BACTERIAL CONTAMINATION OF WATER IN AGRICULTURAL INTENSIVE REGIONS OF OHIO, USA

DISSERTATION

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By

Gayeon Won

Graduate Program in Veterinary Preventive Medicine

The Ohio State University

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Dissertation Committee:

Dr. Jeffrey T. LeJeune, Advisor

Dr. Gireesh Rajashekara

Dr. Rebecca Garabed

Dr. Song Liang

Abstract

Water related disease outbreaks threaten public health and safety worldwide. In the United Sates, notwithstanding public drinking water systems strictly regulated, acute gastrointestinal illnesses (AGI) are continuously reported to health agencies . In agricultural intensive areas, surface and ground water resources are more likely to be exposed to be contaminated with zoonotic bacteria, given the close proximity to sources of feces from livestock, dairy farms and wildlife. The aim of this dissertation was to determine a role of drinking and irrigation water as a vehicle for the transmission of zoonotic bacteria of fecal origin and the need of risk management in rural areas. First, we investigated the microbial quality of private well drinking water system located in six Townships in northeastern Ohio, regions with high concentration of dairy farms. Water samples were collected in 180 households (summer, 2009) and processed to detect fecal indicative organisms, *E. coli* O157 and *Campylobacter jejuni* by using commercial MPN methods and quantitative PCR analysis. Around 46%, 9 % and 4% of wells were contaminated with coliforms, *E. coli* and *E. coli* O157 respectively. There were no positives for *C. jejuni*. Second, current guidelines for microbial irrigation water quality recommended by relevant agencies were evaluated in the regard with their practicality and feasibility to detect water quality deterioration in practical applications. Water samples (n=227) were collected in six surface water sources providing irrigation water to

each six farm located in Northeastern Ohio over one irrigation season (Apr to Nov ,2010). Bootstrap analysis was applied to estimate optimal water testing frequency compared to those in current guidelines based on the value of fecal indicators detected in the water samples. Current guidelines for microbial quality of irrigation water imprecisely reflected the quality of water over one irrigation season in the context of sampling frequency recommended in those guidelines. Third, the association of microbial quality between irrigation water and fresh produce during pre-harvest was investigated at 120 farms located in Ohio, Kentucky and Indiana. Nonparametric correlation analysis and Repetitive-PCR (Rep-PCR) was performed on *E. coli* from water and vegetables isolated on the same farm in the cross-sectional study to determine statistical association and genetic relatedness respectively. Both statistically and genetically associations were not detected between irrigation water and vegetables. In conclusions, water sources used in agricultural intensive areas which are currently not protected under federal regulation are needed to be managed regarding likelihoods of human illnesses for a drinking purpose. For irrigation water, a practical regulation which can focus on preventing pathogen transmissions between water and fresh produce just prior to harvest, is required to be developed.

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Vita

2001-2007 .....DVM, Chonbuk National University

Fall 2007 to present .....Graduate Research Associate, The Ohio State University

Publications

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Chapter 1: Literature review

1.1 Introduction

Access to safe water supplies is a critical human need. However, consumption of contaminated water causes an estimated 88 percent of Disability-Adjusted Life Years (DALY) from diarrheal disease, and is responsible for 1.8 million deaths and 4.1 percent of total DALY global burden of diseases (GBD) (Mathers, Fat, & Boerma, 2008). Environmental and anthropogenic factors such as climate changes and high growth rate of the population have contributed to continuing water scarcity and degradation (Vörösmarty, Green, Salisbury, & Lammers, 2000; Delpla, Jung, Baures, Clement, & Thomas, 2009). The United Nations (UN) has defined a water stressed area as regions where water consumption surpasses ten percent of renewable fresh water resources (Gleick & Palaniappan, 2010). Fresh water paucity and land use changes following rapid urbanization (Foley et al., 2005) have also led to a need for increased capacity of treating pollutants in the water bodies.

Around 97 percent of water on earth is salt water in the oceans and fresh water comprises only three percent of total water on earth (Marks, 2011). Around 68.7 percent of fresh water is reserved in glaciers. Approximately 30.1% and 0.3 % of fresh water is ground water and surface water respectively. 0.9% of the remaining is water in other forms including soil moisture, swamp water and permafrost (Marks, 2011). A primary use of fresh water use is for irrigation; seventy percent of fresh

water withdrawal on earth has been used for this purpose (Fry, 2005). The demand for irrigation water has steadily grown in parallel with increasing food needs from a growing world population (Rosegrant, Cai, & Cline, 2002). In face the of fresh water scarcity, raw, treated, and partially diluted waste water is used to irrigate approximately 20 million hectares of agricultural land in the world (future harvest, 2001; Keraita, Jiménez, & Drechsel, 2008).

Around 0.8 percent of fresh water on earth is used for drinking. Safe and sufficient drinking water supply is one of the fundamental needs for human survival. However, inadequate drinking water supply and issues of quality is one of the world's major concerns, particularly in low income countries (World Health Organization, 2002). In many countries, ingestion of unsafe water, lack of access to sanitation and limited availability of water for hygiene ranks the third out of top twenty significant health risk factors (Ezzati, Lopez, Rodgers, Vander Hoorn, & Murray, 2002). For example, 90 percent of the deaths among children younger than 5 years old was attributed to contaminated drinking water in 42 developing countries (i.e.10.8 million children deaths worldwide in 2001). Poor water quality, combined with inadequate sanitation and poor hygiene, accounted for 88% of diarrheal diseases, the primary cause of these preventable childhood deaths (Black, Morris, & Bryce, 2003). Seckler *et al* defined this situation as severe 'economic water scarcity' which means that the countries have to develop water quality improvement plan even if population has sufficient water resources to meet water needs (Seckler, Barker, & Amarasinghe, 2001).
Even in developed regions, drinking water quality has been threatened by human activities and environmental changes (Leclerc, 2003). For instance, in the US,

contaminated groundwater sources were associated with 64 percent of the drinking water outbreaks between 1989 and 2002 (Blackburn et al., 2004), not accounting for the vast underreporting of waterborne gastrointestinal (GI) illnesses. The emergence and reemergence of waterborne pathogens (World Health Organization, 2003 ;Szewzyk, Szewzyk, Manz, & Schleifer, 2000) and the growth of susceptible population such as the elderly, the immune-compromised (C. P. Gerba, Rose, & Haas, 1996) contribute to the increased potential risk of waterborne diseases' infection in the developed.

Characteristics and the causative agents responsible for water-associated diseases vary according to socio-environmental factors and are considerably different in developing and developed countries (Yang et al., 2012). Diseases associated with water contamination have been categorized into four groups (Ashbolt, 2004) (Table1.1). Risk of water-washed, water-based and water-related diseases is more likely to occur in developing countries, especially in Africa, where warm water bodies have been both favorable habitat and the intermediate part of transmission of tropical parasites such as Hookworm, *Ascaris lumbricoides*, *Schistosoma* spp., *Trichuris* spp.. The diseases caused by these parasites affect millions of individuals annually (Fenwick, 2006; Patz, Graczyk, Geller, & Vittor, 2000).

'Water-borne' infections are characterized by disease outbreaks related with microbial water contamination in developed countries. Water-borne pathogenic agents are transmitted by a fecal-oral route (Ford, 1999). Animal and human wastes originating from septic tanks, domestic animals and wildlife may be moved into nearby fresh water sources (Pye & Patrick, 1983; Sayah, Kaneene, Johnson, & Miller, 2005). Microbial agents that survive in water are ingested by humans through drinking,

recreational activities or on produce that has been contaminated. In rural areas of the US, drinking water is provided to households from community water systems after appropriate treatments, non-community water system or from untreated private water system. Irrigation water is used for growing produce in fields. Zoonotic disease outbreaks related to drinking contaminated water and produce have been steadily increasing in the US. However, the role of water used for drinking and irrigation as transmission routes of pathogens also largely remain unidentified in the epidemiology of most of the major zoonosis (Reilly & Browning, 2008). Because microbial water contaminant may have multiple sources. It is often difficult to separate primary (i.e. drinking) from secondary (i.e. food) transmission (G. F. Craun, Calderon, & Wade, 2006).

The goal of this literature review is to provide a background of water-borne zoonotic diseases and their prevention in the United States by:

1. Summarizing the current burden of waterborne disease in the US and environmental factors affecting disease risk.
2. Describing the ecology of the most concern waterborne bacterial pathogens in water, their survival rate in the environment, and the role of water for human infections.
3. Reviewing current prevention strategies of the risks of waterborne pathogens based on Quantitative Microbial Risk Assessment (QMRA).
4. Outlining the current guidelines for microbial irrigation and drinking water quality.

Upon reading this chapter, the reader should be familiar with the current understanding of the ecology of waterborne zoonotic pathogens and their impact on the disease burden in the US. Current gaps in knowledge on the subject will be highlighted.

1.2 Current Burdens of water-borne disease outbreaks in the US

1.2.1 Drinking Water

Morris *et al* (1995) estimated that waterborne disease outbreaks caused nearly 7.6 million illnesses with high mortality rates (1200 people per a year) in the US. Transmission routes and pathogens of most concern are different from those in developing countries. In the US, around 200 million people draw their household water from treated surface water from 1,671 Community Water Systems (CWS) (i.e. municipal water systems). Ground water treated at 40,301 different CWS is the source of water for approximately 88 million people in (US EPA., 2011; Yoder et al., 2008). Fifteen percent of Americans (15.8 million people), mostly those residing in rural areas, obtain water from their own private well (ground water) or from surface water (US EPA. 2011), which is frequently untreated.

Despite the existence of extensive water supply infrastructures, during 1920 to 2002, almost 900,000 illnesses and 1870 outbreaks related to drinking water were reported to Waterborne Disease and Outbreak Surveillance System (WBDOSS), a partnership between the Centers for Disease Control and Prevention (CDC) and the Council of State and Territorial Epidemiologists (CSTE), and the Environmental Protection Agency (EPA) (M. F. Craun, Craun, Calderon, & Beach, 2006). The outbreaks reported since 1970 have been categorized into the following five water system deficiency types (Blackburn et al., 2004): 1.Water treatment deficiency (i.e. temporary interruption of disinfection, filtration absent or inadequate); 2. Distribution

system deficiency (i.e. contamination of water during construction or repair, or contamination of a storage facility); 3. Untreated ground water deficiency (i.e. from wells or springs source); 4. Untreated surface water deficiency (i.e. from rivers, lakes, or reservoirs); 5. Unknown deficiency.

For example, a water treatment deficiency in CWS was associated with a massive waterborne outbreak of Cryptosporidiosis in Milwaukee, USA (1993). That outbreak affected around 403,000 people and resulted in 104 deaths (Morris, Naumova, & Griffiths, 1998). Water-borne outbreaks of *Escherichia coli* O157:H7 infections that occurred in Missouri (Swerdlow, Woodruff, Brady, Griffin, Tippen, Donnell Jr, Geldreich, Payne, Meyer Jr, & Wells, 1992a), Wyoming (Olsen et al., 2002) and New York state (Bopp, Sauders, Waring, Ackelsberg, Dumas, Braun-Howland, Dziewulski, Wallace, Kelly, & Halse, 2003a) were presumed to be associated with water treatment and distribution system deficiency.

Reynold et al estimated that 19.5 million cases of waterborne illnesses, including AGI occur each year in the US populations served by community and non-community water system (Reynolds, Mena, & Gerba, 2008). Epidemiologic studies found that untreated ground water, particularly supplied by privately owned wells were associated with acute gastrointestinal illnesses (AGI) (Payment et al., 1997; Strauss, King, Ley, & Hoey, 2001; Kuusi et al., 2004). Despite vast underreporting, AGI is one of major concerns for drinking water-borne disease in the US (Colford Jr et al., 2006). Ecological agents are not identified in approximately 30% of outbreaks of acute gastrointestinal illnesses (AGI) associated with contaminated water (M. F. Craun et al., 2006). Further investigation of etiology of AGI from non-community

water system including private well water, is needed to prevent further increases in illnesses.

1.2.2 Irrigation Water

Water used for irrigation account for approximately 65 percent of global water use and 85 percent of the total consumptive water use, water that not directly returned to the original water sources (Berndes, 2002). In the United States, irrigation withdrawals are responsible for 37 percent of all fresh water withdrawals but this varies by regions(Kenny & Geological Survey (US), 2009). Seventeen Western states with low amounts of precipitation (i.e. less than 20 inches per a year) use the majority of the withdrawals (85%) of both surface water and ground water for irrigation (Kenny & Geological Survey (US), 2009). In water scarce areas in the US, waste water after treatments has been used for irrigation to satisfy increasing demands for agricultural use (Crook & Surampalli, 1996). About 2.5 billion gallons/day of municipal wastewater was reclaimed in the US (Crook & Surampalli, 1996) . Arizona, California, Florida, and Texas account for more than 90% of the reclaimed water use (Crook, 2002).

