR product.
9. Add 300µL of ethanol based wash buffer (aka Spin Column Buffer) to each column.
10. Centrifuge for 1 min @ 10,000 RPM.
11. Pour Off Supernatant.
12. Repeat steps 10 & 11.
13. Transfer columns to new collection tubes.
14. Add 50µL of elution buffer to tube from step 13.
15. Centrifuge for 1 min @ 10,000 RPM.
16. Remove column and discard.
17. Repeat steps 13-16 for other column from sample. (Ex: use column for 1& 2, discard, use column for 3 & 4, discard)
18. Make a 40 mL 1% Agarose/ TAE gel.
a. Place .4g Agarose into 40mL of TAE.

b. Microwave for 30s and then swirl. Microwave for and additional 30s and then swirl. Microwave for 10s and then swirl. Pour gel and let cool until solidified (~15 minutes). Add enough TAE to pour over the gel up to the fill line.
c. Place .5µL dots of cleaned, combined PCR products on filter paper.
c. Add .5µL of loading dye to each dot (~3µL total) and then add each dye/sample combination to the wells in the gel. Make sure to place the marker first (3µL of the loading dye) on both the top and the bottom rows of the gel.
d. Connect (+) and (-) connections {Red to red, black to black}.
e. Set meter to 110 V for 45 minutes.
f. Remove gel and place in Ethidium Bromide solution (50µL EtBr in 200mL of water) for 5 minutes.
g. Detain in water solution for 10 minutes.
h. Image products and obtain printout of picture.

Day 4: Restriction Digest
1. Obtain clean PCR samples from fridge.
2. Prepare Reaction Mix for each Restriction Enzyme (RSA1, MSP1, HHA1):
For RSA1:
a.10µL Reaction Buffer #1
b. No BSA
c. 59µL PCR grade water
d. 30µL DNA
e. 1µL of restriction enzyme per reaction

For HHA1:
a.10µL Reaction Buffer #4
b. 1µL BSA
c. 58µL PCR grade water
d. 30µL DNA
e. 1µL of restriction enzyme per reaction

For MSP1:
a.10µL Reaction Buffer #2
b. No BSA
c. 59µL PCR grade water
d. 30µL DNA
e. 1µL of restriction enzyme per reaction

For 4 samples, the reaction mix is the following:
For RSA1:
a.40µL Reaction Buffer #1
b. No BSA
c. 236µL PCR grade water
d. 30µL DNA
e. 4µL of RSA1

For HHA1:
a.40μL Reaction Buffer #4
b. 4μL BSA
c. 232μL PCR grade water
d. 30μL DNA
e. 4μL of HHA1
For MSP1:
a.40μL Reaction Buffer #2
b. No BSA
c. 236μL PCR grade water
d. 30μL DNA
e. 4μL of MSP1
3. Add 30μL of DNA samples to each enzyme digest reaction mix.
4. Place samples in 37°C incubator overnight.

Day 5: Nucleotide Removal
1. Obtain and label spin columns and 1.5mL Centrifuge Tubes for each sample.
2. Heat treat combined PCR samples @ 60°C for 20 min.
3. Add 10 times the volume of Buffer PN to each sample (Ex: if sample is .1μL, add 1μL of Buffer PN).
4. Add ½ of the new volume (~550μL) to the spin column.
5. Spin columns for 1 min @ 6000 RPM.
6. Pour off supernatant.
7. Add remaining ½ of new volume (~550μL) to the spin column.
8. Repeat steps 5 & 6.
9. Add 750μL of Buffer PE (wash buffer).
10. Centrifuge @ 6,000 RPM for 1 min.
11. Pour off wash buffer.
12. Repeat step 10. Change speed to 13,000 RPM for 1 min. Transfer column to next centrifuge tube.
13. Add 50μL of warmed (60°C) PCR grade water to column. {Note: Use this instead of the EB Buffer supplied in the kit}.
14. Allow to sit on bench top for 5 minutes.
15. Centrifuge at 13,000 RPM for 1 minute.
16. Discard spin column.
17. Aliqout 25μL into shipping tubes.
18. Wrap with parafilm and ship to Michican State University for fragment analysis.
19. Make Sure you tell MSU to post the results in an excel file.

