Evaluating practical approaches for on-farm water testing to meet the FSMA Produce Safety Rule requirements

by

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Abstract

The Produce Safety Rule, a part of the FDA Food Safety Modernization Act (FSMA), requires produce farmers to monitor the quality of irrigation and wash water used on the farm for generic *E. coli* in an effort to prevent microbial contamination of fresh produce grown for human consumption. The objectives of this study were to (1) compare alternative methods against the EPA Standard Method in determining *E. coli* levels in surface waters of varying chemical characteristics and (2) determine the effects of transport time on *E. coli* concentrations determined by the EPA standard method. Results indicated Coliscan Easygel 5 mL had the highest correlation ($r = 0.72$, $p = 0.01$) to the EPA standard method, while Coliscan Membrane Filter had the second highest correlation ($r = 0.70$, $p = 0.01$). Results from the prolonged sample holding times indicated significant variation between sample holding times ($p = 0.01$).

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1. Introduction and Literature Review

1.1 Introduction

Water is a scarce resource often used for municipal and agricultural activities with its use and consumption essential for anthropogenic and ecological health. The contamination of water resources in the United States has resulted in severe economic and environmental loses estimated to be around $13 billion a year (Batz et al., 2011). Specifically, outbreaks related to raw produce consumption have also increased ten-fold in the past two decades, contributing a significant amount to economic and environmental losses (Lee and Lee, 2013). Reasons for the increase in outbreaks is believed to be due to improper use of human and animal waste as fertilizers, urban overflow and agricultural runoff (Arnone and Perdek Walling, 2007; Lee and Lee, 2013). There have been 30 reported cases of *Escherichia coli* related foodborne outbreaks since 2006 (CDC, 2018b). One of the most severe outbreaks occurred in the Yuma region of Arizona in 2018 that affected 210 individuals and resulted in five fatalities (CDC, 2018a). The outbreak was found to be from pathogen contaminated canal water used for irrigation (CDC, 2018a).

In the United States about 80% of total consumptive water is used for agricultural production (Falkenmark and Lannerstad, 2005; USDA, 2017, 2019). That however accounts for irrigation in only 7.6 % of the total US cropland and pastureland (Nickerson and Borchers, 2012; USDA, 2019), and most of that water (57%) comes from rivers and streams (USGS, 2010). Crops most commonly irrigated were corn, fruit and vegetables, with 25% of the total irrigated crop land for corn production and 8% for vegetable and

fruit production (USDA, 2019). Agricultural production and processing in the United States contributes a significant amount to the GDP and supplies a large portion of the world with food supplies (Millennium Ecosystem Assessment, 2005; Gordon et al., 2010). Anthropogenic processes are threatening water resources for agricultural production and processing though, which is critical for sufficient food supply and economic development (Millennium Ecosystem Assessment, 2005).

Water quality and scarcity is a major issue facing agricultural production in the United States (Tong and Chen, 2002; Scanlon et al., 2007; Duran-Encalada et al., 2017). The three largest aquifers in the United States contain half of the total groundwater supply in the US and are also the most overdrawn and depleted aquifers (Reilly, 2008; Scanlon et al., 2012; Konikow, 2015). Streamflow depletion is also an increasing environmental issue in the United States and in some areas, streamflow reduction has been reduced to nearly half of the total river's water supply (Simons, 1953; Ruhl, 2009). In addition, disputes over water rights in river basins is largely due to agricultural production, which demonstrates the increased scarcity for water in the US (Feldman, 2008; Ruhl, 2009). Furthermore, nitrogen contamination of groundwater and surface water has posed issues to several water sources in the past decade, resulting in adverse effects in humans and animals (Mueller et al., 1995; Puckett, 1995; Dubrovsky and Hamilton, 2010). Groundwater contamination from nitrogen was most common in areas of agricultural production due to seepage and excess fertilizer use (Mueller et al., 1995; Dubrovsky and Hamilton, 2010). In urban areas, groundwater contamination due to nitrogen is also most commonly related to the over application of fertilizer (Mueller et al., 1995; Dubrovsky and Hamilton, 2010). High phosphorus concentrations were also found

in agricultural streams and was primarily related to the over application of fertilizer in urban and agricultural waterways (Dubrovsky and Hamilton, 2010). Additionally, pathogens are a major concern in water quality and were found to be the leading cause of water impairment in the US (Arnone and Perdek Walling, 2007; Pandey et al., 2014). 45% of lakes, reservoirs and ponds were impaired due to pathogens in the United States, which is a major source of water for agricultural producers (Arnone and Perdek Walling, 2007; Pandey et al., 2014). Furthermore, streams and waterways impaired due to pathogens were primarily from agricultural production and urban runoff, sewage leaks and contaminated sediment (Arnone and Perdek Walling, 2007; Pandey et al., 2014). In lakes, ponds and reservoirs, sources of pathogens generally originated from sediment after heavy storm events (Pandey et al., 2014). Of water-related gastrointestinal diseases in the United States, 11% of the cases were due to *Cryptosporidium parvum* and 8% were due to *E. coli* O157:H7 (Pandey et al., 2014). Due to a large number of contaminated water resources in the United States, there is a high potential for a farmer to use contaminated water sources potentially resulting in foodborne gastrointestinal diseases (USEPA, 2016).

With the increase in foodborne disease outbreaks, contamination of raw produce is becoming a public health concern (Batz et al., 2011; FDA, 2015; CDC, 2018a). To address that concern, the U.S. Food and Drug Administration (FDA) developed the Food and Safety Modernization Act's Produce Safety Rule (FSMA-PSR) and set two goals (FDA, 2015). The first one is to prevent the introduction of foreseeable hazards into produce and the second is to provide reasonable assurance that produce has not been contaminated upon handling (Gradl and Worost, 2017). According to FSMA, eligible

agricultural producers are required to conduct an initial survey on water quality using a minimum of 20 samples collected throughout a two to four year period (FDA, 2015). If the microbial criteria are not met by the agricultural producers, they have three options to then utilize their water supply (FDA, 2015). First, they may let the bacteria die off before harvest by allowing four days between irrigation and harvest (FDA, 2015). Second, they may use commercial activities or washing to remove bacteria from contaminated produce (FDA, 2015). The last option is to treat the contaminated water to remove potentially dangerous bacteria (FDA, 2015).

Challenges with the FSMA Produce Safety Rule begin with the large amount of impaired water sources due to pathogens in the United States, the high cost of analysis required by the recommended enumeration methods and the transportation limitations for many rural agricultural producers (Simons, 1953; Puckett, 1995; Arnone and Perdek Walling, 2007; Dubrovsky and Hamilton, 2010; Scanlon et al., 2012; Pandey et al., 2014). The FDA has anticipated these issues and has allowed the use of alternative methods as long as the alternative methods have sufficient scientific support and provide identical levels of public health protection as compared to the EPA Method 1603 (USHHS, 2015). Several alternative methods have the potential to satisfy the FDA's criteria, reduce the cost of analysis and also eliminate transportation limitations. The costs of analysis for the EPA recommended methods can reach more than $30 dollar a sample. The alternative enumeration methods are considerably more cost-effective with the Coliscan® Easygel method being the least expensive ($3/sample) of those evaluated in this study followed by the 3M™ Petrifilm EC method and the Coliscan® Membrane

Filter method. In addition, alternative methods can potentially eliminate transportation requirements due to the ability to be incubated at room temperature.

A few prior studies have compared alternative enumeration methods to standard methods and have found strong correlations between them (Noble et al., 2003; Vail et al., 2003; Beloti et al., 2003; Schraft and Watterworth, 2005; Olstadt et al., 2007; Stepenuck et al., 2011). The method most commonly compared to the EPA method 1603 (or MPN method) throughout the previous studies is the 3M™ Petrifilm *E. coli*/coliform (EC) count plates. In addition, the precision or accuracy of enumeration methods have been seldom researched (Noble et al., 2003; Vail et al., 2003; Beloti et al., 2003; Olstadt et al., 2007; Stepenuck et al., 2011). Past research has indicated favorable results for 3M™ Petrifilm EC count plates and Coliscan® Membrane Filter in terms of accuracy and precision (Dufour et al., 1981; Noble et al., 2003; Vail et al., 2003; Olstadt et al., 2007). Furthermore, the effects of a 24-hour delay on *E. coli* enumeration has been seldomly researched but have shown significant differences in relation to different sample holding times (McCarthy et al., 2008; Harmel et al., 2016). Therefore, comparing various alternative methods used by citizen volunteers, i.e., Coliscan® Easygel, 3M™ Petrifilm EC, and Coliscan® MF with the EPA Method 1603 would allow for alternative methods for *E. coli* enumeration to be available to agricultural producers and citizen monitoring programs for *E. coli*. enumeration.

1.2 Food Safety Issues

In the United States, the contamination of produce from foodborne outbreaks has caused severe economic and environmental losses throughout the past decade (Batz et al., 2011; CDC, 2018b). From this, gastrointestinal diseases related to foodborne outbreaks

affected 60 to 70 million individuals since 2010 and resulted in 245,921 fatalities (NIH, 2014). Additionally, *Salmonella, Campylobacter, Listeria, Toxoplasma* and norovirus have caused $12.7 billion in annual economic loss (Batz et al., 2011). *E. coli* is closely followed behind with economic losses of $500 million annually (Batz et al., 2011).

Water-related foodborne outbreaks have also resulted in serious public health concerns. Outbreaks associated with raw produce consumption increased from 0.7% to 6% since the 1990s (Lee and Lee, 2013). Raw produce contamination is among the top three causes of foodborne disease outbreaks with 19% of the total following multi-ingredient foods with 28% and seafood with 30% (Lee and Lee, 2013). More recently, fresh produce has been implicated with *E. coli* O157:H7 and *Salmonella* which are transported through fecal-oral routes (Lee and Lee, 2013). The improper use of composted human and animal waste as fertilizers have resulted in the contamination of fresh produce with a high level of *E. coli* and *Salmonella* (Lee and Lee, 2013). Furthermore, *Salmonella*-contaminated produce, meat and dairy have resulted in 67 outbreaks since 2006 (CDC, 2018b). The last reported case of *Salmonella*-related foodborne outbreaks occurred in July of 2018 where raw turkey products were linked to the infections (CDC, 2018c). The case resulted from unsanitary working conditions and caused infections in 26 states, affecting 90 individuals (CDC, 2018c). In addition, a *Salmonella* outbreak in treated recreational water occurred where five individuals were infected (Hlavsa, 2018).

Since 2006, the Center for Disease Control and Prevention (CDC) has reported 30 separate cases of *Escherichia coli* related foodborne disease outbreaks (CDC, 2018b). The last reported outbreak occurred in 2018 where 210 individuals contracted

gastrointestinal diseases, resulting in five fatalities (CDC, 2018a). The outbreak occurred in the Yuma region of Arizona where water from the canal was used to irrigate romaine lettuce. The outbreak from *E. coli* contaminated romaine lettuce affected 36 states and was identified as *E. coli* strain O157:H7 (CDC, 2018a). In addition, an outbreak from 2017 which resulted from the contamination of spinach affected 25 individuals from 15 different states (CDC, 2017). Of these 25 individuals, nine resulted in hospitalizations and one fatality occurred (CDC, 2017). Another contamination from irrigation water containing Shiga toxin producing *E. coli* occurred in May of 2014 where raw clover sprouts were traced back as the source of contamination (CDC, 2014). From this case, 19 individuals were affected in six different states and resulted in no major illnesses or fatalities (CDC, 2014).

1.3 Importance of Water for Agricultural Production

The recreational and agricultural uses of surface water and groundwater is becoming increasingly problematic (USEPA, 2016). United States about 80% of total consumptive water is used for agricultural production (Falkenmark and Lannerstad, 2005; USDA, 2017, 2019). That however accounts for irrigation in only 7.6 % of the total US cropland and pastureland (Nickerson and Borchers, 2012; USDA, 2019), and more than half of that water (57%) is withdrawn from rivers and streams (USGS, 2010). Of the estimated 1.1 billion acres of cropland and pastureland, an estimated 350 million acres (31%) was farmed for agricultural production (Nickerson and Borchers, 2012; USDA, 2019). Of this, only an estimated 7.6% of the US cropland and pastureland was irrigated in 2012 (Nickerson and Borchers, 2012; USDA, 2019). Therefore, the consumptive use of irrigation water for agricultural production is contained to only a small area of the US

land area but accounts for 80% of the total water use (Falkenmark and Lannerstad, 2005; USGS, 2010; Nickerson and Borchers, 2012; USDA, 2017, 2019). The crops most commonly irrigated in the United States consist of corn, hay, soybeans and vegetables (USDA, 2019). In 2019, 25% of the total irrigated cropland was from corn production and 8% was from vegetable and fruit production (USDA, 2019). However, sources of water for agricultural production are becoming depleted and contaminated by anthropogenic processes (Falkenmark and Lannerstad, 2005; USGS, 2010; Gordon et al., 2010). The Ogallala Aquifer is one of the most depleted water sources in the United States and provides one-fifth of the water supplied for cropland (Falkenmark and Lannerstad, 2005). This demonstrates the increased scarcity of water supply for agriculture production.

The economic value of agricultural production in the United States is estimated to be $132 billion/year and accounts for 1% of the Gross Domestic Product (USDA, 2017). Also, agricultural production accounts for 11% of the US employment rate with 21.6 million full-time and part-time jobs (USDA, 2017). Direct on-farm employment was significantly lower than the total US agricultural production employment rate of 1.3% (USDA, 2017). Nevertheless, agricultural production and processing in the United States supplies a large portion of the world with food supplies and contributes a significant amount to state GDP (Millennium Ecosystem Assessment, 2005; Gordon et al., 2010). Agricultural production is strongly dependent on water quality and quantity to sustain food supplies (USGS, 2010; USDA, 2017, 2019). Therefore, the development in precision agriculture to reduce water consumption is a highly researched area and has been shown to greatly reduce on-farm water usage (Millennium Ecosystem Assessment,

2005; USGS, 2010; Gordon et al., 2010; USDA, 2019). In conclusion, the importance of adequate amounts of water for agricultural production and processing is critical for sufficient food supply and economic development but anthropogenic processes are threatening these resources (Millennium Ecosystem Assessment, 2005). Furthermore, the bacterial contamination of fresh produce is also of increasing environmental concern (Matyas et al., 2010; EFSA, 2013).

1.4 Water Issues

In the United States, water quantity and quality have decreased in the past decades threatening agricultural production (Tong and Chen, 2002; Scanlon et al., 2007; Duran-Encalada et al., 2017). Groundwater depletion has been the primary cause of the decrease in water quantity (Tong and Chen, 2002; Konikow, 2015). In the United States, the High Plains, Gulf Coastal Plain, and California Central Valley aquifers contain nearly half the total groundwater supply (Reilly, 2008). They are also the most depleted aquifers in the United States (Reilly, 2008; Scanlon et al., 2012; Konikow, 2015). The High Plains aquifer is the most depleted at 10.2 km^3/year of water loss (Reilly, 2008), and the Gulf Coastal Plain aquifer is the second most depleted at 8.0 km^3/year (Reilly, 2008). Therefore, the over-pumping of wells from major aquifers is resulting in adverse effects on farming communities such as increased irrigation costs and subsidence (Scanlon et al., 2012; Konikow, 2015). In addition, the low recharge rates of aquifers have resulted in water scarcity (Reilly, 2008; Konikow, 2015).

Streamflow depletion is also an increasing water issue in the United States and many states have resorted to legal aid (Simons, 1953; Ruhl, 2009). Most streamflow reductions are due to agricultural practices (Simons, 1953; Feldman, 2008; Ruhl, 2009). In some

areas, streamflow reduction has been nearly half of the total river's water supply (Simons, 1953). One example is the Columbia River Basin in Oregon which has seen a streamflow reduction of up to 56% in heavily farmed areas (Simons, 1953). Furthermore, the water disputes of Alabama, Georgia and Florida primarily resulted from streamflow reductions downstream (Feldman, 2008; Ruhl, 2009). Consumptive water use in the disputed river basins is 80% agriculture, demonstrating the increase in water scarcity in the United States (Feldman, 2008; Ruhl, 2009).

