EVALUATION OF A LOW-COST COMPARTMENT BAG TEST TO QUANTIFY HYDROGEN SULFIDE-PRODUCING BACTERIA IN DRINKING WATER

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ABSTRACT

Claire Carter Tipton: Evaluation of a low-cost compartment bag test to quantify hydrogen sulfide-producing bacteria in drinking water
(Under the direction of Jill Stewart)

Tests for detecting hydrogen sulfide (H_2S)-producing bacteria as fecal indicators have been proposed to assess drinking water safety in low-resource settings. This study compared a semi-quantitative compartment bag test (CBT) to the EPA- and FDA-approved multiple test tube (MTT) method to quantify H_2S-producing bacteria in drinking water sources. Both methods used PathoScreen™ medium to detect target bacteria in 60 surface water samples collected from North Carolina drinking water reservoirs. Samples were subjected to paired levels of incubation temperatures (25° C, 35° C) and numbers of incubation days (1, 2, 3). Results indicated a significant positive correlation between methods, particularly at 25° C and 2 days incubation (r=0.78). However, the CBT tended to underestimate H_2S-producing bacteria concentrations in samples. The CBT shows promise as a microbiological drinking water test for low-resource environments, particularly where quantitative information is preferable to presence/absence results. However, further calibration is recommended to improve test performance.

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LIST OF ABBREVIATIONS AND SYMBOLS

° C	Degrees Celsius
CBT	Compartment bag test
CC	Cane Creek Reservoir
CI	Confidence interval
DO	Dissolved oxygen
E. coli	*Escherichia coli*
EPA	United States Environmental Protection Agency
° F	Degrees Fahrenheit
FC	Fecal coliform
FDA	United Stated Food and Drug Administration
FeS	Ferrous sulfide
FIB	Fecal indicator bacteria
FIO	Fecal indicator organisms
FNR	False negative rate
FPR	False positive rate
H_2S	Hydrogen Sulfide
ISO	International Organization for Standardization
Ln	Natural log
Log_{10}	Log base 10
MDG	Millennium Development Goals
mg/L	Milligrams per liter
mL	Milliliter
MPN	Most Probable Number

MTT	Multiple test tube method
NGO	Non-governmental organizations
NPV	Negative predictive value
OR	Odds Ratios
P	P-value
PA	Presence/Absence
PBS	Phosphate Buffered Solution
pH	Potential of Hydrogen
PPV	Positive predictive value
r	Spearman's rho
r^2	Coefficient of determination
SRB	Sulfate reducing bacteria
TC	Total coliform
TRFLP	Terminal Restriction Fragment Length Polymorphism
UL	University Lake
UNICEF	United Nations International Children’s Fund
USD	United States Dollar
USAID	United States Agency for International Development
W	Shapiro-Wilk normality test statistic
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

Safe drinking water is a fundamental human right and requirement for good health. Despite this, fecal contamination in drinking water affects over 1.8 billion people worldwide and is estimated to cause over 500,000 diarrheal deaths each year. Young children are particularly vulnerable, with approximately 361,000 diarrheal deaths occurring each year to children under five due to unsafe drinking water conditions (WHO 2016). Many of the world's population impacted by poor microbial water quality reside in rural or low-resource environments (Anwar et al. 1999). While many technologies exist to detect fecal contamination in drinking water, the high level of human, financial, and technological capacity required to conduct such tests poses logistical challenges to routine monitoring in these settings (Crocker & Bartram 2014). Similar challenges arise during humanitarian emergencies such as natural disasters and wartime conflict, which often jeopardize utilities and drinking water supplies (Adams 1999).

To overcome this problem, field tests that detect hydrogen sulfide (H_2S)-producing bacteria as indicators of fecal contamination in water have been proposed for use in low-resource settings. The H_2S-producing bacteria field test (H_2S test) has been compared to traditional methods for detecting fecal indicator bacteria (FIB), and has demonstrated relatively good correlation with conventional indicator organisms (Ratto et al. 1989; Castillo et al. 1994; Venkobachar et al. 1994; Genthe & Franck 1999; Rijal & Fujioka 2001; Manja et al. 2001; Sobsey & Pfaender 2002; McMahan 2011; Tambi et al. 2016; Sivaborvon 1988; Martins et al. 1997; Dufour et al. 2013; Manja et al. 1982; Anwar et al. 1999; Nair et al. 2001; Hirulkar & Tambekar 2006; Gupta et al. 2007; Eun & Hwang 2003; Kromoredjo & Fujioka 1991; McMahan et al. 2012). The H_2S test has many optimal traits for use in resource-limited environments,

including simple format, low-cost of materials, and ease of isolating, identifying, and enumerating target organisms (McMahan 2011).

The H_2S test is based on the reaction of iron in the medium with hydrogen sulfide gas produced by sulfate reducing bacteria (SRB), causing the formation of the black insoluble precipitate ferrous sulfide. H_2S-producing bacteria are found in a wide variety of habitats, including freshwater, and include many enteric pathogens such as *Salmonella*, *Proteus*, *Edwardsiella*, *Yersinia, Aeromonas, Clostridium, Staphylococcus, Peptococcus, and Campylobacter,* fecal coliforms *(Citrobacter freundii, Klebsiella pneumoniea*, *K. oxytoca, Enterobacter cloacae),* and H_2S-producing variants of *Escherichia coli* (*E. coli).* Although widely distributed in the environment, H_2S-producing bacteria have been found to be consistently associated with fecal contamination (McMahan 2011; Manja et al. 1982; Manja et al. 2001; Nagaraju & 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 & Fujioka 1991; Genthe & Franck 1999; Sivaborvon 1988; Sobsey & Pfaender 2002; McMahan et al. 2012). They also meet other criteria required for ideal fecal indicator bacteria (FIB), such as similar survival and transport in the environment compared to pathogens (Martins et al. 1997; Castillo et al. 1994; Nagaraju & Sastri 1999), present in greater numbers than pathogens (Nagaraju & Sastri 1999; Castillo et al. 1994; Manja et al. 1982), broad applicability (Ratto et al. 1989; Castillo et al. 1994; Martins et al. 1997; Kromoredjo & Fujioka 1991; Genthe & Franck 1999; Sivaborvon 1988), quantifiable (McMahan 2011; McMahan et al. 2012; Venkobachar et al. 1994; Rijal & Fujioka 2001; Manja et al. 2001), adequate sensitivity (Ratto et al. 1989; Anwar et al. 1999; Genthe & Franck 1999; Rijal & Fujioka 2001; Roser et al. 2005), and logistic feasibility (Genthe & Franck 1999; Bain et al. 2012; Mosley & Sharp 2005; Venkobachar et al. 1994; Castillo et al. 1994; Ratto et al. 1989; Anwar et al. 1999; S P Pathak & Gopal 2005; Hirulkar & Tambekar 2006; Khush et al. 2013; Weppelmann et al. 2014a; Walker et al. 2013; Tambi et al. 2016; Sivaborvon 1988). While more research

is needed, studies by McMahan (2011) and Kush et al. (2013) show potential for the H_2S test to be associated with risk of diarrheal illness.

The H_2S field test was first developed in the early 1980's as a simple and reliable presence/absence (PA) test for village health workers in India (Manja et al. 1982). Since then, many modifications to the test have been developed, including changes to medium composition, medium preparation, sample volume, incubation times and temperatures, test formats, and methods to score results. The H_2S test, particularly PA versions of the test, has received widespread use and commercialization in recent years (Bain et al. 2012).

While the H_2S test has been used globally for over three decades, the method is still under debate among scientists and regulatory agencies. One of the main concerns regarding the test is the lack of standardization across lab- and commercially-made tests (Sobsey & Pfaender 2002). The proliferation of presence/absence tests vs. more quantitative tests is also a concern, especially in light of the World Health Organization's (WHO) Guidelines for Drinking Water Quality shifting towards quantifiable risk-based data (Bain et al. 2012). Differing sensitivity and specificity results among studies has also raised concerns (Wright et al. 2012; Izadi et al. 2010; Tewari et al. 2003; Desmarchelier et al. 1992; Yang et al. 2013a). Until more rigorous research can be done to generate method consensus, as well as provide further evidence to support H_2S-producing bacteria as a viable FIB, regulatory agencies such as the United States Environmental Protection Agency (EPA) and the WHO will not accept the H_2S test for microbial water quality purposes.

In 2007, researchers developed a microbial water quality field test kit for enumerating *E. coli* concentrations in water using a compartmentalized bag test (CBT) and most probable number (MPN) format (Stauber et al. 2014). The *E. coli* CBT compares relatively well to standard FIB methods, consistently correlates with diarrheal illness, and has been tested globally in low-resource settings (Murcott et al. 2015; Weiss et al. 2016; Stauber et al. 2014; Heitzinger et al. 2016; Adank et al. 2016;

Morrison 2016; Gerges et al. 2016; McMahan et al. 2017). The kit is currently manufactured and distributed by Aquagenx, LLC (Chapel Hill, NC, USA).

In an effort to further validate H_2S-producing bacteria as alternative fecal indicator organisms, McManhan (2011) assessed the feasibility of a combined H_2S compartment bag test (H_2S CBT) using lab-made and proprietary H_2S substrates. Researchers ran a cost analysis of the H_2S CBT method against other common microbial water quality tests, including MI Agar, BioRad Rapid *E. coli* 2 Agar, Coliert, Petrifilm *E. coli*, EasyGel, and the *E. coli* CBT. The H_2S CBT was judged to be the most cost-effective, at $0.40 per sample (McMahan 2011). While results were promising, no further work has been done to evaluate and compare the capabilities of the H_2S CBT to standard semi-quantitative methods.

The purpose of this study was to compare the compartment bag test to the EPA- and United States Food and Drug Administration (FDA)-approved multiple test tube (MTT) method to enumerate H_2S-producing bacteria in drinking water sources using a most probable number (MPN) format. Development of an inexpensive, simple, and semi-quantitative H_2S field test would provide more quantitative information on human health risk related to microbial water quality than more common PA H_2S tests. Validation of a reliable and semi-quantitative H_2S test would also aid in the effort to standardize the H_2S test method, by making it more comparable to semi-quantitative and quantitative methods using traditional fecal indicator organisms.

CHAPTER 2: OBJECTIVES

There were two primary objectives for this study:

1. Compare the compartment bag test (CBT) to the multiple test tube (MTT) method to detect and quantify H_2S-producing bacteria in drinking water sources using a most probable number (MPN) format.

2. Determine the effect of incubation time and temperature on test results within and between methods.

CHAPTER 3: LITERATURE REVIEW

Introduction

Fecal contamination of drinking water is a major cause of waterborne illnesses in humans worldwide, with microbial contamination responsible for the great majority of the water-related health burden (WHO 2008). Since the early 1980's, methods for detecting fecal contamination using hydrogen sulfide (H_2S)-producing bacteria as alternative fecal indicator bacteria (FIB) have been developed (Manja et al. 1982). The H_2S-producing bacteria field test (H_2S test) has been advocated for use in low-resource and humanitarian emergency settings due to its low-cost and user friendly format. Additionally, there is a growing body of evidence to support H_2S-producing bacteria as credible alternative fecal indicator organisms.

One of the challenges facing the H_2S test is the lack of standardization across the method. Multiple versions of the H_2S test exist, with differences in medium composition, sample volumes, test formats, and methods to score results limiting the ability to validate and compare the H_2S test to more traditional FIB methods (Sobsey & Pfaender 2002). In addition, there are few semi-quantitative or quantitative H_2S tests on the market compared to presence/absence (PA) tests. PA tests cannot determine microbial concentrations nor estimate health risks related to microbial water quality, which are important factors for determining water safety in resource-limited environments.

One promising semi-quantitative H_2S test uses a compartmentalized bag test (CBT) format along with a proprietary H_2S detection substrate to enumerate H_2S concentrations using a most probable number (MPN) approach. While the feasibility and cost-effectiveness of the H_2S CBT have been

confirmed (McMahan 2011), more comparisons of the test to standard microbial water quality methods that estimate bacteria concentrations by quantal methods are needed.

The goal of this review is to discuss the published literature on H_2S field tests, including the usefulness of H_2S-producing bacteria as indicators of fecal contamination in low-resource settings, the history of method development and subsequent modifications, and the introduction and initial validation of the compartment bag test as a means of quantifying target bacteria in water.

Fecal contamination of drinking water and diarrheal illness

Fecal contamination in drinking water impacts over 25% of the world's population and is estimated to cause over 500,000 diarrheal deaths each year (WHO 2016). Diarrheal disease is a major cause of morbidity and mortality in all age groups, but especially among young children. For instance, unsafe drinking water conditions are estimated to cause over 360,000 diarrheal deaths ever year in children under five (WHO 2016). While mortality from diarrheal illnesses has decreased over the past fifty years, a study conducted in 2003 suggests there has not been an accompanying decrease in morbidity on the global burden of disease (Kosek et al. 2003).

Water is one of the primary pathways for transmission of diarrheal illnesses. Transmission of disease via water can be classified into four categories: waterborne, water-washed, water-based, and water-related (White et al. 2002). Waterborne diseases occur when pathogens are ingested via the fecal-oral route and are the source of illnesses such as gastroenteritis, giardia, cholera, and infectious hepatitis (Cairncross & Feachem 1993). Waterborne pathogens comprise a broad range of microorganisms, ranging from viruses to bacteria to protozoan parasites. Many are considered enteric pathogens because they infect the gastrointestinal tract and are capable of infecting others once shed into the environment via excreta (White et al. 2002).

Fecal indicator organisms

Detecting and monitoring fecal pathogens in drinking water is crucial to managing water systems and minimizing disease risk as well as protecting local and global public health. However, methods to detect the full spectrum of pathogens that may occur in water are currently cost prohibitive and impractical to implement on a widespread scale (US EPA 2009). For decades, regulatory agencies and scientific governing bodies around the world have promoted the use of fecal indicator organisms as surrogates for potential pathogens and subsequent health risks in recreational and drinking water sources (US EPA 2009). Fecal indicator organisms (FIO) are microorganisms found in the intestines of warm-blooded animals, including humans, and are shed in feces. While FIO are generally not hazardous to human health, their presence and density in water indicate the possible presence of pathogenic organisms due to fecal contamination. To qualify as an FIO, candidate microbes and the tests that detect them ideally meet the following criteria according to The Routledge Handbook of Water and Health (Bartram et al. 2015):

- Be present whenever enteric pathogens are present
- Be absent whenever enteric pathogens are absent, or at levels that pose no increased risk
- Be present in greater numbers than pathogens
- Have similar or greater survival rates than pathogens in the environment
- Have broad applicably and detectability in all types of water that humans may encounter
- Be specific to a fecal source with humans or species who share fecal-oral pathogens with humans
- Do not multiply independently in the environment
- Be reliably, rapidly, and distinctly detectable at low-cost
- Be randomly distributed in a given sample

While no fecal indicator organisms to date satisfy all requirements under all circumstances, many regulatory agencies and scientific governing bodies consider *Escherichia coli* (*E. coli*), *enterococci*, and members of the fecal coliform group as the "gold standards" of microbial water quality testing (US EPA 2009). Total coliforms, fecal streptococci, *Clostridium perfringens,* and coliphages are also fecal indicator organisms.

Many presence/absence (PA), semi-quantitative, and quantitative methods have been developed to detect FIO in water resources. PA tests provide simple positive-negative results and are most applicable in situations where water is usually uncontaminated and when most samples provide negative test results. On the other hand, semi-quantitative and quantitative methods provide both positive-negative results and microbial concentration estimations. These features are useful for categorizing water safety levels and estimating potential human health risk. Methods of this nature are most applicable in situations where fecal contamination is likely (WHO 1996). Furthermore, quantitative methods are often desired to satisfy the information and monitoring needs of operational, compliance, and surveillance sampling regimes (Bain et al. 2012). Widely recognized microbial water quality methods approved by organizations such as the United States Environmental Protection Agency (EPA), the World Health Organization (WHO), and the International Organization for Standardization (ISO) include most probable number (MPN), membrane filtration, quantitative polymerase chain reaction (qPCR), and use of defined substrates such as the Coliert Quanti-Tray MPN test (Fewtrell & Bartram, 2001; U.S. EPA, 2015).

Challenges monitoring water quality in low-resource settings

While many methods for detecting and enumerating FIO in water have been developed, implementing them in many parts of the world proves logistically challenging (Bain et al. 2012). Many communities impacted by poor microbial water quality reside in rural and/or resource-limited

environments (Anwar et al. 1999). Furthermore, acute diarrheal infections are one of the most frequent childhood illnesses and reasons for visits at health clinics in low- and middle-income countries (Walker et al. 2013). The lack of human, financial, and technological capacity in low-resource settings limits the ability of countries and communities to monitor water resources (Crocker & Bartram 2014). Similar obstacles arise during humanitarian emergencies such as natural disasters or wartime conflict, which often put utilities and local water supplies in jeopardy. Safe water and sanitation, along with food and shelter, receive the highest priority as first-phase interventions during emergency situations (UNICEF 2012). Due to logistical challenges frequently encountered in emergency and low-resource settings, the need for inexpensive, rapid, and reliable fecal contamination detection methods is paramount.

H_2S-producing bacteria and the H_2S field test

The hydrogen sulfide (H_2S) field test was first developed by Manja et al. (1982) as a low-cost, reliable, and simple microbiological water quality test to detect bacteria that produce hydrogen sulfide (H_2S) gas. There are several genera and species of bacteria that can produce hydrogen sulfide. A major group of environmental bacteria are referred to as sulfate reducing bacteria (SRB). SRB play a key role in the global sulfur cycle and can be found in many habitats, including marine and fresh waters, soils and sediments, biofilms and intestinal contents, and hot springs and hydrothermal sea vents. SRB include both non-pathogenic and pathogenic groups, such as *Desulfovibrio*, *Bacillus*, *Pseudomonas,* fecal coliforms (*Citrobacter freundii, Klebsiella pneumoniea*, *K. oxytoca, Enterobacter cloacae), Salmonella*, *Proteus*, *Edwardsiella*, *Yersinia, Aeromonas, Clostridium, Staphylococcus, Peptococcus, Campylobacter,* and H_2S-producing variants of *E. coli* (Sobsey & Pfaender 2002; Bartram et al. 2015). Hydrogen sulfide production by SRB frequently occurs in anaerobic environments were oxygen is not readily available. In the H_2S test, sulfate reducing bacteria in the media reduce inorganic sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S), which combines with iron (Fe) in the test medium to form ferrous sulfide (FeS). Ferrous sulfide is a

non-soluble black precipitate that is readily distinguishable and denotes a positive reaction (Madigan et al. 2008).

Overview of H_2S test history, method development, and subsequent modifications

In the initial 1982 report, Manja et al. compared a novel H_2S presence/absence (PA) paper strip test to a standard *E. coli* MPN test to detect fecal contamination in drinking water samples in several cities in India. Drinking water samples were added to sterilized bottles containing a reagent of ferrous iron, sulfate salts, and nutrients to promote the growth and metabolism of the bacteria of interest. Samples were observed for black color change over 12-18 h and 24-48 h periods at ambient temperatures (30-37° C). When *E. coli* was detected at levels greater than or equal to 10 MPN/100 mL, the sample was also tested using the H_2S test. Researchers observed the presence of coliform bacteria in drinking water was consistently associated with organisms that produced H_2S. They also reported good agreement between the two methods at higher levels of *E. coli* contamination (> 40 MPN/100 mL) (Manja et al. 1982).

Over the past three decades, many versions and modifications of the H_2S test have been described in the literature. Modifications to the H_2S test include changes in medium composition, preparation of the medium and supporting materials, test format, sample volumes, incubation time, incubation temperature, and scoring of results. Many investigators have evaluated the H_2S method by comparing it to traditional fecal indicator bacteria (FIB) methods under controlled lab conditions or in tropical and subtropical regions such as Indonesia, Peru, Paraguay, Chile, Nepal, and South Africa (Ratto et al. 1989; Kromoredjo & Fujioka 1991; Kaspar et al. 1992; Castillo et al. 1994; Venkobachar et al. 1994; Rijal & Fujioka 2001; Genthe & Franck 1999).

Since the original H_2S test, the addition of cystine or cysteine to the medium composition has been found to increase test sensitivity (Pillai et al. 1999; Venkobachar et al. 1994; Manja et al. 2001; S. P.

Pathak & Gopal 2005; Shahryari et al. 2014; Sobsey & Pfaender 2002). Additionally, longer incubation periods between 24 h to 48 h and incubation temperatures in the range of 25-35° C have shown best results in terms of detecting low levels (5 CFU per sample) of H_2S-producing bacteria (Pillai et al. 1999; Gawthorne et al. 1996; Ratto et al. 1989; Castillo et al. 1994; Genthe & Franck 1999; Manja et al. 2001; Tambekar & Neware 2012; Gupta et al. 2007; Sobsey & Pfaender 2002). The H_2S presence/absence test format has been evaluated extensively, with many versions experiencing widespread commercialization and field use (Bain et al. 2012). More recently, semi-quantitative methods using MPN or membrane filtration formats have been developed (Venkobachar et al. 1994; Rijal & Fujioka 2001; McMahan 2011; Roser et al. 2005; McMahan et al. 2011; McMahan et al. 2012). The H_2S test is typically performed using 10-100 mL sample volumes. Methods to score results typically employ presence/absence (positive-negative) results or MPN or CFU per sample volume concentration estimations. The H_2S test has been frequently evaluated via comparison with traditional FIO including *E. coli*, fecal coliforms, total coliforms, fecal streptococci, and enterococci (Pillai et al. 1999; Ratto et al. 1989; Venkobachar et al. 1994; Gawthorne et al. 1996; Rijal & Fujioka 2001; Nair et al. 2001; McMahan 2011; Tambekar & Neware 2012; Khush et al. 2013; Yang et al. 2013a; Weppelmann et al. 2014a; Shahryari et al. 2014; Roser et al. 2005; Sivaborvon 1988; Martins et al. 1997). As none of these indicators are ideal at detecting fecal contamination in water, the results of such comparisons are open to interpretation. However, most investigators assume that if the H_2S test gives positive results at rates similar to or greater than the reference test, its performance is acceptable (Sobsey & Pfaender 2002). Frequently used fecal indicator reference tests include the Coliert Quanti-tray defined substrate MPN test (McMahan 2011; Khush et al. 2013; Chuang et al. 2011), the multiple fermentation tube test (Anwar et al. 1999; Hirulkar & Tambekar 2006; Shahryari et al. 2014), membrane filtration (Rijal & Fujioka 2001; Hirulkar & Tambekar 2006; Tambi et al. 2016; Weppelmann et al. 2014a; Gupta et al. 2007; S. P. Pathak & Gopal 2005), and the Eijkman test (Hirulkar & Tambekar 2006; Manja et al. 1982).