Over the last several decades, zoonotic pathogens have been isolated from a wide range of raw vegetables in the US (Taormina, Beuchat, & Slutsker, 1999; Mouzin et al., 1997; Rude, Jackson, Bier, Sawyer, & Risty, 1984). In addition, a growing number of food-borne disease outbreaks related to fruit and vegetable have raised concerns of public health in the US (Steele & Odumeru, 2004). Irrigation water quality degradation have been suspected as one of several potential transmission routes of zoonotic pathogens on fresh produce during pre-harvest stages of production (Lynch, Tauxe, & Hedberg, 2009; Beuchat, 2002; Rose et al., 2001). These situations

have prompted relevant agencies to establish water use and reuse guidelines (U.S. Environmental Protection Agency, 2004). However, the role of irrigation water as the conclusive source of contamination has remained uncertain because exposures to sources of contamination can occur during multiple stages of production including during harvest or at post- harvest handling and storage (Delaquis, Bach, & Dinu, 2007). The difficulty of tracing contamination original sources still have posed challenges to public health and resulted in economic burdens of water treatment costs to irrigators (Gómez-Limón & Riesgo, 2004) without certainty that the added expense of production have any measurable effects on the safety of the final product. Hence, the extent that irrigation water contributes to the burdens of contamination of fresh produce is a critical gap in knowledge that needs to be addressed before the feasibility and effectiveness of irrigation water intervention strategies for food safety improvement can be recommended.

1.3 Waterborne transmission of zoonotic pathogens

Waste from humans and other animals seem to be the largest source of zoonotic pathogen loads into environment which could be the origin of water-borne pathogenic agents. Waterborne disease outbreaks occurred during 1986 to 1998 mostly are attributed to confined animal feeding operations (C. P. S. Gerba & James, 2005). This chapter addresses zoonotic pathogens originating from the feces of agricultural animals and potentially causing enteric infections in humans acquired. Among the microbial agents, bacteria are widely known as the most successful form of life in the environmental habitat due to mainly their phenotypic plasticity (Leclerc, 2003). This

intrinsic property of bacteria contributes to resisting environmental insults and or adapting to a wide range of environmental conditions (Browner et al., 2001). Transmission is initiated by the pathogens being excreted in animals' feces into the environment. In the United States, it has been estimated that food animals grown in Concentrated Animal Feeding Operations (CAFOs) produce 1.6 billion tons of manure annually (Altekruse, Swerdlow, & Wells, 1998), which includes the liquid and solids discharged from the animals along with bedding materials (Browner et al., 2001).

1.3.1 Transmission pathway into the environment

Pathogens excreted from infected animals and humans that have been directly introduced to water sources, impact microbial water quality. Feces excreted from infected animals may be transported through overland flow (Cole et al., 1999; Tyrrel & Quinton, 2003). Suspended sediments may play an important role of transporting fecal bacteria into water (Characklis et al., 2005). Sinclair *et al.* also found the positive association between total suspended solids (TSS) and fecal bacteria loads into water sources (Sinclair et al., 2009). Diffuse pollution sources such as livestock grazing, wastes and wildlife, is called nonpoint source (NPS) contamination (Baker, 1992). Discharges from nonpoint sources are usually sporadic, linked with surface runoff, precipitation event and atmospheric deposition. NPS contamination makes it difficult to identify dominant sources of this contamination in water (Meays, Broersma, Nordin, Mazumder, & Samadpour, 2006). Land use management within catchment areas such as setting a buffer zone area may be important to prevent direct agricultural and diffuse microbial input into water sources (Blackwell, Hogan, Pinay, & Maltby, 2009).

In the other hand, contaminants are also transported into water through distinct and confined routes, which can easily identify and treat sources (Parveen et al., 1997). Municipal or industrial wastes and treatment failures in municipal water system are generally related to this type of contamination.

1.3.2 Survival in the environment

Roszak *et al.* defined 'survival' as maintenance of viability under adverse circumstances (Roszak & Colwell, 1987). In comparison to 'normal' status of pathogen which are able to reproduce themselves (i.e. culturable), the Viable but Non Culturable (VBNC), is a strategy for survival of those pathogens in water under stress conditions (Colwell, 2000). In contrast to dead cell, which cannot be revived, cells in VBNC state can be resuscitated and then cultivatable. Water-borne pathogens in VBNC status may pose a potential risk to consumers of drinking water because some of waterborne pathogens including *Campylobacter* spp., *E. coli* including EHEC strains, *Salmonella* spp*, Vibrio cholerae, Vibrio parahaemolyticus*, and *Vibrio vulnificusare* are able to enter VBNC status (Oliver, 2005) and survive for extended periods and are undetectable by traditional microbial methods. This is a more serious issue in public health because some VBNC pathogens retain virulence and infectivity and have a low infectious dose to human (Oliver, 2005).

Despite their survival strategy in water sources, the microbial agents are vulnerable under stress conditions and eventually die. How long enteric pathogens can survive in water generally relies on numerous factors including temperature, moisture, pH, nutrition and the competing microflora, predation, light, other physical chemical parameters (Gonzalez, 1995; Wesche, Gurtler, Marks, & Ryser, 2009).

Temperature differences significantly influenced survival rate of waterborne pathogens and fecal indicators in water sources. A decrease in water temperature may prolong the ability of *E .coli* O157 agents to survive in water (Sampson et al., 2006; Easton, Gauthier, Lalor, & Pitt, 2005). *Campylobacter jejuni*, also can survive at stream water held at low temperature (4 °C) for more than four months (Rollins & Colwell, 1986). These two review papers described gene specific adaptation against stress factors occurs in *E. coli, Salmonella* spp and *Campylobacter* spp. (Murphy, Carroll, & Jordan, 2006; Winfield & Groisman, 2003).

The survival rates of fecal bacteria present in water sources often show a biphasic pattern in which initial decay is fast, followed by slower die-off (Winfield & Groisman, 2003; Hellweger, Bucci, Litman, Gu, & Onnis-Hayden, 2009). Several theories including population heterogeneity may explain this biphasic die-off rate of fecal bacteria in non-host environments (Balaban, Merrin, Chait, Kowalik, & Leibler, 2004; Lewis, 2006).

1.3.3 Environmental Factors influencing microbial water quality

Climate change The World Health Organization estimated that climate changes induced by humans, including global warming and precipitation trends contribute to annual rates of mortality (i.e.15, 000 per a year) worldwide (Houghton, 2001; Patz, Campbell-Lendrum, Holloway, & Foley, 2005). Particularly, heavy rainfall events, flooding and temperature have been associated with an increased number of waterborne disease outbreaks in the world (Hunter, 2003). Global average temperature is expected to increase between 1.4 and 5.8 ˚C during this century (IPCC, 2007). Theoretically, these three events are interrelated (Trenberth, 1999); 1.Increase

in evaporation followed by the increase in temperature affect water-holding capacity of the atmosphere, which accompany intense precipitation. 2. Enhanced precipitation rate and latent surface heating also influence the high frequency of storm events. Intensity of precipitation leads to the increased quantity of surface runoff loading contaminants into surface water bodies (Milly, Dunne, & Vecchia, 2005). This increase of loading and discharge in water bodies may exceed their infiltration capacity and make new directions of path of water flow. In addition to this metrological theory, several studies also reported that these climate changes are associated with microbial quality deterioration in water sources (Kistemann, Classen, Koch, Dangendorf, Fischeder, Gebel, Vacata, & Exner, 2002a; Lerner & Harris, 2009). Curriero et al (2001) found the significant link between waterborne disease outbreaks caused by contaminated drinking and recreational water and extreme precipitation during 1948 to 1994 in the US (Curriero, Patz, Rose, & Lele, 2001). Another recent study also described that fecal streptococci and fecal coliform significantly reflect the variation of microbial quality in surface water caused by storm events in the US (Pan & Jones, 2012).

Land use Land use change is one of the factors influencing water quality deterioration over the past several decades. Industrialization and urbanization have resulted in environmental problems (Foley et al., 2005). Expansions of urban area have contributed to increasing impervious spaces on lands, which impede storm water discharge to vegetation areas and produce more surface runoff(Arnold Jr & Gibbons, 1996; Piao et al., 2007). Increase in surface runoff volume generates higher contaminant loads into nearby water sources (Charbeneau & Barrett, 1998). Smith *et*

al. (2001) reported that in the US, higher number of fecal coliform were observed in watersheds with high proportion of urban areas and agricultural land area on steep slope than those near lands used solely for agricultural practices (Smith, Wickham, Norton, Wade, & Jones, 2001). In addition, the increase in impervious spaces also results in degradation of ground water quality as well as reduction of ground water infiltration (Lerner & Harris, 2009).

1.4 Waterborne zoonotic pathogens and fecal indicative organisms

1.4.0 Excretion from domestic and wild animals

Food animals are considered the primary reservoir of *Escherichia coli* O157:H7, *Campylobacter* spp and *Salmonella enterica*. Pathogenic *Escherichia coli, Campylobacter* spp and non-typhoid *Salmonella* spp. have annually contributed 0.27 million, 1.4 million and 2.4 million cases of gastrointestinal illness (GI) in the United States, respectively (Mead et al., 1999).

Cattle are generally considered the primary reservoir for *E. coli* O157:H7 (Dorn & Angrick, 1991; Slutsker et al., 1998). For an infected cow, *E. coli* O157 is excreted in the range between 10^2 and 10^5 cfu per a gram of feces with cattle shedding less than 10^2 cfu per a gram most of the time (Slutsker et al., 1998). Reports of the prevalence of *E. coli* O157 in cattle ranged between 0 to100 percent in feedlot cattle, from 0.7 to 27.3 percent in cattle on irrigated pasture, and from 0.9 to 6.9 percent in grazing cattle during last three decades (Hussein & Bollinger, 2005). Jones et al indicated that the rate of fecal shedding of *E. coli* O157 was the highest in spring and late summer, in which human infection cases also increased (Jones, 1999).

Healthy cattle may be a reservoir of *Campylobacter jejuni* (Garcia et al., 1985; Humphrey & Beckett, 1987;Wesley et al., 2000). The prevalence rate varied between 10 and 70 percent according to differences of geographic region, herd size species and ages. Fecal material from birds or bats was also suspected as a most likely source of water-borne campylobacteriosis (Palmer et al., 1983). Human infections of *Campylobacter jejuni* also may be temporally linked to seasonal peaks (spring and fall) in fecal shedding rate in cattle (Stanley, Wallace, Currie, Diggle, & Jones, 1998; Tauxe, Nachamkin, Blaser, & Tompkins, 1992).

Salmonella species are known as 'universal pathogen' due to the fact that this microorganism has been isolated from all tested vertebrates (Cole, Hill, Humenik, & Sobsey, 1999).

1.4.1 Pathogens

1.4.1.1 *Escherichia coli* O157:H7

Enterohemorrhagic *E .coli* (EHEC) is the only zoonotic origin pathogen among the six different phathogroups of *E. coli* (Caprioli, Morabito, Brugere, & Oswald, 2005). *Escherichia coli* O157:H7 is the prototype pathogen of Enterohemorrhagic *Escherichia coli* (EHEC) group (Tarr, Gordon, & Chandler, 2005). In North America, *E. coli* O157:H7 annually contribute to around 75,000 infections and 17 outbreaks in human (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005). Ruminants, particularly cattle and sheep and other domestic animals including goats, pigs, poultry, cats and dogs, can harbor these bacteria (Fagan, Hornitzky, Bettelheim, & Djordjevic, 1999). These virulence factors have caused a wide range of clinical signs in human

including bloody or non-bloody diarrhea, abdominal cramps, and hemolytic-uremic syndrome (HUS) in human (Gransden, Damm, Anderson, Carter, & Lior, 1986). *E. coli* O157:H7 is a Gram-negative bacillus (Colwell, 2000). The letter O and H refer to the somatic antigen and the flagella antigen respectively. The majority of *E. coli* O157:H7 strains can be distinguished from most *E. coli* by the inability to ferment sorbitol rapidly and by a lack of production of beta-glucuronidase (Karch et al., 1993). All EHEC strains including *Escherichia coli* O157:H7 produce cytotoxins called shiga toxins (*stx1 and stx2*) or vero toxins (Levine et al., 1987), hemolysin encoded by *hlyA* (H. Schmidt et al., 1994) and intimin encoded by *eaeA* (Louie et al., 1993).

E. coli O157 can survived longer in water than in bovine feces (Wang, Zhao, & Doyle, 1996; Wang & Doyle, 1998). In reservoir water, *E. coli* O157 survived for 13 and 11 weeks at 8 and 25 °C with the decrease of around 1.5 and 3 $\log_{10}$ respectively, which indicated that *E .coli* O157 may have the ability to entering VBNC status in water .