Nutrient and pathogen contamination due to anthropogenic processes has resulted in the degradation of several waterways in the United States (Falkenmark and Lannerstad, 2005; Scanlon et al., 2007; Gordon et al., 2010; USEPA, 2016). Researchers found that most agricultural and urban streams in the United States were in exceedance of the EPA recommended criteria for nutrient loads (Dubrovsky and Hamilton, 2010). Furthermore, nitrogen contamination of groundwater and surface water has posed issues to several water sources in the past decade (Mueller et al., 1995; Puckett, 1995; Dubrovsky and Hamilton, 2010). For example, the contamination of the Floridan aquifer due to nitrite has resulted in cases of blue-baby syndrome and adverse environmental effects (Mueller et al., 1995). Typically, groundwater contamination from nitrogen was most common in areas of agricultural production due to seepage and excess fertilizer use (Mueller et al., 1995; Dubrovsky and Hamilton, 2010). In surface waters, nitrogen contamination was most common in agricultural land use areas and urban areas due to runoff and the over application of fertilizer (Mueller et al., 1995; Dubrovsky and Hamilton, 2010).

Phosphorus contamination of water sources has also caused adverse environmental effects and many of these issues are focused around the eutrophication of waterways and

their effects on aquatic life (Dubrovsky and Hamilton, 2010). Urban waterways were the most affected and were found to have the highest concentrations of phosphorus as compared to all other land use areas (Dubrovsky and Hamilton, 2010). High phosphorus concentrations were also found in agricultural streams and was primarily related to over application of fertilizer in urban and agricultural waterways (Dubrovsky and Hamilton, 2010).

Atmospheric deposition was also a major contributor to nutrient contamination in water sources (Mueller et al., 1995; Puckett, 1995; Butler et al., 2007). For example, nutrient contamination of waterways from atmospheric deposition of mercury is the biggest contributor to mercury contamination in the United States (Butler et al., 2007). In addition, the atmospheric deposition of nitrogen contributed a small amount to waterway contamination which was mostly driven by anthropogenic processes (Mueller et al., 1995; Puckett, 1995). In conclusion, the contamination of water sources from nutrients is an environmental concern that is being addressed at multiple levels of the state and federal government (Mueller et al., 1995; Puckett, 1995; Butler et al., 2007; Dubrovsky and Hamilton, 2010; Gordon et al., 2010).

Anthropogenic processes in the United States have caused increases in pathogens in water sources which have posed multiple issues for urban and agricultural waterways (USEPA, 2016). There are 480,000 km of rivers and shoreline and 2 million ha of lakes and ponds impaired due to pathogens in the United States (Arnone and Perdek Walling, 2007; USEPA, 2016). In addition, pathogens are the leading cause of stream impairment and for lakes, reservoirs and ponds, 45% were found to be impaired in the United States due to pathogens (Arnone and Perdek Walling, 2007; Pandey et al., 2014). Furthermore,

pathogens were found to have a major contribution to lake, pond and reservoir impairment (Arnone and Perdek Walling, 2007; Pandey et al., 2014). Primary sources of impairment due to pathogens depends strongly on the land use area and site location (Arnone and Perdek Walling, 2007; Cabral, 2010). In streams and waterways, pathogen impairment was primarily from agricultural production and urban runoff, sewage leaks and contaminated sediment (Arnone and Perdek Walling, 2007; Pandey et al., 2014). In lakes, ponds and reservoirs, sources of pathogens generally originated from sediment after heavy storm events (Pandey et al., 2014). Additionally, sources of pathogen impairment depended greatly on the influx of contaminants from tributaries (Pandey et al., 2014). Multiple pathogens are responsible for water-related gastrointestinal diseases. *Shigella spp.* was responsible for 27% of the water-related gastrointestinal diseases in the United States (Pandey et al., 2014). Additionally, 11% of the cases were due to *Cryptosporidium parvum* and 12.6% were due to *E. coli* O157:H7 (Pandey et al., 2014). Due to the large number of contaminated water sources in the United States, the potential for a farmer to use contaminated irrigation water is of increasing environmental concern (USEPA, 2016).

1.5 FSMA Produce Safety Rule

The U.S. Food and Drug Administration (FDA) developed the Food and Safety Modernization Act's Produce Safety Rule (FSMA-PSR) due to the reoccurrence of water-related foodborne outbreaks (FDA, 2015). In doing so, the U.S. Food and Drug Administration set two goals (FDA, 2015). The first is to prevent the introduction of foreseeable hazards into produce and the second is to provide reasonable assurance that the produce has not been contaminated upon handling (Gradl and Worost, 2017). With

the increase in foodborne disease outbreaks, contamination of raw produce is becoming a public health concern. Therefore, through FSMA-PSR the FDA is requiring produce farmers to monitor water quality for potential pathogens (USHHS, 2015). Implemented in 2016, the FSMA-PSR established science-based minimum standards for the safe growing, harvesting, packing and holding of produce for human consumption (USHHS, 2015). The final rule established two sets of criteria for microbial water quality, based on the presence of generic *E. coli* (FDA, 2015). Criteria one states that no detectable generic *E. coli* are allowed for particular uses of water for agricultural purposes in which it is likely to contact dangerous pathogens (FDA, 2015). The second set of criteria states numerical criteria, geometric mean and statistical threshold value, for agricultural water directly applied to growing produce (FDA, 2015). For freshwater assessment, the USEPA recommends the use of *E. coli* for bacterial analysis (USEPA, 2012a). A waterway is considered impaired if the *E. coli* concentration exceeds a geometric mean (GM) of 126 CFU/100 mL or a statistical threshold value (STV) of 410 CFU/100 mL (USEPA, 2012a). Also, waterways are considered impaired if concentrations exceed a geometric mean of 35 CFU/100 mL during a 30 day sampling period (USEPA, 2012a). The rule applies to farmed produce that is typically raw before human consumption and does not apply to produce that is cooked before consumption (USHHS, 2015). Within four years of the Final Rule implementation, those farms with more than $25,000 but no more than $250,000 in average produce sales must be in compliance (FDA, 2015). Within three years, farms with more than $250,000 but no more than $500,000 in average annual produce sales must be in compliance ((FDA, 2015). All other farms that do not meet the two prior circumstances, must be in compliance within two years of the Final Rule

implementation (FDA, 2015). The final rule bases testing frequency on the type of water source utilized (FDA, 2015). For surface water, the FDA requires farms to conduct an initial survey on water quality using a minimum of 20 samples. Collected as close to harvest as practicable, the collection should take place throughout a two to four year period (FDA, 2015). For groundwater that is typically directly applied to growing produce, the FDA requires a minimum of four samples collected as close to harvest as is practicable during the growing season (FDA, 2015). Furthermore, there is no requirement for agriculture water to be tested if received from treated water supplies such as public water supply systems (FDA, 2015). If the water does not meet the water quality criteria, corrective actions are required as soon as possible but can be no later than one year after the criteria were not met (FDA, 2015). If the microbial criteria are not met, farmers have three options to utilize their water supply. First, they may allow time for potentially dangerous microbes to die off by allowing no more than four consecutive days between last irrigation and harvest (FDA, 2015). Second, farmers may allow time for microbes to die off between harvest and storage either by washing or commercial activities (FDA, 2015). The last option is to treat the contaminated water to remove potentially dangerous microbes (FDA, 2015). Assessment of microbial water criteria is required by the Final Rule of Food Safety Modernization Act implemented by the FDA to remove potentially dangerous microbes.

Challenges with the FSMA-PSR begin with the large number of impaired water sources in the United States for agricultural production (Simons, 1953; Puckett, 1995; Arnone and Perdek Walling, 2007; Dubrovsky and Hamilton, 2010; Scanlon et al., 2012; Pandey et al., 2014). With roughly 50% of the United States water sources impaired there

is, therefore, a high probability for an agricultural producer to use a contaminated water source making the monitoring of water quality necessary (Puckett, 1995; Arnone and Perdek Walling, 2007; Pandey et al., 2014). The implementation of the FSMA-PSR is also challenging to agricultural producers due to the constraints of the Environmental Protection Agency's (EPA) recommended *E. coli* enumeration methods (FDA, 2018). Currently, the EPA recommends the use of modified membrane filtration techniques (USEPA, 2014) to determine *E. coli* concentrations. In compliance with the EPA Method 1603, water samples are required to be processed by a professional laboratory within six hours of collection, which is often not possible for many rural agricultural producers (USEPA, 2014). For example, in Florida many of the agricultural producers live further than six hours from a professional laboratory. Furthermore, the added cost of analysis and transportation to the laboratory can reach hundreds, if not thousands of dollars. The implementation of FSMA-PSR is therefore problematic due to the high cost and transportation limitations. The FDA has anticipated these issues and has allowed the use of alternative methods as long as the alternative methods have sufficient scientific support and provide identical levels of public health protection as compared to the EPA Method 1603 (USHHS, 2015). Several alternative methods have potential to satisfy the FDA's criteria. For the past 20 years, the Alabama Water Watch (AWW) Program has used Coliscan® Easygel, which render results that repeatedly correlate to the EPA Method 1603 for determining the presence/absence and enumeration of *E. coli.* The Coliscan® Easygel can be and has been easily used by volunteers in the AWW Program for field and on-farm testing. In addition, the Coliscan® Easygel is one the least expensive methods available and is therefore a logical choice to comply with PSR requirements. By

comparing the accuracy and precision of various methods used by citizen volunteers, i.e., Coliscan® Easygel, 3M™ Petrifilm EC, and Coliscan® MF with the accuracy and precision of the EPA Method 1603, would allow for alternative methods to be available to produce farmers for the quantification of *E. coli*.

1.6 Indicator Bacteria

A. Indicator bacteria for the identification of fecal contamination

Gastrointestinal diseases are caused by several different types of waterborne pathogenic organisms and can sometimes be fatal. From 2000-2014, 27,219 individuals contracted gastrointestinal diseases associated with treated recreation water (Hlavsa, 2018). The enumeration of all species of bacteria from water samples would be considerably time-consuming and expensive. Therefore, fecal indicator bacteria are used to assess the microbiological quality of water due to the correlation between fecal bacteria and waterborne disease-causing organisms (Myers et al., 2014). The density of indicator bacteria is a direct measurement of water safety for consumption and body-contact recreation (Myers et al., 2014). Fecal indicator bacteria are a group of organisms that exist in the intestinal tract of warm-blooded animals and enter the secondary habitat through defecation. The presence of fecal indicators in irrigation or surface water is direct evidence of fecal contamination from warm-blooded animals (Myers et al., 2014). In the United States, the most commonly used fecal indicators for surface water contamination are fecal coliform bacteria, *Escherichia coli*, fecal streptococci and enterococci.

The use of indicator organisms to identify fecal contamination in surface waters has been used for more than a century (Gurian and Tarr, 2011). The first scientifically documented case of a waterborne pathogen outbreak occurred in 1854 by physician John

Snow. This early study determined that the consumption of sewage-contaminated water led to human disease due to the presence of cholera (Snow, 1855). This connection was the fundamental step in relating contaminated water to human diseases and therefore set the foundation for epidemiology. With the development of epidemiology, Theodor Escherich isolated a bacterium in 1885 named *Bacillus coli*, later renamed *Escherichia coli* in 1919, that lived in both water and the intestinal tract of animals (Brenner et al., 2005). By 1914, the federal regulation of drinking water by the U.S. Public Health Services was established setting standards for the bacteriological quality of drinking water (USEPA, 2000). The first indicator organism used was *E. coli* by utilizing the Multiple-Tube Fermentation test method, later renamed the Most Probable Number (MPN) procedure. At the time, this procedure required a small amount of lab work but required 48 hours to obtain the results along with several identification tests to confirm the presence of *E. coli*. Several years later the Total Coliform (TC) group was implemented to replace the use of *E. coli*. The TC group was used for 40 years until it was deemed inaccurate due to the large amount of false positive results. Eventually, *E. coli* and *Enterococcus* species were selected as indicators of fecal contamination and remain the current indicator organism today (USEPA, 2017).

B. Major types of indicator bacteria

B.1 Total coliforms (TC): The total coliforms group consists of 19 genera, including *Escherichia, Klebsiella, Enterobacter* and *Citrobacter,* and 80 other species. TC contain facultative anaerobes and aerobes that are rod-shaped, Gram-negative, and non-spore forming bacteria. These microbes typically live in the intestinal tract of warm-blooded animals but also reside in secondary habitats such as soil, surface water and on leafy

plants. TC bacteria ferment lactose with gas formation at 35°C within 48 hours (Britton and Greeson, 1988). The currently used standard method for quantifying TC in water samples is the Most Probable Number (MPN) method and Membrane Filtration (MF) method (Britton and Greeson, 1988; USEPA, 2002). Based on the MF method, coliforms are defined as colonies with a golden-green metallic sheen when incubated on M-Endo medium at 35°C for 24 hours (Britton and Greeson, 1988).

B.2 Fecal coliform (FC): Fecal coliforms (also known as thermotolerant coliforms) include the genera *Escherichia, Klebsiella* and *Citrobacter*, and are a sub group of total coliforms. Coliform group organisms present in the intestines and feces of warm-blooded animals are considered fecal coliforms. FC are non-spore forming, Gram-negative rods with the ability to ferment lactose on suitable culture media, producing both acid and gas at 44.5°C (Britton and Greeson, 1988; Reynolds, 2003). Although the USEPA recommends *E. coli* as the standard indicator bacteria to identify fecal contamination, some states still use FC as the indicator bacteria in recreational water. The most probable number method (MPN) and membrane-filtration method are the currently recommended standard methods to quantify FC in water samples (Britton and Greeson, 1988; USEPA, 2002). The membrane-filtration method FC includes all organisms that exhibit blue colonies when incubated at 44.5±0.2°C for 24 hours on aniline blue containing M-FC medium (Britton and Greeson, 1988). In the MPN method, FC determination is based on cultures showing gas production in the lauryl tryptose broth, incubated at 35°C for 24 hours. Positive cultures are then transferred to EC broth, incubated at 44.5°C for 24 hours and examined for gas production.

B.3 *Enterococcus*: *Enterococcus* species are Gram-positive, non-spore forming, non-motile bacteria, existing mainly in the intestinal tract of animals. *Enterococcus* can also exist in secondary habitats such as soil, water, food and plants due to its tolerances to a wide range of pH (4.5-10), extreme temperatures (10-45^{o}C) and high salt concentrations. Due to the difficulty of distinguishing *Streptococcus* from *Enterococcus* from physical characteristics alone, *Enterococcus* was classified as *Streptococcus* until 1984 when genomic DNA analysis indicated a separate genus classification was needed (Schleifer and Kilpper-Balz, 1984). This resilience of *Enterococcus* in a wide range of environmental conditions makes it favorable for the biological assessment of marine waters. Traditionally, the MPN method and modified *Enterococcus* procedure are being used in enumerating *Enterococcus* from marine waters (USEPA, 2010). In the modified *Enterococcus* method, colonies that exhibit a blue halo on modified mEI agar are considered *Enterococcus* (USEPA, 2010). In addition, the USEPA Method 1611, released in 2012, describes the quantitative polymerase chain reaction (qPCR) procedure in enumerating all known *Enterococcus* species from water (USEPA, 2012a; b).

B.4 *Escherichia coli*: *E. coli* is a rod-shaped, Gram-negative facultative anaerobe living in the intestinal tract of mammals. Most *E. coli* strains are harmless but some serotypes such as O157:H7 strain can cause gastrointestinal diseases. Harmless strains of *E. coli* from a synergistic relationship with their host by producing vitamin K_2 and preventing pathogenic colonization within the lower intestinal tract (Bentley and Meganathan, 1982). *E. coli* accounts for 0.9% of gut microbiota in adult mammals (Eckburg et al., 2005). With a biphasic lifestyle, *E. coli* has a host independent and host-associated phases. Stomach pH of mammals has a pH as low as 2.5, therefore, causing

severe stress when entering the host-associated phase. This harsh environment favors *E. coli* due to the low pH environment, often killing off competing microbes promoting the persistence and growth of *E. coli.* Once *E. coli* has passed through the host-associated phase, they enter the host-independent phase where survival is mainly based on nutrient levels and energy sources (Van Elsas et al., 2011).