Strengths of the H_2S field test

Several investigators have attempted to determine the reliability of the H_2S test for the detection of fecal contamination in drinking water. Overall, their research indicates there is a strong correlation between the H_2S test and traditional fecal indicator bacteria, and that the H_2S method detects fecally contaminated water with about the same frequency and magnitude as traditional comparison methods (Ratto et al. 1989; Castillo et al. 1994; Venkobachar et al. 1994; Genthe & Franck 1999; Rijal & Fujioka 2001; Manja et al. 2001; Sobsey & Pfaender 2002; McMahan 2011; Tambi et al. 2016; Sivaborvon 1988; Martins et al. 1997; Dufour et al. 2013; Manja et al. 1982; Anwar et al. 1999; Nair et al. 2001; Hirulkar & Tambekar 2006; Gupta et al. 2007; Eun & Hwang 2003; Kromoredjo & Fujioka 1991; McMahan et al. 2012).

Additionally, multiple studies have demonstrated the H_2S test's ability to meet many of the criteria required for consideration as an ideal fecal indicator. Similar or greater survival of H_2S-producing organisms to pathogens has been demonstrated (Martins et al. 1997; Castillo et al. 1994; Nagaraju & Sastri 1999), along with greater numbers of H_2S-producing bacteria than pathogens found in sample waters (Nagaraju & Sastri 1999; Castillo et al. 1994; Manja et al. 1982). While the H_2S test does not consistently measure the presence of total coliforms, fecal coliforms, or *E. coli*, many members of the fecal coliform group are known H_2S-producers, including *Klebsiella pneumoniae, K. oxytoca*, *Enterobacter cloacae, and Citrobacter freundii* (LeClerc et al. 2001). Moreover, Sobsey and Pfaender (2002) suggest that organisms-producing positive H_2S results may not all be coliforms but are typically associated with the intestinal tracts of warm-blooded animals. Castillo et al. (1994) found a large variety of bacteria in samples giving positive reactions in the H_2S test, primarily *Clostridium perfringens,* Clostridia, and members of *Enterobacteriaceae* (i.e. *Enterobacter, 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. Many other studies have

found H_2S-producing bacteria to be specific to a fecal source or identifiable as to a source of origin via comparison to standard fecal indicators (McMahan 2011; Manja et al. 1982; Manja et al. 2001; Nagaraju & 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 & Fujioka 1991; Genthe & Franck 1999; Sivaborvon 1988). The H_2S test demonstrates broad applicability, as it has been applied to diverse global water sources including groundwater, surface water, bore wells, dug wells, rainwater cisterns, and municipal water supplies (Ratto et al. 1989; Castillo et al. 1994; Martins et al. 1997; Kromoredjo & Fujioka 1991; Genthe & Franck 1999; Sivaborvon 1988). Adequate or superior detectability has been documented in many cases (Ratto et al. 1989; Anwar et al. 1999; Genthe & Franck 1999; Rijal & Fujioka 2001; Roser et al. 2005), and results can be rapidly obtained in 24 h for heavy-to-moderate contamination and 48 h for light contamination (Manja et al. 2001; Manja et al. 1982; Nagaraju & Sastri 1999; Venkobachar et al. 1994; Castillo et al. 1994; Martins et al. 1997; Genthe & Franck 1999; Rijal & Fujioka 2001; Weppelmann et al. 2014a; Izadi et al. 2010). While PA H_2S tests do not provide quantifiable results, recently developed H_2S MPN tests have shown similar detection and agreement with standard FIB methods (McMahan 2011; McMahan et al. 2012; Venkobachar et al. 1994; Rijal & Fujioka 2001; Manja et al. 2001). Precision of results among samples has been documented, though not between labs (Martins et al. 1997; Rijal & Fujioka 2001). Genthe and Franck (1999) were also able to demonstrate measures of viability and infectivity with H_2S-producing bacteria. While more research is needed, studies by McMahan (2011) and Kush et al. (2013) show potential for the H_2S test to be associated with risk of diarrheal illness.

In some cases, the H_2S test may be more applicable than traditional fecal indicator tests based on other criteria for evidence of fecal contamination. For instance, Gawthorne et al. (1996) suggested the H_2S test works well as a presumptive test for the detection of Salmonella. Furthermore, unlike traditional indicators such as fecal coliforms, *E. coli,* and enterococci, the H_2S test is able to detect spores

of *Clostridium perfringens* and related sulfite-reducing clostridia, which serve as better indicators of protozoan parasites such as Giardia cysts and Cryptosporidium oocysts (McMahan 2011). Roser et al (2005) also contended that the H_2S test appears much more sensitive than measurements of somatic and male-specific (F+) coliphages and protozoan pathogens in their study, and that overall the H_2S test shows fairly high sensitivity, specificity and precision when comparing results across studies (Roser et al. 2005).

Perhaps one of the most promising aspects of the H_2S test is its practicality for low-resource and emergency settings. H_2S test kits are relatively easy to manufacture and are often made locally at lower cost than standard methods (Genthe & Franck 1999). Bain et al. (2012) estimated the cost per test of four common commercialized H_2S PA tests to range from $0.60 to $2.40 USD per sample. The H_2S test has been applied in many developing countries, in emergencies (Mosley & Sharp 2005), and in remote areas of developed countries (UNICEF 2008). The test's simple training and personnel needs, utility in the field, low-cost, and moderate volume requirements are reasons researchers have justified continued evaluation of the method (Genthe & Franck 1999; Venkobachar et al. 1994; Castillo et al. 1994; Ratto et al. 1989; Anwar et al. 1999; S. P. Pathak & Gopal 2005; Hirulkar & Tambekar 2006; Bain et al. 2012; Khush et al. 2013; Weppelmann et al. 2014a; Walker et al. 2013; Tambi et al. 2016; Sivaborvon 1988; Mosley & Sharp 2005), despite notable test weaknesses.

Weaknesses of the H_2S field test

While a promising alternative to traditional fecal indicators, regulatory agencies and scientific governing bodies such as the WHO and EPA have not accepted or recommended H_2S-producing bacteria as alternative fecal indicators (Sobsey & Pfaender 2002). One of the primary reasons is the inability of the H_2S test to meet all criteria for ideal FIO. For example, an ideal fecal indicator should pose no risk to human health. However, H_2S-producing organisms may themselves be pathogenic depending on the concentration present in water samples (McMahan 2011). Inconsistent precision, specificity, and

sensitivity have also been documented among labs (Wright et al. 2012; Izadi et al. 2010; Tewari et al. 2003; Desmarchelier et al. 1992; Yang et al. 2013a). Ideal fecal indicators should also be absent in unpolluted water and present only when waters are fecally contaminated. However, studies have shown that multiple microorganisms produce H_2S, many of which occur naturally in waters that are not fecally contaminated (Sobsey & Pfaender 2002; Ratto et al. 1989; Kaspar et al. 1992; Venkobachar et al. 1994; Sivaborvon 1988; Martins et al. 1997). Similar concerns arise when the H_2S test is conducted in waters with higher levels of naturally occurring iron or sulfide, potentially leading to false positive results. 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). In this case, the rapid reaction of iron with sulfide already present in water samples could produce a darkening of H_2S tests almost immediately upon addition of samples. For this reason, Sobsey & Pfaender (2002) advise visual inspection of H_2S tests for quick or early positive reactions, between a few minutes to an hour of incubation.

While there appears to be no reasonable way to preclude all non-fecal H_2S producers from water sources, understanding the ecology of H_2S producers (i.e. sulfate reducing bacteria, SRB) may explain the likelihood of false positives of this nature. Wetzel (2001) noted that there would be little sulfate for bacteria to use if concentrations are low in freshwater (Wetzel 2001). On the other hand, in settings where sulfate concentrations are high, such as in geothermal environments, SRB could give false positive results in H_2S tests. Another point to consider is that sulfate reducers do not metabolize complex organic compounds such as those used as substrates in H_2S test mediums. Rather, they require short chain organic acids and other products of fermentation. Therefore, it is possible SRB alone would not grow and give positive results in H_2S tests (McMahan 2011). However, Widdel (1988) cautions that in mixed communities of microorganisms, SRB could give a positive result due to the fermentation of sugars by heterotrophic bacteria, thus providing the organic acids used by SRB to give a positive result

(Widdel 1988). However, 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 substrates leading to the growth of SRB in a 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 (Sobsey & Pfaender 2002). Furthermore, McMahan et al. (2012) demonstrated that a semi-quantitative H_2S compartment bag test was not impacted by high sulfur and high iron levels in well-water samples. In addition, they demonstrated a consistent association between positive H_2S test results and species identified in positive samples with fecal indicator organisms and enteric pathogens present in natural waters (lake, wells, and cistern rainwater) in the United States (McMahan et al. 2012).

Another major concern regarding the H_2S test is the lack of standardization across tests. As previously described, numerous modifications and versions of the H_2S test have been developed. Variations include medium composition, medium preparation (dried at elevated temperature, lyophilized, autoclaved only, etc.) sample volume (20 mL, 100 mL, etc.), paper use, paper type, and paper size to which the medium is absorbed, incubation times and temperatures, test formats (PA, semi-quantitative MPN, membrane filter enumeration), and methods to score results (Sobsey & Pfaender 2002). The multitude of different H_2S test versions, as well as the variety of ways they have been evaluated in field and lab studies, makes comparisons across tests difficult (Wright et al. 2012) . While efforts have been made in India and the United States to make commercially prepared medium and implement performance criteria, the test is not standard worldwide and there has been no effort to achieve a standard test procedure (Sobsey & Pfaender 2002).

The proliferation of H_2S PA tests vs. quantitative tests poses another problem for method validation by regulatory agencies. At this time there are no widespread or commercially available quantitative H_2S tests on the market, although there are multiple H_2S PA tests available (Bain et al. 2012). With the basis of WHO Guidelines for Drinking Water Quality shifting towards risk-based data,

the absence of a microbial risk data for H_2S PA tests raises concerns about their validity and interpretation in judging drinking water quality (Bain et al. 2012). This shift increases the need for development of a reliable and affordable quantitative H_2S method. Semi-quantitative test formats provide more information than standard PA tests, as they provide a concentration estimate 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 microbial contamination in water to waterborne disease risk. Semi-quantitative H_2S tests can also be more easily compared to standard semi-quantitative fecal indicator methods, such as the multiple test tube (MTT) and Quanti-tray defined substrate MPN tests.

Recommendations for best use of H_2S test

Current recommendations for best application of the H_2S test vary. Most studies agree the H_2S test is a viable option when no other options exist in emergency or low-resource settings. Promoting the test as a motivational, educational, and empowerment tool on the community- and individual-level has also been advised (Sobsey & Pfaender 2002; Kaspar et al. 1992). Others have recommended conducting H_2S testing in tandem with standard FIO methods (Gawthorne et al. 1996), comparing it to standard methods before widespread deployment (Yang et al. 2013a; Wright et al. 2012; Kaspar et al. 1992; Weppelmann et al. 2014a) or testing in conjunction with other inexpensive FIO field tests (Chuang et al. 2011). Nair et al. (2001) advocated for using the H_2S test in developing countries where acceptable levels of fecal indicators in drinking water are <10 MPN/100 mL (Nair et al. 2001). Gawthorne et al. (1996) recommended using the H_2S test as a presumptive test for *Salmonella* in drinking water in conjunction with coliform testing (Gawthorne et al. 1996).

While more research is needed on certain aspects of the H_2S field test, the test's consistent association with fecal contamination, correlation with standard FIB methods, and ability to easily,

rapidly, and affordably detect a variety of fecal indicator organisms makes studying the H_2S test a worthy endeavor in the effort to provide microbiologically safe drinking water for all.

Compartment bag test method, format description, and history of validation

In 2007 researchers at the University of North Carolina at Chapel Hill and Duke University developed a simple kit for enumerating *E. coli* concentrations in water that is portable, relatively inexpensive, and provides easy-to-interpret results (Stauber et al. 2014). This kit, commonly referred to as the compartment bag test (CBT), is currently manufactured and distributed by Aquagenx, LLC (Chapel Hill, NC, USA). The CBT consists of a clear plastic multi-compartment bag into which 100 mL of water sample is distributed. Bacteria are detected using an *E. coli* growth medium containing a chromogenic substrate (*5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid*). The CBT does not require an incubator if ambient temperatures remain between 25-44.5° C. Positive results are indicated by a blue color-change in one or more of the compartments. Users can estimate *E. coli* concentrations by matching up the number and order of positive and negative compartments with a user-friendly table that follows a Poisson probability distribution assumption to generate discrete MPN/100 mL values and 95% confidence intervals (Appendix 1). Concentration estimates for the CBT are generated based on conventional MPN methods, calculating quantiles of the likelihood function of *E. coli* concentrations, and employing Bayesian Markov chain Monte Carlo (MCMC) analysis methods, as described by Gronewold et al. (2017). Bayesian analysis considers the probability distribution curves and likelihood functions of target microbes in samples to infer bacteria concentrations.

McMahan (2011) estimated the cost of the *E. coli* CBT to be around $1.70 USD per sample, while 2017 cost estimates per test range from $5.00-$10.00 USD. Additionally, the shelf-life of the *E. coil* CBT is approximately 13 months (Aquagenx, 2017). The *E. coli* CBT has been compared to standard methods and popular field tests and has demonstrated consistent and reliable results compared to traditional fecal indicators in water sources (Murcott et al. 2015; Stauber et al. 2014; Weiss et al. 2016). The test

has grown in popularity as a user-friendly and cost-effective microbial detection kit and has been applied successfully in countries such as India, Vietnam, Nicaragua, Peru, Haiti, Ethiopia, Ghana, and Vanuatu (Weiss et al. 2016; Murcott et al. 2015; Stauber et al. 2014; Heitzinger et al. 2016; Adank et al. 2016; Morrison 2016; Gerges et al. 2016; McMahan et al. 2017).

Combining H_2S detection medium with the compartment bag test

In a PhD dissertation McMahan (2011) studied the feasibility of a novel semi-quantitative H_2S test which combined a commercial H_2S detection substrate and culture medium with the compartment bag test. Several field and lab studies were conducted to compare the new test to traditional H_2S and FIB detection methods. Comparisons included lab-made H_2S medium vs. a commercial substrate (PathoScreen™ by HACH, Loveland, CO), Whirl-pak plastic bags vs. plastic bottles, CBT versus Coliert Quanti-Tray to detect H_2S-producing bacteria and *E. coli*, and H_2S CBT vs. six popular field tests (MI Agar, BioRad Rapid *E. coli* 2 Agar, Coliert, Petrifilm *E. coli*, EasyGel, and *E. coli* CBT) for a cost per sample analysis. Overall, McMahan found the proprietary H_2S powder to work as well as lab-prepared medium, the Whirl-pak bags to detect H_2S on par with plastic bottles, and no significant difference between the CBT's ability to detect H_2S and Quanti-Tray's ability to detect *E. coli* at similar incubation temperatures. The H_2S CBT was also found to be the most cost-effective field test, at an estimated $0.40 USD per sample (other tests ranged from $1.70 - $15.00 USD per sample).

A study by McMahan et al. (2011) used biochemical and molecular methods to determine whether the H_2S CBT test could correctly identify sewage-contaminated waters. Researchers used culture-based (spread-plating with differential and selective agar) and molecular (Terminal Restriction Fragment Length Polymorphism, TRFLP) methods to identify types and numbers of fecal indicator organisms, pathogens, and other microbes present in sewage samples with positive H_2S test results. Isolates identified from each method were tested to confirm their ability to produce H_2S and were identified to the genus and species level. The study found that positive H_2S tests consistently contained

fecal bacteria and pathogens. They also found strong relationships of agreement between organisms identified by both methods tested. Researchers concluded the study provided an important step towards determining the H_2S tests' accuracy and specificity (McMahan et al. 2011).

Another study by McMahan et al. (2012) used biochemical (spread-plating with differential and selective agar) and molecular (TRFLP) methods to evaluate the ability of the H_2S CBT test to associate with fecal indicator organisms, pathogens, and other microbes present in natural waters (lake, wells, and cistern rainwater) in North Carolina, United States. Researchers showed that water samples testing positive for H_2S-producing bacteria also had bacteria of likely fecal origin and waters containing fecal pathogens were also positive for H_2S bacteria. They also found that greater than 70% of isolates from natural waters were identified using TRFLP analysis and revealed a relatively stable group of organisms whose community composition differed with water source over time. The study further documented the validity of the H_2S test for detecting and quantifying fecal contamination in water (McMahan et al. 2012).

Study niche and objectives

Since McMahan's initial feasibility studies, no further work has been done to validate the H_2S CBT as a new semi-quantitative H_2S field test. The many promising aspects of the test, including its informative semi-quantitative format, affordability, ease-of-use, and longer shelf-life (approximately 3 years) compared to the *E. coli* CBT make the H_2S CBT worth evaluating as a breakthrough microbial water quality test for emergency and low-resource environments. To better determine the reliability and quantification capabilities of the H_2S CBT, the test must be compared with standard semi-quantitative microbial water quality tests. The new test must also be subjected to incubation times and temperatures that both reflect real-world conditions and optimal growth conditions cited in the literature. The main objective of this study was to compare the compartment bag test (CBT) to the

multiple test tube (MTT) method to quantify H_2S-producing bacteria in drinking water sources using a most probable number (MPN) format. The study will also seek to determine the effect of incubation time and temperature on test results within and between methods. Validation of a reliable, semi-quantitative H_2S test would go a long way towards helping standardize the H_2S method by allowing for comparison with other standard methods and collection of risk-based microbial water quality data.

CHAPTER 4: METHODS

The compartment bag test (CBT) and multiple test tube (MTT) technique were compared by collecting and testing lake water samples over the course of three months from July 2016 to September 2016 at the University of North Carolina at Chapel Hill in Chapel Hill, North Carolina, United States.

Sample Sites

Surface water samples were collected from two reservoirs, University Lake (UL) and Cane Creek Reservoir (CC), that are used as municipal drinking water sources. University Lake is located in Chapel Hill, North Carolina and holds 450 million gallons of water and covers a surface area of 213 acres. The lake provides habitat for terrestrial and aquatic wildlife and is fed by five tributaries primarily passing through agricultural, suburban, and forested areas (Figure 1). Cane Creek Reservoir is located in Orange County, North Carolina and holds three billion gallons of water and covers a surface area of 540 acres. The lake is also a wildlife habitat and is fed by four tributaries passing primarily through agricultural and forested areas (Figure 2).

At each reservoir, five sites were selected as sample collection locations. For University Lake, one site was located directly upstream of the reservoir and four sites were located throughout the reservoir. For Cane Creek Reservoir, one site was located directly upstream of the reservoir, one site directly downstream of the reservoir, and three sites dispersed around recreational access points. Selection of sampling sites was based on location within reservoir (upstream, downstream, middle), ease of access from shoreline, and proximity to factors that may influence microbial water quality (e.g. resident geese colony, public access points, forested areas).