1.4.1.2 Salmonella spp.

Salmonella spp. is Gram- negative, facultative bacillus. This genus, *Salmonella* have two species; *Salmonella enteria* and *Salmonella bongori* (Tindall, Grimont, Garrity, & Euzeby, 2005) . The species *Salmonella enterica* is divided into six subspecies by current view of taxonomy; enterica, salamae, arizonae, diarizonae, indica, and houtenae. *S. enterica* subspecies enterica which consists of more than 2300 serovar (i.e.serotype, subspecies) causes vast majority of food-borne and waterborne salmonellosis in animals and humans (Landeras, González-Hevia, & Mendoza, 1998). Some of S. enterica subspecies enterica serotypes, Typhi and Gallinarum, are adapted

to specific hosts, human and poultry respectively (Pascopella et al., 1995), while other salmonella serotypes show a wide range of host adaptation but severity of symptom are all different among serotypes (Coburn, Grassl, & Finlay, 2006). Serotype Typimurium and Enteritis cause infection to almost hosts but symptom usually self-limited to GI, except in immune-compromised host (Uzzau et al., 2000; Ohl & Miller, 2001). Harley et al reported that 13 different serotype of Salmonella were detected in a rural watershed in the US (Haley, Cole, & Lipp, 2009). Salmonella can be adapted to extreme condition and survive resiliently in the environment (Foster & Spector, 1995).

1.4.1.3 *Campylobacter* spp.

Disease Active Surveillance Network reported that approximately 13 per 10,000 infants (i.e. younger than 1 year age) are annually diagnosed with campylobacteriosis in the US (Fullerton et al., 2007). Campylobacter infection can be manifested in several symptoms; abdominal pain, fever, nausea, vomiting and guillain-barre syndrome as post infectious infection. But human infections are commonly asymptomatic and self-limited (Frost, 2001; Blaser et al., 1979).

This organism is Gram- negative, motile rods and microaerophilic which means that reduced oxygen (3 to 5%) and carbon dioxide (2 to 10%) are required for being grown with neutral pH between 30 and 47 ˚C but has optimal growth temperature at 42 ˚C (Stintzi, 2003). This microaerophilic characteristic makes it hard to culture this organism and diagnose campylobacteriosis in human, which may affect substantial underreporting (Allos & Blaser, 1995). Despite low tolerance in extreme condition, campylobacter is the predominant food-borne and waterborne pathogen in the western world, causing acute diarrhea in human (Frost, 2001). The genus of campylobacter

currently includes 14 species, of which *C. jejuni* and *C .coli* are known as the most cause of campylobacter enteritis (FETUS, 2000). Campylobacter spp. survived in stream water during more than 4 months at 4°C (Rollins & Colwell, 1986)

1.4.2 Pathogen Detection in Water

After representative sampling have been pulled out of the population (i.e. water body), approaches for target microorganisms' detection can be subsequently applied. These tests have played a pivotal role in guaranteeing a certain level of water quality. Even though the technologies for detecting food-borne pathogens have been considerably developed over the last several decades (Shi, Long, & Suo, 2010), the direct application of same protocols to waterborne pathogens' detection has the limitation due to relatively low number of pathogens in water and the difficulty to detect stressed cells (Straub & Chandler, 2003).

Filtration is the initial process of concentrating the low number of pathogens from water. For water samples highly contaminated, this step can be passed over. Water samples are commonly tested with filter membranes with 0.01 to 100 μm pore size, according to an average size of target microorganism (C. Gerba, 1996). The optimal volume of sample a water sample for filtration depends on a type of water sources and target pathogens, turbidity and expected concentrations of target pathogens in the water source. In a field study, 500ml of water was collected to test *E. coli* O157 from private drinking well water which is of a low level of turbidity (Schets et al., 2005).

For irrigation water quality monitoring study, 100ml of the water was collected from the creek (Shelton et al., 2011).

1.4.2.1 Qualitative detection

The purpose of qualitative detection is to determine presence/absence or prevalence rates of a target pathogen in water samples. Pre-enrichment steps are often applied to resuscitate injured and weakened bacteria cell or recover the cell from their defense status such as VBNC and biofilm in samples (Bonetta et al., 2011). But both target cells and non- target cells in samples can be enriched during this procedure, causing a low level of specificity in detection, particularly under the circumstance when a low numbers of a target pathogen are present in water.

Pathogen presence in samples is determined based on the growth of a predominant pathogen in selective cultivation-based methods where selective media are used for suppression of competitive agents in the sample (Quilliam, Williams, Avery, Malham, & Jones, 2011). In the case of presumptive-positive, additional biochemical and/or serological tests are required to avoid false-positive or false-negative results of conventional culture-based assays (J. Y. M. Johnson et al., 2003; Fong et al., 2007). In order to increase qualitative sensitivity of detection, conventional PCR or real-time PCR analysis combined with selective cultural methods is generally used for increasing the number of target cells without DNA purification process (Hörman et al., 2004).

1.4.2.2 Quantitative detection

Total direct microbial counts methods using selective media provide viable bacterial counts in water without prior enrichment steps (Brichta-Harhay et al., 2007). The

Most Probable Number (MPN) method has been traditionally used to enumerate low numbers of bacteria present in water samples (Cochran, 1950; Jenkins, Endale, & Fisher, 2008; Rompré, Servais, Baudart, de-Roubin, & Laurent, 2002). Due to the low sensitivity in these methods, direct counting methods have been used combined with concentration process of samples such as filtration and centrifugation. For instance, in membrane filtration methods, the filter is directly placed to a selective agar plate after filtration and cultured to enumerate viable target pathogens present in water samples. The limitations of these cultural-based quantification methods are false negatives caused by undetectable of cells of water-borne pathogens in a VBNC status, which may be infectious when ingested by human (Oliver, 2005). False positives may be concerns, which are caused by growth of non-target bacteria in the media.

The recent development of real-time PCR (qPCR) analysis has facilitated the enumeration of the initial concentrations of the target bacteria in water (Valasek & Repa, 2005). Targeted DNA amplification can be continuously monitored with fluorescence emitted by DNA binding molecules (Sybr green) (Morrison, Weis, & Wittwer, 1998) or DNA probe (taqman or molecular beacon) (Whitcombe, Theaker, Guy, Brown, & Little, 1999).

While conventional PCR provides measurement amplified DNA by an end-point analysis (Clark et al., 2011). For the absolute quantification, a standard curve has been applied to measure an unknown amount of target nucleic acids in samples (Rutledge & Cote, 2003) . Generally, 10-fold serial dilutions of genomic DNA or cell

equivalents (i.e. a known amount) originated from positive controls are provided as a template (usually in triplicate) for setting up a standard curve. Cell equivalents are measured based on the number of colony forming unit (cfu) from direct plating enumeration methods. The copy number of genomic DNA was calculated by using the following equation (Fitzmaurice et al., 2004):

$$\frac{\{6 \times 10^{23}\ (copy\ number\ mol^{-1})\} \times concentration\ (gram\ \ \mu l^{-1}\ )}{molecular\ weight\ (g\ mol^{-1}\ )}$$

$$= copies/\mu l$$

A standard curve generally generates a linear regression line and a correlation coefficient (R^2), showing the relationship between the reference amplicon or cell equivalents and the number of cycle where fluorescence emitted from amplification of the template exceeds the threshold level (i.e. *Ct* value)(Heid, Stevens, Livak, & Williams, 1996). Hence, initial concentration of a target pathogen in samples (i.e. absolute quantification) and the limit of detection (i.e. baseline sensitivity) can be calculated based on standard curves.

In the comparison to other quantification methods, the number of cell (cell number /ml) quantified by qPCR was significant higher than those (CFU/ ml) enumerated by culture based methods (Haugland, Siefring, Wymer, Brenner, & Dufour, 2005; Khan et al., 2007; Lleo et al., 2005). Josephson *et al* reported that PCR technique based on DNA analysis may result in false positives regarding viable cells in water (Josephson, Gerba, & Pepper, 1993), which make the interpretation of the result more complicated. However, given detection of nucleic acids in VNBC status by PCR and potential

infectivity of pathogens in VBNC status (Oliver, 2005), pathogen quantifications by qPCR is useful for the next step, quantitative microbial risk assessment (QMRA) (Loge, Thompson, & Douglas, 2002; Malorny, Lofstrom, Wagner, Kramer, & Hoorfar, 2008).

1.4.3 Fecal indicative organism

Total coliforms Total coliforms are a closely related group of bacteria, which have the following characteristics: rod-shaped, gram-negative and oxidase-negative, aerobic or facultative anaerobic bacteria (Tallon, Magajna, Lofranco, & Leung, 2005). Generally, this group of bacteria can ferment lactose and beta-galactosidase-positive (Tallon et al., 2005). Some bacteria that belong to a total coliform group do not originate from warm-blooded animal feces but heterotrophic bacteria (EDITION, 2006). Hence, total coliform have often considered unreliable fecal indicative organism because of their capability of re-growth and occurrence in environments (Leclerc, Mossel, Edberg, & Struijk, 2001; Szewzyk et al., 2000). Despite the limitation, total coliform present in water sources has been widely used as an indicator of environmental contamination and the failure of disinfection or effective treatment in the water source (Bennear, Jessoe, & Olmstead, 2009a).

Thermotolerant coliform and Generic *Escherichia coli* Thermotolerant coliforms is a subset of a total coliform group which can ferment lactose to acid and gas at 44.5 ± 0.2°C within 48 hours. *Citrobacter, Enterobacter, Klebsiella* and Generic *E. coli* are included in this group fecal coliform (EDITION, 2006). Generic *E. coli* are indigenous to the intestinal tract of humans and other warm-blooded animals. Even if

E. coli belongs to a coliform group, it can be differentiated from other coliforms because most strains can hydrolyze 4-Methylumbelliferyl beta-D-galactoside (MUG) present in the media and the media appears fluorescent blue under the UV light (Farnleitner et al., 2001). *E. coli* are the most prevalent coliform feces, accounting for more than 94% of coliform identified in human feces (Leclerc et al., 2001). Generic *E. coli* presence in water is also considered the most consistent predictor of GI illness among other fecal indicative organisms (Wade, Pai, Eisenberg, & Colford Jr, 2003). However, recent studies explained that *E. coli* may lack the predictability due to its ability of regrowth in contaminated soil in tropical areas and a low survival rate less than other enteric pathogens in non-host environments (Desmarais, Solo-Gabriele, & Palmer, 2002).

Fecal Streptococci Fecal Streptococci belongs to a group of gram-positive and catalase-negative cocci bacteria (Facklam & Elliott, 1995; Geldreich & Kenner, 1969). Antibiotic resistance patterns in fecal streptococci have been used to differentiates between fecal sources of animal and human and between animal sources (B. Wiggins, Andrews, Conway, Corr, Dobratz, Dougherty, Eppard, Knupp, Limjoco, & Mettenburg, 1999a; Hagedorn et al., 1999; Harwood, Whitlock, & Withington, 2000).

Enterococci Enterococci have been considered a subgroup of the fecal streptococcus. Enterococci are distinguished from other streptococci by their ability to be cultured in 6.5% NaCl and high PH (9.6) at 45°C(EDITION, 2006). *Enterococcus faecalis* have been considered a good candidate of fecal indicator due to their host rage limited to human and a few warm-blood animals (Wheeler, Hartel, Godfrey, Hill, & Segars,

2002) and in marine environment (Edberg, Leclerc, & Robertson, 1997; Colford et al., 2012).

Host specific Bacteriophage Bacteriophage is a virus that can infect bacteria. Three groups of bacteriophage;1. Somatic coliphages 2.Male-specific RNA coliphages (FRNA phages) and 3. Phages infecting *Bacteroides fragilis* have been used as a fecal indicator (Berger & Oshiro, 2002). Bacteroides 16S r RNA host-specific genetic marker has been applied to track the host of fecal contamination (Savichtcheva, Okayama, & Okabe, 2007).

1.4.4 Limitations in fecal indicator use

Despite the usefulness and accessibility of these fecal indicators, their reliability to predict the presence of pathogens in water and potential outbreaks is controversial due to fluctuations in the ratio of pathogens to fecal indicators (Dechesne & Soyeux, 2007; Brown, Proum, & Sobsey, 2008). Odgen *et al*. showed that when decay rate between *E. coli* O157 and generic *E. coli* originated from same source was compared, the rate of *E. coli* O157 was same or slightly faster than that of generic *E. coli* (Ogden, Fenlon, Vinten, & Lewis, 2001). Because of the lack of consistent the absence of proportional relationships between different fecal indicators (i.e. fecal coliform , *E. coli*) and pathogen, there have been also caused ongoing controversy over usefulness and effectiveness of indicators in the prediction of the presence of pathogens (Dechesne & Soyeux, 2007).