In 1986, the USEPA conducted a series of studies to distinguish the relationship of indicator organisms to the incidence of intestinal illnesses of recreational water users (USEPA, 1986). In this study, a significant finding of *E. coli* in recreational fresh water is a direct relationship between the density of *E. coli* and the risk of gastrointestinal illness (Dufour, 1984). Therefore, the USEPA recommends the use of *E. coli* as an indicator organism for freshwater (USEPA, 1986). The USEPA established recreational water quality criteria (RWQC) in 1986 where the single sample maximum (SSM) for designated freshwater swimming areas should be no more than 235 CFU/100 mL, and the long-term steady state geometric mean should be less than 126 CFU/100 mL. The RWQC was revised in 2012 but the long-term steady state geometric mean remained the same. The SSM was revised and replaced with the statistical threshold value of no more than 410 CFU/100 mL. With the new revision criteria, 10% of the samples from a 30-day sample period are allowed to exceed the statistical threshold value. In 2017, the USEPA reviewed the 2012 RWQC revision but no changes were made for the geometric mean and statistical threshold value criteria (USEPA, 2017).

1.7 Detection

Several methods have been developed for the enumeration of *E. coli*. The current MPN method, which produces indole from tryptophan, has been used since 1986 but

requires the use of additional media and incubation time. Due to this, Dufour developed a filtration technique based on the catabolism of glucuronic acid (Dickinson, 2003). While many other methods exist, the methods discussed below will be based on the catabolism of glucuronic acid by the enzyme β-D-glucuronidase. Limitation to these procedures is specific strains of *E. coli* (O157:H7) which do not produce the enzyme β-D-glucuronidase, therefore resulting in false negatives for many of the enumeration methods (Hayes et al., 1995; Beneduce et al., 2003; Micrology Labs, 2018a; b).

A. EPA Method 1603: Due to the length of incubation when using indole production as an indicator of *E. coli* presence, Dufour produced the membrane thermotolerant *E. coli* (mTEC) method (Dickinson, 2003). While this method allowed for the quantification of *E. coli* within 24 hours, it required the transfer of the membrane filter onto a secondary substrate. Therefore, the USEPA developed the modified mTEC (method 1603) procedure in 1998 and is referred to as the EPA Method 1603 today. The EPA Method 1603 procedure is a single-step procedure that does not require the transfer of a membrane filter on to another substrate. The modified mTEC agar contains sufficient nutrients for the selective growth of *E. coli*. As a source of nitrogen, amino acids are included in the agar (Dickinson, 2003) and as a source of carbon, lactose is included in the agar. The yeast extract found in the agar provides necessary vitamins and trace elements. In addition, sodium chloride maintains the osmotic equilibrium while monopotassium and dipotassium provide buffering capacity. Sodium lauryl sulfate and sodium deoxycholate are added to select for Gram-negative bacteria. The agar contains the chromogen, 5-bromo-6-chloro-3-indolyl-β-D-glucuronide, which turns a red or magenta color when catabolized into glucuronic acid by the enzyme β-D-glucuronidase

(Dickinson, 2003). The EPA Method 1603 requires sample volumes to be based on half-log intervals and should use no less than three varying volumes (USEPA, 2014). The water samples are then filtered through a 0.45 μm pore-size membrane filter under vacuum. After filtration, membranes are placed on modified mTEC agar, incubated at 35°C for two hours to resuscitate stressed organisms and then incubated at 44.5°C for 24 hours. Colonies that exhibit a red or magenta color are considered *E. coli*. Results are reported as Colony Forming Units (CFU) per 100 mL of water.

B. Most Probable Number: The most probable number method is one of the most commonly used bacterial enumeration methods and is particularly useful for low concentrations of target organisms (Blodgett, 2010). The theory behind the Most Probable Number (MPN) method is to dilute samples to such a degree that inocula will sometimes, but not always, contain viable bacterial counts (Blodgett, 2010). The number of dilutions and the number of tubes with growth at each dilution implies an estimate of the original concentration in the undiluted sample (Blodgett, 2010). Of the various MPN enumeration methods, the IDEXX method is one of the most widely used. The IDEXX Colilert-18/Quanti-tray has been approved as an alternative method to the standard ISO 9308-1 for the enumeration of coliform bacteria, including *E. coli* (IDEXX, 2014). Similar to the EPA method 1603, the IDEXX method incorporates two chromogenic substrates which are activated by the enzymes β-galactosidase and β-glucuronidase (IDEXX, 2014). The IDEXX Colilert-18/Quanti-tray uses a tray of 51 sterile wells onto which a 100 mL sample is poured (IDEXX, 2014). Those wells that exhibit a yellow color are considered total coliforms and those wells exhibiting a yellow/fluorescent color are considered *E. coli* (IDEXX, 2014). Results from this method are obtained after 18

hours of incubation at a temperature of 35℃ (IDEXX, 2014). The IDEXX method is one of the cheapest approved alternative enumeration methods for *E. coli* enumeration. Additionally, this method provides timely results as compared to other approved enumeration methods.

C. Coliscan® Easygel: The patented Coliscan® Easygel method incorporates two chromogenic substrates, which are activated by the enzymes galactosidase and glucuronidase. β-galactosidase is an enzyme produced by general coliforms during lactose fermentation and exhibits a pink color when activated (Micrology Labs, 2018a). β-glucuronidase is selective to *E. coli* due to it being the only general coliform to produce this enzyme. When β-glucuronidase is activated the colonies exhibit a blue-green colony. Since *E. coli* produces β-glucuronidase and β-galactosidase colonies exhibit a purple colony. In addition, a sample volume of one mL to five mL is recommended for recreational water assessments. The sample is then mixed with the Easygel® solution and poured on a 90mm petri plate coated with a proprietary substrate which solidifies after 45 minutes. Solidified petri plates are then incubated at 30-37℃ for 24 hours or at room temperature (22-27℃) for 48 hours. Colonies that exhibit a blue to purple color will be considered generic *E. coli* and are reported in CFU per 100 mL.

D. Coliscan® Membrane Filter (MF): The Coliscan® MF method medium is a nutrient liquid formulation that contains two color producing chemicals that are activated by specific enzymes. This method is similar to the EPA Method 1603 in that it requires a filtration apparatus. It is also similar to the Coliscan® Easygel method in that it uses identical substrates to enumerate general coliforms and *E. coli.* In addition, the Coliscan® MF method uses the production of the enzyme β-galactosidase for general coliform

identification which results in a pink colored colony. The Coliscan® MF method also uses the production of the enzyme β-glucuronidase for the identification of *E. coli* which produces a teal-green color. Since *E. coli* produces both β-glucuronidase and β-galactosidase those colonies will exhibit a combination of pink and teal-green pigments resulting in a blue-purple pigment. Cells that exhibit blue to purple colonies are considered *E. coli* and reported in CFU per 100 mL. Limitation to this procedure is that *E.* coli 0157:H7 does not produce the enzyme β-glucuronidase thus resulting in a false negative reaction (Micrology Labs, 2018b). In the Coliscan® MF method, a sample volume of one to five mL for recreational water assessment is recommended. The chosen aliquot is placed onto a membrane filtration apparatus and then filtered. After filtration, the membrane is positioned onto a presoaked pad containing the patented medium. The membrane containing medium plate is suggested to be incubated for 18-20 hours at 34-37°C. If an incubator is unavailable, then incubation at room temperature for 24-48 hours is recommended. Cells that exhibit blue to purple colonies will be considered *E. coli* and reported in CFU per 100 mL.

E. 3M Petrifilm *E. coli*/coliform: The 3M™ Petrifilm *E. coli*/Coliform (EC) method is a culture medium system which provides a cost-effective, convenient and reliable way for the enumeration of fecal coliform in food and beverage production. The 3M™ Petrifilm EC method contains proprietary nutrients, a cold-water-soluble gelling agent, 5-bromo-4-chloro-3-indolyl-D-glucuronide and tetrazolium indicator that facilitates colony enumeration (3M, 2017). The procedure for the Petrifilm EC method uses a sample volume of 1 mL pipetted onto a premade card and sealed. The card is then incubated at 35°C for 18-24 hours. Colonies exhibiting blue-green coloration are considered *E. coli*

and reported in CFU per 100 mL.

F. **Importance of Alternative Methods:** Decreasing sampling costs and transportation requirements will greatly improve the feasibility of the Produce Safety Rule. Currently, the FDA has an approved list of enumeration methods in which many are expensive and time-constraining (FDA, 2018). Therefore, there is a need for a more cost-effective and timely enumeration methods. The EPA Method 1603 is considerably more expensive than the alternative methods at $1,343 per a 500g container (>$30/sample). Many laboratories in the Georgia and Alabama area use the IDEXX method due to its simplicity and cheap operation costs. The IDEXX method for *E. coli* enumeration costs around $20 a sample and is significantly cheaper than the EPA method 1603. The Coliscan® Easygel method consists of 10 tests at $30 per a kit which makes each test $3 per a sample making it the least expensive of the enumeration methods. Coliscan® Membrane Filter kits consist of 10 tests at $54. Each test costs $5.40 per sample and is, therefore, the most expensive of the alternative enumeration methods. Finally, the 3M™ Petrifilm EC kit costs $78 and contains 25 tests, making each test $3.10 per sample. The Coliscan® Easygel method is the least expensive of the proposed enumeration methods followed by the 3M™ Petrifilm EC method and the Coliscan® Membrane Filter method, respectively.

1.8 Method Comparison Studies

In the past, researchers have not directly compared the Coliscan® MF, Coliscan® Easygel or 3M™ Petrifilm EC to the EPA Method 1603. Six prior studies have been conducted comparing various other coliform enumeration methods to the EPA recommended methods. One study on beach bacterial water quality compared the

IDEXX, multiple-tube fermentation and membrane filter method. Results indicated all methods were comparable, but the membrane filtration method underestimated the bacterial counts (Noble et al., 2003). Another study conducted by Jeremy Olstadt and colleagues compared ten USEPA approved methods for enzyme-based total coliform and *E. coli* detection tests (Olstadt et al., 2007). These tests included the Colilert®, Colilert-18®, Colisure®, m-Coli Blue 24®, Readycult® Coliforms 100, Chromocult®, Coliscan®, E Colite®, Colitag™ and MI Agar tests. Results indicated that the enumeration from the Colilert® and Coliscan® tests were statistically identical to the recovery percentage of the spiked samples (Olstadt et al., 2007). The third study utilizing volunteer monitoring compared Coliscan® Easygel and 3M™ Petrifilm EC enumeration methods to the approved EPA Method 1603. The results indicated an 80% accuracy in correctly identifying the water conditions between the Coliscan® Easygel and 3M™ Petrifilm EC when compared with the EPA Method 1603 (Stepenuck et al., 2011). The manufacturer recommends using large sampling volumes and, therefore, high concentrations of target organisms due to increased accuracy (Stepenuck et al., 2011; 3M, 2017; Micrology Labs, 2018a). In addition, another study compared 3M™ Petrifilm EC count plates to commonly used methods, mTEC, m-Coliblue and IDEXX Quanti-Tray 2000 (Vail et al., 2003). The results indicated 3M™ Petrifilm EC was statistically correlated to the commonly used methods (Vail et al., 2003). Furthermore, a study comparing mFC-agar and 3M™ Petrifilm EC count plates found there was no statistical difference in coliform counts between the two methods (Schraft and Watterworth, 2005). Additionally, Beloti *et al* (2003) evaluated 3M™ Petrifilm EC count plates for enumeration in water. Results were obtained from 145 water samples and compared to the Most Probable Number

method (Beloti et al., 2003). The results indicated a strong correlation between the 3M™ Petrifilm EC count plates and the MPN method (Beloti et al., 2003). Lastly, a tracking fecal contamination study utilizing 3M™ Petrifilm EC plates narrowed the search area within a watershed, and found the method to be practical and useful for high enumeration counts (Harmon et al., 2014). While this study was not a method comparison study, it illustrates the simplicity and practicality of using 3M™ Petrifilm EC plates for monitorization.

In addition, the precision or accuracy of enumeration methods have been seldomly researched (Noble et al., 2003; Vail et al., 2003; Beloti et al., 2003; Olstadt et al., 2007; Stepenuck et al., 2011). Past research has indicated favorable results for 3M™ Petrifilm EC count plates and Coliscan® Membrane Filter in terms of accuracy and precision (Dufour et al., 1981; Noble et al., 2003; Vail et al., 2003; Olstadt et al., 2007). In addition, the limited research conducted on the effects of a 24-hour delay on *E. coli* enumeration has shown significant variations in results (McCarthy et al., 2008; Harmel et al., 2016).

By comparing of various methods used by citizen volunteers, i.e., Coliscan® Easygel, 3M™ Petrifilm EC, and Coliscan® MF with the EPA Method 1603 would allow for alternative methods to be available to produce farmers and volunteer monitoring programs for *E. coli* enumeration. The Coliscan® Easygel method can be and has been easily used by volunteers in the AWW Program for field and on-farm testing. In addition, the Coliscan® Easygel method is one the least expensive methods available and is therefore a logical choice to comply with PSR requirements.

1.9 Objectives

1. Conduct concurrent analysis of generic *E. coli* in waters of varying chemical characteristics using Coliscan® Easygel, 3M™ Petrifilm, Coliscan® MF, and the EPA Standard Method and statistically compare each of these methods.
2. Conduct concurrent analysis of generic *E. coli* from water samples, spiked at two different *E. coli* concentrations, using Coliscan® Easygel, 3M™ Petrifilm, Coliscan® MF, and the EPA Standard Method to statistically compare the accuracy and precision for each of these methods.
3. Simulate standard shipping of water samples to a laboratory and analyze samples for generic *E. coli* (after a 24 h delay) using the EPA Standard Method. Statistically compare these results against the samples analyzed immediately (and concurrently) after collection using the Coliscan® Easygel, 3MT™ Petrifilm, Coliscan® MF, and the EPA Standard Method to quantify potential errors resulting from the transport of samples.

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2. Alternative methods for determining generic *E. coli* in waters of varying chemical characteristics

Abstract

Given that the Food and Drug Administration Food Safety Modernization Act (FSMA) requires produce farmers to monitor the quality of pre- and post-harvest water used on the farm for generic *E. coli*, a practical means to implement the requirements is needed. Use of the Environmental Protection Agency (EPA) Standard Method (EPA Method 1603) requires farmers to ship water samples from the farm to a laboratory, which presents both an excessive financial burden on produce farmers and the problem of getting the samples analyzed within an acceptable holding time (a maximum of a 6 h transport time and 2 h analysis time). Anticipating these issues, we are seeking the use of alternative measures for meeting the FSMA requirements. Several *E. coli* testing methods are available for citizen monitoring programs, including the Coliscan® Easygel method that has been used, following EPA-approved QAP, by the Alabama Water Watch program for the past 20 years. The goal of this research was to compare alternative methods including Coliscan® Easygel, 3M™ Petrifilm, and Coliscan® MF against the EPA Method 1603 in determining *E. coli* levels in ambient waters of varying chemical characteristics. In addition, a comprehensive study was conducted to determine precision and accuracy of the EPA Method 1603 and alternative methods. Testing was also conducted to simulate the effect of a 24 h transport time to a laboratory on concentrations of *E. coli* determined by the EPA Method 1603. Water samples were collected over a

one-year period from 12 sites on five streams and four lakes. A total of 144 samples were collected and enumerated resulting in the EPA Method 1603 having *E. coli* concentration range of 0 – 3197 CFU/100 mL with a mean of 194 CFU/100 mL. The Coliscan® Easygel method at 5 mL had the strongest correlation ($r = 0.72$, $p = 0.01$) to the EPA Method 1603, while Coliscan® Membrane Filter had the second strongest correlation ($r = 0.70$, $p = 0.01$). At a high dosage, results from accuracy and precision indicated Coliscan® Membrane Filter had a recovery percentage of 98% followed by 3M™ Petrifilm at 73%. All methods had less variation in the samples than did the control (Standard Deviation = 6.49 CFU/mL) with the exception of Coliscan® Membrane Filter (Standard Deviation = 13.75 CFU/mL). Results for accuracy and precision determination at the low dosage indicated the EPA method 1603 had the highest recovery at 86% followed by 3M™ Petrifilm at 80%. All methods had less of a variation than did the control at a low dosage (Standard Deviation = 3.61 CFU/mL). There was a significant variation ($p=0.01$) between a 6-hour holding time and a 24-hour holding time ($r = 0.85$, $p = 0.0001$). In addition, the 6-hour holding time *E. coli* concentrations were significantly higher than the *E. coli* concentrations from samples analyzed after 24-hours. Our study showed that, the alternative enumeration methods had highly significant correlations when compared to the EPA Standard Method. In addition, the 6-hour sample holding time significantly differed from the 24-hour sample holding in *E. coli* concentrations.