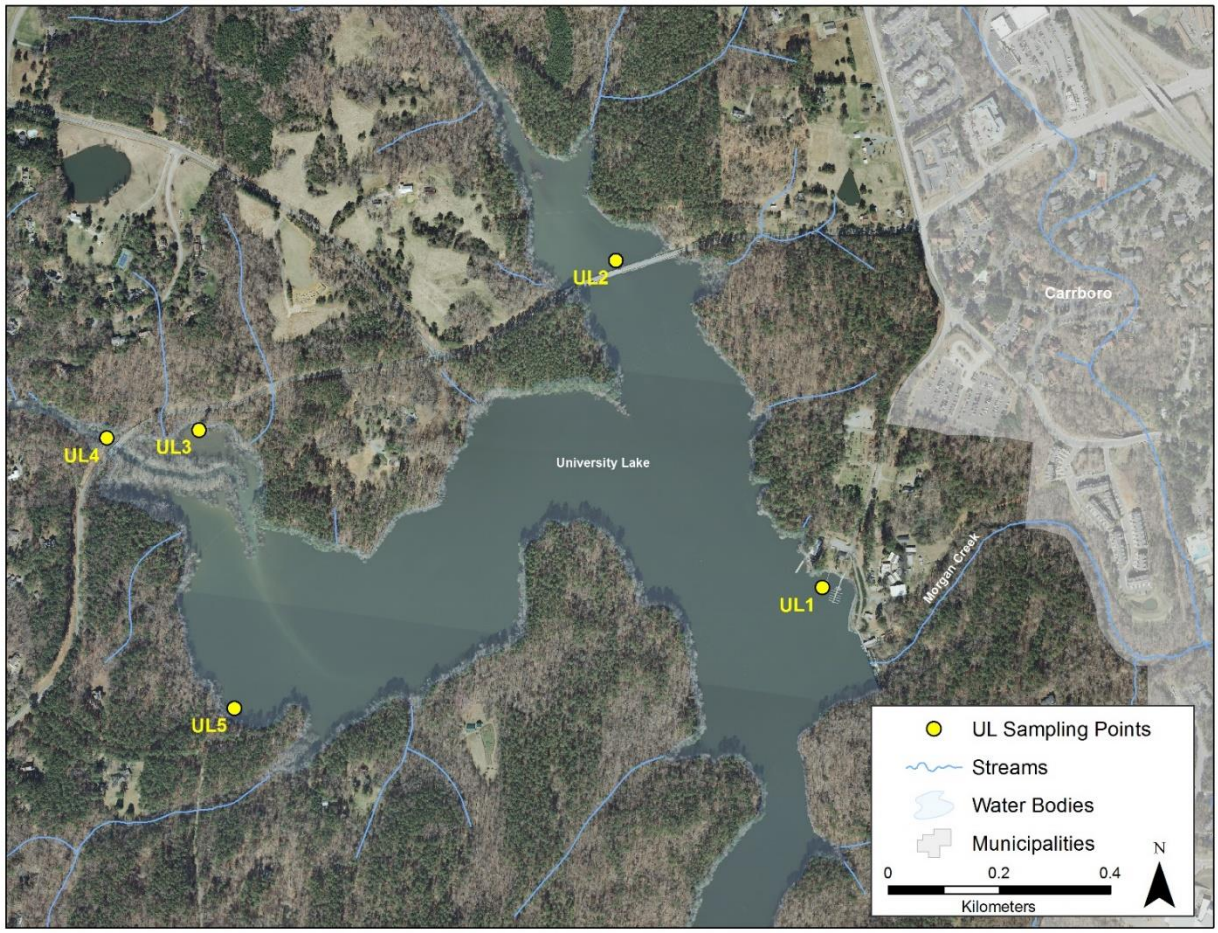


Figure 1: Map of sampling locations at University Lake (UL) located in Chapel Hill, North Carolina

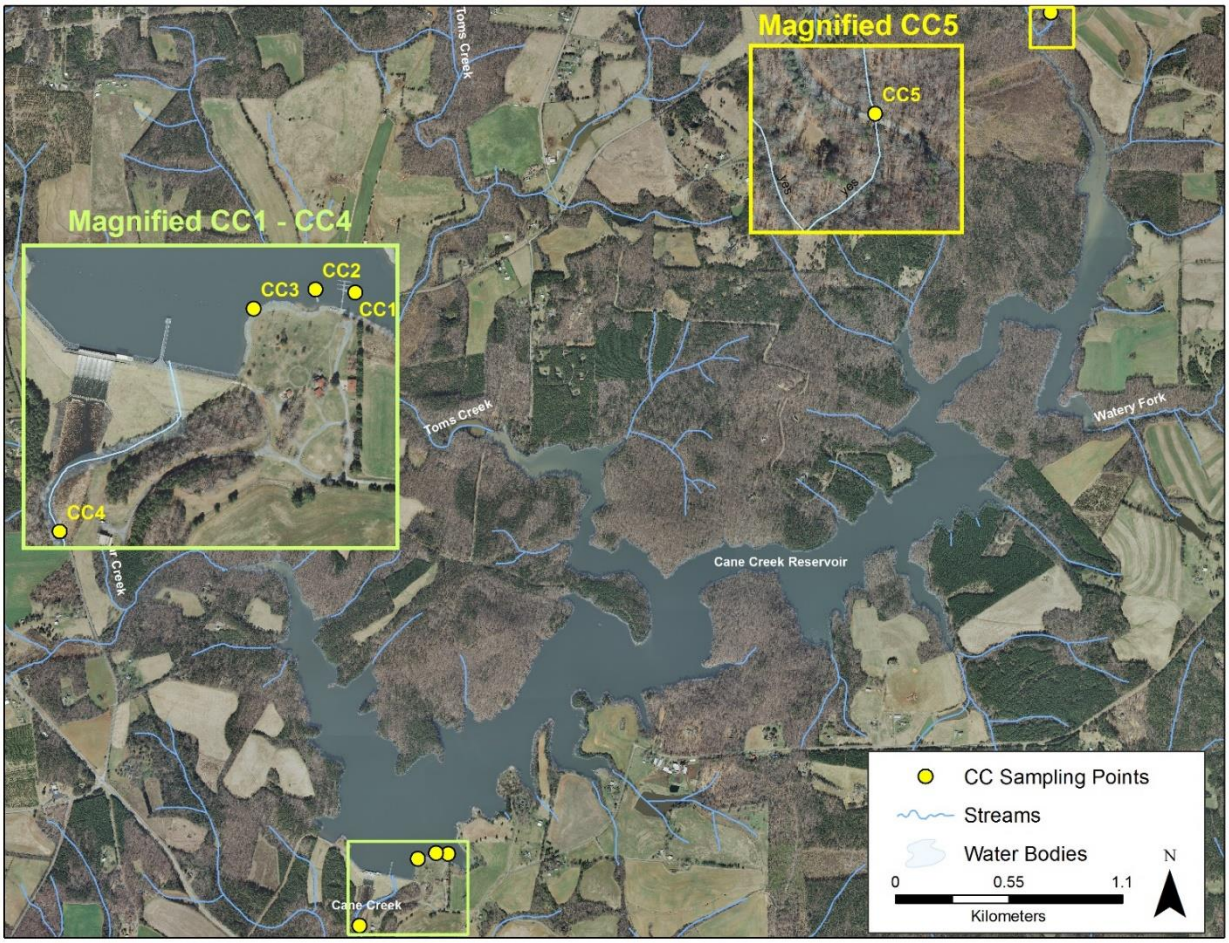


Figure 2: Map of sampling locations at Cane Creek Reservoir (CC), located in Orange County, North Carolina. Sample sites CC1-CC5 have been magnified to (images within boxes) to show landscape around sites.

Sample Collection

Two replicate lake water samples were collected from each sampling site three times between July 2016 to September 2016, for a total of 60 field samples collected. Sampling alternated each week between University Lake and Cane Creek Reservoir so that each site was visited every other week.

Lake water samples were collected in autoclaved 1-L polypropylene containers using aseptic techniques and sampling methods adhering to the Environmental Protection Agency's (EPA) Region 4 surface water sampling methods (Decker & Simmons 2013). Upon collection, samples were stored in insulated containers filled with ice and transported immediately to a lab for processing. All samples were processed within six hours after field collection.

Physical and chemical environmental parameters were also collected at each sampling site during each sampling event using a YSI Professional Plus Multiparameter Instrument (Xylem Inc.). Parameters collected included air temperature (° C), water temperature (° C), pH, specific conductivity (µS/cm), and dissolved oxygen (% and mg/L). Current weather conditions and cloud cover (sunny, partially cloudy, and cloudy) were also recorded at each sampling site. In addition, total precipitation (cm) up to 72 h prior to sampling was collected for each sampling event using Weather Underground rain gauges stationed at the Horace Williams Airport in Chapel Hill, NC. The rain gauge is approximately three miles from University Lake and ten miles from Cane Creek Reservoir.

Sample Processing

All samples were diluted 1:10 and 1:100 in sterile phosphate buffered saline (PBS; Fisher Scientific) to achieve quantification of a countable range of bacteria. Volumes of 100 mL of each sample-dilution were then analyzed by paired compartment bag test (CBT) and multiple test tube (MTT) tests, and were then incubated at either 25° C or 35° C over the course of three days. All tests were checked for the presence of black ferrous sulfide (FeS) at 20-24 h (1 day), 44-48 h (2 days) and 68-72 h (3 days). Incubation temperatures and times were selected based on optimal growth conditions for H_2S-

producing bacteria cited in the literature. In total, 60 tests were conducted per sample dilution-temperature combination, resulting in 120 tests per method by temperature, 120 tests per method by dilution, and 240 tests total per method (Table 1).

Table 1: Number of samples per CBT and MTT method based on dilution and temperature combinations.

Dilution	Temperature (° C)	Number of CBT Tests	Number of MTT Tests	Total
1:10	25	60	60	120
	35	60	60	120
1:100	25	60	60	120
	35	60	60	120
Total		240	240	480

The CBT method consisted of a clear polyethylene bag divided into five compartments of 1, 3, 10, 30 and 56 mL sample volumes (100 mL total) to allow for MPN enumeration. The method was conducted using aseptic techniques adhering to the manufacturer's user manual for drinking water testing in the field (Aquagenx 2015). Briefly, 100 mL of diluted sample water was added to a sterile plastic collection bottle. One HACH PathoScreen™ powder pillow was cut and poured into the collection bottle and swirled to dissolve. Once dissolved, the sample solution was poured into each compartment of a sterile compartment bag test. The compartment bag was then sealed off to isolate the compartments and placed in an incubator to promote bacterial growth.

The MTT method consisted of ten 16x150 mm glass test tubes from Fisher Scientific, each holding 10 mL sample volumes (100 mL total) to allow for MPN enumeration. The method was conducted using aseptic techniques adhering to the FDA's Bacteriological Analytical Manual (Blodgett 2010). Briefly, 100 mL of diluted sample water was added to a sterile plastic collection bottle. One HACH PathoScreen™ powder pillow was cut and poured into the collection bottle and swirled to dissolve. Once

dissolved, the sample solution was added in 10 mL volumes to ten sterile glass test tubes. Tubes were capped and placed in an incubator to promote bacterial growth.

A set of positive and negative controls were included during each sampling event. The positive control (PC) consisted of a pair of CBT and MTT tests containing 100 mL sterile PBS inoculated with *Salmonella enterica* serovar Typhimurium strain LT2, a known H_2S-producer. The negative control (NC) consisted of a pair of CBT and MTT tests containing only 100 mL sterile PBS media. PC and NC tests were diluted to 1:10, incubated at 35° C, and checked over the course of three days alongside field samples.

On days 1, 2, and 3 all tests were temporarily removed from incubators to check for growth. Any black liquid or solid color-change in compartment bags or test tubes indicated the presence of H_2S-producing bacteria. The combination of positive tubes or compartments for each test were recorded in a lab notebook and used to calculate MPN/100 mL and corresponding 95% confidence intervals (CI) as described below.

In addition to the CBT and MTT tests, 100 mL from each sample was processed through a Coliert-18 Quanti-Tray/2000 test (IDEXX Laboratories Inc., Westbrook, ME) to detect concentrations of total coliform and *Escherichia coli (E. coli)* in sample waters. The method was conducted using aseptic techniques adhering to the manufacturer's guidelines (IDEXX 2013). Samples were diluted 1:10 before processing and were incubated at 35° C for 18-20 h before checking for signs of bacterial growth. Results were computed as MPN/100 mL and reported with 95% CI.

Data Analysis

To obtain a single concentration estimate per sample, MPN results from each sample's dilution (1:10 and 1:100) were consolidated using the FDA-approved formula of Thomas (1942):

$$\mathrm{MPN/mL} = (\sum \mathrm{gj}) \,/\, (\sum \mathrm{tjmj} \sum (\mathrm{tj} - \mathrm{gj})\,\mathrm{mj})\ (½)$$

Figure 3: Formula of Thomas (1942) equation

Where the summation is over each dilution (1:10, 1:100) and $\sum g_j$ denotes the number of positive tubes in the selected dilutions, $\sum t_j m_j$ denotes the grams of sample in all tubes in the selected dilutions, and $\sum (t_j - g_j)\, mj$ denotes the grams of sample in all negative tubes in the selected dilutions. To obtain approximate 95% confidence intervals, the FDA-approved method of Haldane (1939) was used to estimate the standard error of $\log_{10}$(MPN) of each sample:

$$\text{Standard Error of Log10(MPN)} = 1/(2.303 * \text{MPN} * (\text{B\^{}0.5}))$$

Figure 4: Part I of Method of Haldane (1939) equation

Where B equals the sum of the exponents of each dilution's negative MPN, multiplied by the dilution amount. Finally, 95% lower and upper confidence intervals were obtained using the following equation:

$$\text{Log10(MPN)} \pm 1.96 * (\text{Standard Error})$$

Figure 5: Part II of Method of Haldane (1939) equation

Applying the Thomas (1942) and Haldane (1939) equations resulted in 48 discrete MPN/100 mL and 95% CI outcomes for the CBT method, and 51 discrete MPN/100 mL and 95% CI outcomes for the MTT method (Appendix 3). The lowest level of detection for both tests was 9 MPN/100 mL, while the highest level of detection was 1812 MPN/100 mL. Discrete values were assigned to left- censored results to allow for inclusion in data analyses. Left-censored (all negative) results were assigned 4.5 MPN/100 mL, or half (½) the value of the lowest level of detection (9 MPN/mL). Right-censored (all positive) results were not included in data analyses, due to the inherent difficulty of determining true value of right-censored results according to Gronewold et al. (2017). A total of 10 right-censored results were removed, resulting in 350 observations of CBT-MTT paired tests over the course of three incubation days.

Data analysis was conducted using Excel 2016 and Statistical Analysis Systems (SAS 9.4). SAS data steps and proc steps were used to manage, transform, and compare paired CBT and MTT MPN/100 mL concentration estimates and 95% confidence intervals. Proc univariate was used to conduct Shapiro-Wilk normality tests on untransformed data and natural log (ln) and log base 10 (log_{10}) transformed data. Descriptive statistics (proc means) were generated to compare CBT vs. MTT means, medians, and median differences and spearman's correlation coefficients (proc corr) were run to test for direction and strength of method association. Contingency tables (proc freq) analyzed percent overlap of positive-negative results, risk-based categorical results, and acceptable drinking water range results. Frequency counts (proc freq) assessed percent overlap of CBT MPN estimates to paired MTT 95% upper and lower confidence intervals. CBT and MTT Log_{10}(MPN) transformed values were compared to each other and the effect of days and temperature using a linear regression model (proc glm). A logistic regression model (proc logistic) was also used to evaluate the effects of incubation (temperature, days) and environmental (precipitation, air temperature, water temperature, pH, specific conductivity, and dissolved oxygen) conditions on the odds of CBT MPN values falling within paired MTT 95% CI. Spearman's correlation coefficients (proc corr) and linear regression (proc glm) were also used to compare replicate sample concentration estimates. Finally, spearman's correlation coefficients (proc corr) were generated to observe relationships between CBT and MTT concentration estimations and total coliform and *E. coli* concentration estimations obtained from the IDEXX Coliert-18 Quanti-Tray/2000 test.

Presence/absence (PA) 2x2 contingency tables were generated to compare the alignment of paired CBT and MTT positive-negative results. Using results from the 2x2 table, CBT sensitivity, specificity, false positive rate (FPR), false negative rate (FNR), positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated. Definitions for each parameter in relation to the CBT are described below:

- **Sensitivity:** percentage of water samples that have a presence of detectable H_2S-producing bacteria that CBT correctly identifies as having H_2S-producing bacteria
- **Specificity:** percentage of water samples that have absence of detectable H_2S-producing bacteria that CBT correctly identifies of having absence of H_2S-producing bacteria
- **False Positive Rate:** percentage of water samples that have absence of H_2S-producing bacteria that CBT identifies having presence of detectable H_2S-producing bacteria
- **False Negative Rate:** percentage of water samples that have presence of detectable H_2S-producing bacteria that CBT identifies having absence of H_2S-producing bacteria
- **Positive Predictive Value:** percentage of water samples identified by the CBT as having presence of detectable H_2S-producing bacteria that truly have presence of H_2S-producing bacteria
- **Negative Predictive Value:** percentage of water samples that the CBT identifies as having absence of detectable H_2S-producing bacteria that truly have absence of H_2S-producing bacteria
- **Accuracy:** percentage of samples classified correctly by CBT

The 4x4 contingency table was generated by categorizing paired CBT and MTT results based on the World Health Organization's (WHO) health risk categories for *E. coli* in drinking water. Currently the WHO does not have similar categories for H_2S-producing bacteria, otherwise those definitions would have been used. The WHO categorizes *E. coli* MPN/100 mL concentrations based on the following:

- **Safe:** water sample contains less than 1 MPN/100 mL of *E. coli* bacteria
- **Intermediate Risk:** water sample contains greater than 1 MPN/100 mL but less than or equal to 10 MPN/100 mL of *E. coli* bacteria
- **High Risk:** water sample contains greater than 10 MPN/100 mL but less than or equal to 100 MPN/100 mL of *E. coli* bacteria
- **Very High Risk**: water sample contains greater than 100 MPN/100 mL of *E. coli* bacteria

In addition, some developing countries accept drinking water with *E. coli* bacteria as long as levels are less than 10 MPN/100 mL. To account for regulations in these countries, a 2x2 contingency table comparing paired tests within these parameters was generated as well.

Quality control

Quality control was maintained throughout the study by rigorous training and practice of all research staff in field and lab processing techniques. The project manager supervised the process, and repeated verbal and visual confirmation of data and data-entry were practiced by all team members. Microsoft Excel was used to check for erroneous data entries using filters, sorting, and find/replace commands on each data column and row. Statistical consultants advised on data analysis frameworks and reviewed analysis process for soundness.

CHAPTER 5: RESULTS

Overview of key findings

Several statistical comparisons were made between CBT and MTT results to determine the ability of the CBT to detect and quantify H_2S-producing bacteria at similar rates and magnitudes to the MTT. A strong positive correlation was found between the CBT and MTT, particularly at 25° C incubation temperature over a 2 day period (44-48 h). Overview of descriptive statistics and linear regression analyses indicated the CBT consistently underestimated H_2S concentrations as determined by the MTT. The odds that CBT MPN results would fall within paired MTT 95% CI bounds increased with a one unit increase in field water temperature (° C), and decreased with a one unit increase in field air temperature and pH. Additionally, a significant positive correlation was found between MTT H_2S concentrations and *E. coli* concentrations determined by the Coliert Quanti-Tray method. No significant relationship was found between the CBT and Quanti-Tray methods.

Normality tests for untransformed and transformed data

To determine if data were normally distributed, Shapiro-Wilk tests were applied to untransformed and transformed data. Results indicated that both untransformed and transformed data were not normally distributed. The untransformed dataset containing CBT and MTT MPN/100 mL values displayed highly significant p-values ($P<0.0001$) and normality values of $W=0.57$ (CBT) and $W=0.59$ (MTT). For natural log (ln) and log base 10 ($\log_{10}$) transformed data, normality values of the CBT ($W=0.91$) and MTT ($W=0.95$) were much closer to being normally distributed. However, a highly significant p-value ($P<0.0001$) indicated transformed data were not normally distributed. Q-Q plots verified a closer alignment but still non-normally distributed datasets.

Correlation coefficient analysis of method pairs

To test the direction and strength of association between the CBT and MTT methods, a Spearman Rank Correlation analysis was run on $\log_{10}$ transformed paired CBT-MTT MPN values. Correlation coefficients were generated comparing CBT and MTT results overall, by incubation temperatures (25° C, 35° C), and by numbers of incubation days (1, 2, 3). Correlation coefficients were also generated for every combination of incubation temperature and numbers of incubation days (Table 2). Finally, coefficients were generated for CBT and MTT pairs when H_2S bacteria concentrations in tests were less than or equal to 100 MPN/100 mL. All correlations for every group were positive and highly significant ($P<0.0001$), indicating the CBT and MTT methods were significantly positively correlated. A relatively high correlation ($r = 0.72$) was found when paired CBT and MTT tests were compared overall. A higher correlation was found when samples had been incubated at 25° C ($r=0.76$) vs. 35° C ($r=0.66$). Incubating samples for 2 days had a higher correlation ($r=0.71$) than incubating for 1 day ($r=0.67$) or 3 days ($r=0.43$). When methods were compared based on combinations of incubation times and temperatures, the highest correlation occurred when samples had been incubated for 2 days at 25° C ($r=0.78$) (Figure 3). Incubating samples for 1 day at 25° C ($r=0.66$) (Figure 4) and 1 day at 35° C ($r=0.66$) (Figure 5) also demonstrated good correlations. The lowest correlation occurred when samples had been incubated for 3 days at 25° C ($r=0.48$) (Figure 6). When paired tests containing target bacteria concentrations less than or equal to 100 MPN/100 mL were compared, a moderately high correlation ($r=0.55$) was found. When these tests were compared by incubation temperature and time, samples incubated for 2 days at 25° C had the highest correlation ($r= 0.55$), while samples incubated for 3 days at both 25° C and 35° C had the lowest correlations ($r=0.32$).

Table 2: Spearman correlation coefficients (r) of compartment bag test (CBT) vs. multiple test tube (MTT) $\log_{10}$(MPN/100 mL) values for every combination of incubation temperature and numbers of incubation days.

Temp (° C)	Day	N Obs	Correlation (r) [a]
25	1	60	0.66
	2	58	0.78
	3	56	0.48
35	1	60	0.66
	2	58	0.58
	3	58	0.61

[a] *All p-values highly significant (P<0.0001).*

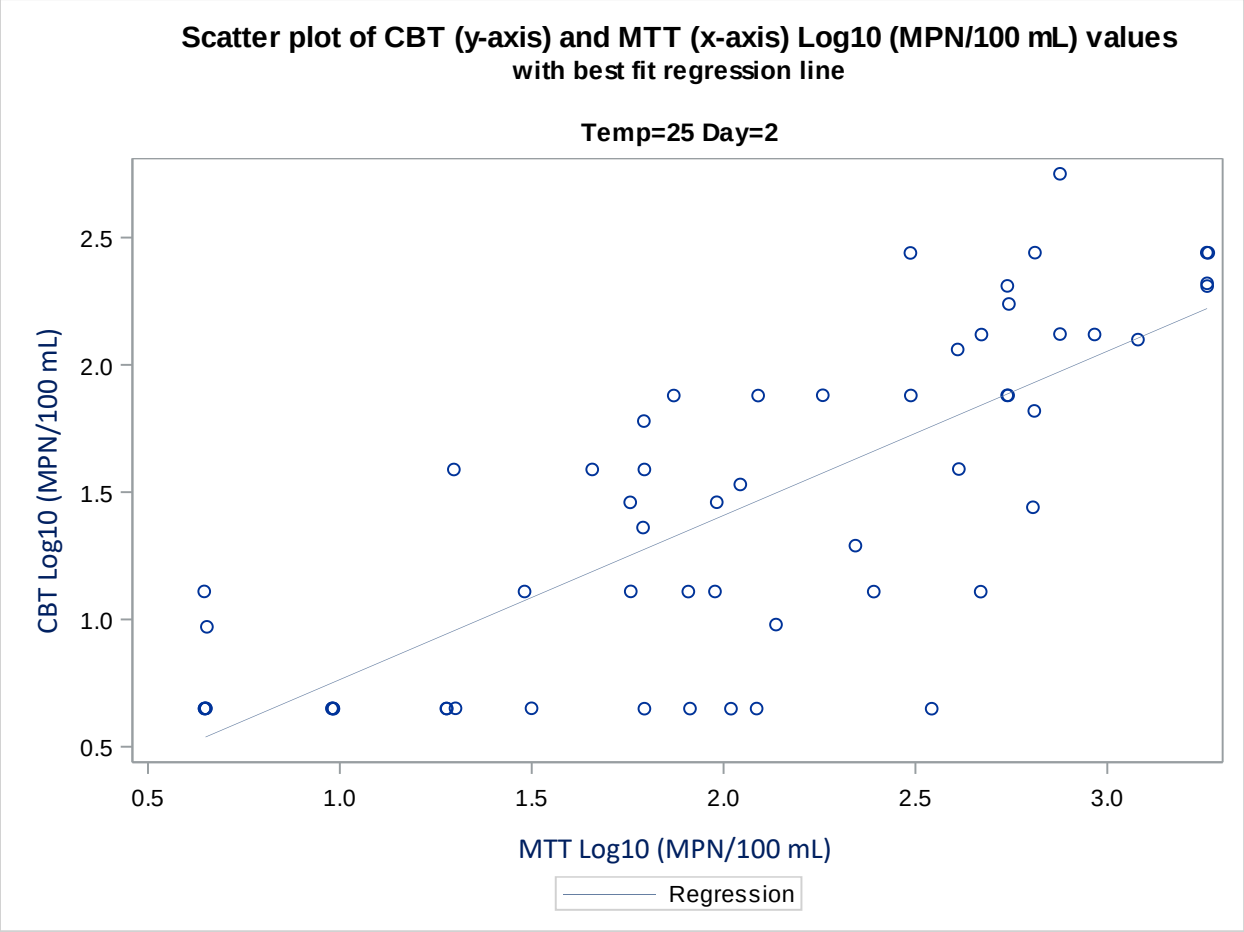


Figure 6: Scatter plot of the highest correlation (r = 0.78) between compartment bag test (CBT) log transformed (Log_{10}) most probable number (MPN) per 100 mL values vs. paired MTT Log_{10}(MPN/100 mL) values with best fit regression line. Paired samples had been incubated for 2 days at 25° C.