1.5 Fecal source tracking

Positive occurrence of fecal indicators in water was used to trace the origin of fecal sources. Historically, the ratio of fecal streptococci and fecal coliform detected in environmental samples has been used to the fecal source tracking (Messley & Kingsbury, 1973). If the ratio is over 4 or between 0.1 and 0.6, this ratio indicated sample contaminated from animal sources. The ratio less than 0.1 showed the source may be originated from wild animals (Messley & Kingsbury, 1973). This FS/FC ratio was currently no longer used for the recommendation due to a lack of the validation by sufficient empirical evidence (American Public Health Association,). Host specific *Bacteriodales* 16s rRNA genes also have been widely applied to discriminate among fecal sources of ruminants, domestic animals and human in fresh water sources (Bernhard & Field, 2000; Dick et al., 2005; Gilpin et al., 2003). In addition, the desire of identification of sources of fecal contamination in water sources has led to the development of diverse fecal source tracking (FST) methods using genotypic and phenotypic characteristics of fecal indicative(Bernhard & Field, 2000) organisms. If fecal indicators are detected in targeted water bodies, the detection have generally informed the potential fecal source intrusion into water, not where they derived. Fecal source tracking (FST) methods have been developed to understand dominant sources of fecal contamination, particularly in nonpoint source contamination to establish strategies for controlling microbial contamination in water.

Microbial Source Tracking (MST) methods generally contain of three parts; 1.Source identifiers 2.Detection methods 3.Analytical approaches (Stoeckel, 2005).

Source identifier Fecal indicator microorganisms used for parameters in regulation of water quality have been generally chosen for investigating both the magnitude of fecal contamination of water sources and the source of the contamination (Stoeckel, 2005). Pathogenic microorganisms also have been widely used to trace the origin of specific target pathogen detected in water sources (Bopp, Sauders, Waring, Ackelsberg, Dumas, Braun-Howland, Dziewulski, Wallace, Kelly, & Halse, 2003b; Hänninen et al., 2003).

Detection Methods Detection methods are chosen based on characteristics of selected source identifier such as genotype and phenotype to recognize source identifier among the various putative sources (Stoeckel, 2005) .

Genotypic methodologies can be used to distinguish genetic fingerprint of organisms detected from different animal hosts or environments. It is assumed that certain organisms or strains have become adapted to particular hosts, species or environments, and subsequently the genetic types have been passed to their replicates (i.e. offspring) (Stanier, Ingraham, Wheelis, & Painter, 1987). Genotyping methods include Pulse-Field Gel Electrophoresis (PFGE), Repetitive element PCR, ribotyping and Host-specific molecular marker and performed to elucidate genotypic differences within organisms traced (Simpson, Santo Domingo, & Reasoner, 2002). Phenotypic characters of microorganisms such as physical or biochemical traits also have been used in identification(Stanier et al., 1987). Antibiotic Resistance Analysis (ARA) has been widely applied to discriminate human and animal sources of fecal pollution (B. Wiggins, Andrews, Conway, Corr, Dobratz, Dougherty, Eppard, Knupp, Limjoco, &

Mettenburg, 1999a; Reneau et al., 2002). The methods commonly used for MST as outlined in table 1.2.

MST methods are also classified as library-dependent (generally validation of isolate by isolate) and library-independent (generally in the sample level) methods (Meays, Broersma, Nordin, & Mazumder, 2004). Library-dependent methods require the construction of a library which is compared to unknown environmental isolates to confirm the fecal origin (Ahmed et al., 2007).

Analytic Approaches All library-dependent methods require the procedure of accuracy evaluation, comparing between isolates from unknown sources and the database of known sources. By using the power of the discriminant analysis (i.e. multivariate statistical methods)(R. A. Johnson & Wichern, 2002), isolates from known and unknown sources are classified based on different profiles according to the applied methods. When ARA was applied to distinguish the each origin of fecal streptococci (FS) among cattle, human, poultry and wild life, the average rate of correct classification (ARCC) varied according to studies, ranging from 64 to 84percent (B. A. Wiggins, 1996; B. Wiggins, Andrews, Conway, Corr, Dobratz, Dougherty, Eppard, Knupp, Limjoco, & Mettenburg, 1999a; B. Wiggins, Andrews, Conway, Corr, Dobratz, Dougherty, Eppard, Knupp, Limjoco, & Mettenburg, 1999b). In comparison of rate of correct classification (RCC) between rep-PCR and ribotyping, rep-PCR had significantly a higher power than ribotyping performed for discriminating the sources of generic *E. coli* isolates from humans and seven different animals(Carson, Shear, Ellersieck, & Schnell, 2003). Combination of two different

MST methods has been applied to enhance typability of methods since any single MST method application has not shown an ideal result (Field & Samadpour, 2007).

1.6 Waterborne pathogen sampling and detection

1.6.1 Sampling strategies in water sources

1.6.1.1 Power analysis

Detection of pathogens in water begins with designing an appropriate sampling plan. The use of statistic methods may differ depending upon the main purposes for monitoring and intention of the sampling plan. Generally, statistical power analysis has been applied to determine the required sample size to have a certain level of precision and sufficient statistical power in scientific fields (Kraemer & Thiemann, 1987). Statistically, power $(1 - \beta)$ means the probability of rejecting the null hypothesis when it is false (i.e. true rejection of H_o). Null hypothesis (H_o) refers to the concept of no difference, assumed to be false while alternative hypothesis (H_1) is assumed to be true (Pagano, Gauvreau, & Pagano, 2000). Type 1 error (α) happens when null hypothesis is rejected when it is true, which also refer to *P* value. Type 2 error (β) indicates the probability of not rejecting the null hypothesis when it is false (i.e. false positive) (Zar, 1999). These concepts are described in Figure 1.1 within two independent populations. Herein, effect size refers to the difference of value between true population and population hypothesized in H_o (Cohen, 1992). The power is proportional to the effect size multiplied by, α level multiplied by the square root of the sample size, and divided by the standard deviation (i.e. sample variance) based on

an assumed parametric distribution (US EPA, 2010). Hence, a larger sample size would be required when effect size is decreased and sample variance is increased in same levels of statistical power and α level. However, Mumby *et al* indicated that power calculation used for non-parametric distribution is less straightforward (Mumby, 2002).

1.6.1.2 Sample size determination in the water quality monitoring study

The aim of water monitoring is to acquire quantitative and representative information on characteristics of microbial quality of the water sources including temporal and spatial variation of the quality (Sanders, 1983). Several researches have employed statistical approaches to calculate sample size required (Dunnette, 1980; Antelo, Arce, & Carballeira, 1998). However, caution is needed when applying power analyses to calculate sample size required in this type of study.

First, factors affecting microbial water quality can lead to the unexpected increase of parameters. There are external factors influencing dispersion of data collected in water sources regarding temporal and spatial fluctuation of pathogen concentrations in water. Some examples include; 1.Random changes due to storms, precipitation or non-point source contamination, 2. Fixed changes due to seasonal or diurnal changes of contamination, survival rates of targeted pathogens or types of water body such as stream, ocean and lake, etc. (Loftis & Ward, 1980; Kistemann, Classen, Koch, Dangendorf, Fischeder, Gebel, Vacata, & Exner, 2002b). To evaluate the impact of the fluctuation of the quality parameter, various sampling designs have been applied: A nested design (hierarchical) sampling design is used for detecting temporal variation of contaminants in the water between times such as seasons, months and

days or parameters (Elsdon & Gillanders, 2006; Hatje, Rae, & Birch, 2001; Reischer et al., 2011).

Second, samples should be independently and randomly collected. However, water sampling is often repeatedly (i.e. non-randomly) performed at the same locations at different time points, particularly in long-term trend monitoring studies. In these cases, therefore, different approaches of sample size calculation have been applied to measure autocorrelation effects (H. O. Johnson, Gupta, Vecchia, & Zvomuya, 2009; Whitman & Nevers, 2003).

Third, for the studies determining compliance with specific water quality guidelines, the sample size should be sufficiently large enough to ensure a reliable verification of the quality parameter at or below the level required in the guidelines. Whitman *et al* evaluated a sample size required to achieve a certain level of precision of mean value of *E. coli* in beach water at a 95% confidence level for a month (Whitman & Nevers, 2004). Brauer *et al* also investigated an optimal interval of water sampling to achieve 20 percent of accuracy level of mean value of a quality parameter in watersheds (Brauer, O'Geen, & Dahlgren, 2008). These approaches may help determining an optimal sampling frequency at the acceptable level of precision or accuracy to achieve goals of water quality monitoring.

In statistics, precision refer to how close values measured in samples are to each other while accuracy means that how an estimated value from sample can predict the true value of a population. Sampling precision is associated with the variability of samples used for parameter estimation and commonly calculated by the coefficient of variation (CV) (Zar, 1999). CV is equivalent to standard deviation divided by mean

value in samples. Confidence Interval (CI) explains uncertainty associated with sample statistics of parameters in the population (Briggs, Wonderling, & Mooney, 1997).

Collectively, power analysis, traditional statistic tools cannot be solely applied to determine sample size (i.e. sampling frequency in water samples during a specific period) due to temporal and spatial variation of the parameter, non-parametric and dependency among data. Practical approaches and empirical data need to be combined with statistical tools in order to make informed water quality management decision.

1.7 Quantitative microbial risk assessment

Factors including initiation of human infections caused by waterborne pathogens basically depends a number of organism ingested, exposure route, the median infectious dose of pathogens, immune status of humans (A. J. Hamilton, Stagnitti, Premier, & Boland, 2006). Hence, in order to predict final risks of water-borne pathogen to consumers, comprehensive tools are required. Quantitative Microbial Risk Assessment (QMRA) has been increasingly applied to estimate final adverse health outcomes following the exposure to pathogens in drinking water and irrigation water (A. J. Hamilton, Stagnitti, Premier, Boland, & Hale, 2006; Medema & Smeets, 2009). This tool also has been used to monitor the efficacy of pathogen removal from drinking water and has provided a theoretical framework and establishment for threshold levels of pathogens or fecal indicators in water (Eisenberg, Bartram, & Hunter, 2001).

QMRA is the process which includes abstraction and utilization of relevant data and incorporation of all available information into tools of deterministic or stochastic forms. Deterministic approaches include probabilistic models in which each parameter has a single value (i.e. point estimate), while each parameter is represented by probability function in stochastic approaches with Monte Carlo simulation which can explain uncertainty and variability in the risk model (A. Hamilton & Stagnitti, 2006).

The process formally contains of four steps; Hazard Identification, Exposure Assessment, Dose-Response Assessment, Risk Characterization.

1.7.1 Drinking water

1.7.1.1 Hazard Identification

The aim of this problem formulation step is to identify the link between microbial pathogens in drinking water and related human illnesses and to investigate frequency and magnitude of water-borne outbreaks. Epidemiological investigations (Swerdlow, Woodruff, Brady, Griffin, Tippen, Donnell Jr, Geldreich, Payne, Meyer Jr, & Wells, 1992b) and clinical surveillances are general sources of data to populate the aspect of the model (Lammerding & Fazil, 2000; Swerdlow, Woodruff, Brady, Griffin, Tippen, Donnell Jr, Geldreich, Payne, Meyer Jr, & Wells, 1992b).

1.7.1.2 Exposure assessment

The aim of this step is to determine the number of pathogens ingested by typical consumers (Strachan, Dunn, & Ogden, 2002). The average volume of drinking water intake (V) is multiplied by mean concentration of pathogens (μ) in the water to calculate the total exposure under the assumption that microorganism would be

evenly distributed. Probability that water sample (V) that contain N organisms can be followed by Poisson probability distribution, assuming that pathogen is distributed randomly in water (Haas, Rose, & Gerba, 1999).

Schmidt *et al* indicated that measurement analytical error in enumerating pathogen or indicator concentration in the lab can lead to the biased exposure assessment in QMRA process and that enumeration methods need to include temporal and spatial variability of microorganism concentration (P. Schmidt & Emelko, 2011). Bayesian framework was developed and applied to improve reliability of the model when used qualitative (i.e. presence/absence) observations for *E. coli* O157 in water sources are used for the input (S. Petterson, Dumoutier, Loret, & Ashbolt, 2009).

1.7.1.3 Dose-response Assessment (Hazard Characterization)

The aim of dose-response assessment is to determine the association between the dose ingested and the probability of illness or infection in exposed populations. Infectious doses of water-borne pathogens substantially vary according to pathogens (Table1.3). However, infectious dose of each pathogen as a single input parameter cannot fully explain the association since the likelihood of adverse health effects also depends on variability among the exposed population including host ages and the immune status of host (Buchanan, Smith, & Long, 2000).