2.1 Introduction

Fecal contamination of produce from irrigation sources has resulted in numerous foodborne disease outbreaks. In 2006, 30 separate cases of *E. coli* related foodborne disease outbreaks were reported by the Center for Disease Control (CDC) (CDC, 2018).

Annually, *E. coli* related outbreaks cause economic losses of $272 million (Batz et al., 2011). Furthermore, the annual economic losses due to out breaks from *Salmonella, Campylobacter, Listeria, Toxoplasma* and norovirus amount to $12.7 billion annually (Batz et al., 2011). *Salmonella* contaminated produce, meat and dairy have resulted in 67 outbreaks since 2006 (CDC, 2018). Therefore, the Federal Drug Administration (FDA) is requiring produce farmers to monitor irrigation water for potential pathogens due to the likelihood of a farmer to use contaminated water sources.

The Food Safety Modernization Act (FSMA) set forth by the FDA was implemented in 2016 and established science-based minimum standards for the safe growing, harvesting, packing and holding of produce for human consumption (USHHS, 2015). The final rule established two sets of criteria for microbial water quality, based on the presence of generic *E. coli* (FDA, 2015). Criterium one states that no detectable generic *E. coli* are allowed for uses of water for agricultural purposes in which it is likely to contact dangerous pathogens, such as water used for hand washing before and after harvest, water used on food-contact surfaces, and water used to directly contact produce (FDA, 2015). The second set of criteria states numerical criteria, geometric mean and statistical threshold value, for agricultural water directly applied to growing produce (FDA, 2015). The final rule does not apply to small produce operations that generate less than $25,000 in revenue during a three year period and produce that are typically cooked before consumption (USHHS, 2015). The produce safety rule first applied at the beginning of 2017 to produce farms that generate more than $500,000 on an annual basis. In 2018, the rule began applying to farms that generated more than $250,000 but less than $500,000. Lastly, in 2019, the produce safety rule applied to produce farms whose

monetary value exceeded $25,000 during the past three years. In order to assess irrigation and wash water for potential pathogens, fecal indicator bacteria are most commonly used.

Gastrointestinal diseases are caused by several different types of waterborne pathogenic organisms. A total of 27,219 individuals contracted gastrointestinal diseases associated with treated recreational water from 2000 to 2014 (Hlavsa, 2018). Due to the correlation between fecal bacteria and waterborne disease-causing organisms, fecal indicator bacteria are used to assess the risk of pathogens in recreational and drinking water (Myers et al., 2014). Furthermore, the density of indicator bacteria is a direct measurement of water safety for consumption and body-contact recreation (Myers et al., 2014). Fecal indicator bacteria are a group of organisms that exists in the intestinal tract of warm-blooded animals and enter the secondary habitat through fecal matter. Therefore, the presence of fecal indicators in irrigation or surface water is direct evidence of fecal contamination from warm-blooded animals (Myers et al., 2014). In the United States, the most commonly used fecal indicators for surface water contamination are fecal coliforms, *Escherichia coli*, fecal streptococci and enterococci with *E. coli* being the most commonly used for freshwater.

Methods to enumerate fecal indicator bacteria are limited to expensive, time-restricted analyses through standard methods (USEPA, 2014). The widely used standard, the EPA method 1603, recommends an analysis time within no more than six hours after collection which is often expensive and not feasible for remote farm operations due to transportation limitations (USEPA, 2014). In addition, the EPA method 1603 requires a professional laboratory to conduct sample analysis, increasing the cost of analysis. The FDA requires a minimum of 20 samples for surface waters and is therefore expensive to

conduct the survey. Additionally, the EPA method 1603 costs more than $30 per sample making the implementation of the Produce Safety Rule problematic. There is, therefore, a need for alternative enumeration methods that do not have transportation limitations and are more cost-effective than the standard methods. This research found alternative enumerations methods that are significantly more cost-effective as compared to the EPA method 1603. The least expensive method is Coliscan® Easygel, at $3 per sample which provides results within 48 hours at room temperature, eliminating the need for transportation. The most expensive alternative method is the Coliscan® Membrane Filter at $5.40 per sample. The Coliscan® Membrane Filter method also provides results within 48 hours at room temperature eliminating the need for transportation and, in turn, reducing costs of analysis. A few prior studies have compared alternative enumeration methods to standard methods and have found strong correlations between them (Noble et al., 2003; Vail et al., 2003; Beloti et al., 2003; Schraft and Watterworth, 2005; Olstadt et al., 2007; Stepenuck et al., 2011). The most commonly compared methods throughout the previous studies are 3M™ Petrifilm *E. coli*/coliform (EC) count plates compared to the EPA method 1603 or MPN method. The IDEXX method is also used in most of the previous method comparisons studies and have shown high correlations to alternative methods and standard methods (Noble et al., 2003; Vail et al., 2003; Beloti et al., 2003). Only a few studies have compared Coliscan® Easygel to standard methods and have indicated high correlations between Coliscan® Easygel and standard methods (Stepenuck et al., 2011). In addition, the precision or accuracy of enumeration methods have been seldom researched. Past research has indicated favorable results for 3M™ Petrifilm EC count plates and Coliscan® Membrane Filter in terms of accuracy and precision (Dufour

et al., 1981; Noble et al., 2003; Vail et al., 2003; Olstadt et al., 2007). Furthermore, the effect of a 24-hour delay on *E. coli* enumeration has been seldom researched but have shown significant variations in relation to different sample holding times (McCarthy et al., 2008; Harmel et al., 2016). Therefore, the objectives for this study were to:

4. Conduct concurrent analysis of generic *E. coli* in waters of varying chemical characteristics using Coliscan® Easygel, 3M™ Petrifilm, Coliscan® MF, and the EPA Standard Method and statistically compare.
5. Conduct concurrent analysis of generic *E. coli* from water samples, spiked at two different *E. coli* concentrations, using Coliscan® Easygel, 3M™ Petrifilm, Coliscan® MF, and the EPA Standard Method to statistically compare the accuracy and precision.
6. Simulate standard shipping of water samples to a laboratory and analyze samples for generic *E. coli* (after a 24 h delay) using the EPA Standard Method. Statistically compare these results against the samples analyzed immediately (and concurrently) after collection using the Coliscan® Easygel, 3MT™ Petrifilm, Coliscan® MF, and the EPA Standard Method to quantify potential errors resulting from the transport of samples.

2.2 Materials and Methods

The FSMA Produce Safety Rule is requiring produce farmers to monitor on-farm water for potential pathogens (FDA, 2015). The EPA recommend enumeration method for monitoring potential pathogens in water is expensive to conduct, require the use of a professional laboratory and have time constraints (USEPA, 2014). Therefore, we conducted analysis using three alternative enumeration methods and compared them to

the EPA method 1603. In addition, we analyzed a 24-hour sample holding time for generic *E. coli.* A total of 146 samples were collected and enumerated using the EPA Method 1603, Coliscan® Easygel, 3M™ Petrifilm and Coliscan® Membrane Filter methods. Water samples were collected over a one-year period from 12 sites on five streams and four lakes. Comparison of these methods was done using simple linear regression analysis. In addition, the accuracy and precision of each method was determined based on the enumeration of a known concentration of *E. coli.* Accuracy was based on the percent recovery of *E. coli* from the control as compared to the enumeration method. Precision was based on the standard deviation within each method. Additionally, the effects of a 24-hour holding time on the enumeration of *E. coli* using the EPA Method 1603 was determined using simple linear regression and two-sample t-tests. Furthermore, an additional six months of samples were collected for the 24-hour sample holding time analysis.

Study Area. Sampling sites were located in two different watersheds, the Sougahatchee Creek watershed and the Parkerson Mill Creek watershed. The Sougahatchee Creek watershed is located in Lee County, Alabama, where it resides in the Piedmont Plateau physiographic region. A tributary to the Tallapoosa River, Sougahatchee Creek has a drainage area of 559 km^2 (ADEM, 2008). The water use classification for Sougahatchee Creek is fish and wildlife, swimming, and public water supply. Due to nutrient enrichment and elevated concentrations of pathogens, Sougahatchee Creek is listed on the Alabama 303(d) list (ADEM, 2018). Land use for the watershed mainly consists of forest (76.8%), agriculture (11.7%), and urban (8.4%) (ADEM, 2008). Potential sources of contamination include urban runoff, pasture and agricultural runoff, sewer line leakage

and septic tank malfunctions.

The Parkerson Mill Creek watershed is also located within Lee County, Alabama, where it lies partially in the Piedmont Plateau and partially in the Coastal Plains physiographic region. A tributary to Chewacla Creek, Parkerson Mill Creek has a drainage area 24 km^2 (ADEM, 2011). The present use classification for Parkerson Mill Creek is fish and wildlife and is included on the Alabama's 303(d) list as impaired due to elevated levels of pathogens (ADEM, 2018). The watershed has mainly urban land uses but also contains agricultural and forested areas (ADEM, 2011). The main contributions to stream impairment consist of urban runoff, leaking septic systems, and leakage from sewer lines (ADEM, 2011). Rainfall data during the study period were obtained from Alabama Mesonet Weather Data (www.awis.com/mesonet), USGS Precipitation Station (nwis.waterdata.usgs.gov/usa/nwis) and National Oceanic Atmospheric Administration's (NOAA) website (www.w1.weather.gov/data).

Collection of water samples. Water samples were collected monthly from September 2017 to August 2018 from eight locations on five different streams (Parkerson Mill Creek, Chewacla Creek, Town Creek, Sougahatchee Creek, and Pepperell Branch) and four lakes (Sougahatchee Lake, Agricultural Heritage Pond, Willow Lake, and EW Shell pond S8) which had varying chemical characteristics (Fig. 2.1). The first location (AgS01) was at the headwaters of Sougahatchee Creek where a dam was constructed to form Sougahatchee Lake. The second site (AgS02) was from a tributary (Pepperell Branch) which feeds into Sougahatchee Creek. Just below the confluence of Pepperell Branch and Sougahatchee Creek was the third sampling site (AgS03). Auburn University's fisheries pond S8 was the second sampling lake (AgS04) followed by a

residential lake, Willow Creek Lake (AgS05). Four sampling sites (AgS06, AgS07, AgS08, and AgS11) were located along Parkerson Mill Creek, a tributary to Chewacla Creek. One sampling location (AgS09) was located upstream of the confluence of Parkerson Mill Creek and Chewacla Creek. Another sampling site, Town Creek (AgS10) was selected as it is a tributary to Chewacla Creek. The Agricultural Heritage Pond on Auburn University's campus was also sampled (AgS12). Using sterile 250-mL and two 500-mL high-density polyethylene bottles, samples were collected 6 – 12 inches below the water surface and placed in an ice-filled cooler. The 250-mL samples were then transported to the Alabama Water Watch laboratory where they were then analyzed for *E. coli* using the alternative methods within six hours of collection. The two 500-mL samples were transported to Auburn University's Soil Microbiology lab where they were analyzed for *E. coli* using the EPA method 1603.

Evaluation of Water Characteristics. During water sample collection, chemical characteristics of water each site were measured at the time of collection. A wide range of chemical characteristics was needed to represent a broad spectrum of water conditions. Utilizing the Alabama Water Quality Monitoring Kit (LaMotte, Chestertown, MD), dissolved oxygen (DO) (ppm), pH, alkalinity ($CaCO_3$ mg/L), hardness ($CaCO_3$ mg/L), turbidity (JTU) and water temperature (^oC) were determined. DO was measured by titration of sodium thiosulfate in duplicates and averaged for each site. Characteristics were averaged amongst each site for all sampling collection dates.

Enumeration of *E. coli* in water samples. *E. coli* concentrations in water samples were analyzed using the EPA Method 1603, Coliscan® Easygel, 3M™ Petrifilm and Coliscan® Membrane Filtration methods. The EPA Method 1603 (USEPA, 2014) was used as a

standard method. From each sample, three varying volumes (3, 10, 30 mL for creeks and 10, 30, 100 mL for lakes) were filtered through 0.45 μm membrane filters in duplicates. The phosphate-buffered (2.2 mM $MgCl_2$ and 0.65 mM KH_2PO_4) dilution water (pH=7.0±0.2) was used to bring volumes less than 10 mL to a total volume of 10 mL for filtration. Approximately 10 mL of phosphate-buffered dilution water was used to rinse the walls of the filter funnel to wash any cells that might be adhered. The membrane filter was then cultivated on modified membrane-thermotolerant *Escherichia coli* agar (mTEC) media (Difco, Detroit, MI) for two hours at 35°C and then 24 hours at 44.5°C. Those colonies exhibiting a red/magenta color were considered *E. coli* and reported as colony forming units (CFU) per 100 mL. 3M™ Petrifilm EC count plates were also used for enumeration and required a volume of one mL. That sample was placed onto the Petrifilm card (3M, 2017), sealed and incubated at 35°C for 24 hours. Colonies exhibiting a blue/green color were considered *E. coli* and reported as CFU per 100 mL. For the Coliscan® Easygel method, two sample volumes (1 and 5 mL) were plated onto the proprietary agar (Micrology Labs, 2018a) and incubated at 35°C for 24 hours. Colonies exhibiting a purple color were considered *E. coli* and reported as CFU per 100 mL. Lastly, for the Coliscan® Membrane Filter method, 5 mL of water was filtered through a membrane and placed onto the Coliscan® Membrane Filter plates (Micrology Labs, 2018b). The plates were then incubated at 35°C for 24 hours; colonies exhibiting a blue-purple color were considered *E. coli* and reported as CFU per 100 mL.

Determination of accuracy and precision for each method. Accuracy determination was based on the enumeration of a known concentration of *E. coli* for each method. Bioball Multishot 550 (*E. coli* NCTC 12923, ATCC 8739) was rehydrated in 1.1 mL of

Bioball rehydration fluid. Aliquots of 11 CFU/100 μL and 55 CFU/100 μL were extracted and plated on nutrient agar as a positive control. Stock solutions of two dosages (11 CFU/mL and 55 CFU/mL) were prepared through dilution using phosphate-buffered dilution water from the EPA Method 1603 procedure. In replicates of three, 1 mL aliquots from each dose were enumerated using the EPA Method 1603 and the alternative methods. For the EPA Method 1603 and the Coliscan™ Membrane Filtration methods, 1 ml aliquots were added to 20 mL of phosphate-buffered dilution water before filtration. The resulting plates were then incubated at 35ºC to 37ºC for 18 – 24 hours. Colonies exhibiting the previously mentioned colorizations were considered *E. coli* and reported in CFU/mL. A total of three experiments were performed for a total of 90 samples (45 at the high concentration and 45 at the low concentration). Precision was calculated based on the standard deviation within each method and also reported as a percentage of coefficient of variation.

Determination of the effects of a 24-h holding time on the enumeration of *E. coli* using the EPA Method 1603. The effect of a 24-hour delay on enumeration was assessed using the modified mTEC method. One set of the duplicate samples were first analyzed within the six-hour time frame as recommended by the EPA Method 1603. The second set of 500-mL duplicate samples were kept in an ice-filled cooler for a duration of 24-hours and then enumerated using the EPA Method 1603. Additional samples were collected bi-weekly for six more months after the initial one-year monthly sampling period to obtain a total of 96 samples.