Once Samples have returned:
1. Copy only the green data (G, ...) into a new data file and label it and save.
2. Rename files as Sample Name/Number_restriction enzyme.txt.

For unique unclassified organisms:
1. Run unclassified organism info through NCBI: http://www.ncbi.nlm.nih.gov/ using the Nucleotide database search

2. Get sequence info and paste into: http://rdp.cme.msu.edu/classifier/classifier.jsp
3. Classify the organism and change the In silico results to include this new information.

Appendix 5. Summary of Fragment Data for Sewage and Natural Water TRFLP Experiments

Site	Reciprocal Simpson	Simpson	Shannon Evenness	Total Frag Utilization	#Patterns Detected	RSA I	HHaI	MSPI frags	Agree-ment between methods (1=Y)
Cistern 1	4.031	0.2481	0.6594	69.23	13	3	4	6	-
Cistern 2	13.32	0.0751	0.8667	97%	30	8	9	13	-
Cistern 3	4.721	0.2118	0.6143	40%	25	5	15	1	1
DF 1	5.566	0.1797	0.6957	60%	15	3	10	2	1
DF 2	6.146	0.1627	0.8247	0%	12	7	1	4	-
NCSU 1	5.354	0.1868	0.6622	39%	18	2	3	6	0
Sewage 2	16.22	0.06166	0.7471	77.78%	63	12	23	28	1
Sewage 3	27.26	0.03668	0.818	92%	88	24	28	36	1
Sewage 4	21.73	0.04601	0.7959	89%	83	23	27	33	1

UL 1	8.281	0.1208	0.7763	65%	23	4	8	11	1
UL 2	5.159	0.1938	0.6348	58.60%	29	10	2	0	1
UL 3	3.925	0.2548	0.6926	67%	12	4	5	3	1

Literature Cited

Anwar, M. S., N. A. Chaudhry, and M Tayyab. 1999. Bacteriological quality of drinking water in Punjab: evaluation of H2S strip test. J Pak Med Assoc 49(10):237-241.

Blackwood, C.B., D. Hudleston, D.R. Zak, and J. S. Buyer. 2007. Interpreting Ecological Diversity Indices Applied to Terminal Restriction Fragment Length Polymorphism Data: Insights from Simulated Microbial Communities. Appl Environ Microbiol. 73(16): 5276–5283.

Blackwood, C. B., T. Marsh, S.-H. Kim, and E. A. Paul. 2003. Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. Appl. Environ. Microbiol. 69:926-932

Broderick, N.A, Raffa K.F., Goodman R. M., and J. Handelsman. 2004. Census of the Bacterial Community of the Gypsy Moth Larval Midgut by Using Culturing and Culture-Independent Methods. Appl. Environ. Microbiol. 70(1):293-300

Brown, J. 2003. Evaluation of point-of-use microfiltration for drinking water treatment in rural Bolivia, Department of Geography, University of Cambridge.

Brown, J. and M.D. Sobsey (2007) Use of Ceramic Water Filters in Cambodia. Field Note. Water Sanitation Programme, World Bank, Phnom Penh, Cambodia.

Brown, J., Proum, S. and Sobsey, M. 2008. "E. coli in household drinking water and diarrheal disease risk: evidence from Cambodia". *Water Science and Technology* 58(4): 757-763.

Castillo, G., R. Duarte, Z. Ruiz, M. T. Mauricic, B. Honorato, R. Mercado, V. Coloma, V. Lorca, M. T. Martins, and B. K. Dutka. 1994. Evaluation of disinfected and untreated drinking water supplies in Chile by the H_2S paper strip test. Water Research 28:1765-1770.

Clasen, T.; Schmidt, W. P.; Rabie, T.; Roberts, I.; Cairncross, S., Interventions to improve water quality for preventing diarrhoea: systematic review and meta-analysis. Bmj 2007, 334, (7597), 782.