In quantitative dose response assessment, several Probability Density Functions (PDF) have been applied to fit available data in a statistically acceptable sense (Haas, Thayyar-Madabusi, Rose, & Gerba, 2000; Teunis, Nagelkerke, & Haas, 1999). Experimental data such as human feeding trials and outbreak data are incorporated into those function (Fig 1.1). PDF parameters have been determined based on

preliminary data or previous reports. The PDF selection could dominate the final risk estimated in QMRA (Smeets, Dullemont, Van Gelder, Van Dijk, & Medema, 2008) . PDF selected for dose-response assessment of each reference pathogen are present in Table 1.4. However, there is still a need for more empirical data to validate these dose response models due to uncertainty (i.e. a lack of knowledge about factors affecting exposure or risk) and variability (i.e. heterogeneity across the people, place and time) in those models (Strachan, Doyle, Kasuga, Rotariu, & Ogden, 2005). Particularly, different models tend to provide very different risk estimates at the exposure at the low dose of pathogen, which is often extrapolated from response of high-dose oral challenge data in tail parts in the PDF.

1.7.1.4 Risk Characterization

The aim of this process is to integrate all information such as exposure profile and a host/pathogen profile and synthesize the actual risks from previous sequential steps. The estimated risk is generally shown as a PDF or histogram of the probability of infection for a person exposed to pathogens. An acceptable risk, a level of 10^{-6} (i.e. probability in which one person get infected in 10^{6} people by the hazard) as a gold standard, have been accepted to evaluate this characterized risk for development of water quality criteria (Fewtrell & Bartram, 2001).

This characterization has contributed to developing drinking water quality criteria and risk management decisions (Neumann & Foran, 1997; Zmirou-Navier, Gofti-Laroche, & Hartemann, 2006).

1.7.2 Irrigation Water

The QMRA tool has been used to estimate the final risk of contaminated fresh produce consumption (Danyluk & Schaffner, 2011). Pathogens remaining on fresh produce, have been estimated based upon pathogen concentrations in irrigation water, volume of the water on produce and decay rate of the pathogen (A. J. Hamilton et al., 2006). Petterson *et al*. reported that the risk estimates were much more sensitive to the inactivation rate of the pathogens on fresh produce originated from irrigation water than the number of pathogens in the water (S. R. Petterson, Ashbolt, & Sharma, 2001; A. J. Hamilton et al., 2006). This tool also has been applied by World Health Organization (WHO) to develop guidelines for the microbiological quality of reclaimed irrigation water (Blumenthal, Mara, Peasey, Ruiz-Palacios, & Stott, 2000). The acceptable annual risk (i.e. 1:10,000 annual risk of infection from pathogens in drinking water provided by US EPA) was determined by regulators. QMRA models were used and back calculated based upon knowing decay rate to generate the maximum acceptable contamination rate in water (i.e. a minimum allowable number of the target pathogen in the water) that would not result in the produce being contamination about acceptable limit (Fig 1.4) (Bastos, Bevilacqua, Silva, & Silva, 2008)

1.8 Risk Management: Regulations and Guidelines for microbial water quality

Risk management is the decision-making process in dealing with the characterized risks (Fig 1.4) by establishing regulations or guidelines based on priority settings (Hrudey & Leiss, 2003). Poland *et al*. indicated that this process was challenging

because multi-stakeholders should be satisfied with this management in water utility sector(Pollard, Strutt, MacGillivray, Hamilton, & Hrudey, 2004).

Currently, Total Coliform Rule (TCR) as US federal regulation covers approximately 154, 000 public water systems including community and non-community water system in the US, which serve 307 million people (Bastos et al., 2008; US EPA, June 2010). This rule basically requires the absence of detectable total coliform in the water systems and has strict fines of violations (Table 1.5). US EPA estimated that the water systems pay approximately $210−230 million per a year to comply with TCR (Bennear, Jessoe, & Olmstead, 2009b).

However, for individual drinking water system such as private drinking well water supplies where fifteen percent of US populations obtain their drinking water in the households, local authorities in each state are responsible for monitoring private supplies under the no federal regulation (US EPA., 2011).

At present, there are no guidelines for microbial quality for fresh irrigation water, regulated by the federal authority in the US. Relevant produce organizations have published guidelines to minimize the risk of fresh produce-borne disease outbreaks, which substantially vary according to the agencies (table 1.6). For instance, The California Leafy Green marketing Agreement

(LGMA) established water quality criteria for irrigation water (California leafy green marketing agreement. 2010.) , which is similar to the guideline for recreational waters by the US EPA (U.S. Environmental Protection Agency Office of Water (4305T), 2004).

1.9 Summary and Questions remained

Water-washed, water -based, water-related and water-borne infections caused by pathogenic microorganisms pose substantial health burdens to populations in developing countries. Water scarcity in semi- arid, arid areas, environmental changes influencing water quality deterioration have also contributed to an increase in waterborne disease outbreaks worldwide. In the United States, one of the most developed countries, fresh water contamination with human and animal feces is still a significant risk to human health. Despite low mortality rate in waterborne diseases, the proportion of the incidence and prevalence of acute gastrointestinal illness (AGI) attributed to microbial water contamination have increased.

Fecal origins zoonotic bacterial pathogens are excreted from hosts into the environment, transmitted to fresh water sources by surface run off and ingested by the population through drinking water or fresh produce irrigated by the contaminated water sources. The development of improved detection methods has led to increased isolations of pathogenic microorganisms and consequently an increase in the identification and reporting of waterborne disease outbreaks. Diverse bacterial source tracking methods contribute to confirming agricultural or anthropogenic practices as main causes of microbial contamination in fresh water sources. These risk factors highlight the needs for additional source water protection and the establishment of prevention strategies to mitigate direct or indirect release of contaminants into surface or groundwater resources (Ivey, de Loė, Kreutzwiser, & Ferreyra, 2006). Applications of QMRA to estimate and

predict the risk from waterborne disease outbreaks also is helpful in design of the implementation and the mitigation plans. Despite source water quality degradations, several massive water-borne disease outbreaks caused by treatment or distribution deficiencies in public drinking water systems may have facilitated promulgation of the regulation for drinking water quality in the US such as Total Coliform Rule, which cover only public water systems and its use for drinking.

Given the proximity of animal agriculture, rural residents in agricultural intensive areas may be more likely to be exposed to risks of microbial contaminations in their water used for drinking and irrigation purposes. However, guidelines which is advisory, voluntary and non-enforceable have been recommended to protect rural residents. Because of the magnitude of agricultural production in the state of Ohio, the hazards associated with microbial contamination of water, the following chapters of this dissertation should be thoroughly assessed. The following chapters of this dissertation focus on the following areas:

1. In the second chapter, microbial quality of private well water in individual drinking water systems most commonly used in these areas was investigated to determine the magnitude and frequency of fecal contaminations and the pathogens in these water sources.

2. In the third chapter, the effectiveness of current guidelines for microbial quality of irrigation water which are substantially different according to agencies was assessed to determine how practically these guidelines reflect the field situation during pre-harvest to protect the zoonotic bacterial pathogens' transmission to fresh produce.

3. In the fourth chapter, the association of microbial quality between irrigation water and fresh produce during pre-harvest was identified to estimate the potential risk of human infection caused by the pathogen remaining on fresh produce irrigated with contaminated water source.

4. In the final chapter, we summarize and synthesize the new information presented in the proceeding chapters and make recommendations of risk management.

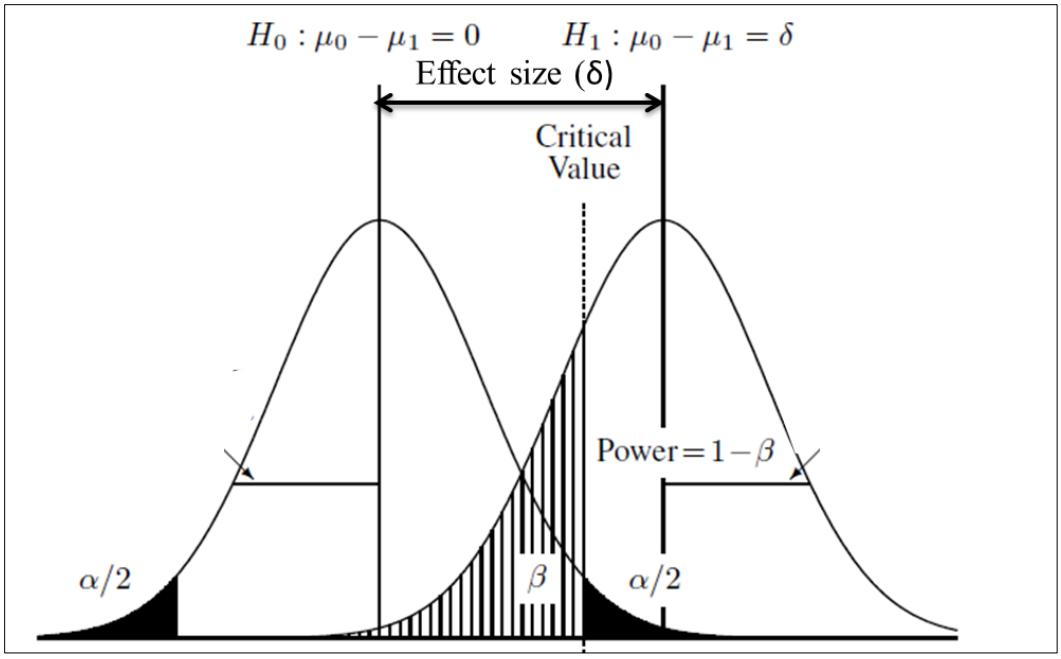

Figure 1. 1 Errors in hypothesis testing in two different normal populations (van Belle & Millard, 1998)

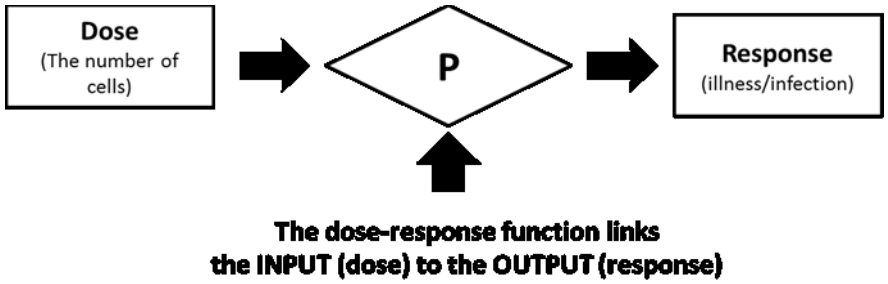

Figure 1.2 Illustration of the association between dose-response function and dose-response information (Amzal et al., 2009)

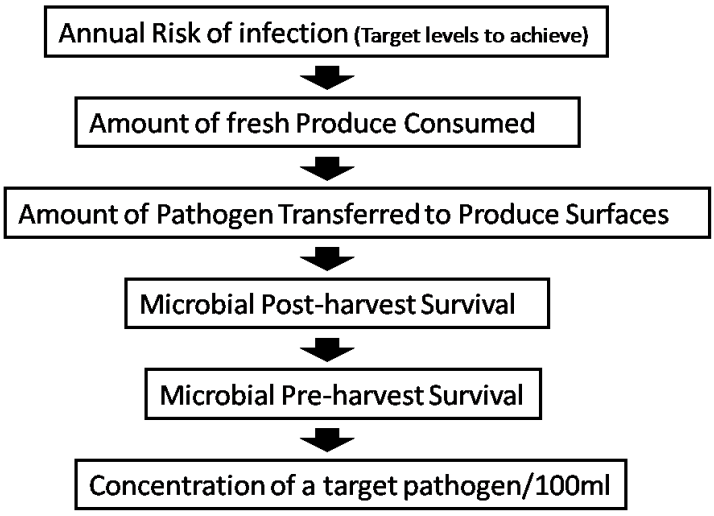

Figure 1.3 QMRA steps used for the calculation of threshold levels of pathogen to achieve acceptable annual risk of infection, modified from (Stine, Song, Choi, & Gerba, 2005)

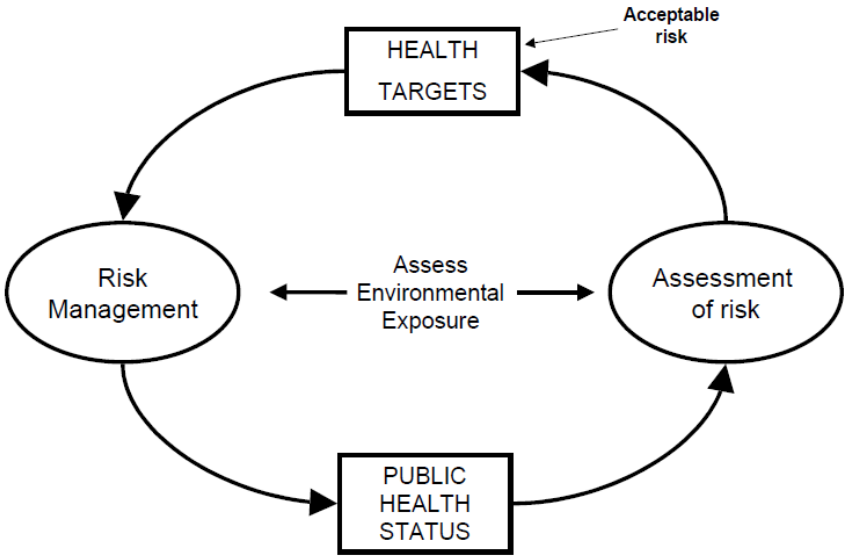

Figure 1.4 Risk analysis processes (Fewtrell, L., & Bartram, J.,2001)

Table 1.1 Relative risk of four categories of water-associated infectious diseases in different regions in the world, modified from (Ashbolt, 2004;Yang et al., 2012).