Data Analysis

Evaluation of *E. coli* enumeration methods. Samples were duplicated in analysis and

averaged amongst site for each method tested to prevent pseudo-replication. Utilizing Simple Linear Regression, analysis of method comparisons were performed using R version 3.5.2 software (R Core Team, 2013). Regression analysis was used to identify the relationship between two separate methods. First, the assumptions of the simple linear regression model were assessed. The independence assumption was satisfied due to the design of the study. Analysis of the other three assumptions of the model was done using fit diagnostics from the statistical program R. Based on initial simple linear regression analysis, it was obvious the data did not satisfy the normality assumption, linearity assumption and constant variance assumption. This was due to QQ plots following a non-linear pattern, right-skewed histograms and residual plots with funneled residuals. Therefore, log transformations on the data set were needed to satisfy these assumptions. Before log transformations, all data points were accredited with an additional value of one in order to prevent log transformations on values of zero (Vail et al., 2003; Beloti et al., 2003; Schraft and Watterworth, 2005). After log transformations on data points, histograms showed normal distributions, QQ plots resembled linearity and normality and residual plots showed equal variances which satisfied the simple linear regression model assumptions. The EPA Method 1603 was considered our standard method and was compared with each alternative method (Coliscan® Membrane Filter, Coliscan® Easygel, and 3M™ Petrifilm EC). In addition, alternative methods were compared with one another using linear regression models. In the accuracy and precision study, accuracy determination was based on the enumeration of a known concentration of *E. coli* for each method and reported as a percentage of recovery. Precision was defined as the standard deviation within each method and reported as CFU/mL. For the sample holding time

analysis, simple linear regression models were used in the software program R. Log transformations on the 24-hour sample holding time data set were made as described earlier and compared with the log-transformed six-hour sample holding time data set. Additionally, a two-sample t-test was conducted to evaluate the difference in group means from 6-hour and 24-hour sample holding times.

2.3 Results

A. Water Chemistry Characteristics During sample collection, water chemical characteristics of sampling sites were determined (Table 2.1). Twelve samples were obtained for each of the 12 sites for a total of 144 samples. Water temperature values ranged from 5.5 – 33℃ with the lowest site average at 16.17℃ for site AgS09 and the highest site average at 21.46℃ for site AgS12. Average pH values ranged from 6.96 for site AgS09 and 7.79 for site AgS11. Sites AgS05 and AgS09 had the lowest measured pH value at 6.5 and site AgS12 had the highest measured pH value at 9. Dissolved oxygen concentrations ranged from the lowest at 1.2 ppm for site AgS08 and the highest at 11.2 ppm for site AgS10. Average dissolved oxygen concentrations ranged from 7.35 ppm for site AgS05 and 8.71 ppm for site AgS10. Alkalinity values ranged from the lowest at 20 mg/L $CaCO_3$ for site AgS01 to the highest at 210 mg/L $CaCO_3$ for site AgS11. Average alkalinity values ranged from 30.00 mg/L $CaCO_3$ for site AgS01 to 164.58 mg/L $CaCO_3$ for site AgS11. Hardness values ranged from the lowest at 10 mg/L $CaCO_3$ for site AgS01 and 210 mg/L $CaCO_3$ for site AgS11. Average hardness values ranged from 23.30 mg/L $CaCO_3$ for site AgS01 and 148.33 mg/L $CaCO_3$ for site AgS11. Turbidity values ranged from 2 – 50 JTUs with the lowest average at 2.67 JTUs for site AgS10 and 17.67

mg/L $CaCO_3$ for site AgS04. Through this, a varying range of chemical characteristics based on site location can be observed.

B. Enumeration of *E. coli* from water samples. A total of 144 water samples were collected from 12 different sites for a 12-month period. The results indicated that Coliscan® Easygel at 5 mL had the highest correlation (r=0.7241, p=0.01) with the modified mTEC (EPA Method 1603) method (Fig. 2.2). This was closely followed by Coliscan® Membrane Filter (r=0.6986, p=0.01) (Fig. 2.3). Coliscan® Easygel at 1 mL (r=0.6530, p=0.01) and 3M™ petrifilm (r=0.6287, p=0.01) had lower correlations potentially due to the small (1 mL) volume of sample used for these two tests (Fig. 2.4 and Fig. 2.5). All four citizen monitoring methods had a significant correlation (p=0.01) to the EPA Method 1603 (Table 2.2).

Citizen monitoring methods were also compared with one another using regression analysis (Table 2.3). Results indicated Coliscan® Membrane Filter had the strongest correlations to the other three citizen monitoring methods. Coliscan® Easygel 5 mL had the second highest correlations to the other three citizen monitoring methods. Coliscan® Easygel 1 mL and 3M™ Petrifilm had the third and fourth lowest correlations to the other three citizen monitoring methods. In addition, the EPA Method 1603 had 13% of the samples exceed the statistical threshold value (STV) (Table 2.5) (Appendix 2.1). The Coliscan® Easygel method at 5 mL had the largest percentage (27%) of samples exceeding the STV (Appendix 2.2). Coliscan® Easygel at 1 mL had the second largest percentage (22%) of samples exceeding the STV (Appendix 2.3). The 3M™ Petrifilm had the third largest percentage (19%) (Appendix 2.4) and Coliscan® Membrane Filter had the lowest (17%) (Appendix 2.5).

C. Determination of accuracy and precision. Nutrient agar recoveries were within the range of expected recovery rates for the Bioball Multishot 550. The results from the accuracy and precision indicated the Coliscan® Membrane Filter had the highest accuracy (recovery) percentage at 98% for high dose when compared to the nutrient agar control (Table 2.4). 3M™ Petrifilm had the second highest recovery percentage at 73%. The EPA Method 1603 and Coliscan® Easygel method had the third and fourth highest recovery percentage at 54% and 31%, respectively, at a high dosage. At the low dose, the EPA method 1603 had the highest recovery percentage at 86% when compared to the nutrient agar. Coliscan® Membrane Filter had the second highest recovery at 80% at low dose. The 3M™ Petrifilm method and the Coliscan® Easygel method had third and fourth lowest recovery percentage at 64% and 28%, respectively. The precision of each method was measured by the standard deviation within each method. At the high dose, the EPA Method 1603 had the lowest standard deviation of 4.77 CFU/mL. 3M™ Petrifilm had the second lowest standard deviation at 5.04 CFU/mL. Coliscan® Easygel had a standard deviation of 5.24 CFU/mL followed by the Coliscan® Membrane Filter at 13.75 CFU/mL. Our control, the nutrient agar, had a standard deviation of 6.49 CFU/mL at the high dose which is in the range of acceptable variation according to Bioball's information. At the low dose, all four enumeration methods had less of a standard deviation than did our control at 3.61 CFU/mL. Therefore, the standard deviation in each method was consistent with or exceeded our control with the exception of Coliscan® Membrane Filter (Table 2.4).

D. Determination of the effects of a 24-h holding time on enumeration of *E. coli* using the EPA Method 1603. The effects of a 24-hour delay on *E. coli* enumeration

indicated that there was a significant correlation (r=0.7273, p=0.001) between the 24-hour and 6-hour holding times (Fig. 2.6). A two-sample t-test, however, showed that there was a significant difference (p=0.001) between means from the 6-hour holding time (μ = 66.55, SD = 8.11) and 24-hour holding time (mean = 49.801 CFU/100 mL, SD = 7.348 CFU/100 mL). Results from alternative methods when compared to 24-hour sample holding time indicated the alternative methods overestimated the *E. coli* concentrations when concentrations were around the STV and GM. Above the STV, the alternative methods underestimated the *E. coli* concentrations when compared to the *E. coli* concentrations after a 24-hour sample holding time (Fig. 2.7).

2.4 Discussion

This study compared the analysis of generic *E. coli* in waters of varying chemical characteristics using three citizen monitoring methods and the EPA Method 1603. The method comparison study was conducted for a one-year period from September 2017 to August 2018. Sampling sites were selected based on previously reported water chemistry characteristics and *E. coli* counts. Rainfall (Appendix 2.6) also had a direct correlation to enumeration levels as shown in previous studies (Kistemann et al., 2002; Kleinheinz et al., 2009; Pandey et al., 2012). During the study period, the two methods using 5 mL sample volumes had higher correlations than those with 1 mL sample volumes. This is believed to be due to an increased probability of collecting the target organism (Vail et al., 2003). Furthermore, the results from this experiment are consistent with past research in indicating a correlation of r=0.60 or higher when compared to a standard method (Noble et al., 2003; Vail et al., 2003; Beloti et al., 2003; Schraft and Watterworth, 2005; Olstadt et al., 2007; Stepenuck et al., 2011; Harmon et al., 2014). When the alternative

enumeration methods were compared to the each other, they had stronger correlations to each other than to the EPA method 1603. This suggest the alternative enumeration methods are more correlated to each other than to the EPA method 1603 which believed to be due to differences in incubation temperature and media composition (Beloti et al., 2003; Harmel et al., 2016). Within the alternative method comparison, Coliscan® Membrane Filter method had the highest correlations to the other alternative enumeration methods at $r > 0.76$. This is possibly due to the fact that the Coliscan® Membrane Filter method required the use of a membrane filtration, similar to the modified mTEC method. In addition, this method used a sampling volume of 5 mL which is thought to increase the enumeration precision (Vail et al., 2003). Therefore, the high correlation of Coliscan® Membrane Filter shows its favorability in the application of monitoring *E. coli* in streams and waterways. Additionally, this method is more practical than the EPA Method 1603 for citizen monitoring and on-site microbial analysis due to its low cost of operation and time effective analysis. The 3M™ Petrifilm method has been one of the most compared enumeration methods due to its simplicity and practicality (Vail et al., 2003; Beloti et al., 2003; Schraft and Watterworth, 2005; Stepenuck et al., 2011; Harmon et al., 2014). Results from our experiment showed consistent results with past research ($r = 0.6 – 0.8$) in achieving a relatively high correlation ($r = 0.63$) to the EPA Method 1603 (Noble et al., 2003; Vail et al., 2003; Beloti et al., 2003; Schraft and Watterworth, 2005). This study, therefore, strengthens its validation as a practical initial on-site *E. coli* enumeration method. Lastly, the Coliscan® Easygel method had significant correlations to the EPA Method 1603. With this method, 1 mL and 5 mL samples were used. Five mL sample volumes had a greater correlation to the standard method as compared to a 1 mL

sampling volume. Therefore, it is recommended to use the larger sampling volume as compared to a smaller one for *E. coli* enumeration when counts are low (Vail et al., 2003). The Coliscan® Easygel method is simple to use and is cost-effective, which, in addition to high correlations, displays its potential to be an on-site *E. coli* enumeration method.

The results from this study revealed the accuracy and precision of each enumeration method was highly variable and potentially depended on the *E. coli* strain utilized. For recovery, 3M™ Petrifilm (73%) and Coliscan® Membrane Filter (98%) methods performed well while modified mTEC (54%) and Coliscan® Easygel (31%) performed poorly. The Bioball manufacturing procedure requires droplets of cells to be frozen in liquid nitrogen, freeze-dried and vacuum-sealed. A possible reason for larger differences is the susceptibility of lab-grown strains of Bioball supplied *E. coli* to be stressed during the manufacturing procedure and therefore do not grow well on selective media. The modified mTEC method requires an incubation temperature of 35°C and then 44.5°C. During the study, we found the high incubation temperature of 44.5°C to be stressful to the *E. coli* NCTC strain 12923. Incubation temperature was then lowered to 37°C used by the alternative enumeration methods and growth resumed as expected. This potentially demonstrates the susceptibility of *E. coli* NCTC strain 12923 to perform poorly on selective media. Nevertheless, Coliscan® Membrane Filter and 3M™ Petrifilm methods again demonstrated their practicality and favorability as on-site *E. coli* enumeration methods with high recovery percentages. In terms of variation within each method, all methods, except the Coliscan® Membrane Filter method, had lower variations within replicates than did the control (SD < 6.49 CFU/ mL). The control had a standard

deviation within the acceptable range (<8.1 CFU/mL) found in Bioball's certificate of analysis. The Coliscan® Membrane Filter method had a standard deviation of 13.75 CFU/ mL and was twice of the control. This is possibly due to sampling volumes of 1 mL which is thought to decrease the precision of bacterial counts (Vail et al., 2003). Nonetheless, these results are consistent with past research indicating significant differences between sample holding times, 1-20% (McCarthy et al., 2008; Harmel et al., 2016).

The 24-hour and 6-hour sample holding times were found to be significantly correlated with each other but had significantly different means. Past research was highly variable in conclusion about sample holding times and show a 5% change in enumeration counts while others showed up to 20% change in enumeration counts (Harmel et al., 2016). This, therefore, suggests that a holding time of no more than six hours should be further studied, and careful consideration of the analysis time frame should be reconstructed.

Results from the comparison of *E. coli* concentrations at a 24-hour sample holding time to the *E. coli* concentrations from the alternative methods at a 6-hour sample holding time revealed the alternative methods over-estimated the *E. coli* concentrations when near the GM and STV. For farmers who must comply with the Produce Safety Rule, the use of the alternative methods may classify the water as impaired when the water was, in fact, below the GM and/or the STV. Therefore, the use of alternative methods could potentially result in increased costs to the farmer to maintain sanitary water supplies. On the contrary, if samples were held for 24-hours and enumerated using the EPA Method 1603 then missed-risk is possible. Especially for *E. coli* concentrations close to the STV

and GM. Therefore, it would be beneficial to farmers to utilize alternative enumeration methods to screen water sources for potential pathogens.

This study revealed that the alternative enumeration methods are practical for initial microbial screenings. In addition, the alternative enumeration methods are cost-effective for citizen monitoring programs and farmers who must comply with the Produce Safety Rule. From this study, the alternative methods were significantly correlated to the EPA Method 1603 and, therefore, demonstrate their practicality and efficiency in the enumeration of generic *E. coli* from water sources. This is especially true for rural agricultural producers with limited access to professional laboratories. In addition, a sample holding time of six hours needs to be further studied to properly identify the variation within the sample holding times.

Potential drawbacks to the alternative enumeration methods consist of large variations in *E. coli* concentrations within each method and the potential for the alternative methods to over-estimate *E. coli* concentrations. When compared to the EPA Method 1603, the alternative methods over-estimated *E. coli* concentrations but rarely underestimated the *E. coli* concentrations. This is mostly due to the large amount of variation within each method (Table 2.5). The variation within samples for the alternative methods was nearly twice the standard deviation for the EPA Method 1603, with the Coliscan® Easygel at 5 mL having the largest amount of variation (Table 2.5). This could be problematic to farmers who must comply with the PSR. Enumeration methods that over-estimate *E. coli* concentrations could classify clean water as impaired, increasing the likelihood a farmer would have to develop a water sanitation system.

2.5 Summary

The comparison of alternative enumeration methods to the EPA Method 1603 indicated significant correlations between them. Results suggest the alternative enumeration methods would be practical and efficient in conducting initial *E. coli* enumeration tests for water testing. The accuracy and precision of the alternative methods were also consistent with previous studies and suggest the alternative methods are comparable to the EPA Method 1603. The comparison of sample holding times indicates the need for further research into the variability of *E. coli* enumeration due to varying sample holding times. Results suggested there were significant differences in *E. coli* concentrations from different sample holding times. This result from sample holding time differences was consistent with past research, but further examination should be taken. Lastly, the results of the alternative enumeration methods comparisons indicated that the alternative methods were more correlated to each than to the EPA method 1603.