Colford JM Jr, Wade TJ, Schiff KC, Wright CC, Griffith JF, Sandhu SK, Burns S, Sobsey M, Lovelace G, Weisberg SB. (2006) Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. Epidemiology. 18(1):27-35 Committee on Indicators for Waterborne Pathogens, National Research Council (2004) Indicators for Waterborne Pathogens The National Academies Press, Washington, DC

Committee on Indicators for Waterborne Pathogens. 2004. Indicators for Waterborne Pathogens. National Academies Press

Craun, M. F., Craun, G. F., Calderon, R. L., & Beach, M. J. (2006). Waterborne outbreaks reported in the United States. J Water Health*, 4 Suppl*

Desmarchelier, P., Lew, A., Caique, W., Knight, S., Toodayan, W., Isa, A.R., and A. Barnes. (1992). An evaluation of the hydrogen sulphide water screening test and coliform counts for water quality assessment in rural Malaysia. Transactions of the Royal Society of Tropical Medicine and Hygiene. 86, 448-450

Dillon JG, Fishbain S, Miller SR, Bebout BM, Habicht KS, Webb SM, Stahl DA. High rates of sulfate reduction in a low-sulfate hot spring microbial mat are driven by a low level of diversity of sulfate-respiring microorganisms. Applied and Environmental Microbiology, August 2007, p. 5218-5226, Vol. 73, No. 16

Dennehy, P. H. (2005). Acute diarrheal disease in children: epidemiology, prevention, and treatment. Infect Dis Clin North Am*, 19*(3), 585-602.

Eaton, A.D., L.S. Clesceri, E.W. Rice, A.E. Greenberg, M.A.H. Franson, (editors). 2005. Standard Methods for the Examination of Water and Wastewater: Centennial Edition. 21st Edition. ISBN: 0875530478. American Public Health Association. Washington, D.C. 1368 p.

Eisenberg, J. N., Cevallos, W., Ponce, K., Levy, K., Bates, S. J., Scott, J. C., et al. (2006). Environmental change and infectious disease: how new roads affect the transmission of diarrheal pathogens in rural Ecuador. *Proc Natl Acad Sci U S A, 103*(51), 19460- 19465.

Fewtrell L., Kaufmann R.B, Kay D., Enanoria W., Haller L., Colford J. M., Water, sanitation, and hygiene interventions to reduce diarrhoea in less developed countries: a systematic review and meta-analysis. Lancet Infect Dis 2005, 5, (1), 42-52.

Fujioka, R. S. & T. M. Unutoa. 2006. Comparative stability and growth requirements of S. aureus and faecal indicator bacteria in seawater. Water Sci. Technol. 54(3), 169 – 175.

Gawthorne, T., Gibbs,R.A, Mathew, K., and G.E. Ho. (1996). H_2s papers as presumptive tests for salmonella contamination in tropical drinking water. Water Science and Technology. 34(7-8):187-194

Genthe, B. and M. Franck (1999). "A tool for Assessing Microbial Quality in Small Community Water Supplies: An H2S Strip Test. to: Water Research Commission.

by: Division of Water, Environment and Forestry Technology, CSIR, Stellenbosch, South Africa." WRC Report: 1.

Gundry, S., Wright, J. & Conroy, R. 2004 A systematic review of the health outcomes related to household water quality in developing countries. J. Water. Health 2(1), 1 – 13.

Gupta, S.K., M.A. Sheikh, M.S. Islam, K.S. Rahman, N. Jahan, M.M. Rahman, R.M. Hoekstra, R. Johnston, P.K. Ram, S. Luby. 2008. Usefulness of the hydrogen sulfide test for assessment of water quality in Bangladesh. Journal of Applied Microbiology. 104(2):388-395.

Tambekar, D.H., Hirulkar, N.B., Gulhane, S.R., Rajankar P.N, and S.S. Deshmukh. 2010. Suitability of the H2S test for detection of fecal contamination in drinking water. African Journal of Biotechnology Vol. 5 (10), pp. 1025-1028

Jensen, P. K., Jayasinghe, G., van der Hoek, W., Cairncross, S. & Dalsgaard, A. 2004 Is there an association between bacteriological drinking water quality and childhood diarrhoea in developing countries? Trop. Med. Int. Health 9(11), 1210 – 1215.

Johnson, M.C., A.A. Devine, J.C. Ellis, A.M. Grunden, V. Fellner. Effects of antibiotics and oil on microbial profiles and fermentation in mixed cultures of ruminal microorganisms. 2009. J Dairy Sci. 92(9):4467-4480.