Group	Diseases	Regions in High Risk
Water-borne diseases	Infection caused by contaminated water ingestion (ex. *E. coli* O157, Cholera)	Europe, Central Africa North India
	Cryptosporidiosis	West Europe
Water-based diseases	Infection by worm which spend their part of life cycle in water (ex. Schistosomiasis)	Brazil, northwest Africa, central Africa, and southeast of China.
Water-related diseases	Diseases spread by vector living in water or closed to the water (ex. Malaria, Dengue fever)	Central Africa in particular Ethiopia and Kenya, and north India
Water-washed diseases	Disease caused by the lack of sufficient quantity of water and low level of hygiene (ex. Conjunctivitis, Trachma)	Brazil, Northwest Africa, Central Africa, Latin America

Table 1.2 Characteristics of current methods commonly used for microbial source tracking (modified from (Scott, Rose, Jenkins, Farrah, & Lukasik, 2002; Dombek, Johnson, Zimmerley, & Sadowsky, 2000; Farber, 1996)

	Target area for discrimination	**Approaches**	**Advantage/ Disadvantage**
Antibiotic Resistance Analysis (ARA)	Phenotypic/ Exposure to different antibiotic regime	Antibiotic resistance indexing	Rapid/ geographical specific
Pulsed-Field Gel Electrophoresis (PFGE)	Genotypic/High molecular weight DNA (10kb to 10Mb)	Restriction	Sensitive/ too sensitive to broadly
Random Amplified Polymorphic DNA(RAPD) PCR	Genotypic/ RAPD	Random Amplification	Fast, high discriminatory power/concerns of reproducibility
Repetitive- PCR	Repetitive extragenic palindromic (REP) sequences	Repetitive element Amplification	Rapid/ concerns of reproducibility
Ribotyping	Ribosomal ribonucleic acids (rRNA)	Restriction	Highly reproducible/Labor intensive, variation in the protocols

Table 1.3 Infectious doses of representative water-borne pathogens (Goss & Richards, 2008)

Waterborne pathogens	**No. of cells to cause illnesses**
Salmonella spp.	$1–10^9$ CFU
Campylobacter spp.	500 CFU
E. coli O157:H7	$<10^3–10^9$ CFU
Giardia	10 Cysts
Cryptosporidium spp.	10–30 Oocysts

Table 1.4 Parameter values in commonly applied probability functions for estimating the association between dose and response in QMRA (modified from (Soller, Bartrand, Ashbolt, Ravenscroft, & Wade, 2010))

Reference pathogen	**Published probability distribution selected (Dose-response)**	**parameter values**	**Percent of infections resulting in illness**
E. coli O157:H7	Beta-Poisson	0.4 45.9	28%
Salmonella enterica	Beta-Poisson	0.3126 2884	20%
Campylobacter jejuni	Hypergeometric	0.024 0.011	2.8%
Cryptosporidium spp.	Exponential	0.09	50%

Table 1.5 Definition of violations in Total Coliform Rule (TCR) modified from (Bennear, Jessoe, & Olmstead, 2009a)

Violation Terms		**Definition**
Acute violation		occurs when any repeat water sample tests positive for *E. coli* or fecal coliforms, total coliforms
Monthly (non-acute) violation	two positive (2P) rule	Occur when at least 2 samples test positive for total coliform in fewer than 40 samples
	five percent (5%) rule	incur if more than five percent of samples test positive for total coliform in at least 40 samples
Repeat samples		required to take three repeat samples
Oversampling	sampling out	oversampling that reduces the probability of a monthly violation
	overcompliance	oversampling that does not reduce the probability of a monthly violation

Table 1.6 Comparisons among guidelines of microbial irrigation water quality recommended by relevant agencies (The growing and harvesting of fresh produce narrative.)

	FSLC[a] standards	**LGMA[b] Guidelines**	**Florida Tomato rule**
Microbial standard	Well water: Generic E. coli : <1.1 MPN/100ml Surface water Generic E. coli :<126 MPN/100ml	≤ 126 MPN of generic *E. coli* for 5 samples ≤ 235 MPN of Generic *E. coli* for any single sample	Meets the US EPA recreational water microbial standard
Periodic water Sampling	Within 60 days of water sources' first seasonal use	Single sample per water source shall be tested prior to use if >60days since last use	Ground water tested at least annually Surface water at least quarterly

a Food Security Learning Center
b California Leafy Green Products Handler Marketing Agreement

Reference

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Chapter 2: Microbial quality of private well drinking water in northeastern Ohio

Abstract

In agricultural intensive areas, drinking contaminated water from private wells is considered an important cause of Acute Gastroenteric Illnesses (AGI), particularly among high-risk populations. In the summer of 2009, the microbial water quality of 180 randomly selected private wells in two northeastern Ohio counties, a region with a high concentration of dairy farms, was assessed. Forty-five percent (82/180) of water samples were contaminated with total coliforms. Generic *E. coli* were present in nine percent (16/180) of samples. Using real-time PCR, *E. coli* O157:H7 was identified in four percent (7/180) of specimens. For *Campylobacter* spp. DNA could not be amplified from 70 of the samples tested for this organism. The frequency of generic *E. coli* contamination varied among townships ($P<0.001$). Well structure (i.e. age and depth) or other common measures of pollution potential (depth of water, hydrology, topography, Net recharge soil media) was not correlated with coliforms and *E .coli* contamination. Importantly, the presence of the pathogen *E. coli* O157:H7 was not associated with the presence of fecal indicators in the water samples: Only one of the seven *E. coli* O157–positive samples was also positive for generic *E. coli*. Appropriate risk management and communication processes are needed to reduce the potential waterborne disease outbreaks.

2.1 Introduction

In agricultural intensive areas, water quality deterioration is a growing concern for public health. Surface waters are often used for recreational, irrigation and drinking purposes. Surface water sources such as ponds, streams and ditches are easily contaminated by surface runoffs originating from non-point sources, animal and human wastes (Ahmed, Sawant, Huygens, Goonetilleke, & Gardner, 2009; Johnson et al., 2003). In contrast, well water (groundwater) is generally perceived safe for drinking by consumers (Jones et al., 2005). However, during 2007 and 2008, contaminated ground water was responsible for 36 percent of all waterborne disease outbreaks in the United States (Craun et al., 2010). Two recent case-control studies in the US also identified the drinking of well water as a risk factor for children's enteric infections (Denno et al., 2009; Gorelick, McLellan, Wagner, & Klein, 2011).

Approximately 15 percent (15.8 million) of the population in the US obtains drinking water from domestic private wells (Hlavsa et al., 2011). Most private well owners reside in rural areas. These wells have the potential to be contaminated with fecal organisms for nearby agricultural and other anthropogenic activities, although the US Environmental Protection Agency recommends that fecal indicators should be completely absent in drinking water from private wells (US EPA., 2011). Krapac *et al*. (2002) reported that ground water near swine manure storage pits was adversely affected (Krapac et al., 2002). Septic tank density (Borchardt, Chyou, DeVries, & Belongia, 2003) and human and animal feces in the vicinity of wells (Licence, Oates, Synge, & Reid, 2001) also affects

microbial quality of private well water. Arnade et al (1999) indicated significant impact of precipitation on microbial quality of well water located near septic tanks (Arnade, 1999). Water also may be contaminated during conveyance in distribution systems (Trevett, Carter, & Tyrrel, 2005; Payment et al., 1997).

Individuals living on a farm, or living in regions with high density of livestock are at higher risk for infections for *Campylobacter* and *E. coli* O157 (Stanley & Jones, 2003; Oliver, Jayarao, & Almeida, 2005). But the routes of exposures to these pathogens among rural residents are not clearly defined. We hypothesized that wells in close proximity to bovine operations might be a source of these pathogens. Thus, the purpose of this study was to determine the frequency and magnitude of private well water contamination with of microbial indicators (Coliforms, generic *E. coli*) and two specific pathogens, *E. coli* O157:H7 and *Campylobacter jejuni* in an agriculture intensive region of Ohio. In addition, the influence of factors anticipated to affect microbial contamination in this agriculturally intensive region were assessed.

2.2 Materials and methods

Sample collection Sampling was conducted in six townships of Wayne and Holmes Counties, Ohio during summer (May to August 2009). This region is characterized by the presence of a large number of dairy farms. (Jill Clark, Holli Kendall, William Flinn, 2008). Three townships were selected from each of these two counties to represent areas of variable dairy farm densities and differing ground water pollution potential as

indicated by the Ohio Department of Natural Resources Division (Ground Water Mapping & Technical Services, ODNR Division of Soil and Water Resources, 2010). This map was developed based on the major hydrological factors (i.e. depth of water, net recharge, aquifer Media, soil media, topography, impact of the vadose zone media Hydraulic conductivity of the aquifer). Thirty households in each township were randomly selected based on addresses present in the Ohio Department of Natural Resources, well log (http://ohiodnr.com/water/maptechs/wellogs/appnew/default.aspx). In total, one hundred and eighty samples were collected. Each sampling location (i.e. households) and the location of dairy farm were plotted on a regional map using Arc GIS program (version 9.2, Esri, CA) (Figure 2.1). Two liters of water was obtained from indoor residential cold water faucets or outside faucets connected to the private well. Samples were kept chilled and analyzed within 12 hours after sampling.

Total coliform and generic *E. coli* were enumerated in 100 ml aliquots of the water sample using a commercial MPN tests following the manufacturer's instructions (Quanti-Tray2000Idexx laboratories, Westbrook, ME). In addition, 2, one-liter aliquots of each water samples were filtered through 0.22 μm pore membrane filters (Millipore Corporation, Billerica, MA, 01821) using a vacuum pump. One filter was transferred into 10 ml of phosphate buffered saline (PBS) and shaken for three minutes on an orbital shaker (250 rpm). Subsequently, the rinsate (i.e. 10 ml of PBS after shaking) was incubated at 37°C overnight (i.e. enriched samples with PBS). This overnight broth culture was frozen at -70°C, with 30% buffered glycerol. The other filter was frozen at -70°C and the DNA from attached organisms was extracted at a later time using a

commercial kit according to the manufacturer's instructions (Rapid Water TM DNA Isolation Kit, MOBIO Laboratories, Inc). These later specimens were called the non-enriched samples.

Statistical analysis Binary logistic regression models (Stata ver.10, Stata Corporation, College Station, TX) were used to assess the association between well attributes collected from Ohio well log such as well age, depth, and spatial factor (i.e. township as a surrogate for pollution index/cattle density) and the presence or absence of fecal indicators (Hosmer & Lemeshow, 2000).

Detection of pathogens by real-time PCR Commercial real-time PCR kits were used to amplify and detect targets specifics for *E. coli* O157 and *Campylobacter jejuni* (MicroSEQ® *E.coli* O157:H7 detection kit, TaqMan®*Campylobacter jejuni* detection kit, Applied Biosystems, Foster City, USA) following the manufacturer's directions. Targeted sequences and primer sets are proprietary. From enriched samples, template DNA was extracted from the frozen stocks stored at −70°C using a simple boiling method: One milliliter of enriched samples was centrifuged at 13,000 rpm during 3 min and cell pellets were resuspended in 300 µl sterile water, boiled for 10 min, and then cooled on ice for 20min. Cellular debris was pelleted by centrifugation (13,000 rpm, 3min) and the supernatant was used as PCR template. Fluorogenic probes included reporter dye (VIC, proprietary), quencher dye (FAM, 6-carboxyfluorescein) and internal positive control (NED) that targeted a synthetic piece of DNA in the sample for *E .coli* O157 detection. The fluorescence intensities of amplification reactions was measured and analyzed using an automated 7500 Real time RT-PCR system (Applied bio system,

Foster City, CA). Standard curves were generated with 10-fold serial dilutions ranged between 10^0 and 10^{-8}cfu per ml (spiked cfu/ml) from *E. coli* O157:H7 strain (American Type Culture Collection strain ATCC 43888). In order to quantify the initial concentration of *E. coli* O157 in the samples, the PCR analysis was re-performed with non-enriched samples that tested positive from enrichments under same conditions. The extracted DNA non-enriched filters from the first 76 collected water samples were also tested for *Campylobacter jejuni* following the manufacture-recommended amplification conditions.