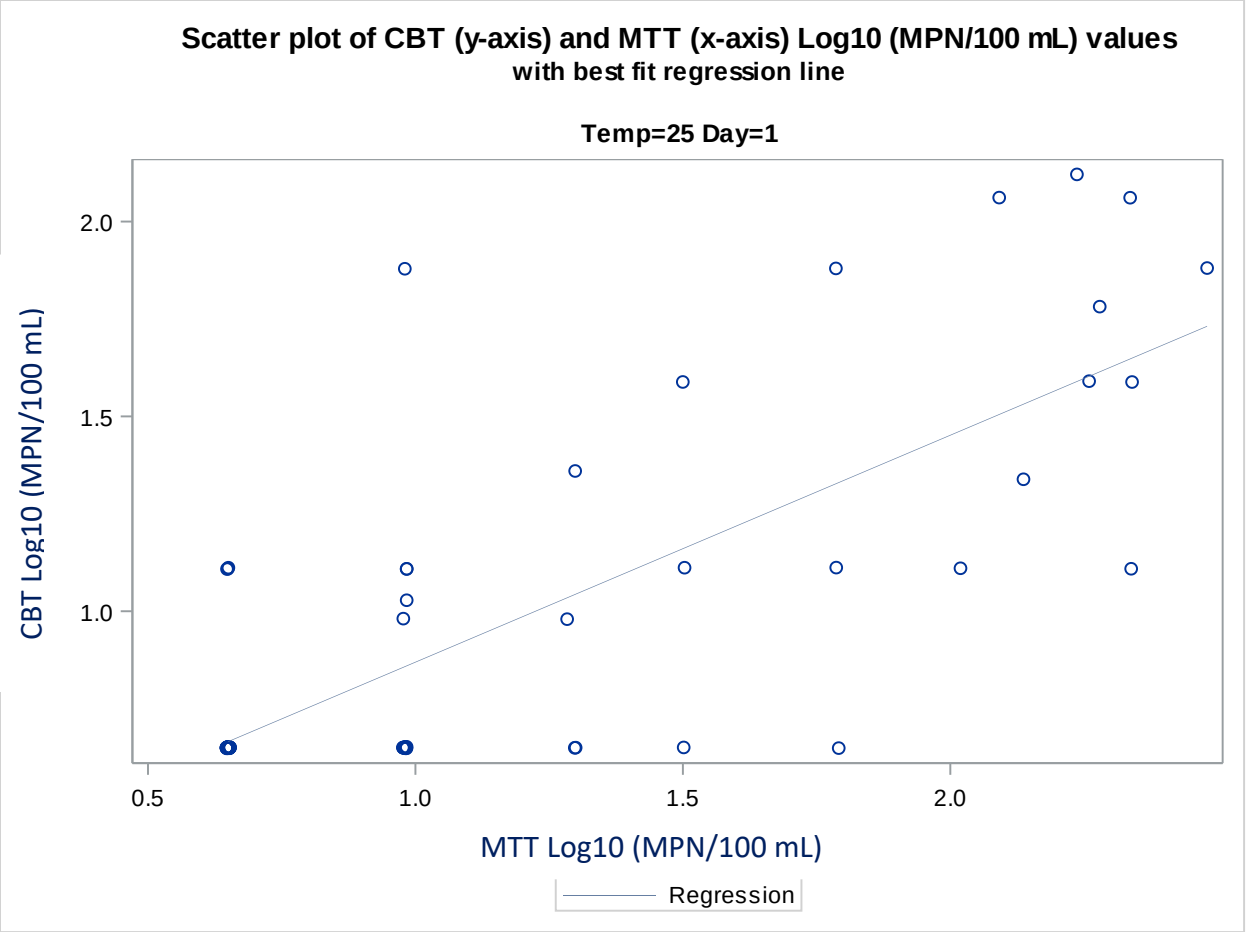


Figure 7: Scatter plot of one of the higher correlations (r = 0.66) between compartment bag test (CBT) log transformed (Log_{10}) most probable number (MPN) per 100 mL values vs. paired MTT Log_{10}(MPN/100 mL) values with best fit regression line. Paired samples had been incubated for 1 day at 25° C.

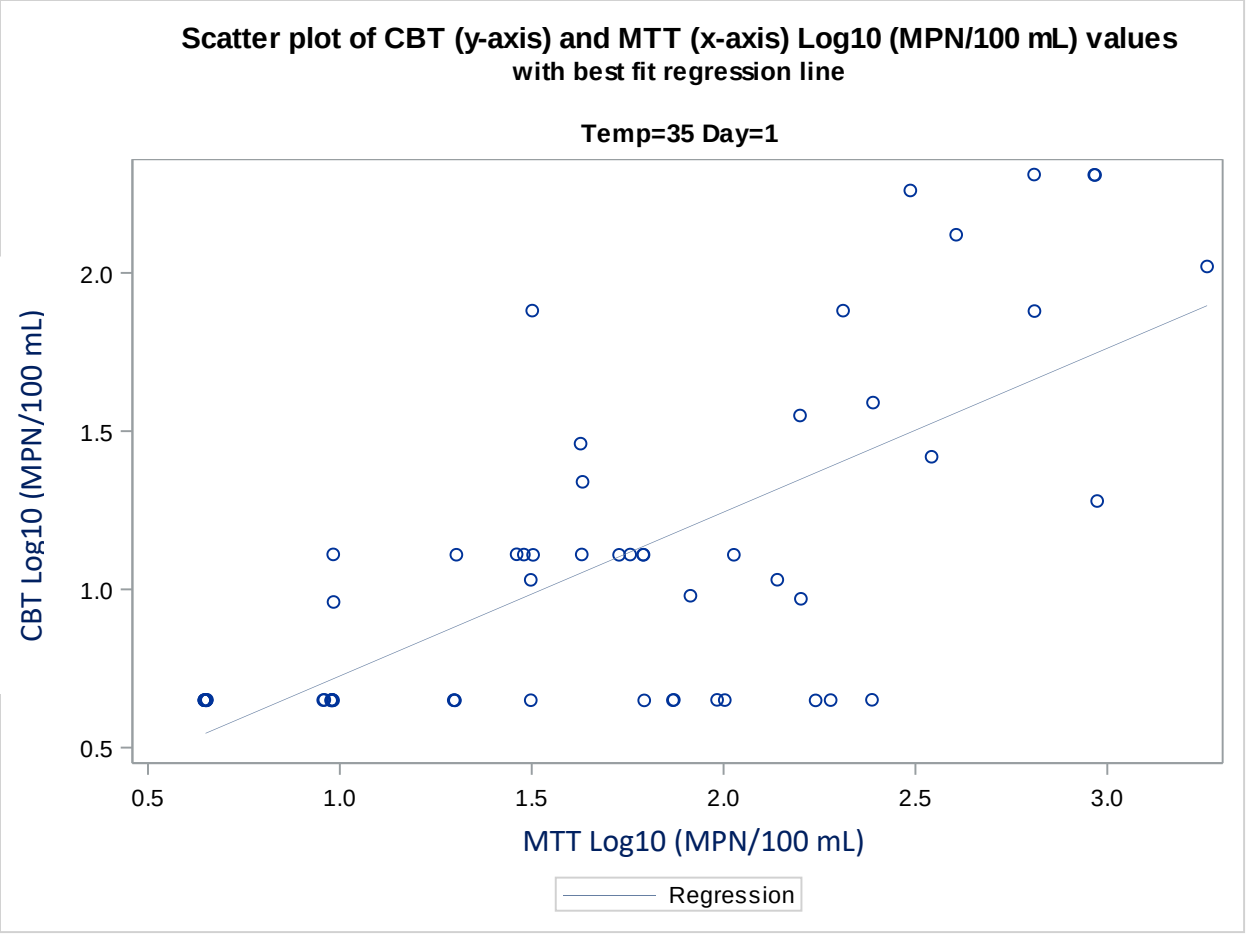


Figure 8: Scatter plot of one of the higher correlations (r = 0.66) between compartment bag test (CBT) log transformed (Log_{10}) most probable number (MPN) per 100 mL values vs. paired MTT Log_{10}(MPN/100 mL) values with best fit regression lin. Paired samples had been incubated for 1 day at 25° C.

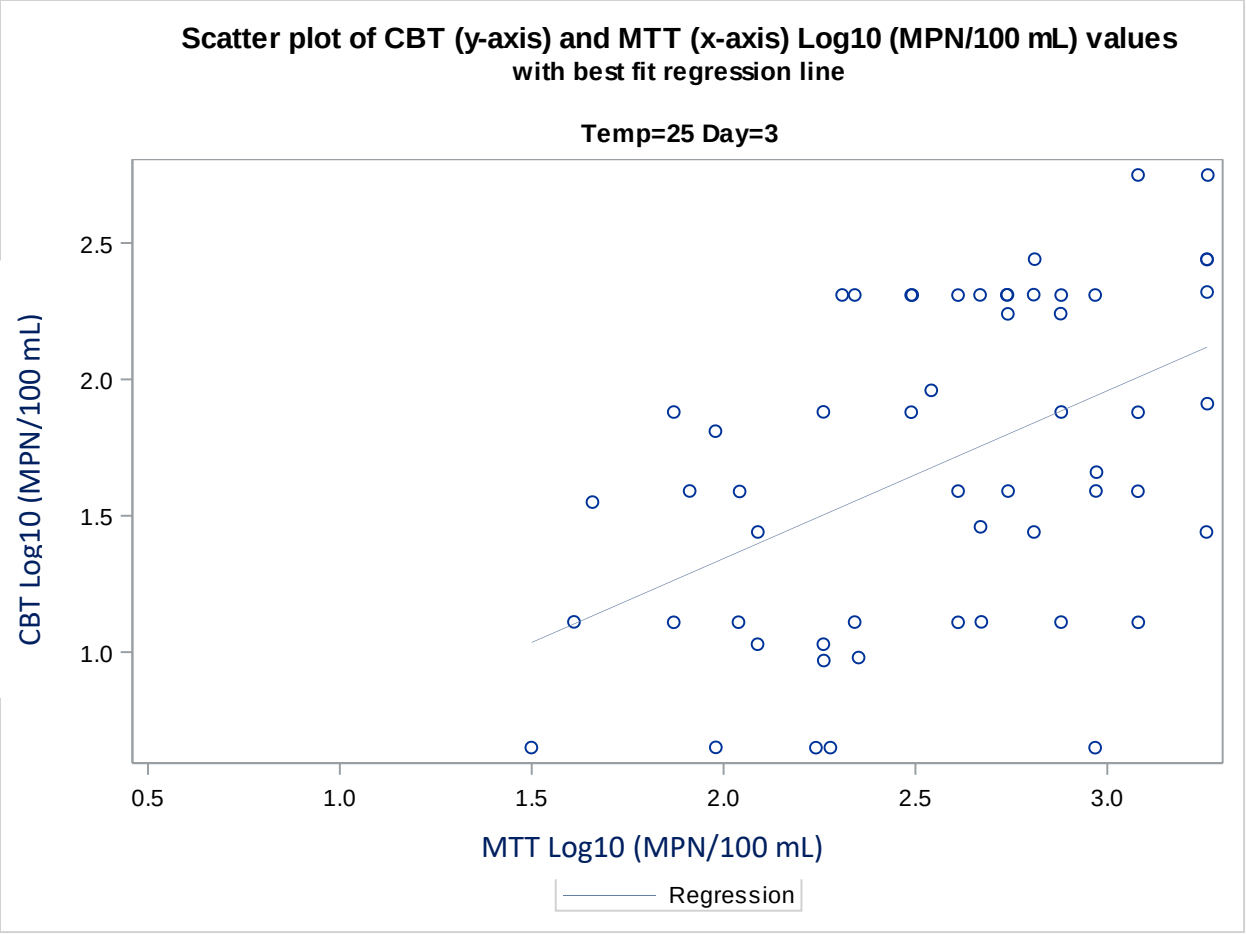


Figure 9: Scatter plot of the worst correlation (r=0.48) between compartment bag test (CBT) log transformed (Log_{10}) most probable number (MPN) per 100 mL values vs. paired MTT Log_{10}(MPN/100 mL) values with best fit regression line. Paired samples had been incubated for 3 days at 25° C.

Comparison of descriptive statistics

To compare similarities and differences in descriptive statistics for CBT and MTT concentration estimations, the mean, median, standard deviation, variance, and median difference for each method overall were calculated (Table 3). The mean, median, standard deviation, variance, and median difference were also calculated for every incubation time and temperature combination (Table 4). Finally, the same statistics were calculated for paired tests when H_2S bacteria concentrations in samples were less than or equal to 100 MPN/100 mL (Table 5). To calculate median differences, a Wilcoxon Singed-Rank test was applied. All median difference results were statistically significant, indicating that there was a significant difference between CBT and MTT MPN medians. Regardless of incubation

conditions, the mean and median for the CBT method were consistently lower than the corresponding MTT mean and median. Visual representations of paired tests were also generated though box & whisker plots showing log_{10} transformed CBT-MTT pairs overall (Figure 7), by incubation temperature (Figure 8), and by numbers of incubation days (Figure 9).

Table 3: Comparison of H_2S MPN descriptive statistics for compartment bag test (CBT) vs. multiple test tube (MTT) test overall, in terms of means, medians, standard deviations, variances, and median differences.

Test	N Obs	Mean MPN/100 mL	Median MPN/100 mL	Std Dev MPN/100 mL	Variance (MPN/100 mL)^2	Median Difference [a] MPN/100 mL
CBT	350	76	27	116	13460	-224
MTT	350	300	106	436	190263	

[a] *Median difference determined by Wilcoxon Signed-Rank Test, results highly significant (P<0.0001).*

Table 4: Comparison of H_2S MPN descriptive statistics of compartment bag test (CBT) vs. multiple test tube (MTT) test by incubation temperature and numbers of incubation days, in terms of means, medians, standard deviations, variance, and median differences.

Temp (° C)	Day	Test	N Obs	Mean MPN/100 mL	Median MPN/100 mL	Std Dev MPN/100 mL	Variance (MPN/100 mL)^2	Median Difference [a] MPN/100 mL
25	1	CBT	60	19	5 [b]	30	882	-23
		MTT		42	10	71	4980	
	2	CBT	58	72	28	103	10697	-273
		MTT		345	108	487	236899	
	3	CBT	56	107	43	125	15555	-488
		MTT		594	437	540	291762	
35	1	CBT	60	29	9	52	2745	-135
		MTT		164	43	316	99577	
	2	CBT	58	76	39	118	13821	-245
		MTT		320	170	392	153398	
	3	CBT	58	160	115	159	25256	-199
		MTT		359	170	452	204035	

[a] *Median difference determined by Wilcoxon Signed-Rank Test, all results significant (P<0.05)*

[b] *This value represents left-censored data, rounded up from 4.5 to 5 MPN/100 mL.*

Table 5: Comparison of descriptive statistics (in terms of mean, median, standard deviation, variance, and median differences) for compartment bag test (CBT) vs. multiple test tube (MTT) test when H_2S bacteria concentrations in tests are less than or equal to 100 MPN/100 mL.

Test	N Obs	Mean MPN/100 mL	Median MPN/100 mL	Std Dev MPN/100 mL	Variance (MPN/100 mL)^2	Median Difference [a] MPN/100 mL
CBT	164	17	5 [b]	21	439	-12
MTT	164	29	20	27	753	

[a] *Median difference determined by Wilcoxon Signed-Rank Test, results highly significant (P<0.0001)*
[b] *This value represents left-censored data, rounded up from 4.5 to 5 MPN/100 mL.*

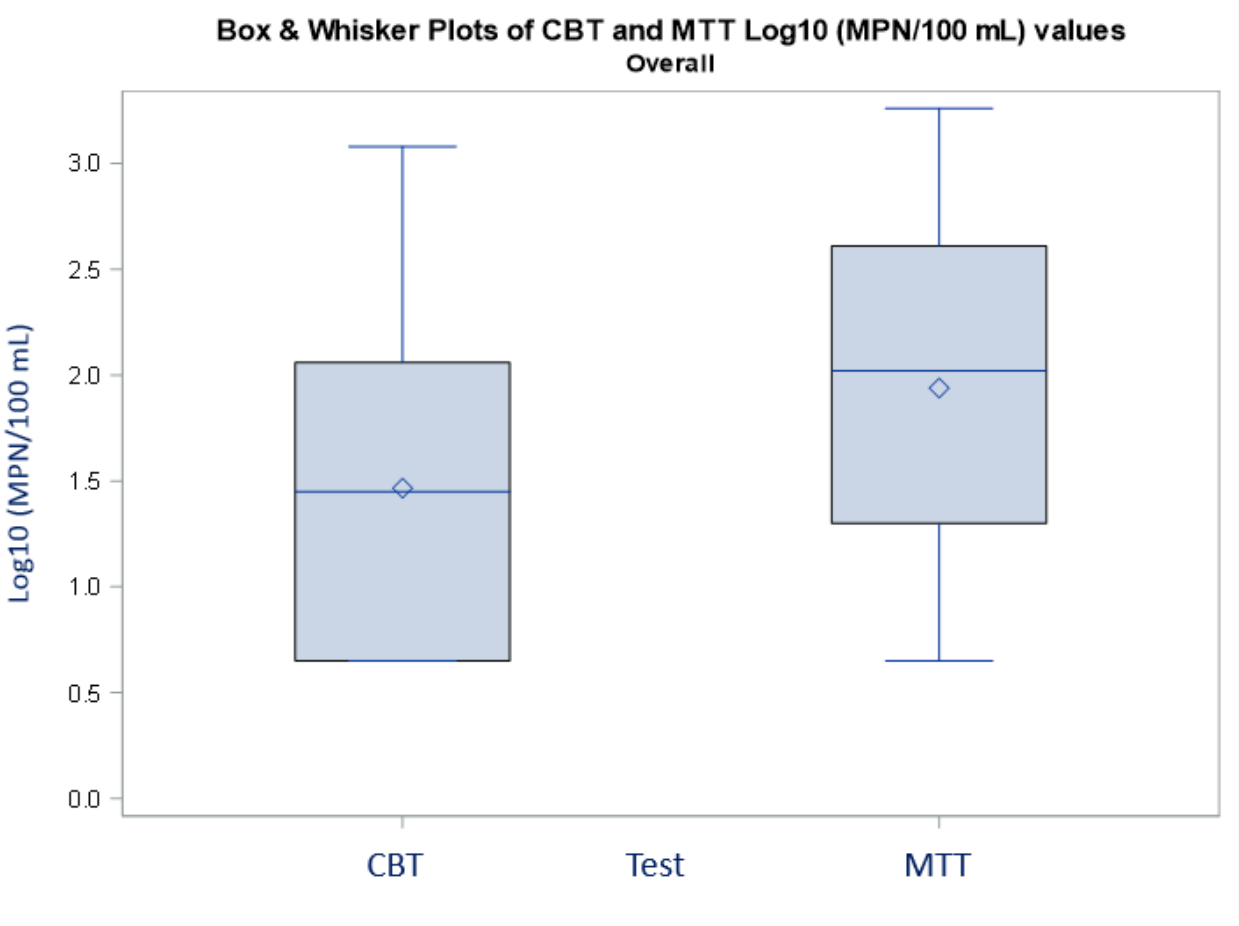


Figure 10: Box & whisker plots of compartment bag test (CBT) log transformed (Log_{10}) most probable number (MPN) per 100 mL values vs. paired MTT Log_{10}(MPN/100 mL) values overall (not separated by incubation temperature or numbers of incubation days).