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Table 2.1 Water chemistry characteristics per sampling site

Site	Water Temperature °C	pH			DO (ppm)			Alkalinity (mg/L $CaCO_3$)			Hardness (mg/L $CaCO_3$)			Turbidity (JTU)		
	mean	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max
AgS01	20.10	7.30	7.0	8.0	7.84	6.0	10.2	30.00	20.0	40.0	23.30	10.0	30.0	9.50	2.0	30.0
AgS02	16.46	7.17	7.0	8.0	8.10	6.2	10.0	57.08	40.0	70.0	57.50	40.0	70.0	8.92	2.0	50.0
AgS03	17.58	7.08	7.0	7.5	8.18	6.0	9.6	48.33	40.0	60.0	40.00	30.0	50.0	6.83	2.0	40.0
AgS04	20.79	7.38	7.0	8.0	7.85	6.0	10.0	37.08	30.0	50.0	30.00	20.0	40.0	17.67	2.0	40.0
AgS05	19.13	7.00	6.5	7.5	7.35	4.6	9.4	34.17	25.0	45.0	29.17	20.0	40.0	12.67	2.0	45.0
AgS06	16.42	7.67	7.0	8.0	8.28	6.0	10.2	92.92	65.0	135.0	95.00	70.0	140.0	5.92	2.0	30.0
AgS07	16.79	7.50	7.0	8.0	8.29	6.2	10.6	68.33	55.0	100.0	67.50	50.0	90.0	4.83	2.0	20.0
AgS08	16.88	7.88	7.5	8.5	8.03	1.2	10.4	70.83	50.0	105.0	60.00	40.0	80.0	4.42	2.0	25.0
AgS09	16.17	6.96	6.5	7.0	7.80	5.0	10.0	39.17	30.0	50.0	33.33	20.0	50.0	9.42	2.0	20.0
AgS10	16.54	7.29	7.0	8.0	8.71	6.8	11.2	53.33	45.0	65.0	52.50	40.0	80.0	2.67	2.0	45.0
AgS11	17.67	7.79	7.0	8.0	7.89	5.8	9.6	164.58	115.0	210.0	148.33	100.0	210.0	4.17	2.0	25.0
AgS12	21.46	7.75	7.0	9.0	7.82	6.0	10.0	52.50	45.0	65.0	44.17	30.0	60.0	16.42	2.0	35.0

Table 2.2 Parameters of linear regression equations for log *E. coli* concentrations determined using citizen monitoring methods vs. the Modified mTEC method

Method	R^2	r	Slope	P-value
Coliscan Easygel 5 mL	0.5243	0.7241	0.7670	<0.01
Coliscan Membrane Filtration	0.4884	0.6986	0.7612	<0.01
Coliscan Easygel 1 mL	0.4265	0.6530	0.5593	<0.01
3M Petrifilm	0.3953	0.6287	0.5456	<0.01

Table 2.3 Correlation coefficients between methods used for *E. coli* enumeration

Method (r)	Coliscan Easygel 5 mL	Coliscan Membrane Filtration	Coliscan Easygel 1 mL	3M Petrifilm
Coliscan Easygel 5mL	1.00			
Coliscan Membrane Filtration	0.82	1.00		
Coliscan Easygel 1mL	0.78	0.80	1.00	
3M Petrifilm	0.73	0.76	0.70	1.00

Note: $p < 0.01$

Table 2.4 Mean, standard deviation (Std. Dev.), percentage of coefficient of variation (% CV) and recovery percentage of known concentrations of *E. coli* NCTC 12923 (CFU/mL) by four enumeration methods

Target Dosage	Nutrient Agar			Modified mTEC				Membrane Filter				EasyGel				3M Petrifilm			
	Mean	Std. Dev.	% CV	Mean	Std. Dev.	% CV	% Recovery	Mean	Std. Dev.	% CV	% Recovery	Mean	Std. Dev.	% CV	% Recovery	Mean	Std. Dev.	% CV	% Recovery
High Dosage	39	6.49	17	21	4.77	23	54	38	13.75	36	98	12	5.24	44	31	29	5.04	17	73
Low Dosage	8	3.61	45	7	2.46	35	86	6	2.52	42	80	2	1.69	85	28	5	1.45	29	64

Note: High dosage (55 CFU/mL) and Low dosage (11 CFU/mL). Mean (n=9) ± Standard Deviation. Samples replicated three times at three runs for a total of nine samples per method and dosage

Table 2.5 Percentages of water samples exceeding the Statistical Threshold Value (410 CFU/100 mL) for *E. coli*

	EPA 1603	Coliscan Membrane Filter	Coliscan Easygel 1 mL	Coliscan Easygel 5 mL	3M Petrifilm
% Exceeding STV	13	17	22	27	19

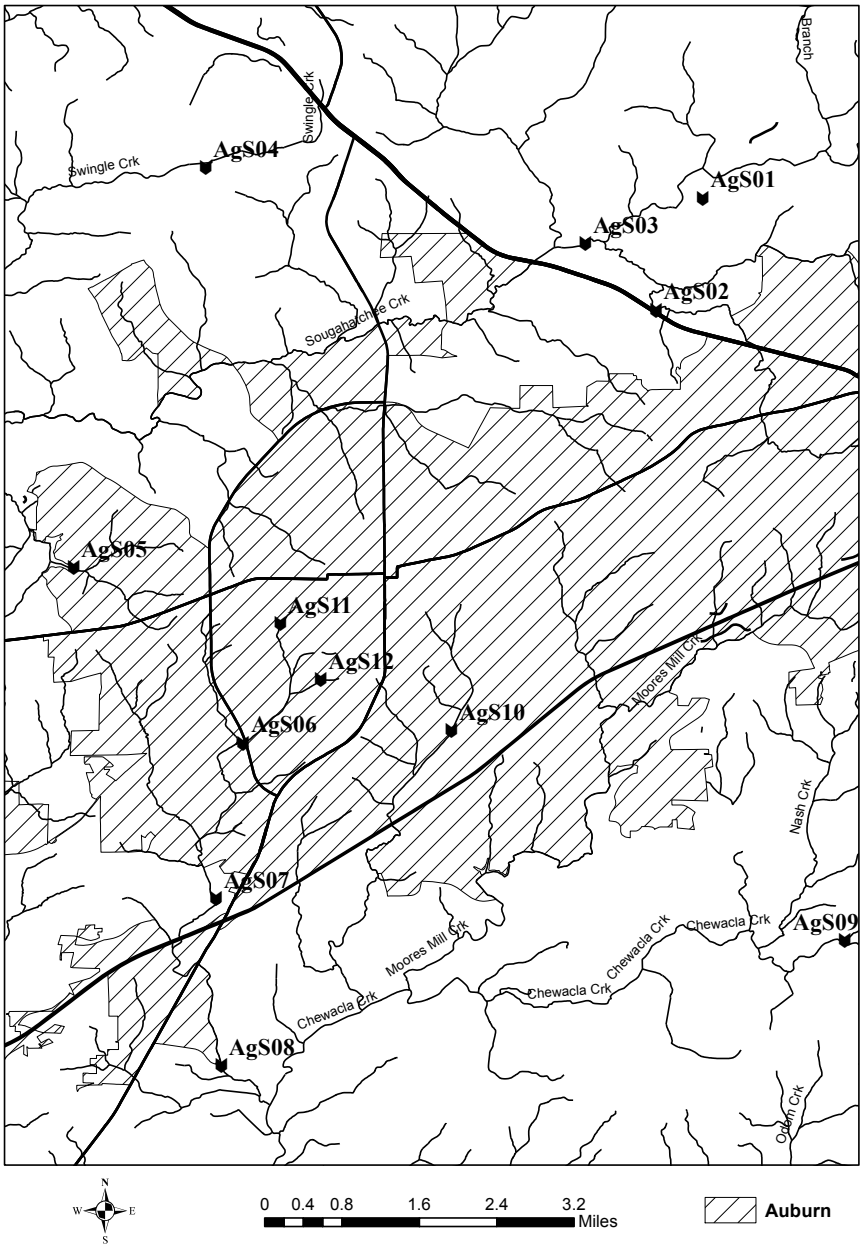

Fig. 2.1 Sampling sites in the Sougahatchee Creek and Chewacla Creek Watersheds: Sougahatchee Lake (AgS01), Pepperell Branch (AgS02), Sougahatchee Creek (AgS03), EW Shell S8 (AgS04), Willow Creek Lake (AgS05), Parkerson Mill Creek (AgS06, AgS07, AgS08, AgS11), Chewacla Creek (AgS09), Town Creek (AgS10), and Ag Heritage Pond (AgS12).

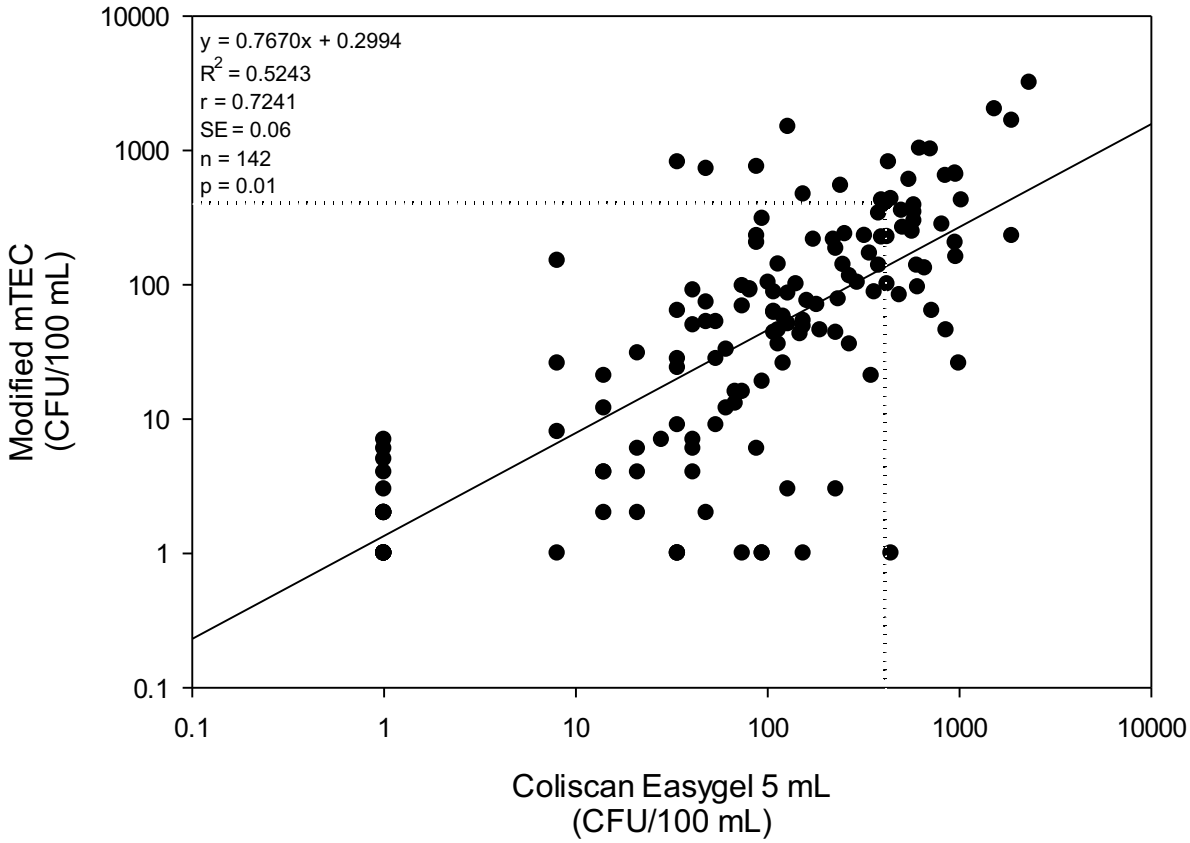

Figure 2.2 Correlation between the Coliscan Easygel at 5 mL and the Modified mTEC method for *E. coli* enumeration

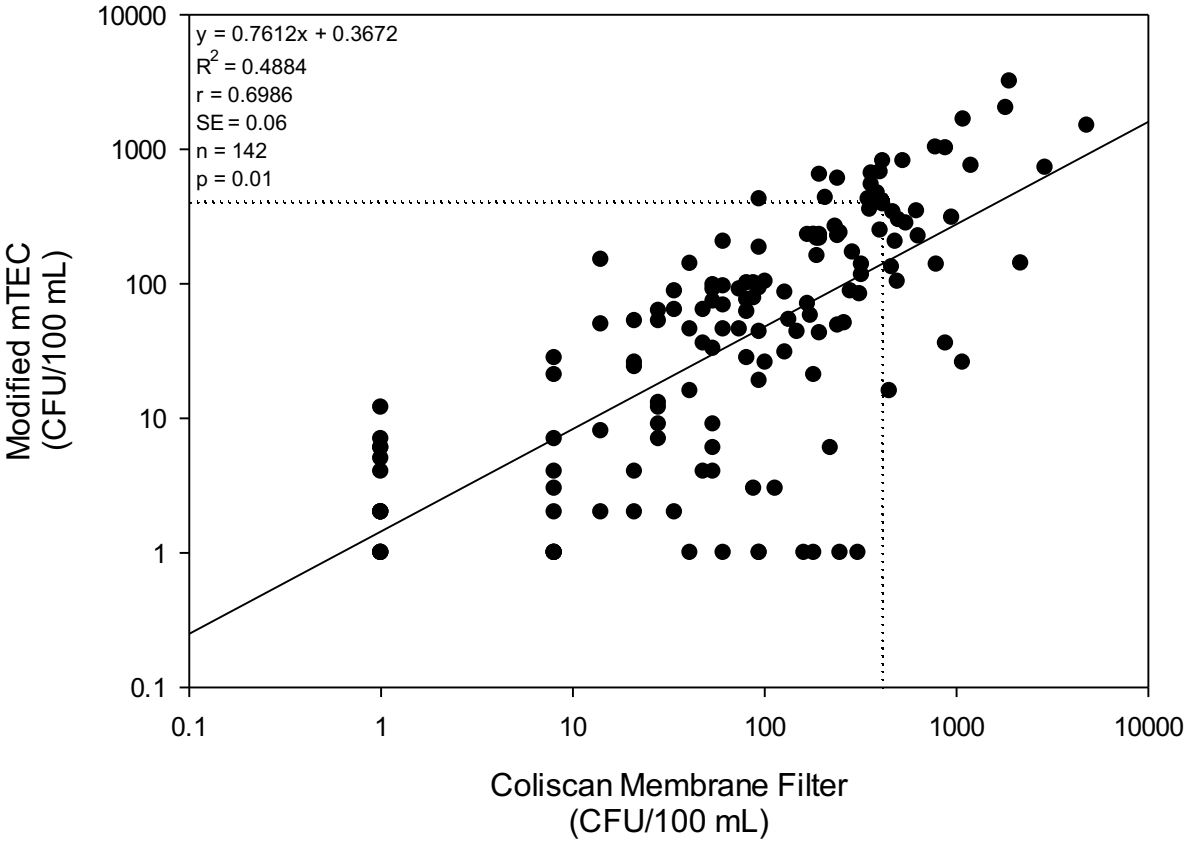

Figure 2.3 Correlation between the Coliscan Membrane Filtration method and the Modified mTEC method for *E. coli* enumeration

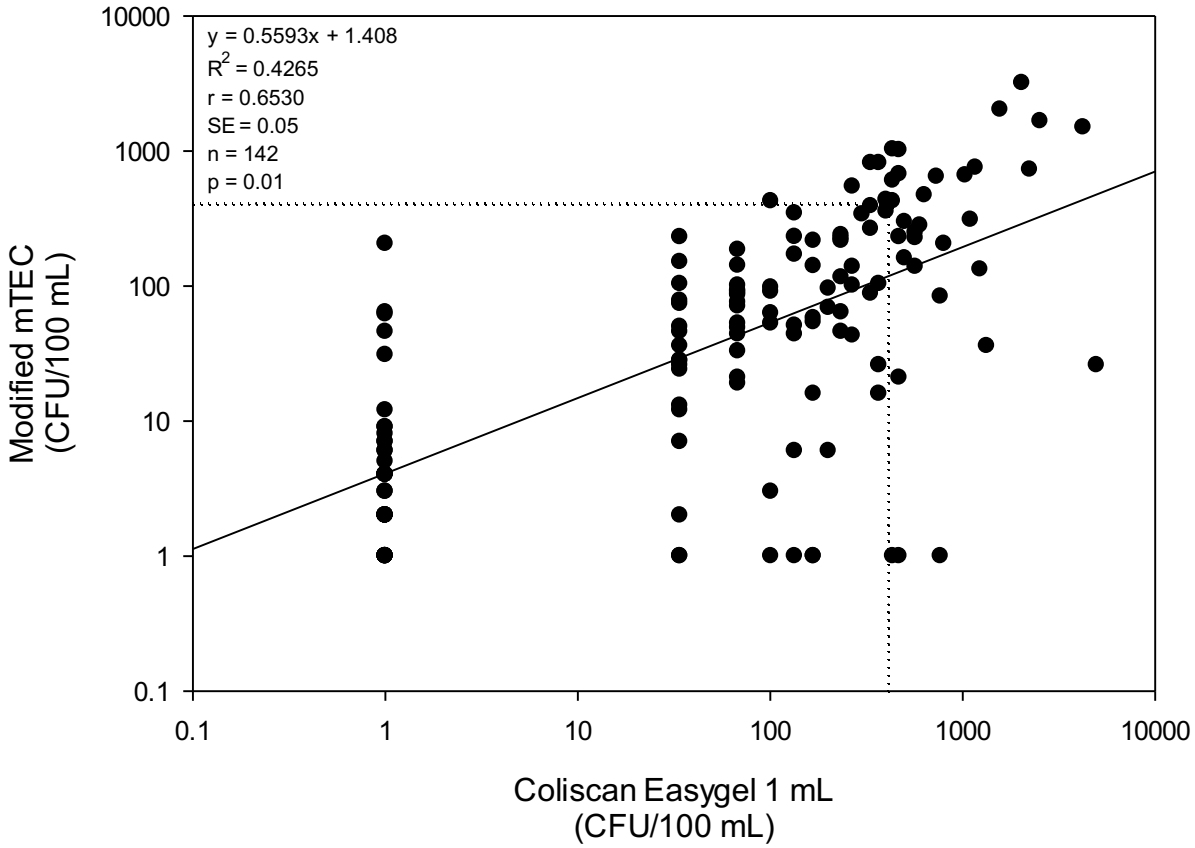

Figure 2.4 Correlation between the Coliscan Easygel at 1 mL and the Modified mTEC method for *E. coli* enumeration