2.3 Results

2.3.1 Fecal Indicator detection

Total coliforms were found in total (82/180, 45.5 %) of sampled well water and nine percent (16/180) of the samples were positive for generic *E. coli*. There were differences in the frequencies of fecal indicators present among townships (Table 2.1). For example, generic *E. coli* was found in seven samples (7/30, 23%) of water samples collected in Sugar Creek township while in Franklin Township, both fecal indicators were detected at the lowest frequency (27% of coliforms; 3.3% of *E. coli*). Total coliforms and generic *E .coli* were moderately correlated (r= 0.463, $P<0.001$).

2.3.2 *E. coli* O157 detection

From our standard curve, as few as 10^{-6} genomic targets were detectable after 38 amplification cycles in this PCR assay (For VIC, y=-3.94312x + 15.742, R^2=0.9527; For FAM, y=-4.38946x + 14.71, R^2=0.9807).

E. coli O157:H7 was detected in seven enrichment samples (Table 2.2). All positive samples showed fluorescent signal from FAM in 24 to 37 cycles, VIC in 17 to 38 cycles of VIC signal and IPC (NED) in 28 to 38 cycles at the 0.2 of cutoff Ct value. Out of seven positive samples, there were two samples that tested positive for *E. coli* O157:H7 from the direct (non-enriched) specimens. These specimens showed fluorescent signals from FAM in 34 and 37 cycles, VIC in 39 and 37 cycles and IPC in around both 34 cycles. The quantity of DNA of *E. coli* O157:H7 calculated by using the standard curve, PCR templates contained DNA of bacterial counts less than 10^{-5}-10^{-6}cfu/ml in the two non-enriched positive samples. If the organism was evenly distributed in the water source, there would be one cfu of *E. coli* O157 in 2 liters of the well water. The amount of amplicon in the five other samples was below detection threshold without enrichment. For *Campylobacter jejuni*, although all positive control samples provided expected results, no fluorescent signals were detected from any of the direct (non-enriched) specimens tested.

2.3.3 Logistic regression model

Presence of total coliforms in water samples was not associated with increase of well depth and age, well location ($P > 0.2$). However, well location significantly affected the presence of *E. coli (*coefficient =0.416, P= 0.038) in the water sample while well age and

depth did not have any relation with the presence of *E. coli*. The logit of presence of *E .coli* increases by 0.009 for a one unit (i.e. meter) increase in well depths (coefficient=0.0091, *P*=0.007).

2.4 Discussion and Conclusions

Rural wells located in the region were frequently contaminated with bacteria indicative of fecal contamination. Water sampled in Saltcreek Township showed the highest frequency of generic *E. coli*. *Escherichia coli* O157:H7 was also detected in seven water samples (3.9%, 7/180). Among the *E. coli* O157-positive samples, one sample was positive for both coliforms and generic *E. coli*; and two samples were positive for coliforms alone, one at high levels. The four remaining samples that tested positive for *E. coli* O157 were negative for both total coliforms and generic *E. coli.* Schets et al (2005) described similar results that *E. coli* O157:H7 was isolated in 2.7 percent of well water samples which had no fecal indicators (i.e. intestinal enterococci, total coliforms and generic *E. coli*) in the Netherlands (Schets et al., 2005).

The detection of *E. coli* O157 in drinking water is of serious concern. One explanation for this observation is the possibility of false positive results on the PCR. However, given the reported sensitivity and specificity of the detection kit used (Sen, L. Sinclair, Boczek, & Rice, 2011), and the consistent negative results on our negative control assays, we considered this unlikely. Sen et al indicated that the *E. coli* O157:H7 detection kit can correctly identify total 37 *E. coli* O157 strains and does not react with a large number of other pathogenic E. coli and three common waterborne bacteria under a cutoff *Ct* value of

35 for FAM and VIC (Sen et al., 2011) . In same study, regarding the sensitivity of the kit, they detected low concentration of *E. coli* O157:H7 in the presence of high concentration of other *E. coli* species (i.e. 10^8 cells).

In the context of waterborne pathogen detection, viable but non culturable (VBNC) complicate the estimation targeted pathogens in water by PCR (Roszak & Colwell, 1987). Since PCR analysis is performed based on the detection of nucleic acids, not a viable cell, nucleic acids in dead cell can result in positive signals in PCR analysis (Fode-Vaughan, Maki, Benson, & Collins, 2003; Josephson, Gerba, & Pepper, 1993). In the consideration of the rate of nucleic acid degradation (i.e. three weeks) (Josephson et al., 1993), residents drinking well water positive for *E. coli* O157, may have been exposed to risks of *E. coli* O157 infection during the period prior to the sampling point. PCR positive samples would still be important as an indication of recent past contaminations with this organism

Total coliform group consist of closely related bacteria which can be inherently found in soil, water and feces (World Health Organization, 2011). All bacteria included in this group are not considered fecal origin. The presence of total coliform in municipal water systems has implicated the deficiency of distribution system or treatment due to the potential of environmental contamination as described in Total Coliform Rule (World Health Organization, 2011)(TCR) regulated by the US Environmental Protection Agency (US EPA)(Bennear, Jessoe, & Olmstead, 2009). Generic *E .coli*, a subset of a total coliform group, is more likely to indicate fecal contamination in water. However, the

capacity of generic *E. coli* to predict the occurrence of pathogens with a fecal origin has not been determined.

Lack of association between the presence of generic *E. coli* and *E. coli* O157:H7 found in the water samples may be attributed to several factors. First, *E. coli* O157:H7 is distinguished from generic *E. coli* by the enzymatic activity of β-glucuronidase (GUD) (Hayes et al., 1995). Generic *E. coli* has the capacity to hydrolyze 4-methylumbelliferyl β-D-glucuronide (MUG) in the media to release 4-methylumbelliferone (MU) but *E .coli* O157 is consistently MUG negative. Thus, the indicator commonly used in *E. coli* detection tests, including the tests employed herein, would not be cleaved to a fluorescent by-product and the test would yield a negative results in the presence of *E. coli* O157. Notwithstanding, contamination of water with E. coli O157, in the absence of other generic *E. coli* is unlikely. *E. coli* O157 does not typically persist longer in water than generic counterparts. A second, and more likely explanation, for the apparent discrepancy in PCR and MPN results for *E. coli* O157 and generic *E. coli* respectively, is the fact that the numbers of *E. coli* O157 estimated in the water were very low: They were detectable only when 2 liters of water was tested whereas generic *E. coli* counts were determined in 100ml aliquots. Had larger volumes of water been tested for generic *E. coli,* low levels of contamination may have been detected.

Disease Active Surveillance Network reported that around 13 per 10,000 infants (i.e. younger than 1 year age) are annually diagnosed with campylobacteriosis in the US (Fullerton et al., 2007). Despite the low survival rate of Campylobacter spp. in environments due to their microaerophilic characteristics (Stintzi, 2003), a low infectious

dose and conversion to a VBNC stage in adverse environmental condition may affect the high prevalence of human campylobacteriosis via water and food. However, in spite that we used TaqMan®*Campylobacter jejuni* Detection Kit (Life Technologies) with high sensitivity for *Campylobacter jejuni* detection (Toplak, Kovač, Piskernik, Možina, & Jeršek, 2012), no positive sample was found.

In the US, the outbreaks of *E .coli* O157:H7 via drinking water sources occurred causing more than one hundred illnesses in each outbreak (Olsen et al., 2002){{217 Bopp, D.J. 2003}}. Even though those outbreaks were likely to be associated with untreated well or surface water supplied from municipal water system, they may implicate a role of untreated private drinking water as a potential route of this pathogen. In addition, reported cases of waterborne disease outbreaks via drinking well water showed that infections occurred in visitors rather than permanent residents when they were exposed same contaminated water sources (Licence et al., 2001; Olsen et al., 2002). Belongia *et al*. found that 59 percent of farm resident children have *C. jejuni* antibodies and 14 percent of *E. coli* O157 LPS antibodies (Belongia et al., 2003). Multidrug resistance *E. coli* was also observed in private well drinking water in Canada (Mataseje et al., 2009). These reports may indicate that residents have acquired a certain level of immunity against the pathogens in the water sources and precautionary measures are strongly required to protect immune-comprised population.

The residents using private well water located in the area were highly and unknowingly exposed to the risk of *E .coli* O157:H7 infection, fecal and environmental contamination. Efforts should be made to prevent contamination of rural wells, as microbiological testing

alone is not a reliable assurance of safety. These studies indicate that AGI disease observed in agricultural intensive regions may be attributed to human exposure to pathogens via drinking water. The factors to identify associations between well characteristics and expected pollution potential index based on hydrologic parameters provides evidence that microbiological contamination of wells is not inevitable in high-risk, agriculturally intensive regions. Instead, contamination potential may be associated with other factors such as well maintenance.

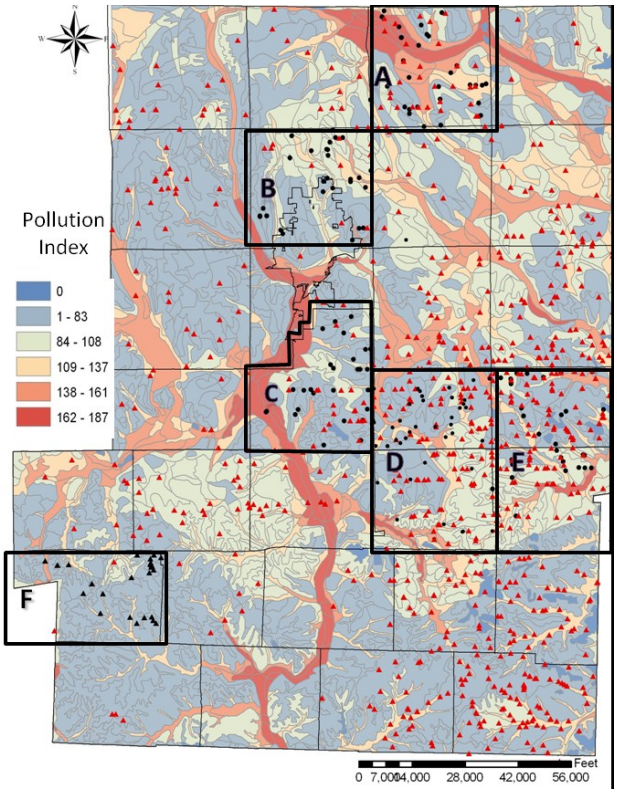

Figure 2.1 locations of sampling (i.e. households, black circle) and dairy farms (red triangle) projected on the ground water pollution indexed map

Table 2.1 Frequency of fecal indicators and *E. coli* O157:H7 detected in well drinking water sample

Township	**Total coliforms (%)**	**Generic *E. coli* (%)**	***E. coli* O157:H7 (%)**	**Dairy Farm desnity**	**Pollution potential**
Milton (A)	43.3 (13/30)	3.3 (1/30)	3.3 (1/30)	High	High
Wayne (B)	40 (12/30)	3.3 (1/30)	3.3 (1/30)	Medium	Medium
Franklin (C)	26.7 (8/30)	3.3 (1/30)	0	high	Medium
Saltcreek (D)	53.3 (16/30)	23 (7/30)	6.7 (2/30)	Medium	High
Paint (E)	46.7 (13/30)	6.7 (2/30)	9.9 (3/30)	Medium	High
Knox (F)	60 (18/30)	13.2 (4/30)	0	Low	Low
Total	45.6 (82/180)	8.9 (16/180)	3.9 (7/180)		

Table 2.2 The number of fecal indicators in water samples that was positive for *E. coli* O157:H7 detected using the PCR analysis

Sample No.	**Sampling location (Township)**	**Sampling date**	***E. coli* O157:H7**	**Generic *E.coli* (MPN/100ml)**	**Total coliforms (MPN/100ml)**
8	Saltcreek	6/4/2009	Presence	0	2
15	Saltcreek	6/5/2009	Presence	1	5
17	Paint	6/8/2009	Presence	0	0
29	Milton	6/12/2009	Presence	0	0
55	Paint	6/22/2009	Presence	0	2419.2
57	Paint	6/22/2009	Presence*	0	0
128	Wayne	7/16/2009	Presence*	0	0

*This water sample showed positive fluorescent signals for *E. coli* O157:H7 from both filter and frozen isolation in bank in the PCR analysis.