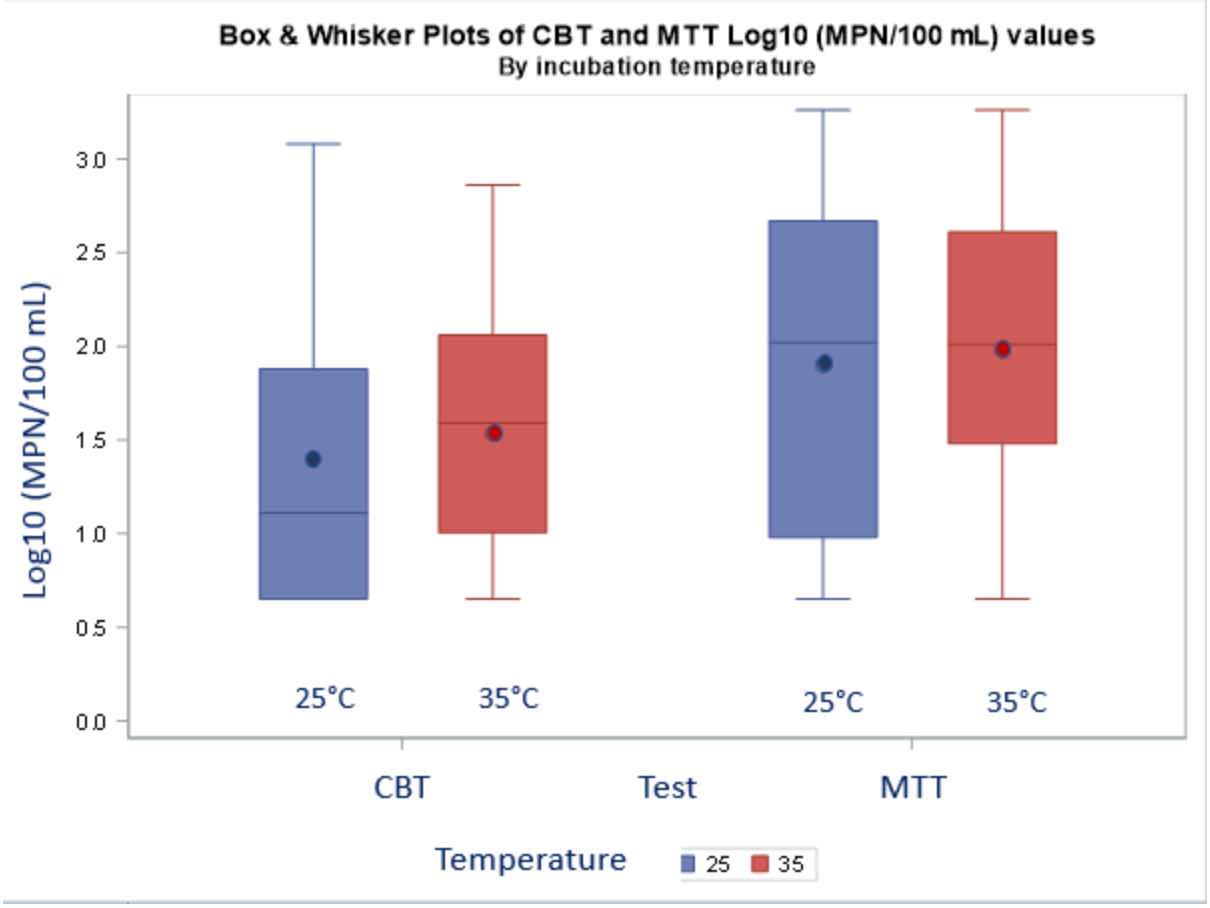


Figure 11: Box & whisker plots of compartment bag test (CBT) log transformed (Log_{10}) most probable number (MPN) per 100 mL values vs. paired MTT Log_{10}(MPN/100 mL) values based on incubation temperature.

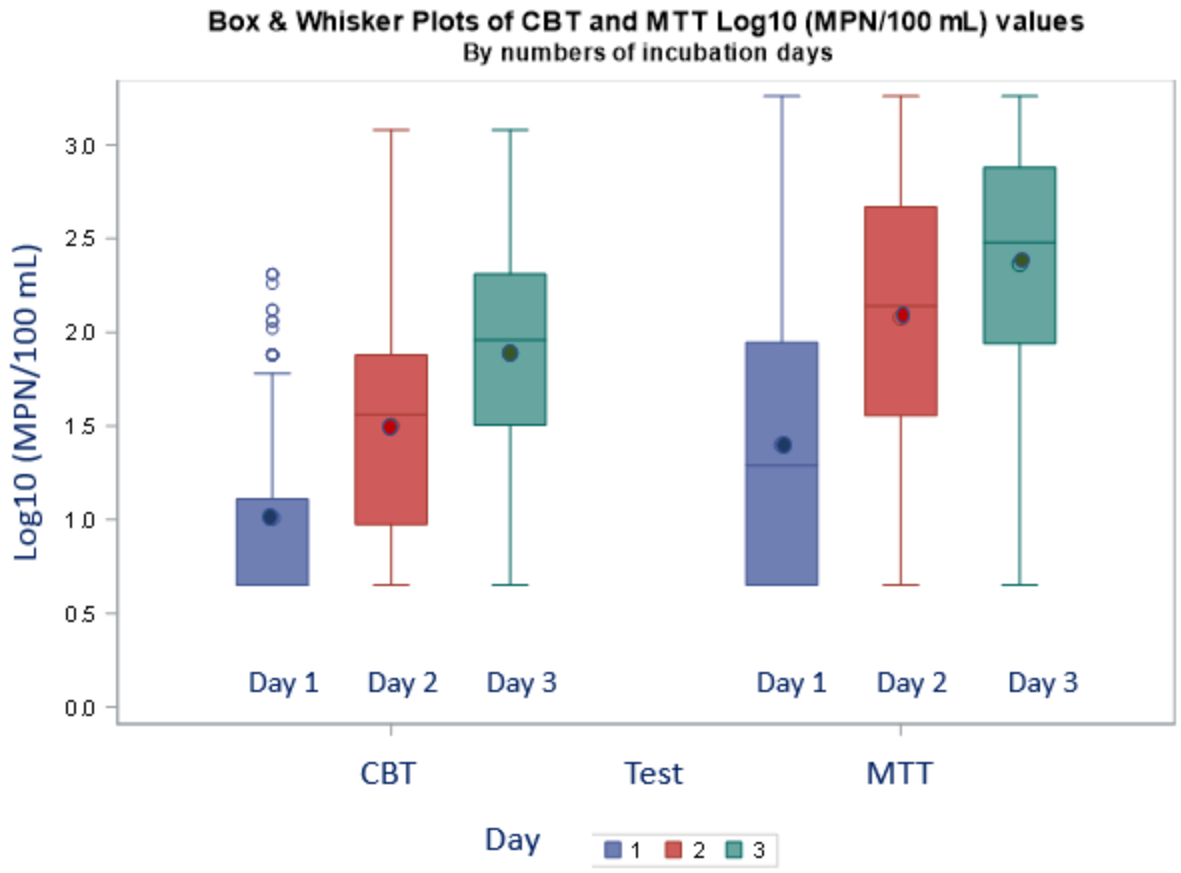


Figure 12: Box & whisker plots of compartment bag test (CBT) log transformed (Log_{10}) most probable number (MPN) per 100 mL values vs. paired multiple test tube (MTT) Log_{10}(MPN/100 mL) values based on numbers of incubation days.

Presence/absence 2x2 contingency table by day

To determine how well the CBT detects H_2S-producing bacteria compared to the MTT, a presence/absence (PA) 2x2 contingency table was generated for each CBT and MTT pair by incubation day (Table 6). From this table, CBT sensitivity, specificity, false positive rate, false negative rate, positive predictive value, negative predictive value, and accuracy were calculated (Table 7). A highly significant ($P<0.0001$) Fisher's exact test and Cohen's Kappa coefficient of 0.43 indicated a significant strength of agreement between CBT and MTT methods identifying PA results. The CBT demonstrated relatively high sensitivity (80%), specificity (86%), and positive predictive (98%) values, while displaying a very low false

positive rate (2%). The CBT also demonstrated a high false negative rate (62%) and low negative predictive value (38%). The overall accuracy of the CBT was 81%.

Table 6: Presence/absence 2x2 contingency table of frequency and row percent overlap of the compartment bag test (CBT) vs. multiple test tube (MTT) method by incubation days.

Test Type [a]			
CBT	MTT +	MTT –	Total
+	246 (98) [b]	6 (2)	252 (100)
–	61 (62)	37 (38)	98 (100)
Total	307	43	350

[a] Fisher's exact tests highly significant (P<0.0001), Kappa coefficient 0.43.
[b] Numbers outside parenthesis denote frequency overlap, numbers inside parenthesis denote row percent.

Table 7: Compartment bag test (CBT) sensitivity, specificity, false positive rate, false negative rate, positive predictive value, negative predictive value, and accuracy compared to multiple test tube method (MTT).

Test	Sensitivity	Specificity	False Positive Rate	False Negative Rate	Positive Predictive Value	Negative Predictive Value	Accuracy
CBT	80%	86%	2%	62%	98%	38%	81%

Presence/absence 2x2 contingency table by sample

In addition to the previous 2x2 PA contingency table, paired CBT and MTT results were also compared by sample (Table 8). From this table, CBT sensitivity, specificity, false positive rate, false negative rate, positive predictive value, negative predictive value, and accuracy were calculated (Table 9). Results were not significant (P>0.05) due to the lack of negative results recorded by either test after 3 days of incubation. Although results were not significant, the false negative rate for the CBT decreased to 5% compared to 62% in Table 7.

Table 8: Presence/absence 2x2 contingency table of frequency and row percent overlap of the compartment bag test (CBT) vs. multiple test tube (MTT) method by sample.

Test Type [a]			
	MTT		
CBT	+	–	Total
+	113 (99) [b]	1 (1)	114 (100)
–	6 (100)	0 (0)	6 (100)
Total	119	1	120

[a] *Fisher's exact test not significant (P>0.05)*
[b] *Numbers outside parenthesis denote frequency overlap, numbers inside parenthesis denote row percent.*

Table 9: Compartment bag test (CBT) sensitivity, specificity, false positive rate, false negative rate, positive predictive value, negative predictive value, and accuracy compared to multiple test tube method (MTT).

Test	Sensitivity	Specificity	False Positive Rate	False Negative Rate	Positive Predictive Value	Negative Predictive Value	Accuracy
CBT [a]	95%	0%	1%	5%	94%	0%	94%

[a] *Results not significant (P>0.05)*

2x2 contingency table using drinking water standards in developing settings by day

Most regulatory agencies focus on levels of fecal indicator bacteria in drinking water that are less than or equal to 100 MPN/100 mL sample. In addition, some developing countries consider the standard for safe drinking water to be when *E. coli* is at or below 10 MPN/100 mL sample. To determine how well the CBT performs within these standards, a 2x2 contingency table was generated comparing the ability of the CBT to MTT to classify samples between 1-10 MPN/100 mL and 11-100 MPN/100 mL of H_2S-producing bacteria (Table 10). A highly significant Fisher's exact test ($P<0.0001$) and kappa coefficient of 0.53 showed a significant strength of agreement between the CBT and MTT tests identifying categorical results. The accuracy of the CBT under these standards was 76%.

Table 10: 2x2 contingency table of frequency and row percent overlap of the compartment bag test (CBT) vs. multiple test tube (MTT) based on concentrations of H_2S-producing bacteria between 1-10 MPN/100 mL and 11-100 MPN/100 mL by day.

Test Type [a]			
	MTT		
CBT	0-10 MPN/100 mL	11-100 MPN/100 mL	Total
0-10 MPN/100 mL	65 (71) [b]	27 (29)	92 (100)
11-100 MPN/100 mL	12 (17)	60 (83)	72 (100)
Total	77	87	164

[a] Fisher's exact tests highly significant (P<0.0001), Kappa coefficient 0.53.
[b] Numbers outside parenthesis denote frequency overlap, numbers inside parenthesis denote row percent.

2x2 contingency table using drinking water standards in developing settings by sample

In addition to the previous 2x2 contingency table, paired CBT and MTT results were also compared by sample comparing the CBT to MTT when H_2S-producing bacteria concentrations were between 0-10 MPN/100 mL or 11-100 MPN/100 mL (Table 11). Results were not significant (P>0.05) due to the lack of test pairs recording results within 0-10 MPN/100 mL for the same sample. The accuracy of the CBT under these conditions was 77%.

Table 11: 2x2 contingency table of frequency and row percent overlap of the compartment bag test (CBT) vs. multiple test tube (MTT) based on concentrations of H_2S-producing bacteria between 1-10 MPN/100 mL and 11-100 MPN/100 mL by sample.

Test Type [a]			
	MTT		
CBT	0-10 MPN/100 mL	11-100 MPN/100 mL	Total
0-10 MPN/100 mL	0 (0) [b]	3 (100)	3 (100)
11-100 MPN/100 mL	2 (11)	17 (89)	19 (100)
Total	2	20	22

[a] Fisher's exact test not significant (P>0.05).
[b] Numbers outside parenthesis denote frequency overlap, numbers inside parenthesis denote row percent.

4x4 contingency table using WHO drinking water risk-based categories

To determine how well the CBT performs when detecting low, medium, high, and very high concentrations of H_2S-producing bacteria, a 4x4 contingency was generated (Table 12). CBT and MTT MPN/100 mL values were categorized based on the World Health Organization's (WHO) health risk guidelines for *E. coli* in drinking water. A highly significant Fisher's exact test (P<0.0001) and weighted kappa coefficient of 0.45 determined the CBT and MTT show significant strength of agreement in identifying categorical results. The accuracy of the CBT under these guidelines was 50%.

Table 12: 4x4 contingency table of frequency and row percent overlap of the compartment bag test (CBT) vs. multiple test tube (MTT) most probable number (MPN) results according to the World Health Organization's health risk categories for *E. coli* in drinking water.

Test Type [a]					
	MTT				
CBT	Safe	Intermediate Risk	High Risk	Very High Risk	Total
Safe	37 (38) [b]	25 (26)	25 (26)	11 (11)	98 (100)
Intermediate Risk	1 (5)	3 (14)	5 (24)	12 (57)	21 (100)
High Risk	5 (3)	6 (4)	57 (39)	80 (54)	148 (100)
Very High Risk	0 (0)	1 (1)	6 (6)	76 (92)	83 (100)
Total	43	35	93	179	350

[a] *Fisher's exact test highly significant (P<0.0001), weighted kappa coefficient 0.45.*
[b] *Numbers outside parenthesis denote frequency overlap, numbers inside parenthesis denote row percent.*

Frequency counts of CBT MPN estimates falling within paired MTT confidence intervals

To determine if CBT MPN values fell within an acceptable range of paired MTT MPN values, a frequency count of instances when CBT MPN values fell within paired MTT lower and upper 95% confidence intervals was calculated (Table 13). Counts and percentages were generated for every combination of incubation temperature and numbers of incubation days. The highest percentage overlap occurred when samples were incubated at 25° C for 1 day (77%). As incubation time and

temperature increased, the counts and percent overlap of CBT to MTT results decreased, with the lowest percentage overlap occurring when samples were incubated at 25° C for 3 days (14%). Results for 35° C at 1 and 3 days were not statistically significant.

Table 13: Frequency counts and percentage overlap of compartment bag test (CBT) most probable number (MPN) estimates falling within the lower and upper 95% confidence intervals of paired multiple test tube (MTT) MPN results.

Temperature (° C)	Day	Count	Percentage (%)	P-value
25	1	46	77	<.0001
	2	20	34	0.02
	3	8	14	<.0001
35	1	28	47	0.61 [a]
	2	19	33	0.01
	3	28	48	0.79 [a]

[a] *Results not significant (P>0.05).*

Linear regression comparing paired CBT-MTT MPN values

Linear Regression Hypothesis Test

y = mx + b
y = MTT MPN
m = slope
x = CBT MPN
b = intercept
H_O: m = 1, b = 0
H_A: m ≠ 1, b≠ 0

Figure 13: Linear regression hypothesis test, based on equation of a line y = mx + b.

To test whether paired CBT and MTT tests reported similar MPN estimates, linear regression was conducted on $\log_{10}$(MPN) transformed data. The effects of incubation temperature and numbers of incubation days were also incorporated into the model. If paired CBT and MTT tests were equivalent, they would support the null hypothesis of a perfect linear relationship by giving a slope estimate of one and an intercept estimate of zero (Figure 10). Results showed an intercept estimate of 0.25 and MTT $\log_{10}$(MPN) slope estimate of 0.5 (Table 14). Results were significant (P<0.05), rejecting the null hypothesis and indicating that for every unit increase in the log transformed value of the MTT, the corresponding CBT log transformed value increased by 0.5 units. While paired CBT-MTT tests trended positively, results indicated there was a significant difference between CBT and MTT MPN values. Factoring incubation temperature and numbers of incubation days into the model, results indicated there was a significant difference between CBT $\log_{10}$(MPN) outcomes at

25° C vs. 35° C. Relative to 25° C, the outcome of the CBT log transformed values at 35° C was 0.12 higher than CBT log transformed values at 25° C. There was no significant difference between CBT log transformed values at incubation day 1 vs. day 2 (P=0.064). However, relative to day 1, the outcome of CBT log transformed values at day 3 was 0.35 higher (P<0.0001). The R^2 value for this linear regression model was 0.56. In addition, a visual representation of the linear regression model was generated by comparing log_{10} transformed CBT and MTT MPN/100 mL paired values overall (not separating by incubation temperate of numbers of incubation days) (Figure 11). A line of equivalence was drawn to show the instance when a CBT-MTT test pair gave equivalent concentration estimations (CBT MPN = MTT MPN) above 0 MPN/100 mL. Besides the 37 instances when both tests scored 0 MPN/100 mL, there was only one instance where both tests generated the same MPN value. This instance occurred after a sample had been incubated for 1 day at 25° C. Both tests for that sample generated a concentration estimate of 9.53 MPN/100 mL, or 0.98 log_{10}(MPN)/100 mL (Figure 11).

Table 14: Linear regression of compartment bag test (CBT) log transformed (Log_{10}) most probable number values (MPN) with paired multiple test tube (MTT) log transformed outcomes, incubation temperature, and numbers of incubation days.

Parameter	Estimate	Standard Error	t Value	Pr > |t|	R^2
Intercept estimate	0.26	0.07	4.47	.0002	0.56
MTT log10(MPN) [b]	0.50	0.03	14.67	<0.0001	
Temp 25 [c]	0.0	.	.	.	
Temp 35	0.12	0.05	2.60	0.0098	
Day 1 [c]	0.0	.	.	.	
Day 2	0.11	0.06	1.86	0.064 [a]	
Day 3	0.35	0.07	5.31	<0.0001	

[a] *Result not statistically significant (P>0.05).*
[b] *MTT log10(MPN) is the slope estimate.*
[c] *Parameter is the reference variable*.

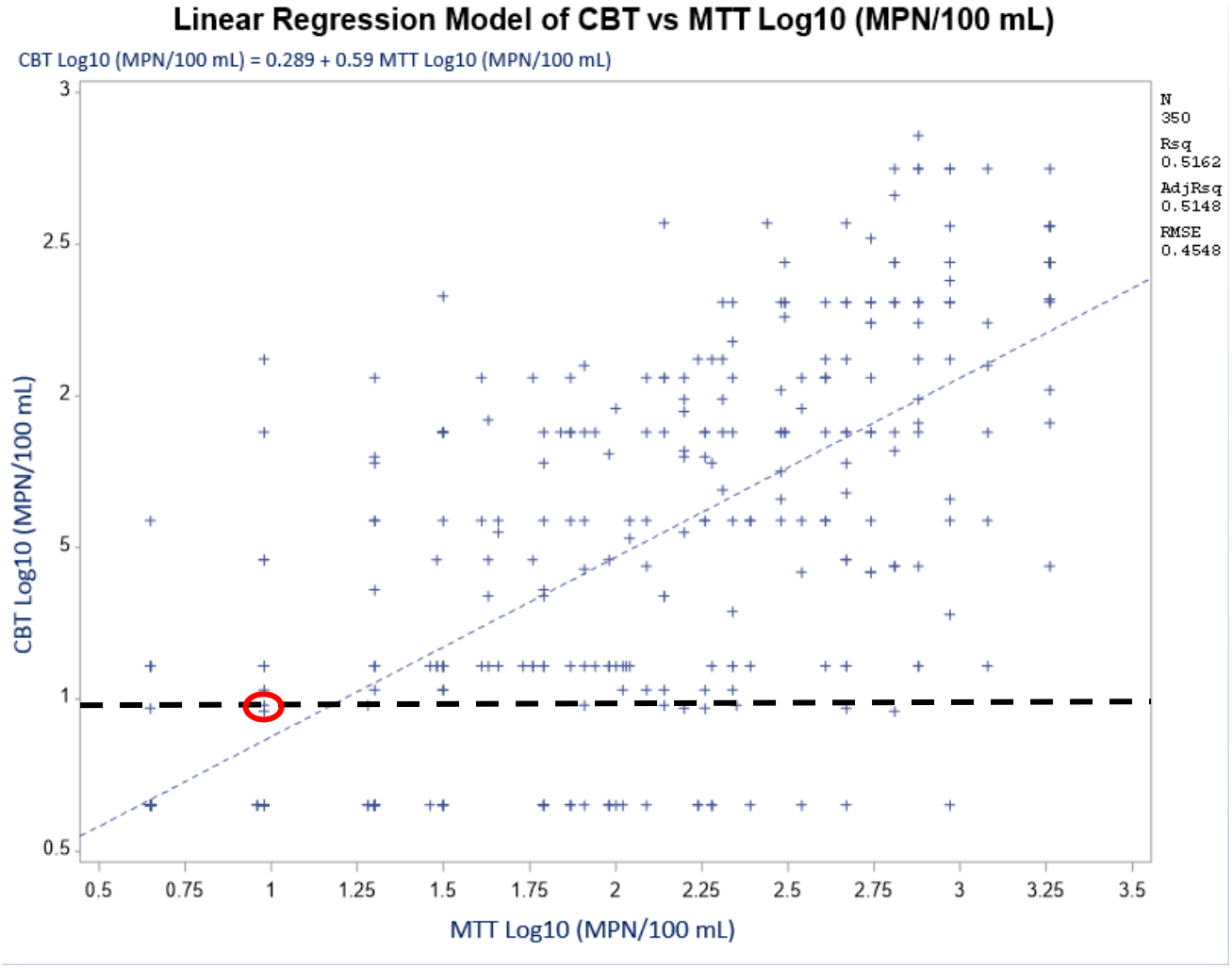


Figure 14: Linear regression of compartment bag test (CBT) log transformed (Log_{10}) most probable number (MPN) values with paired multiple test tube (MTT) Log_{10}(MPN) values overall (not separated by incubation temperature or numbers of incubation days). The dashed line is a line of equivalence showing the one instance where a CBT test gave the same concentration estimate as its paired MTT test. The circle is the sample that generated the same concentration estimation, of 9.53 MPN/100 mL (or 0.98 log_{10}(MPN)/100 mL), by both tests. This sample had been incubated for 1 day at 25° C.