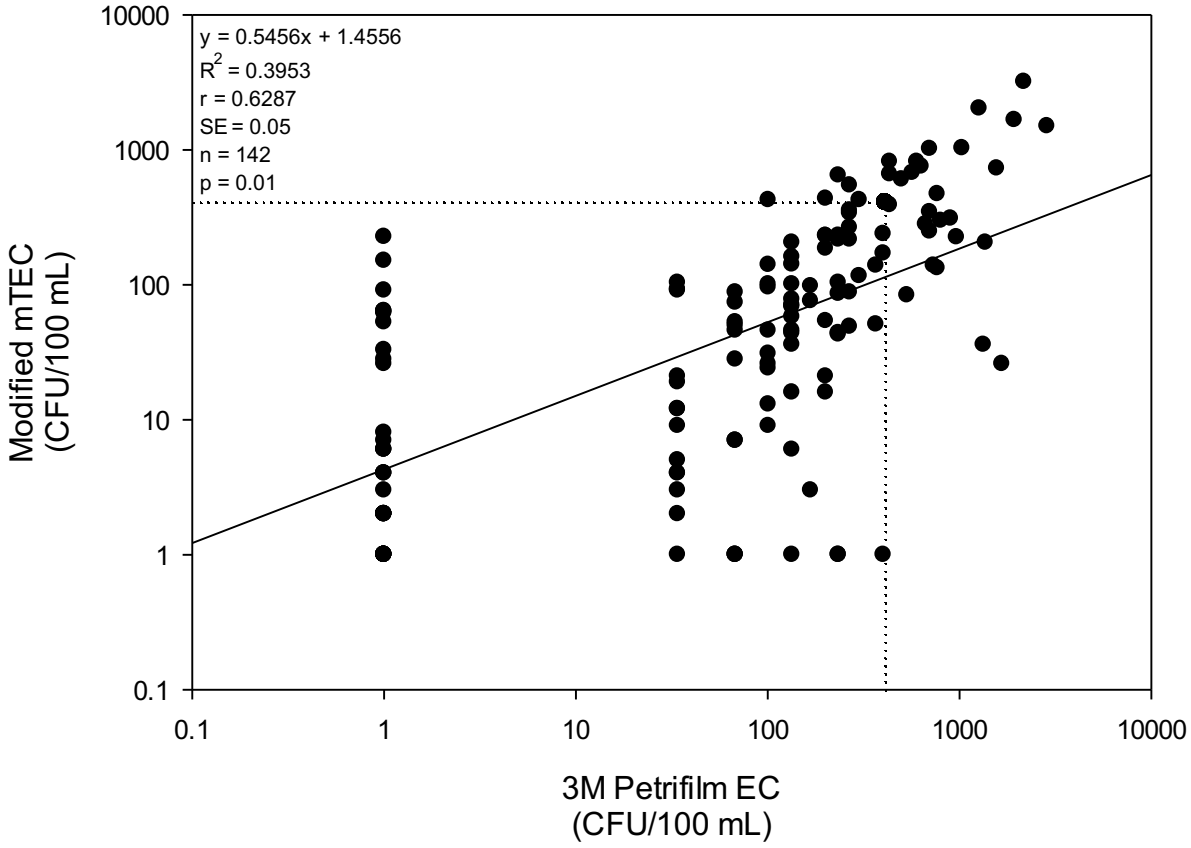

Figure 2.5 Correlation between the 3M^{TM} Petrifilm method and the Modified mTEC method for *E. coli* enumeration

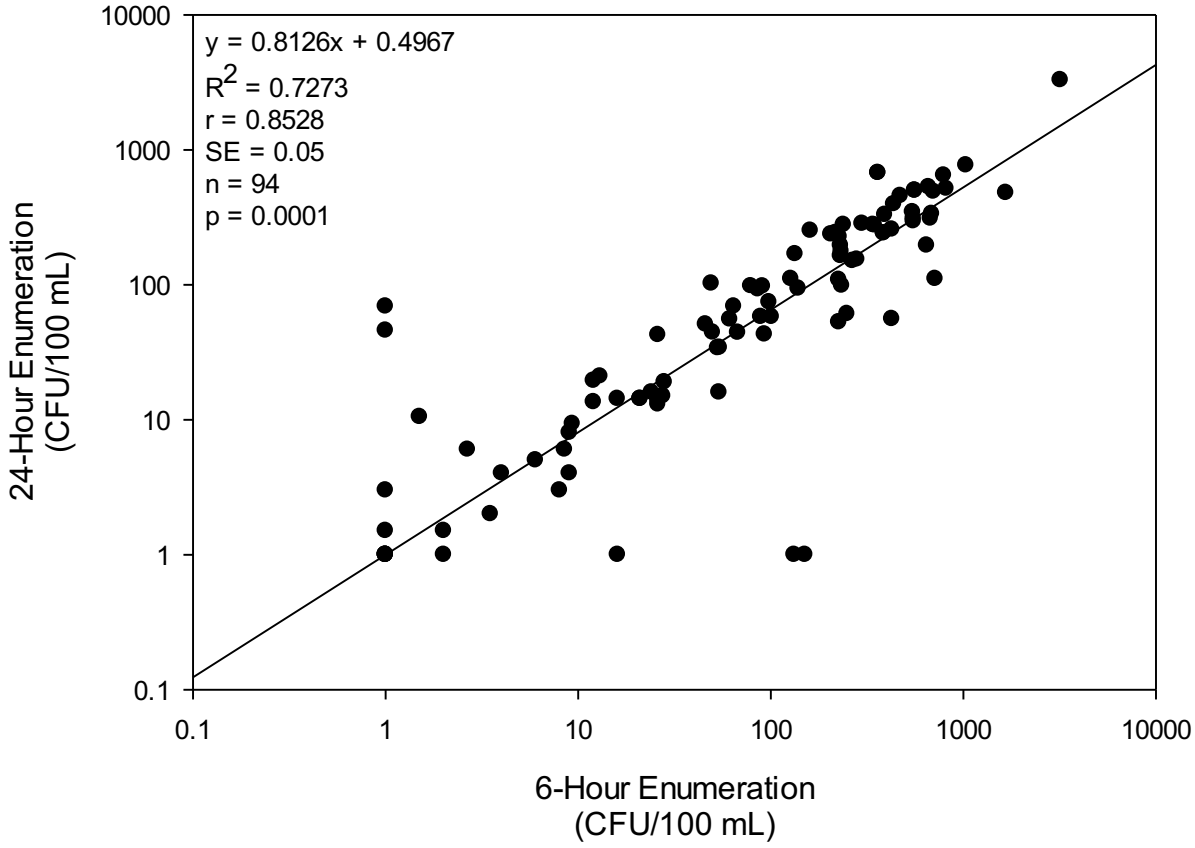

Figure 2.6 Correlation between 24-hour and 6-hour sample holding times for *E. coli* concentrations

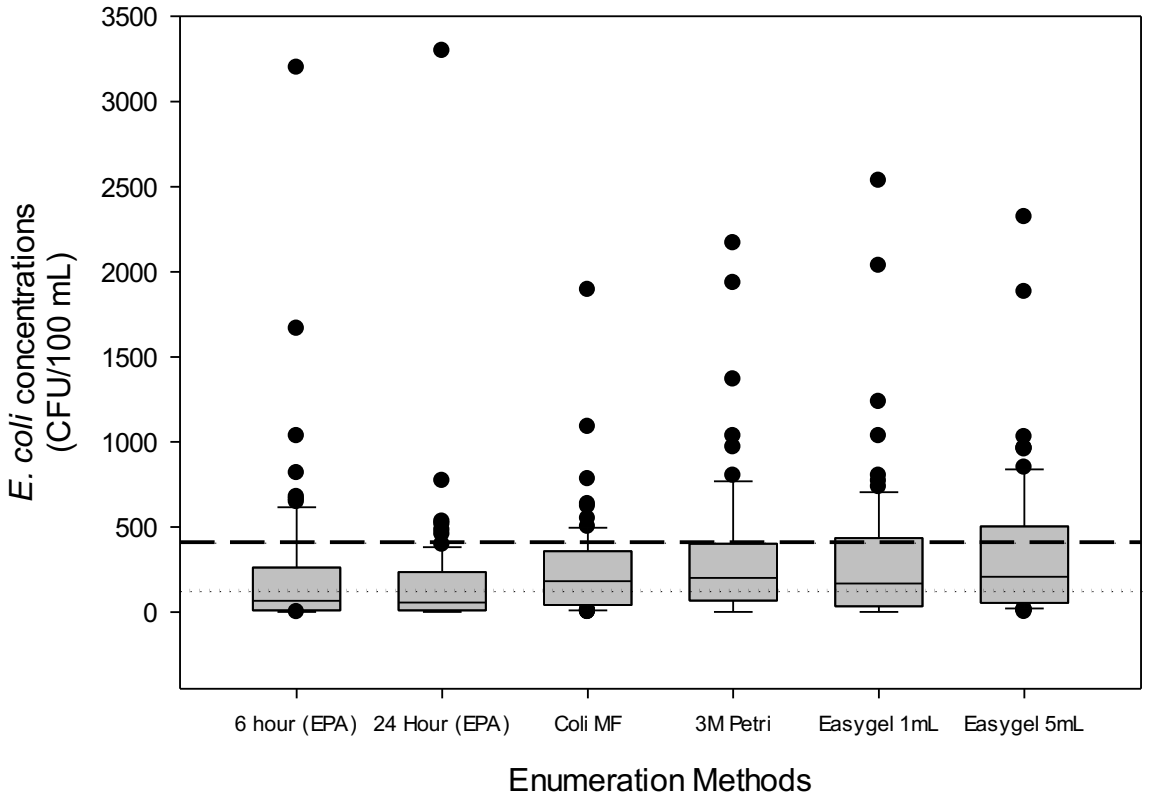

Figure 2.7 Distribution of *E. coli* concentrations from 24-hour sample holding time and *E. coli* concentrations at 6-hour sample holding time

Note: Top dashed line represents the Statistical Threshold Value (410 CFU/100 mL) and the bottom dotted line represents the Geometric Mean (126 CFU/100 mL)

3. Conclusion & Future Research

The Food and Drug Administration's Produce Safety Rule is requiring produce farms to monitor potential pathogens and the water used for processing produce. The standard methods to enumerate for potential pathogens are time-consuming to enumerate and expensive. Alternative enumeration methods have also been seldom researched. This study was therefore conducted to identify the practicality of alternative enumeration methods for on-site *E. coli* enumeration. The EPA Method 1603 modified mTEC, approved to comply with PSR, was compared to three alternative enumeration methods utilizing sampling sites with varying chemical characteristics. The accuracy and precision for each method were then evaluated using a laboratory-grown *E. coli* in order to identify the variation within each method. Lastly, differences in sample holding times were evaluated to establish a firm understanding of the effect of holding time on enumeration by the EPA method.

The comparison of alternative enumeration methods to the EPA approved standard method indicated significant correlations between the two. Results suggest that the alternative enumeration methods would be practical and efficient in conducting initial *E. coli* enumeration tests for water testing which is consistent with past research. The accuracy and precision of the alternative methods were also consistent with previous studies and suggest the alternative methods are comparable to the EPA standard method. The comparison of sample holding times indicates that further research into the

variability of *E. coli* enumeration due to this factor should be investigated. Results suggested there was significant difference in bacterial concentrations from different sample holding times. This result was consistent with past research, but further examination should be taken.

The future of *E. coli* and fecal coliform enumeration lies within qPCR. Researchers with the USEPA are currently developing a qPCR method for *E. coli* enumeration. After the USEPA developed the qPCR method for *Enterococcus* enumeration a revision was made to encourage states to conduct site-specific assessments using qPCR. This is due to potential matrix interference issues in water types used in the National Epidemiological and Environmental Assessment of Recreational Water Study (NEEAR) effluent-affected beach sites. Making varying water types an issue in developing qPCR methods for *E. coli* enumeration.

4. Appendices

Figure S 2.1 *E. coli* concentrations determined using EPA Method 1603 for each site

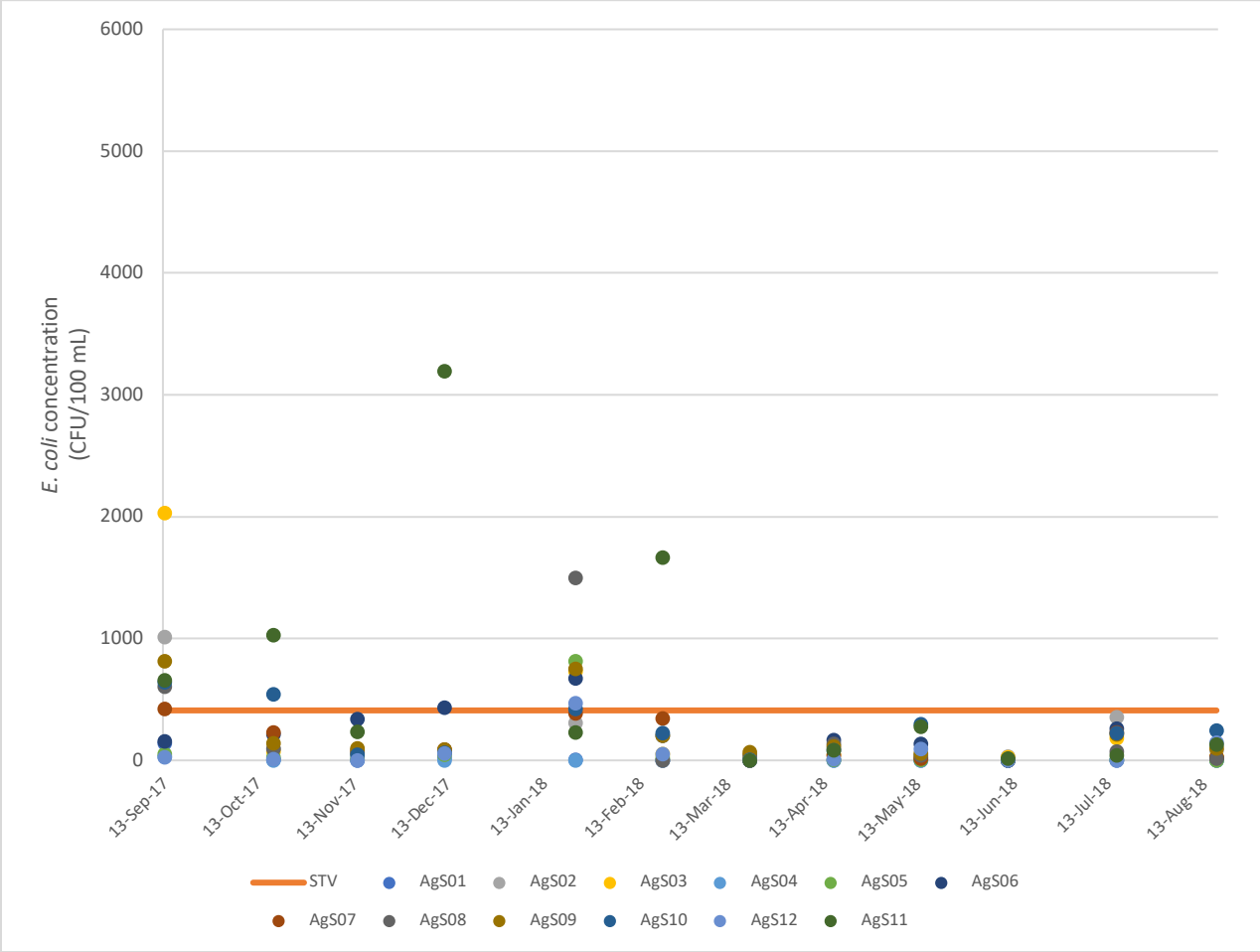

Figure S 2.2 *E. coli* concentrations determined using Coliscan Easygel at 5 mL for each site