Jothikumar N. and K. Subba Rao. 2000. A field test for the assessment of faecal contamination of potable water. J. Environ. Monit. 2:183-185

Kaspar P., I. Guillen, D. Revelli, T. Meza, G. Velazquez, H. Mino de Kaspar, L. Pozolli, C. Nunez, and G. Zoulek. 1992. Evaluation of a simple screening test for the quality of drinking water systems. Trop Med Parasitol 43(2):124-127.

Kent, A.D., D.J. Smith, B.J. Benson, E.W Triplett. 2003. Web-Based Phylogenetic Assignment Tool for Analysis of Terminal Restriction Fragment Length Polymorphism Profiles of Microbial Communities. Appl. Environ. Microbiol. 69:6768-6776

Kim, S. H., and T. L. Marsh. 2004. The analysis of microbial communities with terminal restriction fragment length polymorphism (T-RFLP), p. 789-808. In G. A. Kowalchuk, F. J. de Bruijn, I. M. Head, A. D. Akkermans, and J. D. van Elsas (ed.), Molecular microbial ecology manual, 2nd ed. Springer, New York, NY.

Kosek, M., Bern, C., & Guerrant, R. L. (2003). The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull World Health Organ, *81*(3), 197-204.

Kromoredjo, P. and R. S. Fujioka (1991). "Evaluating three simple methods to assess the microbial quality of drinking water in Indonesia." Environ. Toxicol. Water Qual 6: 259–270.

Landis, J.R., and Koch, G. G. 1977. The measurement of observer agreement for categorical data. Biometrics. 33:159-174

Leclerc, H., Mossel, D. A. A., Edberg, S. C., and C. B. Struijk. Advances in the Bacteriology of the Coliform Group: Their Suitability as Markers of Microbial Water Safety Annu. Rev. Microbiol. 2001. 55:201–34.

Liu, W.T., T.L. Marsh, H. Cheng, and L.J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516-4522

Madigan, M. T., and J. M. Martinko. 2008 Brock Biology of Microorganisms. 12th edition. pp. 617-625

Manja, K. S. and R. K. Kaul (1992). "Efficacy of a simple test for bacteriological quality of water." J. Ind. Assoc. Environ. Manage 19: 18–20.

Manja K.S., M. S. Maurya, and K. M. Rao. 1982. A simple field test for the detection of faecal pollution in drinking water. Bull World Health Organ 60(5):797-801.

McMahan, L., T. Lusk, S. Chung, C. Stauber, L. Casanova, and M. D. Sobsey. 2009. Development and Evaluation of Existing and Newly Emerging Simple, Portable Field Tests to Detect Microbial Indicators of Fecal Contamination in Water. Proceedings of American Water Works Association Global Conference on Microbial Contaminants in Drinking Water in Singapore. American Water Works Association.

Minz D, Fishbain S, Green SJ, Muyzer G, Cohen Y, Rittmann BE, Stahl DA. Unexpected population distribution in a microbial mat community: sulfate-reducing bacteria localized to the highly oxic chemocline in contrast to a eukaryotic preference for anoxia. Applied and Environmental Microbiology, October 1999, p. 4659-4665, Vol. 65, No. 10

Moe, C. L., Sobsey, M. D., Samsa, G. P. & Mesolo, V. 1991 Bacterial indicators of risk of diarrheal disease from drinking-water in the Philippines. Bull. World Health Organ. 69(3), 305 – 317.

Morales, S.E., P.J Mouser, N. Ward, S.P. Hudman, N.J. Gotelli, D.S Ross, and T.A. Lewis. 2006. Comparison of Bacterial Communities in New England Sphagnum Bogs Using Terminal Restriction Fragment Length Polymorphism (T-RFLP). Microbial Ecology. 52:34–44

Nair, J., Gibbs, R., Mathew K., and G.E. Ho. (2001), Suitability of the H2S method for testing untreated and chlorinated water supplies. Water Science and Technology 44(6):119–126

Nikaeen, M., Izadi, M., Sabzali, A., Bina, B., Jafari, N.A.J., Hatamzdeh, M., and Hossein Farrokhzadeh. (2010). The effects of incubation period and temperature on the hydrogen sulphide (H2S) technique for detection of faecal contamination in water. African Journal of Environmental Science and Technology Vol. 4(2)084-091

Pant, H., S. Sarfaraz, et al. (2002). "Evaluation of L-Cystine-amended H 2 S test for the detection of faecal pollution in potable water: comparison with standard multiple tube fermentation method." World Journal of Microbiology and Biotechnology 18(4): 321-324.