Correlation coefficient analysis comparing CBT outcomes based on incubation time and temperature

To determine the strength of association between CBT test outcomes based on factors such as incubation temperature and numbers of incubation days, CBT MPN values were grouped based on incubation temperature and days and were compared to each other using Spearman's correlation coefficients (Table 15). All results were significantly positively correlated. The strongest association between different temperature combinations occurred when the CBT had been incubated at 35° C for 2

days vs. 25° C for 3 days (r=0.76). Several other combinations demonstrated strong associations as well, including 35° C for 1 day vs. 25° C for 3 days (r=0.72), 35° C for 1 day vs. 25° C for 2 days (r=0.71), and 25° C for 2 days vs. 35° C for 3 days (r=0.70). Comparing similar temperature combinations, 35° C for 2 vs. 3 days reported high correlation (r=0.78), along with 25° C at 2 vs. 3 days (r=0.85). The weakest correlation for different temperatures occurred when samples had been incubated at 35° C for 1 day vs. 25° C for 1 day (r=0.50) and for similar temperatures at 35° C for 1 day vs. 3 days (r=0.58).

Table 15: Spearman's correlation coefficients (r) for compartment bag test (CBT) most probable number (MPN) values by every combination of incubation temperature and numbers of incubation days. [a]

CBT [a]		Day					
		1		2		3	
Day	Temperature (° C)	25	35	25	35	25	35
1	25	1	0.50	0.69	0.66	0.69	0.69
	35	0.50	1	0.71	0.69	0.72	0.58
2	25	0.69	0.71	1	0.69	0.85	0.70
	35	0.66	0.69	0.69	1	0.76	0.78
3	25	0.69	0.72	0.85	0.76	1	0.72
	35	0.69	0.58	0.70	0.78	0.72	1

[a] *All correlation coefficients (r) highly significant (P<0.0001).*

Correlation analysis of sample replicates

At each sampling site two sample replicates were collected and processed side-by-side through CBT-MTT pairs. To determine whether CBT sample replicates and MTT sample replicates record similar H_2S concentration estimates, a Spearman correlation and linear regression analyses were conducted. Results showed significant positive correlation between replicates, with CBT replicates 1 and 2 demonstrating a correlation coefficient of 0.86 and MTT replicates 1 and 2 a correlation coefficient of 0.93. Linear regression models also demonstrated closely matching slope estimates of 0.93 for CBT replicates and 1.06 for MTT replicates.

Logistic regression of odds of CBT-MPN alignment based on incubation and environmental conditions

Logistic regression was performed to predict whether CBT MPN values were more or less likely to fall within paired MTT MPN 95% Confidence Intervals based on the following lab and environmental predictor variables: incubation temperature, number of incubation days, precipitation, air temperature, water temperature, pH, specific conductivity (SC), and dissolved oxygen (DO). The logistic regression model generated coefficient estimates, P-values, odds ratios, and odds ratio 95% Confidence Intervals (Table 16). Incubation temperature, precipitation, SC, and DO were not significant ($P>0.05$) in influencing the odds of CBT MPN outcomes falling within paired MTT MPN 95% CI. Numbers of incubation days, air temperature, water temperature, and pH however appeared to influence CBT outcomes when all other variables were held constant. For a one unit increase in water temperature, the odds of CBT MPN falling within paired 95% CI increased 1.71 times. For a one unit increase in air temperature or pH, the odds decreased 0.79 and 0.52 times, respectively. Incubating for 1 day vs. 3 days increased the odds 6.12 times. Incubating for 2 days vs. 3 days increased the odds 1.16 times. Incubating for 2 days vs. 1 day decreased the odds 0.19 times.

Table 16: Logistic regression model of likelihood estimates, P-values, odds ratios, and odds ratio 95% confidence intervals (CI) of the effects of incubation and environmental conditions on the probability of compartment bag test (CBT) outcomes falling within paired multiple test tube (MTT) 95% CI.

Parameter	Estimate	Pr > ChiSq	Odds Ratios	95% Confidence Limits	
				Lower	Upper
Intercept	-5.10	0.197	[a]	[a]	[a]
Incubation temperature 25° C vs. 35° C	-0.02	0.898	[a]	[a]	[a]
Day 1 vs. 3	1.16	<0.0001	6.12	3.17	11.80
Day 2 vs. 3	-0.50	0.006	1.16	0.63	2.15
Day 2 vs. 1	-0.50	0.006	0.19	0.10	0.36
Precipitation (cm)	0.29	0.141	[a]	[a]	[a]
Air temperature (° C)	-0.23	0.001	0.79	0.69	0.91
Water temperature (° C)	0.53	<0.0001	1.71	1.33	2.20
pH	-0.66	0.043	0.52	0.27	0.98
SC (µS/cm)	-0.01	0.437	[a]	[a]	[a]
DO (mg/L)	-0.06	0.942	[a]	[a]	[a]
DO (%)	0.01	0.822	[a]	[a]	[a]

[a] Result not statistically significant (P>0.05), odds ratio and odds ratio 95% confidence intervals omitted.

Spearman correlation coefficients comparing H_2S results *to Quanti-Tray 2000/ results*

To determine whether there was a relationship between concentrations of H_2S-producing bacteria and concentrations of total coliform (TC) and *E. coli*, a Spearman's correlation analysis was conducted comparing CBT and MTT MPN results to Quanti-Tray MPN results. While there was no significant correlation between CBT H_2S results and TC or *E. coli*, there was a significant (P=0.037) positive correlation (r=0.38) between MTT H_2S results and *E. coli* concentrations.

Summary of key findings

Results show the CBT and MTT methods to be significantly positively correlated. The CBT was found to consistently underestimate H_2S concentrations given by paired MTT results. The odds ratio of the CBT aligning with the MTT results were found to be influenced by lab and field parameters such as incubation time, water temperature, dissolved oxygen, and pH. There was also a significant relationship

between concentration estimations of H_2S-producing bacteria and *E. coli* when the MTT was compared to the Quanti-Tray.

CHAPTER 6: DISCUSSION

Summary of findings related to objectives

Our results provide evidence that the compartment bag test consistently detects and quantifies H_2S-producing bacteria in water samples. This is demonstrated through the significant correlation coefficients generated between paired CBT and MTT tests across all levels of incubation temperature and numbers of incubation days (Table 2). Our findings suggest that incubation temperature and time periods between 1-2 days at 35° C and at least 2 days at 25° C promote the highest correlation of method results. Although the CBT consistently reflected changes in concentrations of H_2S-producing bacteria, it also consistently underestimated target bacteria levels in sample water, as indicated by the MTT. This trend was observed across multiple statistical analyses, and warrants further assessment and calibration of the current H_2S CBT method. This study is one of the first to evaluate a semi-quantitative format using a compartmentalized bag for the enumeration of H_2S-producing bacteria in water supplies. Our research illustrates the potential of the H_2S CBT to be a significant breakthrough in the market for semi-quantitative H_2S field tests and a promising candidate for water quality testing and monitoring in low-resource and humanitarian emergency settings.

First major finding– strong positive correlation between CBT and MTT methods

Correlation analyses comparing concentration estimates of CBT and MTT paired results revealed the CBT was positively correlated and strongly associated with the MTT (Table 2). Results were significant and highly correlated overall and across all combinations of incubation temperatures and numbers of incubation days. This is important for evaluating the precision of the CBT, and indicates that regardless of incubation conditions, the compartment bag test consistently reflected changes in target

bacteria concentrations as indicated by the MTT. While many studies have compared H_2S field tests to traditional methods that detect fecal indicator bacteria, few studies have compared two semi-quantitative test formats using the same H_2S medium.

Rijal and colleagues (2001) developed and evaluated two semi-quantitative modifications of the H_2S test. One was a paper strip MPN version using replicate sample volumes of 1, 10, and 100 mL. The other was an enumerative version for H_2S colonies on membrane filters using an agar medium. Both H_2S tests were compared to each other and the occurrence of total coliforms and *E. coli* bacteria in groundwater, stream water and rainwater cisterns. Investigators found the H_2S tests to give comparable results to *E. coli*, although total coliforms were detected in more samples than *E. coli* or H_2S bacteria (Rijal & Fujioka 2001).

McMahan (2011) compared the PathoScreen™ medium with two popular lab-made broths for the detection of H_2S-producing bacteria using spiked sewage and natural water samples. Researchers found no significant difference in levels of H_2S-producing bacteria for the commercially available HACH media vs. the lab-made H_2S broths regardless of incubation temperature ($P=0.49$). McMahan also compared the ability of the H_2S CBT to detect the presence/absence of H_2S-producing bacteria with the ability of the Coliert Quanti-Tray method to detect the presence/absence of *E. coli* in spiked and natural waters. Researchers reported similar detection levels for both fecal indicators ($P<.0001$)(McMahan 2011).

In 2001 Manja et al. compared the original H_2S medium from 1982 with three modified medium compositions containing combinations of additional L-cysteine and decreased peptone. Investigators found the addition of L-cysteine to detection mediums to give the best results in terms of numbers of positive results (Manja et al. 2001).

A study by Murcott et al. (2015) evaluated several microbial water quality field tests side-by-side to determine the best test for development settings, including three popular PA H_2S tests (lab-made H_2S,

TARA Aquacheck, and ORlab H_2S). Researchers judged the *E. coli* CBT, along with TARA Aquacheck and ORLab, to perform the best overall in terms of True Result, False Positive, False Negative, Sensitivity, Specificity, Positive Predictive Value, and Negative Predictive value (Murcott et al. 2015).

Second major finding– recommendations for optimal incubation temperature and time conditions

Correlation analyses comparing CBT and MTT tests indicated that incubation periods of around 1 day (20-24 h) for temperatures around 35° C and around 2 days (44-48 h) for temperatures around 25° C provided the highest correlation between CBT and MTT tests. The strongest strength of association occurred when samples were incubated for 2 days at 25° C (r=0.78) and 1 day at 35° C (r=0.66). Incubation periods of 1 day at 25° C (r=0.66) also resulted in a strong correlation. Furthermore, when CBT and MTT means, medians, and median differences were compared based on different combinations of incubation temperatures and days, it was found that 1 day at 35° C as well as 1 day at 25° C had the smallest difference in medians of -135 and -23, respectively.

Frequency counts analyzing the frequency of CBT results falling within paired MTT 95% CI bounds demonstrated the highest percent overlap when samples were incubated at 25° C for 1 day (77%) (Table 13). While the descriptive statistics and frequency counts may appear to support an optimum incubation time and temperature combination of 1 day at 25° C, the much lower percent overlap witnessed by the rest of the temperature-time combinations calls into question this assumption. With percent overlap tending to decrease as the number of incubation days increase, it appears the gap between CBT and MTT H_2S concentration estimations widens, up to the point where MPN values from the CBT infrequently fell within paired MTT 95% CI. This finding concurs with results examining the effects of incubation time and temperature in the logistic regression model, which suggested that day 1 vs. day 2 or 3 provides a higher likelihood that CBT outcomes will fall within paired MTT 95% CI (Table 16). The fact that there is a high percent overlap at 1 day at 25° C is encouraging, as it suggests the two

tests generate similar concentration estimations during the initial stages of H_2S-producing bacteria growth.

The performance of the CBT based on different combinations of incubation time and temperature was assessed via correlation analyses (Table 15). In general, correlations between CBT tests tended to increase as the number of incubation days increased. The strongest strength of association between different time-temperature combinations occurred when samples were incubated for 2 days at 35° C and 3 days at 25° C (r=0.76). Other highly correlated combinations included 1 day at 35° C and 3 days at 25° C (r=0.72) and 1 day at 35° C and 2 days at 25° C (r=0.71). Linear regression models indicated that incubation temperature significantly influences CBT MPN outcomes. Relative to 25° C, the outcome of the CBT log transformed MPN values at 35° C were slightly higher (0.12) than CBT log transformed MPN values at 25° C. Interestingly, there was no significant difference between CBT log transformed MPN values at incubation day 1 vs. day 2 (P=0.064). However, there was a significant increase in CBT log transformed MPN values at day 3 vs. day 1 (0.35). These observations make sense considering the tendency of H_2S-producing bacteria to increase in number at a faster rate at higher temperatures over longer periods of time.

While an incubator is ideal for obtaining best results, these findings suggest CBT concentration estimations change similarly if samples are incubated at 35° C between 1-2 days and 25° C between 2-3 days. These findings agree with a large body of evidence suggesting that longer incubation periods between 24 h to 48 h coupled with temperatures between 25-35°C give best results in terms of detecting low levels (5 CFU per sample) of H_2S-producing bacteria (Pillai et al. 1999; Gawthorne et al. 1996; Ratto et al. 1989; Castillo et al. 1994; Genthe & Franck 1999; Manja et al. 2001; Tambekar & Neware 2012; Gupta et al. 2007; Sobsey & Pfaender 2002). These optimal incubation conditions are promising for use in tropical and subtropical regions, where ambient temperatures frequently fall within this range and may allow for use of the H_2S CBT in the field without the use of an incubator.

Third major finding– underestimation of H_2S-producing bacteria by the CBT

Despite promising method associations, our results also suggest the CBT consistently underestimated the concentration of H_2S-producing bacteria in sample water as identified by the MTT. A linear regression model of Log_{10}(MPN) data gave a slope estimate of 0.5, meaning that for every log_{10} transformed unit increase in MTT MPN, the corresponding log_{10} transformed CBT MPN only increased by 0.5 units. Consistently lower means, medians, and median differences across incubation time and temperature combinations clearly demonstrated that paired CBT and MTT concentration estimates were different (Tables 3, 4, 5). Contingency tables further illustrated this gap. The presence/absence 2x2 table by day demonstrated that more often than not, samples judged negative by the CBT were judged positive by the MTT (Table 6). This is reflected in the CBT's high false negative rate (62%) and low negative predictive value (38%) (Table 7). The CBT's low false positive rate (2%) and high positive predictive value (98%) are encouraging, as they indicate samples judged positive by the CBT almost always concurred with the MTT. However, this trend is consistent with the conclusion that the CBT underestimates H_2S concentrations. Furthermore, the 4x4 contingency table based on the WHO's health risk guidelines for *E. coli* in drinking water suggest that the MTT tends to score at least one risk category higher than its corresponding CBT test (Table 12).

While discrepancies exist between the methods, when a 2x2 presence/absence (PA) contingency table is applied to paired tests by sample (Table 8), the false negative rate drops from 62% (Table 7) to 5% (Table 9). While results for this analysis were not significant, they demonstrate that the CBT can more accurately reflect PA results determined by the MTT if the CBT is allowed to incubate for longer periods of time (2-3 days).

In addition, the gap between methods lessens when a 2x2 contingency table is applied comparing the CBT's ability to match with the MTT when H_2S bacteria concentrations in tests were between 0-10 MPN/100 mL and 11-100 MPN/100 mL (Table 10). This shows that while there are still

differences between the tests, the H_2S CBT has the potential to be deployed in developing countries where drinking water is acceptable for consumption when *E. coli* in drinking water is less than or equal to 10 MPN/100 mL. A decrease in differences between the CBT and MTT methods is also observable when the mean, median, and median difference were calculated for CBT and MTT pairs when target bacteria concentrations were less than or equal to 100 MPN/100 mL (Table 5).

Few studies have compared two semi-quantitative test formats using the same H_2S detection medium. While adequate or superior sensitivity of H_2S PA tests has been documented in some cases (Ratto et al. 1989; Anwar et al. 1999; Genthe & Franck 1999; Rijal & Fujioka 2001; Roser et al. 2005), other studies have reported inconsistent accuracy, false positive, and false negative rates when compared to traditional FIB tests (Wright et al. 2012; Izadi et al. 2010; Tewari et al. 2003; Desmarchelier et al. 1992; Yang et al. 2013a). Yang et al. (2013) found through a simulation that as the threshold used to define contamination increased from 1 to 100 CFU/100 ml, PA H_2S test sensitivity increased but specificity decreased. They also found that increasing test volumes from 20 to 100 mL increased sensitivity but reduced specificity (Yang et al. 2013b). However, a study by Manja et al. (2001) found that H_2S PA tests did not differ significantly between sample volumes of 20, 55, and 100 mL (Manja et al. 2001).

Others such as Nair et al. (2001), Manja et al. (1982), and Gupta et al. (2007) have found that the H_2S field test reflects FIB concentrations consistently when fecal contamination was moderate (10 MPN/100 mL) or heavy (>40 MPN/100 mL, >100 MPN/100mL) but inconsistent when fecal contamination was low (Manja et al. 1982; Nair et al. 2001; Gupta et al. 2007). Weppelmann et al. (2014) assessed the feasibility of the PA PathoScreen™ H_2S test compared to membrane filtration to detect FIB after the 2010 earthquake and cholera outbreak in Haiti. Investigators collected drinking water samples from a variety of sources and incubated tests for 24 h and 48 h between 25° C-29° C. They found a H_2S test sensitivity of 65% and specificity of 93%, and concluded that the method was

attractive for low-resource settings but questionable due to its low sensitivity (Weppelmann et al. 2014b).

While the H_2S CBT provides a feasible solution for water quality monitoring in low-resource settings, the fact that the CBT underestimates H_2S concentrations is notable. It is critical for microbial water quality tests to be judged on their reliability and predictability, as well as their accessibility and affordability. A test that provides incorrect information such as false negatives undermines the potential uses and benefits of the test (Sobsey & Pfaender 2002). While not absolutely accurate in its current state, the CBT's ability to consistently reflect changes in H_2S concentrations is promising. This indicates H_2S CBT performance and accuracy could be improved upon further evaluation and calibration of the method's physical, chemical, or mathematical formats.

Practical recommendations for future research

Recommendations for future research include eliminating any remaining differences between the H_2S CBT and comparison methods to better understand discrepancies in target bacteria detection and enumeration. While we went to great lengths to minimize differences between tests, differences in H_2S detection and enumeration likely derived from the two methods' differing MPN formats (10 glass test tubes of 10 mL volumes each vs. 5 plastic compartments of 1, 3, 10, 30 and 56 mL each). It is possible the consistent and deep distribution of sample liquid within the MTT's rigid glass test tubes allowed for increased bacteria stratification and/or growth, thus allowing for easier identification and interpretation by researchers. In 2011 McMahan compared the H_2S detection capabilities of the CBT vs. plastic bottles. Although bacterial numbers detected by the CBT were slightly higher than numbers from bottles, after 48 h of indication there was no significant difference ($P= 0.31$) in growth. However, the H_2S CBT has never been compared directly to glass test tubes. Future studies comparing the effects of different test formats (glass vs. plastic, test tubes vs. bottles vs. bags) are recommend.

The use of more modern statistical methods, such as Bayesian statistical analysis, could be used to generate more precise concentration estimations and comparisons between methods. Bayesian analysis considers the probability distribution curves and likelihood functions of target microbes in samples to infer bacteria concentrations. Concentration estimations for the original *E. coli* CBT were generated based on conventional MPN methods, calculating quantiles of the likelihood function of *E. coli* concentrations, and employing Bayesian Markov chain Monte Carlo (MCMC) analysis methods, as described by Gronewold et al. (2017). Similar statistical analyses could be used in conjunction with conventional Thomas (1942) and Haldane (1939) MPN calculation methods to obtain a more comprehensive comparison of the H_2S CBT results to standard method results.

Another recommendation for future research is to use a more sensitive H_2S detection medium, or one that can produce a more easily observed positive result in a shorter period of time. This could be achieved by adding medium ingredients or modifying the test to facilitate a more reduced anaerobic environment, which promotes the growth of H_2S-producing bacteria and inhibits the growth of aerobic bacteria. Interestingly, the ingredients of the PathoScreen™ H_2S detection medium (HACH, Loveland, CO) do not contain cystine or cysteine, amino acids frequently reported to increase the ability of H_2S detection when concentrations of target bacteria are low (Pillai et al. 1999; Venkobachar et al. 1994; Manja et al. 2001; S. P. Pathak & Gopal 2005; Shahryari et al. 2014; Sobsey & Pfaender 2002; McMahan 2011). Cystine or cysteine has also been shown to reduce the incubation period required for comparable results, as documented by Venkobachar et al. (1994) and Pillai et al. (1999) (Venkobachar et al. 1994; Pillai et al. 1999). Similarly, the addition of extra sulfide to the medium could also aid in the H_2S detection process.

Finally, future research should elucidate the correlation between the H_2S CBT and health risk. Studies of this nature would help establish the H_2S CBT as a credible semi-quantitative field test, further

examine the test's capabilities in the field, and increase the body of evidence needed by regulatory agencies to assess and accept the H_2S test as an alternative fecal indicator test.