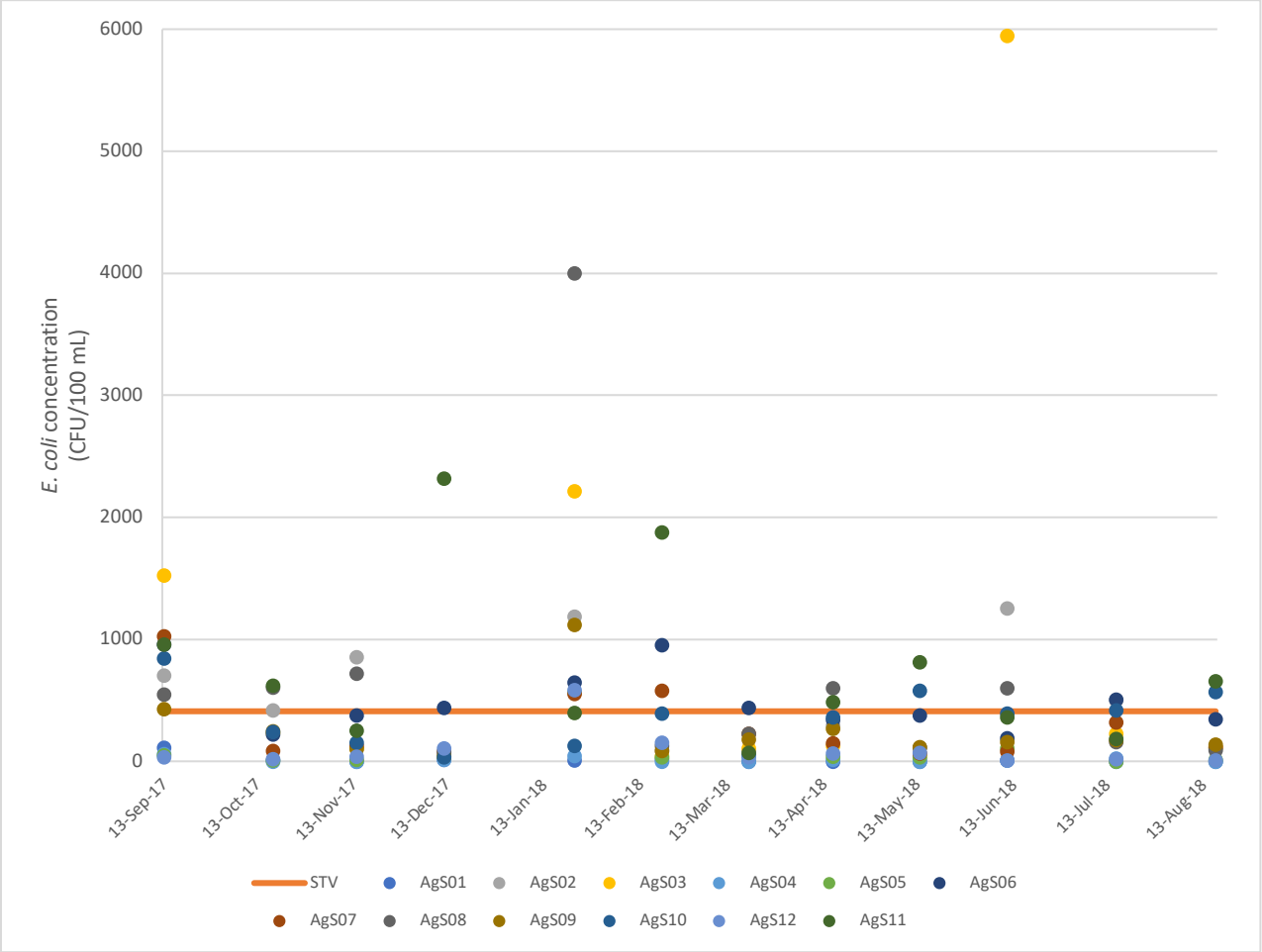

Figure S 2.3 *E. coli* concentrations determined using Coliscan Easygel at 1 mL for each site

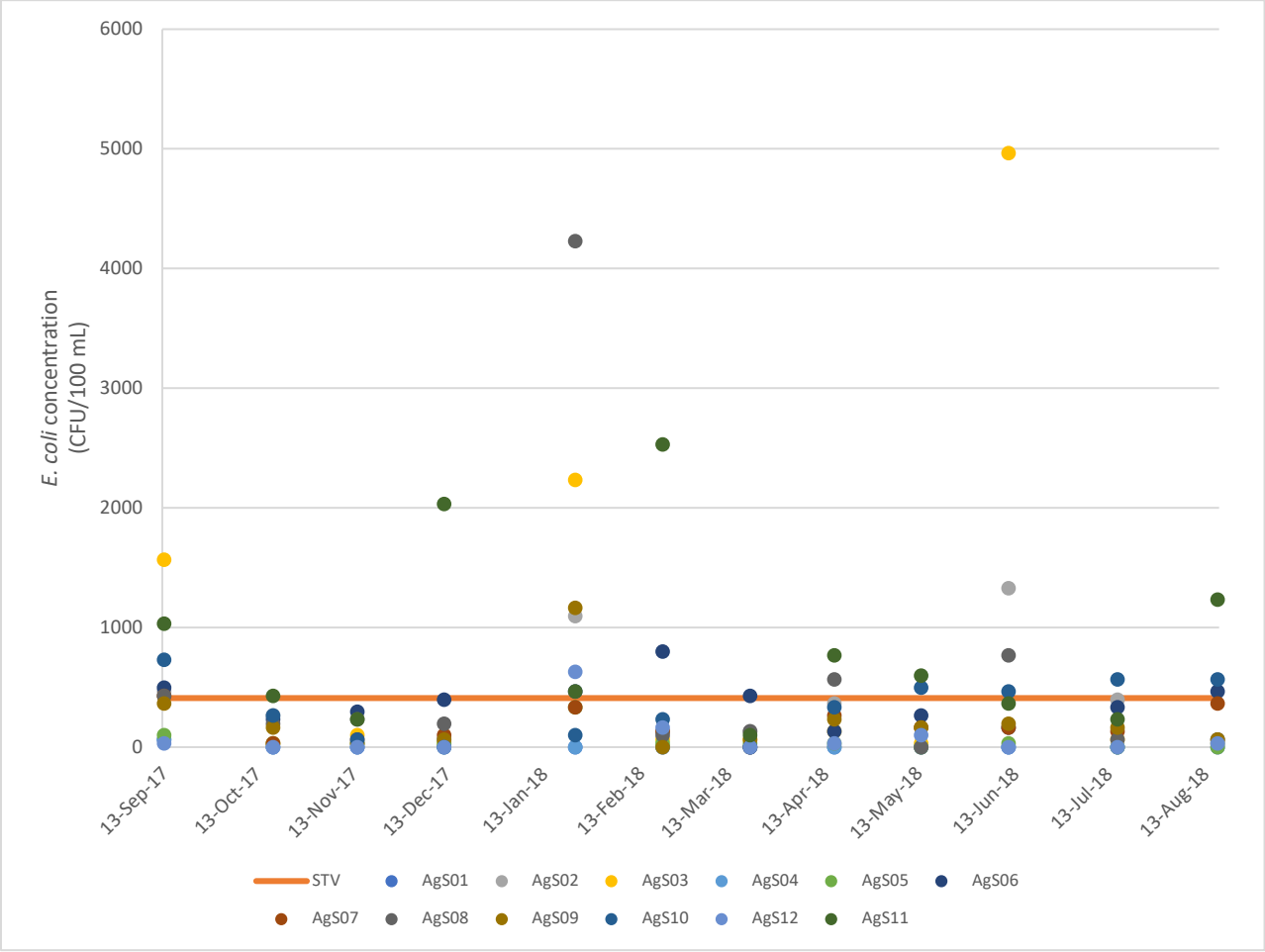

Figure S 2.4 *E. coli* concentrations determined using 3M Petrifilm for each site

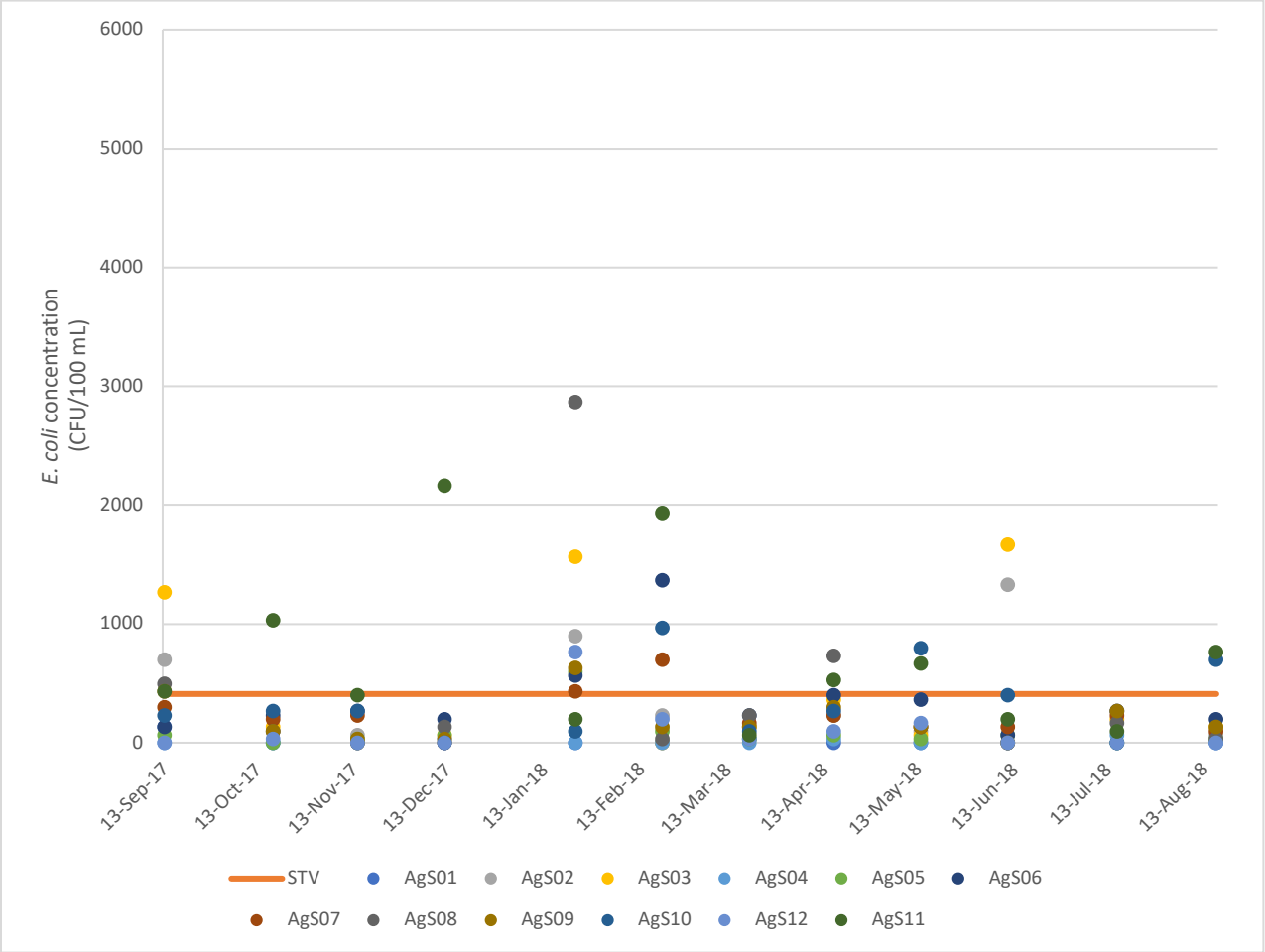

Figure S 2.5 *E. coli* concentrations determined using Coliscan Membrane Filter for each site

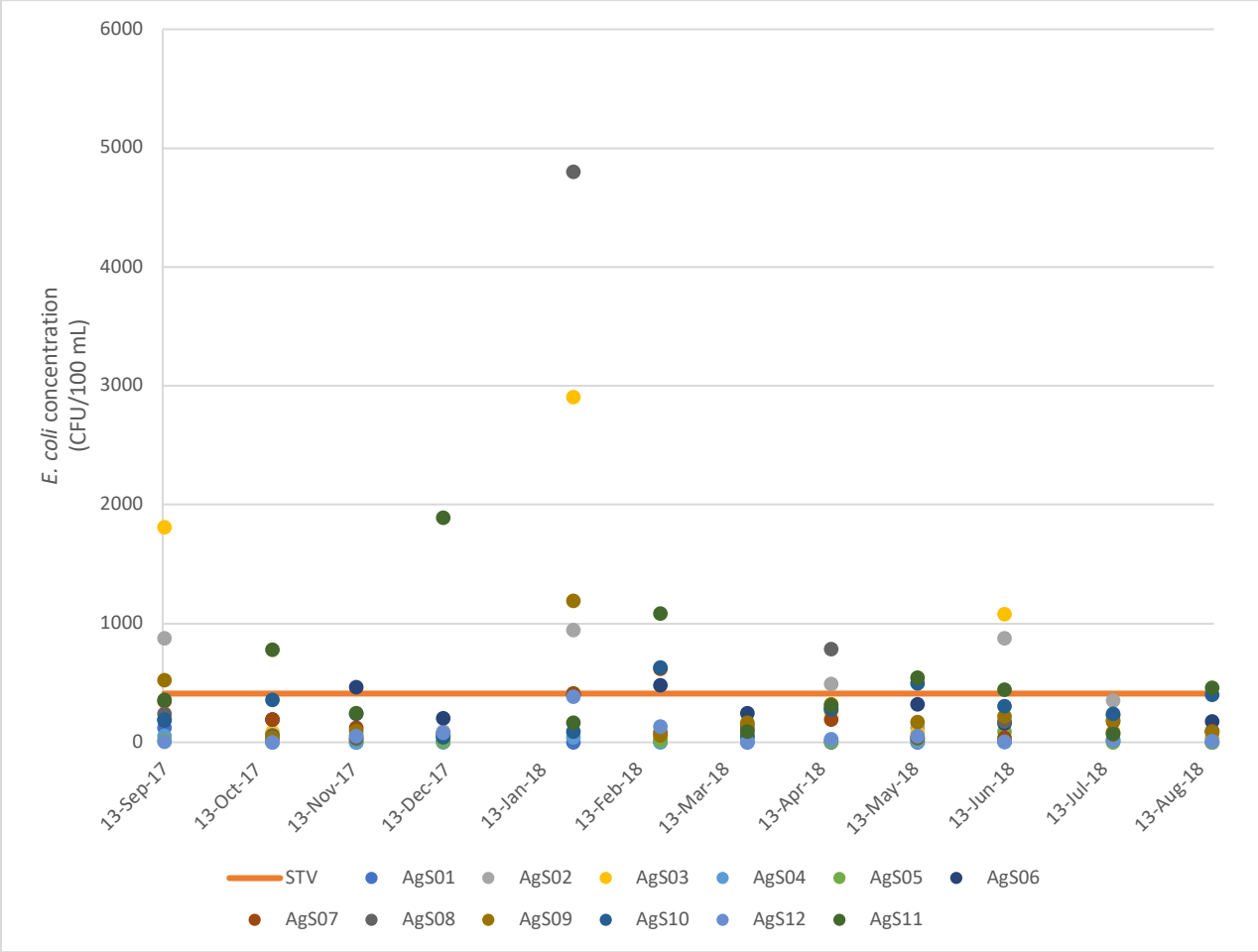

Figure S 2.6 Water temperature averages for each sampling date and precipitation amounts for 5-days before analysis date