Pathak, S. P. and K. Gopal (2005). "Efficiency of Modified H 2 S Test for Detection of Faecal Contamination in Water." Environmental Monitoring and Assessment 108(1): 59-65.

Pidiyar, V.J., K. Jangid, M.S. Patole, Y.S Shouche. 2004. Studies on cultured and uncultured microbiota of wild Culex quinquefasciatus mosquito midgut based on 16S ribosomal RNA gene analysis. Am. J. Trop. Med. Hyg. 70(6):597-603.

Pruss-Ulsten, A., and C. Corvaldn. (2007). How much disease burden can be prevented by environmental interventions? Epidemiology 18:167-178

Ratto, A., B.J. Dutka, C. Vega, C. Lopez, A. El-Shaarawi. 1989. Potable water safety assessed by coliphage and bacterial tests. Water Research 23:253-255.

Reis, M. A. M., J. S. Almeida, P.C. Lemos, and M. J.T. Carrondo. 1992. Effect of Hydrogen Sulfide on Growth of Sulfate Reducing Bacteria. Biotechnology and Bioengineering. 40(5):593-600

Rijal, G. K. and R. S. Fujioka (2001). "Synergistic effect of solar radiation and solar heating to disinfect drinking water sources." Water Sci Technol 43(12): 155-62.

Roser D.J., N. Ashbolt, G. Ho, K. Mathew, J. Nair, D. Ryken-Rapp, and S. Toze. 2005. Hydrogen sulphide production tests and the detection of groundwater faecal contamination by septic seepage. Water Sci Technol 51(10):291-300.

Sobsey, M.D., T. Handzel and L. Venczel (2003) Chlorination and safe storage of household drinking water in developing countries to reduce waterborne disease Water Science & Technology, 47(3): 221–228

Sobsey, M.D and F.K Pfaender (2002) Evaluation of the H2S method for the detection of fecal contamination of drinking-water. WHO/SDE/WSH/02.08, World Health Organization, Geneva.

Solo-Gabriele HM, Wolfert MA, Desmarais TR, Palmer CJ. Sources of Escherichia coli in a coastal subtropical environment. Appl Environ Microbiol. 2000 Jan;66(1):230-7.

Stauber CE, Elliott MA, Koksal F, Ortiz GM, DiGiano FA, Sobsey MD (2006). Characterization of the biosand filter for E. coli reductions from household drinking water under controlled laboratory and field use conditions. Water Sci Technol., 54(3):1-7.

Tewari, S., P.W. Ramteke, and S. K. Garg. 2003. Evaluation of simple microbial tests for detection of fecal coliforms directly at 45.5C. Environmental Monitoring and Assessment. 85:191–198.

UNICEF/WHO. 2009. Diarrhoea: Why children are still dying and what can be done.

Venkobachar, C., D. Kumar, K. Telreja, A. Kumar, and L. Iyengar. 1994. Assessment of bacteriological water quality using a modified H_2S strip test. *J. Water. SRT. Aqua* 43(6):311-314.

Wang, Q., G.M. Garrity, J.M. Tiedje, and J.R. Cole. 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol. 73(16):5261-5267.

WASH Cluster meeting notes. 24 Nov 2010, SERENA Hotel, Islamabad. http://www.pakresponse.info/LinkClick.aspx?fileticket=v2HjB9qAb9o%3D&tabid=85&mid=842. Accessed Jan 2011.

Wetzel, R.G. 2001. Limnology, 3rd Ed., Academic Press, San Diego, CA

White, G. F., Bradley, D. J., & White, A. U. (2002). Drawers of water: domestic water use in East Africa. 1972. Bull World Health Organ, 80(1), 63-73; discussion 61-62.

Widdel, F. 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria, pp 469-586. In: Biology of Anaerobic Organisms, A.J.B. Zehnder (ed.), Wiley-Liss, NY, NY.

World Health Organization. 2006. Guidelines for drinking-water quality: incorporating 1st and 2nd addenda, Vol.1, Recommendations (electronic version). http://www.who.int/entity/water_sanitation_health/dwq/fulltext.pdf

Yasushi, K. 1998 Microbially induced corrosion and biodegradation, Corrosion Engineering, 47:909-913.