Limitations

Possible limitations to this study include the lack of confirmation that positive samples definitively contained H_2S-producing bacteria of fecal origin. While we did not confirm the types nor sources of microbes in positive samples, we did discover a significant (P=0.037) positive correlation (r=0.38) between H_2S-producing bacteria enumerated by the MTT and *E. coli* bacteria enumerated by the Quanti-Tray method. In addition, there is a large body of evidence supporting a strong association between the H_2S field test and H_2S-producing bacteria of fecal origin (Ratto et al. 1989; Castillo et al. 1994; Venkobachar et al. 1994; Genthe & Franck 1999; Rijal & Fujioka 2001; Manja et al. 2001; Sobsey & Pfaender 2002; McMahan 2011; Tambi et al. 2016; Sivaborvon 1988; Martins et al. 1997; Dufour et al. 2013; Manja et al. 1982; Anwar et al. 1999; Nair et al. 2001; Hirulkar & Tambekar 2006; Gupta et al. 2007; Eun & Hwang 2003; Kromoredjo & Fujioka 1991; McMahan et al. 2012). In particular, a study by McManhan et al. (2011) tested samples from a variety of natural waters in North Carolina, United States, including water from one of our sample locations, University Lake. Researchers used culture and molecular analyses to speciate bacteria isolated from positive H_2S samples, and identified H_2S-producing organisms in all positive samples, including *Klebsiella ozonae*, *Proteus mirabilis, K. pneumoniae, C. freundii,* and Salmonella. Furthermore, they were able to demonstrate a strong association between the presence of H_2S-producing bacteria and enteric pathogens in samples, including Salmonella, *Escherichia coli, Citrobacter freundii, Klebsiella pneumoniae, and Proteus* (McMahan 2011). To allow for confirmation of H_2S-producing bacteria of fecal origin in our study, we collected and froze several vials of test media from positive CBT compartments from each reservoir sample site. Future studies could culture and isolate colonies from these vials to confirm the sources and types of bacteria present in positive CBT tests.

More exploratory analyses should be done to investigate the effects of environmental parameters on the ability of H_2S CBT to detect H_2S-producing organisms. While logistic regression models suggest ambient factors such as water temperature may increase the odds of the CBT aligning more closely with the MTT, and air temperature and pH may have the opposite effect, too little data was collected (n= 30) to confidently document these phenomena. A study analyzing relationships between environmental conditions and H_2S-producing bacteria would be an informative direction for future study.

Human error and bias due to lack of blinding during result interpretation is possible when conducting H_2S tests, as discussed by Wright et al. (2012). Furthermore, samples collected from the field often contain a variety of non H_2S-producing bacteria, which can grow excessively under lab conditions and make color-based result interpretation difficult. Mistaking dark-colored debris in samples for a positive result is also a concern, especially for new or untrained test users. Moving forward it will be critical to establish consistent standards for identifying true positive results to benefit H_2S test users. Different interpretations of what makes a sample a true positive will influence test results, and may be one reason why inconsistent precision, sensitivity, and specificity has been documented across labs (Martins et al. 1997; Rijal & Fujioka 2001, Wright et al. 2012; Izadi et al. 2010; Tewari et al. 2003; Desmarchelier et al. 1992; Yang et al. 2013a). These limitations are universal to any H_2S field test regime, as well as other tests that rely on color changes to denote positive/negative results. In our study, we limited human error as much as possible through rigorous training and practice of collecting, processing, and interpreting results before conducting sampling events. It is important to note many H_2S test users are not trained microbiologists. Therefore, it is very important to develop standard and easy-to-use tests to allow for untrained users to correctly identify H_2S positive results.

Additional findings

In addition to our main findings, we also demonstrated a significant ($P<0.0001$) strength of association between sample replicates for both CBT replicates ($r=0.86$) and MTT replicates ($r=0.93$). Linear regression analysis also confirmed closely matching slope estimates of 0.93 for CBT replicates and 1.06 for MTT replicates. This finding was expected and showed that when the CBT and MTT methods processed samples from the same sites, similar H_2S concentration estimations were generated.

Interestingly, logistic regression analyses evaluating the effects of incubation and environmental conditions on CBT MPN outcomes suggest that both lab and field conditions impact the likelihood of CBT MPN outcomes falling within paired MTT MPN 95% confidence intervals (CI) (Table 16). An increase in water temperature for instance increased the odds (OR=1.71) that CBT outcomes would fall within paired MTT 95% CI ranges. Increases in air temperature and pH, on the other hand, decreased the odds 0.79 and 0.52 times, respectively. While intriguing, more exploratory data analysis of this phenomenon is required before making assumptions about relationships between environmental conditions and H_2S-producing bacteria concentrations. As for lab conditions, incubation for 1 day vs. 3 days increased the odds of method alignment 6.12 times, while incubation for 2 days vs. 3 days increased the odds 0.63 times. These results make sense in light of the previously described frequency count analysis, which showed the highest percent overlap (77%) of CBT MPN values falling within paired MTT 95% CI to be at 1 day of incubation at 25° C. These results indicate that CBT and MTT H_2S concentration estimates start closer together on day one, but increase in distance as the numbers of incubation days increase. While incubation time played a significant role in influencing method alignment, incubation temperature (25° C vs. 35° C) was not a significant factor influencing the alignment of the two methods. This is promising, as it indicates that the incubation temperatures used in this study were not the primary sources of method misalignment.

Another interesting finding was a statistically significant (P=0.037) positive correlation (r=0.38) between H_2S-producing bacteria enumerated by the MTT method and *E. coli* bacteria enumerated by the Quanti-Tray method. This finding agrees with the body of literature supporting a strong association between H_2S-producing bacteria, many fecal indicator organisms, and fecal contamination in water (Ratto et al. 1989; Castillo et al. 1994; Venkobachar et al. 1994; Genthe & Franck 1999; Rijal & Fujioka 2001; Manja et al. 2001; Sobsey & Pfaender 2002; McMahan 2011; Tambi et al. 2016; Sivaborvon 1988; Martins et al. 1997; Dufour et al. 2013; Manja et al. 1982; Anwar et al. 1999; Nair et al. 2001; Hirulkar & Tambekar 2006; Gupta et al. 2007; Eun & Hwang 2003; Kromoredjo & Fujioka 1991; McMahan et al. 2012). This finding also adds weight to the argument that H_2S positive samples identified in this study were associated with H_2S-producing bacteria of fecal origin. Interestingly, results from the H_2S CBT did not correlate with any Quanti-Tray results. This suggests the H_2S CBT is currently not as sensitive as the MTT, though further calibration of the method will likely improve correlations.

Summary, significance, and implication of study findings

Overall the H_2S CBT shows promise as a semi-quantitative method for enumerating H_2S-producing bacteria in the field. Compared to the many PA H_2S tests on the market, the H_2S CBT provides a distinct advantage to users who seek microbial concentration data in water resources. Development of a reliable, simple, and semi-quantitative H_2S field test would provide more information on potential human health risk related to microbial water quality than PA H_2S tests. With the basis of WHO Guidelines for Drinking Water Quality shifting towards quantifiable risk-based data (Bain et al. 2012), it will become increasingly important for non-governmental organizations (NGOs), aid workers, utilities, local health workers, and communities in low-resource and emergency settings to collect quantifiable microbial water quality data.

Although the H_2S CBT consistently detects changes in target bacteria concentrations, the method also tends to underestimate MTT MPN results. Further study is recommended to calibrate and enhance the method's ability to quantify H_2S-producing bacteria at a similar level to more traditional semi-quantitative microbial water quality methods. Reasons for differences between methods may derive from the two methods' differing test materials and/or MPN formats (10 glass test tubes at 10 mL volumes vs. 5 plastic compartments at various volumes). Suggestions for improvement include minimizing test differences to determine the source of CBT underestimation and altering the H_2S detection medium to increase sensitivity or produce a more obvious color-change.

This research advances the body of knowledge on H_2S field test by introducing a novel semi-quantitative test that is logistically feasible and has the potential to satisfy shifting water quality testing and monitoring needs in low-resource environments. Once developed, the test could be made accessible and affordable to people and institutions who now lack access to tests to determine the microbial safety of their water. The calibration and validation of a reliable semi-quantitative H_2S test would also aid the efforts to standardize the H_2S method, by making it more comparable to semi-quantitative and quantitative methods using traditional fecal indicator organisms.

CHAPTER 7: CONCLUSION

We found that the compartment bag test (CBT) was significantly positively correlated with the multiple test tube (MTT) comparison method for quantifying H_2S-producing bacteria in lake water samples used as drinking water sources. The association was strongest under incubation temperature and time conditions of around 25° C over the course of 2 days (44-48 h). However, the CBT tended to underestimate the true concentration of H_2S-producing microorganisms in samples as indicated by the MTT.

This study uncovers the potential of the H_2S CBT to be a viable semi-quantitative method for detecting and quantifying hydrogen sulfide-producing bacteria as fecal indicators in drinking water sources. While deployable in any setting, the tendency of the current H_2S CBT to underestimate concentrations of target organisms may not be readily accepted in higher resource settings, where more expensive yet accurate microbial water quality methods exist. Rather, the low-cost of materials, simple format, and consistency of the H_2S CBT to detect and enumerate target organisms lends itself to tropical and subtropical settings where resources are limited and fecal contamination in water supplies is likely. This method also lends itself to humanitarian emergency situations where rapid, cheap, and simple methods are in high demand.

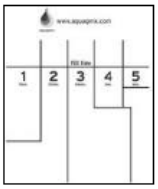
This research uncovers a new and improved H_2S field test that may one day fill a sizable gap in the arsenal of low-resource microbial water quality field tests. Compared to the many presence/absence (PA) H_2S tests dominating the market, the H_2S CBT provides a distinct advantage to NGOs, aid workers, public health officials, and utilities in developing countries and low-resource environments seeking microbial concentration estimations over less informative PA results. The ability of the H_2S CBT to

provide semi-quantitative results proves especially valuable when users seek to correlate water quality with health risk. The test's MPN format also allows for improved comparison between the H_2S method and other traditional semi-quantitative fecal indicator bacteria (FIB) methods.

Overall the CBT proves capable of enumerating H_2S-producing bacteria using a most probable number format. It also provides concentration estimates that consistently reflect changes in concentrations indicated by the comparison standard. Future research should focus on further evaluation and calibration of the H_2S CBT to improve its performance against standard microbial water quality methods such as the MTT. Further research is also needed to correlate H_2S tests such as the H_2S CBT with disease risk to add to the body of evidence needed by regulatory agencies to assess and accept the H_2S test as an alternative FIB test. The H_2S CBT is a promising alterative fecal indicator field test whose semi-quantitative format opens the door for enhanced protection, empowerment, and education of individuals and communities world-wide.

APPENDIX I: CBT REFERENCE RESULT CHART

Appendix I: Compartment bag test (CBT) reference result chart provided by manufacturer (Aquagenx, LLC). Column labels from left to right: compartment number showing all possible compartment positive-negative color change combinations, most probable number per 100 mL water sample (MPN/100 mL), upper 95% Confidence Interval, and qualitative health risk categories based on MPN and Confidence Interval



Compartment #					MPN/100mL	Upper 95% Confidence Interval/100mL	Health Risk Category Based on MPN and Confidence Interval
1	2	3	4	5			
10mL	30mL	56mL	3mL	1mL			
					0.0	2.87	Low Risk/Safe
					1.0	5.14	Intermediate Risk/ Probably Safe
					1.0	4.74	
					1.1	5.16	
					1.2	5.64	
					1.5	7.81	
					2.0	6.32	
					2.1	6.85	
					2.1	6.64	
					2.4	7.81	
					2.4	8.12	
					2.6	8.51	
					3.2	8.38	
					3.7	9.70	
					3.1	11.36	Intermediate Risk/ Possibly Safe
					3.2	11.82	
					3.4	12.53	
					3.9	10.43	
					4.0	10.94	
					4.7	22.75	
					5.2	14.73	
					5.4	12.93	
					5.6	17.14	
					5.8	16.87	
					8.4	21.19	
					9.1	37.04	
					9.6	37.68	
					13.6	83.06	High Risk/Possibly Unsafe
					17.1	56.35	
					32.6	145.55	High Risk/Probably Unsafe
					48.3	351.91	
					>100	9435.10	Unsafe

APPENDIX II: MTT REFERENCE RESULT CHART

Appendix II: Multiple test tube reference result scale provided by the United States Food and Drug Administration's Bacteriological Analytical Manual Appendix 2: Most Probable Number from Serial Dilutions. Columns from left to right: Number of positive tubes per test, most probable number per 100 mL water sample (MPN/100 mL), lower 95% Confidence Interval (CI), and upper 95% Confidence Interval

Table 5
For 10 tubes at 10 ml inocula, the MPN per 100 ml and 95 percent confidence intervals.

		Conf. lim.	
Pos. tubes	MPN/100ml	Low	High
0	<1.1	–	3.3
1	1.1	.05	5.9
2	2.2	.37	8.1
3	3.6	.91	9.7
4	5.1	1.6	13
5	6.9	2.5	15
6	9.2	3.3	19
7	12	4.8	24
8	16	5.9	33
9	23	8.1	53
10	>23	12	–

APPENDIX III: ALL STUDY CBT AND MTT OUTCOMES

Appendix III: All CBT and MTT MPN/100 mL and 95% CI outcomes, based on United States Food and Drug Administration's Bacteriological Analytical Manuel (BAM) MPN calculator applying the Thomas (1942) and Haldane (1939) equations. Column labels from left to right: number of outcome observations (N Obs), CBT most probable number per 100 mL water sample (MPN), CBT lower 95% Confidence Interval (95% CI Low), CBT upper 95% Confidence Interval (95% CI High), number of CBT MPN outcomes recorded (Count), MTT most probable number per 100 mL water sample (MPN), MTT lower 95% Confidence Interval (95% CI Low), MTT upper 95% Confidence Interval (95% CI High), and number of MTT MPN outcomes recorded (Count)

	CBT MPN/100 mL				MTT MPN/100 mL			
N Obs	MPN	95% CI High	95% CI Low	Count	MPN	95% CI High	95% CI Low	Count
1	5 (0)	32	1	98	5 (0)	32	1	43
2	9	66	1	7	9	65	1	2
3	10	68	1	5	10	68	1	33
4	11	76	2	9	19	77	5	3
5	13	92	2	45	20	80	5	18
6	19	77	5	1	29	90	9	2
7	20	78	5	1	30	94	10	3
8	22	88	5	4	32	99	10	16
9	23	91	6	2	41	108	15	3
10	26	105	7	2	43	114	16	4
11	27	110	7	6	46	121	17	3
12	29	115	7	8	54	130	22	1
13	34	105	11	1	57	138	24	4
14	36	111	11	2	62	148	26	13
15	39	156	10	23	69	154	31	1
16	46	143	15	2	74	166	33	7
17	48	128	18	1	81	180	36	6
18	49	130	18	1	82	171	39	1
19	56	148	21	1	88	184	42	2
20	60	185	19	4	95	200	45	6
21	62	194	20	3	101	202	51	3
22	64	171	24	1	106	221	50	3
23	67	207	22	2	106	205	55	1
24	76	237	25	29	110	220	55	3
25	81	195	34	2	122	244	61	6
26	84	201	35	1	139	268	72	4
27	90	240	34	1	139	279	70	4
28	91	244	34	2	157	291	84	1
29	97	259	36	3	159	306	83	6
30	106	282	40	2	175	316	97	3
31	115	307	43	14	180	335	97	7
32	126	302	52	2	192	369	100	5

33	132	351	49	8	202	365	112	5
34	151	362	63	1	219	407	118	9
35	175	421	73	4	224	395	127	1
36	182	405	82	1	247	447	137	2
37	205	493	85	20	248	427	144	2
38	207	435	99	2	277	489	157	1
39	216	482	97	1	302	562	162	7
40	238	574	99	1	310	535	180	6
41	273	608	122	14	345	583	204	1
42	330	693	157	1	350	633	193	4
43	362	761	172	4	405	715	229	9
44	369	825	165	4	468	810	271	14
45	461	970	219	1	545	925	321	12
46	564	1190	267	10	640	1067	384	11
47	727	1466	361	1	763	1255	464	13
48	1206	2476	588	2	936	1521	576	14
49	-	-	-	-	1214	1957	753	6
50	-	-	-	-	1812	2931	112097	16
51	-	-	-	-	>1812	10618	1238	10

APPENDIX IV: COMPLETE DATASET

Appendix IV: Complete dataset of paired CBT and MTT tests post consolidating dilutions and obtaining one MPN value per sample via the Thomas (1942) and method of Haldane (1939) equations. Column labels from left to right: number of sample observations (#), date samples were collected (Date), reservoir where samples were collected (Lake, UL = University Lake, CC = Cane Creek Reservoir), site within reservoir where samples was collected (Site, 1-5), replicate sample ID (Rep, 1 or 2), sample incubation temperature (Temp, 35°C or 25°C), sample incubation number of days (Day, 1-3) CBT most probable number per 100 mL water sample (MPN), CBT lower 95% Confidence Interval (95% CI Low), CBT upper 95% Confidence Interval (95% CI High), MTT most probable number per 100 mL water sample (MPN), MTT lower 95% Confidence Interval (95% CI Low), and MTT upper 95% Confidence Interval (95% CI High).

Paired Sample Descriptors							CBT MPN/100 mL			MTT MPN/100 mL		
#	Date	Lake	Site	Rep	Temp	Day	MPN	CI Low	CI High	MPN	CI Low	CI High
1	8/8/16	UL	1	1	35	1	13	2	92	57	24	138
2	8/8/16	UL	1	1	35	2	13	2	92	95	45	200
3	8/8/16	UL	1	1	35	3	151	63	362	219	118	407
4	8/8/16	UL	1	2	35	1	13	2	92	43	16	114
5	8/8/16	UL	1	2	35	2	39	10	156	122	61	244
6	8/8/16	UL	1	2	35	3	115	43	307	159	83	306
7	8/8/16	UL	1	1	25	1	5	1	32	10	1	68
8	8/8/16	UL	1	1	25	2	76	25	237	122	61	244
9	8/8/16	UL	1	1	25	3	205	85	493	219	118	407
10	8/8/16	UL	1	2	25	1	5	1	32	10	1	68
11	8/8/16	UL	1	2	25	2	29	7	115	95	45	200
12	8/8/16	UL	1	2	25	3	64	24	171	95	45	200
13	8/8/16	UL	2	1	35	1	36	11	111	157	84	291
14	8/8/16	UL	2	1	35	2	132	49	351	202	112	365
15	8/8/16	UL	2	1	35	3	369	165	825	277	157	489
16	8/8/16	UL	2	2	35	1	22	5	87	43	16	114
17	8/8/16	UL	2	2	35	2	62	20	194	159	83	306
18	8/8/16	UL	2	2	35	3	90	34	240	159	83	306
19	8/8/16	UL	2	1	25	1	13	2	92	5	1	32
20	8/8/16	UL	2	1	25	2	273	122	608	310	180	535
21	8/8/16	UL	2	1	25	3	205	85	493	310	180	535
22	8/8/16	UL	2	2	25	1	5	1	32	5	1	32
23	8/8/16	UL	2	2	25	2	76	25	237	180	97	335
24	8/8/16	UL	2	2	25	3	76	25	237	180	97	335
25	8/8/16	UL	3	1	35	1	205	85	493	936	576	1521
26	8/8/16	UL	3	1	35	2	362	172	761	936	576	1521
27	8/8/16	UL	3	1	35	3	362	172	761	1812	1121	2931
28	8/8/16	UL	3	2	35	1	205	85	493	936	576	1521
29	8/8/16	UL	3	2	35	2	273	122	608	1812	1121	2931
30	8/8/16	UL	3	2	35	3	273	122	608	1812	1121	2931

31	8/8/16	UL	3	1	25	1	13	2	92	219	118	407
32	8/8/16	UL	3	1	25	2	273	122	608	3624	1238	10618
33	8/8/16	UL	3	1	25	3	564	267	1190	3624	1238	10618
34	8/8/16	UL	3	2	25	1	76	25	237	62	26	148
35	8/8/16	UL	3	2	25	2	273	122	608	1812	1121	2931
36	8/8/16	UL	3	2	25	3	564	267	1190	3624	1238	10618
37	8/8/16	UL	4	1	35	1	106	40	282	1812	1121	2931
38	8/8/16	UL	4	1	35	2	273	122	608	3624	1238	10618
39	8/8/16	UL	4	1	35	3	564	267	1190	3624	1238	10618
40	8/8/16	UL	4	2	35	1	205	85	493	640	384	1067
41	8/8/16	UL	4	2	35	2	205	85	493	3624	1238	10618
42	8/8/16	UL	4	2	35	3	369	165	825	3624	1238	10618
43	8/8/16	UL	4	1	25	1	76	25	237	302	162	562
44	8/8/16	UL	4	1	25	2	1206	588	2476	3624	1238	10618
45	8/8/16	UL	4	1	25	3	1206	588	2476	3624	1238	10618
46	8/8/16	UL	4	2	25	1	60	19	185	192	100	369
47	8/8/16	UL	4	2	25	2	207	99	435	1812	1121	2931
48	8/8/16	UL	4	2	25	3	207	99	435	1812	1121	2931
49	8/8/16	UL	5	1	35	1	5	1	32	74	33	166
50	8/8/16	UL	5	1	35	2	39	10	156	180	97	335
51	8/8/16	UL	5	1	35	3	62	20	194	180	97	335
52	8/8/16	UL	5	2	35	1	26	7	105	350	193	633
53	8/8/16	UL	5	2	35	2	26	1	65	545	321	925
54	8/8/16	UL	5	2	35	3	26	7	105	545	321	925
55	8/8/16	UL	5	1	25	1	5	1	32	20	5	80
56	8/8/16	UL	5	1	25	2	76	25	237	310	180	535
57	8/8/16	UL	5	1	25	3	76	25	237	310	180	535
58	8/8/16	UL	5	2	25	1	5	1	32	10	1	68
59	8/8/16	UL	5	2	25	2	132	49	351	468	271	810
60	8/8/16	UL	5	2	25	3	205	85	493	405	229	715
61	8/15/16	CC	1	1	35	1	5	1	32	9	1	65
62	8/15/16	CC	1	1	35	2	29	7	115	10	1	68
63	8/15/16	CC	1	1	35	3	132	49	351	10	1	68
64	8/15/16	CC	1	2	35	1	5	1	32	10	1	68
65	8/15/16	CC	1	2	35	2	39	10	156	20	5	80
66	8/15/16	CC	1	2	35	3	115	43	307	20	5	80
67	8/15/16	CC	1	1	25	1	5	1	32	5	1	32
68	8/15/16	CC	1	1	25	2	13	2	92	5	1	32
69	8/15/16	CC	1	1	25	3	13	2	92	74	33	166
70	8/15/16	CC	1	2	25	1	5	1	32	5	1	32
71	8/15/16	CC	1	2	25	2	5	1	32	5	1	32

72	8/15/16	CC	1	2	25	3	39	10	156	405	229	715
73	8/15/16	CC	2	1	35	1	5	1	32	9	1	65
74	8/15/16	CC	2	1	35	2	13	2	92	88	42	184
75	8/15/16	CC	2	1	35	3	76	25	237	69	31	154
76	8/15/16	CC	2	2	35	1	5	1	32	5	1	32
77	8/15/16	CC	2	2	35	2	11	2	76	219	118	407
78	8/15/16	CC	2	2	35	3	115	43	307	57	24	138
79	8/15/16	CC	2	1	25	1	5	1	32	5	1	32
80	8/15/16	CC	2	1	25	2	5	1	32	106	50	221
81	8/15/16	CC	2	1	25	3	205	85	493	468	271	810
82	8/15/16	CC	2	2	25	1	5	1	32	5	1	32
83	8/15/16	CC	2	2	25	2	5	1	32	81	36	180
84	8/15/16	CC	2	2	25	3	27	7	110	122	61	244
85	8/15/16	CC	3	1	35	1	9	1	65	10	1	68
86	8/15/16	CC	3	1	35	2	22	5	87	139	70	279
87	8/15/16	CC	3	1	35	3	62	20	194	20	5	80
88	8/15/16	CC	3	2	35	1	5	1	32	5	1	32
89	8/15/16	CC	3	2	35	2	11	2	76	106	50	221
90	8/15/16	CC	3	2	35	3	60	19	185	20	5	80
91	8/15/16	CC	3	1	25	1	5	1	32	5	1	32
92	8/15/16	CC	3	1	25	2	13	2	92	81	36	180
93	8/15/16	CC	3	1	25	3	39	10	156	81	36	180
94	8/15/16	CC	3	2	25	1	5	1	32	5	1	32
95	8/15/16	CC	3	2	25	2	5	1	32	122	61	244
96	8/15/16	CC	3	2	25	3	13	2	92	219	118	407
97	8/15/16	CC	4	1	35	1	19	5	77	936	576	1521
98	8/15/16	CC	4	1	35	2	273	122	608	936	576	1521
99	8/15/16	CC	4	1	35	3	564	267	1190	936	576	1521
100	8/15/16	CC	4	2	35	1	76	25	237	640	384	1067
101	8/15/16	CC	4	2	35	2	362	172	761	1812	1121	2931
102	8/15/16	CC	4	2	35	3	362	172	761	1812	1121	2931
103	8/15/16	CC	4	1	25	1	13	2	92	10	1	68
104	8/15/16	CC	4	1	25	2	273	122	608	1812	1121	2931
105	8/15/16	CC	4	1	25	3	273	122	608	3624	1238	10618
106	8/15/16	CC	4	2	25	1	76	25	237	10	1	68
107	8/15/16	CC	4	2	25	2	175	73	421	545	321	925
108	8/15/16	CC	4	2	25	3	273	122	608	1812	1121	2931
109	8/15/16	CC	5	1	35	1	39	10	156	248	144	427
110	8/15/16	CC	5	1	35	2	238	99	573	936	576	1521
111	8/15/16	CC	5	1	35	3	273	122	608	936	576	1521
112	8/15/16	CC	5	2	35	1	76	25	237	202	112	365

113	8/15/16	CC	5	2	35	2	115	43	307	545	321	925
114	8/15/16	CC	5	2	35	3	330	157	693	545	321	925
115	8/15/16	CC	5	1	25	1	39	10	156	180	97	335
116	8/15/16	CC	5	1	25	2	205	85	493	545	321	925
117	8/15/16	CC	5	1	25	3	205	85	493	545	321	925
118	8/15/16	CC	5	2	25	1	115	43	307	122	61	244
119	8/15/16	CC	5	2	25	2	273	122	608	640	384	1067
120	8/15/16	CC	5	2	25	3	273	122	608	640	384	1067
121	8/22/16	UL	1	1	35	1	13	2	92	10	1	68
122	8/22/16	UL	1	1	35	2	76	25	237	32	10	99
123	8/22/16	UL	1	1	35	3	76	25	237	32	10	99
124	8/22/16	UL	1	2	35	1	13	2	92	30	10	94
125	8/22/16	UL	1	2	35	2	29	7	115	30	10	94
126	8/22/16	UL	1	2	35	3	84	35	201	43	16	114
127	8/22/16	UL	1	1	25	1	5	1	32	5	1	32
128	8/22/16	UL	1	1	25	2	5	1	32	32	10	99
129	8/22/16	UL	1	1	25	3	36	11	111	46	17	121
130	8/22/16	UL	1	2	25	1	5	1	32	20	5	80
131	8/22/16	UL	1	2	25	2	39	10	156	20	5	80
132	8/22/16	UL	1	2	25	3	76	25	237	74	33	166
133	8/22/16	UL	2	1	35	1	13	2	92	29	9	90
134	8/22/16	UL	2	1	35	2	39	10	156	41	15	108
135	8/22/16	UL	2	1	35	3	115	43	307	41	15	108
136	8/22/16	UL	2	2	35	1	5	1	32	5	1	32
137	8/22/16	UL	2	2	35	2	13	2	92	32	10	99
138	8/22/16	UL	2	2	35	3	216	97	482	32	10	99
139	8/22/16	UL	2	1	25	1	5	1	32	5	1	32
140	8/22/16	UL	2	1	25	2	29	7	115	57	24	138
141	8/22/16	UL	2	1	25	3	29	7	115	468	271	810
142	8/22/16	UL	2	2	25	1	5	1	32	10	1	68
143	8/22/16	UL	2	2	25	2	60	19	185	62	26	148
144	8/22/16	UL	2	2	25	3	91	34	244	345	204	583
145	8/22/16	UL	3	1	35	1	29	7	115	43	16	114
146	8/22/16	UL	3	1	35	2	76	25	237	219	118	407
147	8/22/16	UL	3	1	35	3	115	43	307	405	229	715
148	8/22/16	UL	3	2	35	1	76	25	237	32	10	99
149	8/22/16	UL	3	2	35	2	115	43	307	139	72	268
150	8/22/16	UL	3	2	35	3	369	165	825	139	72	268
151	8/22/16	UL	3	1	25	1	10	1	68	10	1	68
152	8/22/16	UL	3	1	25	2	34	11	105	110	55	220
153	8/22/16	UL	3	1	25	3	205	85	493	202	112	365

154	8/22/16	UL	3	2	25	1	5	1	32	10	1	68
155	8/22/16	UL	3	2	25	2	10	1	68	139	72	268
156	8/22/16	UL	3	2	25	3	205	85	493	545	321	925
157	8/22/16	UL	4	1	35	1	182	82	405	310	180	535
158	8/22/16	UL	4	1	35	2	564	267	1190	640	384	1067
159	8/22/16	UL	4	1	35	3	564	267	1190	763	464	1255
160	8/22/16	UL	4	2	35	1	132	49	351	405	229	715
161	8/22/16	UL	4	2	35	2	461	219	970	640	384	1067
162	8/22/16	UL	4	2	35	3	727	361	1466	763	464	1255
163	8/22/16	UL	4	1	25	1	132	49	351	175	97	316
164	8/22/16	UL	4	1	25	2	205	85	493	1812	1121	2931
165	8/22/16	UL	4	1	25	3	564	267	1190	1812	1121	2931
166	8/22/16	UL	4	2	25	1	115	43	307	219	118	407
167	8/22/16	UL	4	2	25	2	564	267	1190	763	464	1255
168	8/22/16	UL	4	2	25	3	564	267	1190	1214	753	1957
169	8/22/16	UL	5	1	35	1	13	2	92	32	10	99
170	8/22/16	UL	5	1	35	2	39	10	156	74	33	166
171	8/22/16	UL	5	1	35	3	115	43	307	74	33	166
172	8/22/16	UL	5	2	35	1	13	2	92	54	22	130
173	8/22/16	UL	5	2	35	2	76	25	237	82	39	171
174	8/22/16	UL	5	2	35	3	76	25	237	88	42	184
175	8/22/16	UL	5	1	25	1	11	2	76	10	1	68
176	8/22/16	UL	5	1	25	2	39	10	156	62	26	148
177	8/22/16	UL	5	1	25	3	39	10	156	110	55	220
178	8/22/16	UL	5	2	25	1	13	2	92	5	1	32
179	8/22/16	UL	5	2	25	2	39	10	156	46	17	121
180	8/22/16	UL	5	2	25	3	205	85	493	310	180	535
181	8/29/16	CC	1	1	35	1	5	1	32	10	1	68
182	8/29/16	CC	1	1	35	2	5	1	32	5	1	32
183	8/29/16	CC	1	1	35	3	13	2	92	32	10	99
184	8/29/16	CC	1	2	35	1	5	1	32	5	1	32
185	8/29/16	CC	1	2	35	2	5	1	32	5	1	32
186	8/29/16	CC	1	2	35	3	29	7	115	10	1	68
187	8/29/16	CC	1	1	25	1	5	1	32	5	1	32
188	8/29/16	CC	1	1	25	2	5	1	32	10	1	68
189	8/29/16	CC	1	1	25	3	5	1	32	95	45	200
190	8/29/16	CC	1	2	25	1	5	1	32	5	1	32
191	8/29/16	CC	1	2	25	2	5	1	32	10	1	68
192	8/29/16	CC	1	2	25	3	11	2	76	122	61	244
193	8/29/16	CC	2	1	35	1	5	1	32	5	1	32
194	8/29/16	CC	2	1	35	2	5	1	32	5	1	32

195	8/29/16	CC	2	1	35	3	5	1	32	29	9	90
196	8/29/16	CC	2	2	35	1	5	1	32	5	1	32
197	8/29/16	CC	2	2	35	2	5	1	32	5	1	32
198	8/29/16	CC	2	2	35	3	39	10	156	5	1	32
199	8/29/16	CC	2	1	25	1	5	1	32	10	1	68
200	8/29/16	CC	2	1	25	2	5	1	32	5	1	32
201	8/29/16	CC	2	1	25	3	5	1	32	192	100	369
202	8/29/16	CC	2	2	25	1	5	1	32	5	1	32
203	8/29/16	CC	2	2	25	2	9	1	66	5	1	32
204	8/29/16	CC	2	2	25	3	9	1	66	180	97	335
205	8/29/16	CC	3	1	35	1	5	1	32	10	1	68
206	8/29/16	CC	3	1	35	2	5	1	32	468	271	810
207	8/29/16	CC	3	1	35	3	60	19	185	468	271	810
208	8/29/16	CC	3	2	35	1	5	1	32	10	1	68
209	8/29/16	CC	3	2	35	2	13	2	92	101	51	202
210	8/29/16	CC	3	2	35	3	91	34	244	101	51	202
211	8/29/16	CC	3	1	25	1	5	1	32	5	1	32
212	8/29/16	CC	3	1	25	2	5	1	32	5	1	32
213	8/29/16	CC	3	1	25	3	10	1	68	224	127	395
214	8/29/16	CC	3	2	25	1	5	1	32	5	1	32
215	8/29/16	CC	3	2	25	2	5	1	32	20	5	80
216	8/29/16	CC	3	2	25	3	5	1	32	936	576	1521
217	8/29/16	CC	4	1	35	1	11	2	76	139	70	279
218	8/29/16	CC	4	1	35	2	39	10	156	302	162	562
219	8/29/16	CC	4	1	35	3	106	40	282	302	162	562
220	8/29/16	CC	4	2	35	1	9	1	66	159	83	306
221	8/29/16	CC	4	2	35	2	49	18	130	202	112	365
222	8/29/16	CC	4	2	35	3	97	36	259	202	112	365
223	8/29/16	CC	4	1	25	1	5	1	32	10	1	68
224	8/29/16	CC	4	1	25	2	27	7	110	640	384	1067
225	8/29/16	CC	4	1	25	3	27	7	110	1812	1121	2931
226	8/29/16	CC	4	2	25	1	5	1	32	32	10	99
227	8/29/16	CC	4	2	25	2	115	43	307	405	229	715
228	8/29/16	CC	4	2	25	3	205	85	493	640	384	1067
229	8/29/16	CC	5	1	35	1	5	1	32	20	5	80
230	8/29/16	CC	5	1	35	2	9	1	66	468	271	810
231	8/29/16	CC	5	1	35	3	48	18	128	468	271	810
232	8/29/16	CC	5	2	35	1	13	2	92	106	55	205
233	8/29/16	CC	5	2	35	2	13	2	92	763	464	1255
234	8/29/16	CC	5	2	35	3	205	85	493	763	464	1255
235	8/29/16	CC	5	1	25	1	39	10	156	219	118	407

236	8/29/16	CC	5	1	25	2	67	22	207	640	384	1067
237	8/29/16	CC	5	1	25	3	205	85	493	763	464	1255
238	8/29/16	CC	5	2	25	1	22	6	88	139	72	268
239	8/29/16	CC	5	2	25	2	132	49	351	936	576	1521
240	8/29/16	CC	5	2	25	3	205	85	493	936	576	1521
241	9/12/16	UL	1	1	35	1	5	1	32	20	5	80
242	9/12/16	UL	1	1	35	2	76	25	237	139	70	279
243	9/12/16	UL	1	1	35	3	115	43	307	139	70	279
244	9/12/16	UL	1	2	35	1	5	1	32	32	10	99
245	9/12/16	UL	1	2	35	2	67	22	207	159	83	306
246	9/12/16	UL	1	2	35	3	97	36	259	159	83	306
247	9/12/16	UL	1	1	25	1	23	6	91	20	5	80
248	9/12/16	UL	1	1	25	2	23	6	91	62	26	148
249	9/12/16	UL	1	1	25	3	39	10	156	545	321	925
250	9/12/16	UL	1	2	25	1	5	1	32	10	1	68
251	9/12/16	UL	1	2	25	2	13	2	92	57	24	138
252	9/12/16	UL	1	2	25	3	39	10	156	1214	753	1957
253	9/12/16	UL	2	1	35	1	13	2	92	20	5	80
254	9/12/16	UL	2	1	35	2	13	2	92	192	100	369
255	9/12/16	UL	2	1	35	3	132	49	351	192	100	369
256	9/12/16	UL	2	2	35	1	11	2	76	32	10	99
257	9/12/16	UL	2	2	35	2	27	7	107	81	36	180
258	9/12/16	UL	2	2	35	3	126	52	302	81	36	180
259	9/12/16	UL	2	1	25	1	13	2	92	10	1	68
260	9/12/16	UL	2	1	25	2	13	2	92	95	45	200
261	9/12/16	UL	2	1	25	3	39	10	156	936	576	1521
262	9/12/16	UL	2	2	25	1	5	1	32	10	1	68
263	9/12/16	UL	2	2	25	2	13	2	92	30	10	94
264	9/12/16	UL	2	2	25	3	13	2	92	468	271	810
265	9/12/16	UL	3	1	35	1	5	1	32	62	26	148
266	9/12/16	UL	3	1	35	2	76	25	237	302	162	562
267	9/12/16	UL	3	1	35	3	56	21	148	302	162	562
268	9/12/16	UL	3	2	35	1	5	1	32	74	33	166
269	9/12/16	UL	3	2	35	2	76	25	237	468	271	810
270	9/12/16	UL	3	2	35	3	369	165	825	468	271	810
271	9/12/16	UL	3	1	25	1	5	1	32	10	1	68
272	9/12/16	UL	3	1	25	2	5	1	32	350	193	633
273	9/12/16	UL	3	1	25	3	27	7	110	640	384	1067
274	9/12/16	UL	3	2	25	1	13	2	92	5	1	32
275	9/12/16	UL	3	2	25	2	13	2	92	468	271	810
276	9/12/16	UL	3	2	25	3	81	34	195	1812	1121	2931

277	9/12/16	UL	4	1	35	1	5	1	32	192	100	369
278	9/12/16	UL	4	1	35	2	76	25	237	405	229	715
279	9/12/16	UL	4	1	35	3	205	85	493	468	271	810
280	9/12/16	UL	4	2	35	1	13	2	92	62	26	148
281	9/12/16	UL	4	2	35	2	29	7	115	468	271	810
282	9/12/16	UL	4	2	35	3	76	25	237	468	271	810
283	9/12/16	UL	4	1	25	1	13	2	92	106	50	221
284	9/12/16	UL	4	1	25	2	76	25	237	545	321	925
285	9/12/16	UL	4	1	25	3	76	25	237	763	464	1255
286	9/12/16	UL	4	2	25	1	5	1	32	62	26	148
287	9/12/16	UL	4	2	25	2	76	25	237	545	321	925
288	9/12/16	UL	4	2	25	3	175	73	421	545	321	925
289	9/12/16	UL	5	1	35	1	13	2	92	62	26	148
290	9/12/16	UL	5	1	35	2	39	10	156	350	193	633
291	9/12/16	UL	5	1	35	3	115	43	307	350	193	633
292	9/12/16	UL	5	2	35	1	10	1	68	81	36	180
293	9/12/16	UL	5	2	35	2	46	15	143	302	162	562
294	9/12/16	UL	5	2	35	3	205	85	493	302	162	562
295	9/12/16	UL	5	1	25	1	5	1	32	10	1	68
296	9/12/16	UL	5	1	25	2	76	25	237	74	33	166
297	9/12/16	UL	5	1	25	3	76	25	237	1214	753	1957
298	9/12/16	UL	5	2	25	1	10	1	68	19	5	77
299	9/12/16	UL	5	2	25	2	20	5	78	219	118	407
300	9/12/16	UL	5	2	25	3	46	15	143	936	576	1521
301	9/19/16	CC	1	1	35	1	5	1	32	10	1	68
302	9/19/16	CC	1	1	35	2	5	1	32	10	1	68
303	9/19/16	CC	1	1	35	3	11	2	76	20	5	80
304	9/19/16	CC	1	2	35	1	5	1	32	5	1	32
305	9/19/16	CC	1	2	35	2	5	1	32	20	5	80
306	9/19/16	CC	1	2	35	3	13	2	92	20	5	80
307	9/19/16	CC	1	1	25	1	5	1	32	10	1	68
308	9/19/16	CC	1	1	25	2	5	1	32	19	5	77
309	9/19/16	CC	1	1	25	3	13	2	92	41	15	108
310	9/19/16	CC	1	2	25	1	5	1	32	5	1	32
311	9/19/16	CC	1	2	25	2	5	1	32	5	1	32
312	9/19/16	CC	1	2	25	3	5	1	32	32	10	99
313	9/19/16	CC	2	1	35	1	5	1	32	5	1	32
314	9/19/16	CC	2	1	35	2	5	1	32	62	26	148
315	9/19/16	CC	2	1	35	3	22	6	88	62	26	148
316	9/19/16	CC	2	2	35	1	5	1	32	5	1	32
317	9/19/16	CC	2	2	35	2	5	1	32	20	5	80

318	9/19/16	CC	2	2	35	3	11	2	76	32	10	99
319	9/19/16	CC	2	1	25	1	5	1	32	5	1	32
320	9/19/16	CC	2	1	25	2	5	1	32	62	26	148
321	9/19/16	CC	2	1	25	3	13	2	92	110	55	220
322	9/19/16	CC	2	2	25	1	5	1	32	5	1	32
323	9/19/16	CC	2	2	25	2	5	1	32	19	5	77
324	9/19/16	CC	2	2	25	3	5	1	32	175	97	316
325	9/19/16	CC	3	1	35	1	5	1	32	20	5	80
326	9/19/16	CC	3	1	35	2	5	1	32	62	26	148
327	9/19/16	CC	3	1	35	3	13	2	92	46	17	121
328	9/19/16	CC	3	2	35	1	5	1	32	10	1	68
329	9/19/16	CC	3	2	35	2	13	2	92	20	5	80
330	9/19/16	CC	3	2	35	3	76	25	237	32	10	99
331	9/19/16	CC	3	1	25	1	5	1	32	5	1	32
332	9/19/16	CC	3	1	25	2	5	1	32	10	1	68
333	9/19/16	CC	3	1	25	3	13	2	92	405	229	715
334	9/19/16	CC	3	2	25	1	5	1	32	5	1	32
335	9/19/16	CC	3	2	25	2	5	1	32	10	1	68
336	9/19/16	CC	3	2	25	3	11	2	76	180	97	335
337	9/19/16	CC	4	1	35	1	5	1	32	95	45	200
338	9/19/16	CC	4	1	35	2	39	10	156	247	137	447
339	9/19/16	CC	4	1	35	3	115	43	307	405	229	715
340	9/19/16	CC	4	2	35	1	5	1	32	101	51	202
341	9/19/16	CC	4	2	35	2	9	1	65	640	384	1067
342	9/19/16	CC	4	2	35	3	97	36	259	763	464	1255
343	9/19/16	CC	4	1	25	1	13	2	92	32	10	99
344	9/19/16	CC	4	1	25	2	13	2	92	247	137	447
345	9/19/16	CC	4	1	25	3	13	2	92	763	464	1255
346	9/19/16	CC	4	2	25	1	5	1	32	5	1	32
347	9/19/16	CC	4	2	25	2	39	10	156	405	229	715
348	9/19/16	CC	4	2	25	3	13	2	92	1214	753	1957
349	9/19/16	CC	5	1	35	1	5	1	32	175	97	316
350	9/19/16	CC	5	1	35	2	81	34	195	763	464	1255
351	9/19/16	CC	5	1	35	3	564	267	1190	936	576	1521
352	9/19/16	CC	5	2	35	1	5	1	32	248	144	427
353	9/19/16	CC	5	2	35	2	27	7	110	763	464	1255
354	9/19/16	CC	5	2	35	3	175	73	421	1214	753	1957
355	9/19/16	CC	5	1	25	1	39	10	156	32	10	99
356	9/19/16	CC	5	1	25	2	126	52	302	1214	753	1957
357	9/19/16	CC	5	1	25	3	273	122	608	1812	1121	2931
358	9/19/16	CC	5	2	25	1	13	2	92	62	26	148

359	9/19/16	CC	5	2	25	2	132	49	351	763	464	1255
360	9/19/16	CC	5	2	25	3	175	73	421	763	464	1255

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