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Chapter 3: Spatial-temporal variations of microbial quality in surface irrigation water, Ohio, USA

Abstract

Guidelines for microbial irrigation water quality have been proposed by regulatory agencies and organizations to reduce potential risks of waterborne contamination of produce. Most recommendations emphasize threshold values of fecal indicators and have often relied on only a limited number of water tests over the course of irrigation season. In this study, two irrigation ditches and four surface reservoirs located in Ohio, USA were repeatedly sampled (n=227) to investigate fluctuation of fecal indicators concentration over an irrigation season (2010). Bootstrap analysis was applied to determine the sensitivity of the measured parameters as a function of sampling frequency. E. coli counts in water collected from irrigation ditches were approximately one order of magnitude higher (2.48 ± 0.79 log MPN per 100ml than that in reservoirs (1.54± 0.04 log MPN per 100ml) and increased following heavy rainfall events (>20mm) ($P < 0.01$). Sampling frequency was estimated in terms of accuracy and precision. The 95% bootstrap confidence interval width surrounding coliform and *E. coli* estimates based on a single sample were broad, including or exceeding the upper limit for acceptable use standards recommended by several organizations (126 CFU/100ml). In conclusion, a

single water sample imprecisely reflected the quality of water over the course of the irrigation period. Caution should be used when drawing conclusions about the microbial acceptability of water used for irrigation purposes based on a limited number of water quality measurements. Environmental factors influencing the spatiotemporal variation in the water quality (i.e. the type of water source and recent heavy precipitation events) and the expected interval between testing and harvest should be considered in developing irrigation water testing frequency guidelines.

3.1 Introduction

The availability of adequate water of suitable quality for irrigation is one of the most limiting factors for increasing food production worldwide (Postel, 1989). In addition to ground water, surface water, which is more susceptible to contamination, is often used for irrigation. Moreover, in the face of a shortage of fresh water in some regions, reclaimed wastewater is used for agricultural purposes in an effort to conserve hydrological resources (Mara, Sleigh, Blumenthal, & Carr, 2007). The increased reports of foodborne illnesses associated with fresh produce in recent years have spurred interest in the microbial quality of water used for agricultural purposes (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). In an effort to protect human health, numerous regulatory agencies and organizations have developed guidelines for the quality of water used for irrigating edible crops, these recommendations have been developed based on a variety of approaches and differ substantially among agencies. Many of these recommendations focus on chemical contaminants in water and address only superficially microbiological

criteria such as threshold values of fecal indicators in water, despite a potential health risk caused by microbial contaminated fresh produce. A few of the guidelines indicate the need of periodic water testing. For instance, The California Leafy Greens Marketing Agreement (LGMA) and Food Security Learning Center (FSLC) recommend that one sample per irrigation water source should be tested within 60 days before first use(An Initiative of The Pew Charitable Trusts at Georgetown University,). Florida tomatoes rules require an annual test for a ground water and testing quarterly for surface water sources (An Initiative of The Pew Charitable Trusts at Georgetown University,). However, the scientific justification for these threshold values and the frequency of microbial water quality testing in these regulations is rarely provided.

Since spatial and temporal variability are typically observed in microbial parameters of surface water (Nnane, Ebdon, & Taylor, 2011), establishing a sampling frequency that is sufficient to detect important changes in water quality is critical. A parametric estimation has been commonly applied to determine an effective sample size for a water quality monitoring (equation 1) (Antelo, Arce, & Carballeira, 1998; Canter, 1985; Loftis & Ward, 1980). The sample size required to determine within a 95% of confidence interval of a sample mean can be estimated with the following formula.

$$n = \left[\left(t^2_{(\alpha/2,r-1)}\right)\left(\frac{s^2}{d^2}\right)\right] \quad (1)$$

Where n is the number of samples, s^2 is the estimated variance of samples, d is a half-width of desired confidence interval, and t is the Students't-test value. For this formula to be accurate, the underlying assumptions are as follows: 1) the data are normally distributed and 2) the data are not correlated (Zar, 1999). When the assumptions are

violated, alternative approaches for sample size estimation are required. For example, in microbial irrigation water quality monitoring, environmental factors causing the variance of concentration of microbial indicators should be considered in establishing on the optimal sampling frequency. Non-point source (NPS) pollution attributed to runoff coming from manure applied fields and dairy farms located around irrigation water source may play also an important role to deteriorating microbial irrigation water quality in agricultural-intensive areas (Gould, 1989; Jamieson, Gordon, Sharples, Stratton, & Madani, 2002). Moreover, Kistemann et al (2002) reported that rainfall causing extreme runoff event resulted in an increase of total microbial loads in drinking reservoirs (Kistemann et al., 2002). Curriero *et al* (2001) identified that extreme precipitation events were linked to waterborne disease outbreaks caused by surface water contamination (Curriero, Patz, Rose, & Lele, 2001).

Despite the knowledge that irrigation water can be contaminated from multiple sources in agricultural–intensive areas, scientific justification for sampling frequency is still lacking. Sampling too infrequently provides inadequate information to assess risk under highly variable conditions. High testing frequency may lead an economic burden of irrigators, with limited practical increase in the knowledge of the risk profile. The purpose of this study was to explore approaches to rationalize the adequacy of irrigation water test frequency using statistical methods and field trials. In this study, we investigated the trend of microbial water quality in agriculturally intensive regions of Ohio, USA. Based on bootstrap analysis with this empirical distribution, we calculated the error associated single sample based estimates of microbial irrigation water quality.

3.2. Material and Methods

3.2.1 Study Sites

Microbial irrigation water quality parameters were monitored in six irrigation water sources located Ohio, USA over an irrigation season (April to November, 2010). Sample site D1, was an irrigation ditch that received water from a surface reservoir 1.8 km away. Sample site D2 was within the same irrigation system 1.3 km further downstream. Reservoir 1(R1), Reservoir 2(R2) and Reservoir 3(R3), Reservoir 4 (R4) were the main irrigation sources for four vegetable farms respectively. Sample site R1 was a surface reservoir approximately 10 km away from irrigation ditch sampling sites. It was supplied with water from a stream in a completely different watershed than sample sites D1 and D2. Sample sites R2-4 were also surface reservoirs supplied by headwater streams that eventually drained into a common river.

3.2.2 Sample collection and Microbiological Analysis

Sample were collected twice a week in April and May in pre-sterilized plastic bags placed 50 cm below water surface using grab sampling methods during the irrigation season (June to Oct) and samples were collected weekly. Samples were stored on ice immediately after collection and, within 12 hours of sample collection, total coliforms (TC) and generic *E. coli* counts in 100ml aliquots of the water samples were determined by an MPN method using the Quanti-tray 2000 test (Idexx laboratories, Westbrook, ME) following manufacturer's instructions.

Rainfall Data Precipitation data near each water collection site were downloaded from the Ohio Agricultural Research Development Center (OARDC) weather station (http://www.oardc.ohio-state.edu/newweather/). Based on hourly rainfall data, the precipitation amount (mm) during 24 hours preceding each sampling collection was calculated.

3.3 Statistical Analysis

3.3.1 Water quality analysis

The counts (n) of total coliforms (TC) and generic *E. coli* from six water collection sites were logarithmic-transformed ($\log_{10}$ (n+1)). The Anderson-Darling test was applied to check the normality. The Wald-Wolfowitz Runs test was applied to test independence in this non-normally distributed dataset (Wald & Wolfowitz, 1940). Monitored microbiological parameters for all stations were represented using time series plot. The difference of the median concentration of microbial parameters between reservoirs and ditches was compared using Kruskal-Wallis test. All data analysis performed using Minitab software (ver15, Mintab Inc., State College, PA, USA) and STATA (ver10, StataCorp LP, College Station, TX, USA) at the significant level of less than 0.05.

3.3.2Association with precipitation

Kruskal-Wallis test was applied to evaluate the effect among categorized precipitation data and microbial water quality. Subsequently Dunn's multiple comparison test was used to compare the difference among the microbial water quality according to each categorized precipitation data (Minitab Macro).

3.3.3 Bootstrap Analysis

Central Limit Theorem states that the distribution of mean from non-normal population will tend to be distributed normally as sample size (n) increase (Zar, 1999). Thus, a bootstrap resampling method(Efron, 1979) was applied to estimate the water quality and associated error based on various hypothetical sampling frequencies. Sample sets, ranging from 1 to 150, were drawn from original data (resampling) with 1000 reiterations. The mean distribution of each bootstrapped subsample was determined including the 95% confidence interval for each tested resample size. Coefficient of variance (CoV) estimate in each different resample size was also computed in order to assess how well the bootstrap distribution of subsample of different sizes reflected the empirical distribution and how many samples will be required to achieve a given level of precision (1-(CV*100))(Efron & Tibshirani, 1986). All bootstrap analysis was performed with the statistical software R (R version 2.13.2,http://www.R-project.org).

3.3 Results

3.3.1 Assumption test

None of data collected from sites was normally distributed ($P<0.05$). Hence, all water quality data was analyzed using nonparametric tests. Since the counts from each site on

subsequent sample dates were mutually independent (P>0.05), autocorrelation effects were not considered in further statistical analysis.

3.3.2 Spatial variability of irrigation water quality

Total coliforms were detected in all water samples from every reservoir during April to November 2010. Coliform counts ranged between 10^1 and 10^5per 100ml (Fig.1). For irrigation ditches, total coliforms were also present in all water samples: range 10^3 to 10^6 per 100ml (Fig.1). The median coliform count in water collected from reservoirs and irrigation ditches was 3.6 and 4.5 $\log_{10}$MPN/100ml respectively. Most water samples (96.9%, 219/226) from reservoirs and irrigation ditches also tested positive for *E. coli*. For *E. coli*, the median concentration in water collected from the reservoir sources was 1.5 $\log_{10}$MPN/100ml. Median *E. coli* concentration in irrigation ditch water was 2.5 $\log_{10}$MPN/100ml. Collectively, TC and *E. coli* from ditch water were higher than those from water collected from reservoirs (P<0.0001) (Fig. 3.2). The individual measurements were compared to guidelines for microbial irrigation water quality classified by different organizations (Table1). Overall, median concentration of *E. coli* in water from ditches more frequently surpassed microbial quality standards established by LGMA than water from the reservoirs (22.8% v 45.6%, P<0.005).

3.3.3 The association between precipitation and microbial water quality

Although Spearman correlation analysis identified significant association between the coliform (Spearman's rho (ρ) = .188, P=0.045) and *E. coli* (ρ=.218, P=0.0009 respectively) counts in water and the amount of rainfall in the previous 24hrs,

visualization of scatter plots of data, and Kolmogorov-Smirnov analysis clearly indicated heteroscadacity (i.e. non-normally distribution) of residuals and a non-linear relationship between two variables in both models. Thus, precipitation data were divided into 4 categories respectively 1) no rainfall (0mm); 2) light precipitation (up to 5mm); 3) moderate precipitation (between 5 and 20 mm); 4) heavy rain fall (>20mm). In reservoirs, neither coliforms nor *E. coli* counts were associated with the amount of precipitation in the previous 24hr (P>0.5). However, in the ditch samples, both total coliforms and *E. coli* counts in water were higher on sampling dates following greater than of rain (P=0.022) (Fig 3.3).

3.3.4 Bootstrap results

An anticipated decrease in confidence limits was observed with increasing resamples size (Fig 3. 4 A, B). The confidence interval width surrounding the estimated parameters decreased only marginally with increasing resample size above the indicated resample size (Fig 3. 4 A, B). The mean TC and *E. coli* counts in ditches had narrower confidence intervals than those of reservoirs, despite having higher concentration of organisms. In this study, the mean and 95% CI of counts from ditches invariably exceed current guidelines, regardless of number of times of virtual resampling. In contrast, in the reservoir water dataset, increasing resampling frequency above ten resulted in parameter estimates that did not exceed the standard. Sample sizes predicted to achieve various levels of precision can be estimated from Figure 3.5.

3.4 Discussion

In this study, the type of water sources and the amount of precipitation preceding sample collection contributed to the level contamination and the variance of microbial quality of irrigation water sampled over the course of an irrigation season. Bootstrap analysis applied to this study provided evaluation of the adequacy of sampling frequency for detecting the parameter variation based on an empirical distribution with the least bias explained by an acceptable level of precision (i.e. more than 85%) of bootstrapped distribution(Whitman & Nevers, 2004; Whitman & Nevers, 2004)(Fethke, Anton, Cavanaugh, Gerr, & Cook, 2007). For the statistical analysis, both parametric and non-parametric data measured microbial water quality can be applied to this tool without any limitations, based on the central limit theorem. Other non-parametric tests (i.e. Mann-Kendall test) commonly used for detecting the trend of water quality in previous studies (Naddeo, Zarra, & Belgiorno, 2007) was not applied in this study since the data did not demonstrate evidence of autocorrelation. From a statistical standpoint, at least 5 ditch samples and 12 reservoir samples would be required to estimate *E. coli* concentrations to reach the precision level of 85% with 95% confidence interval in ditch and reservoir respectively under same environmental conditions during the period. Moustafa et al (2001) indicated that they observed no significant difference in concentration of nutrition loads and retention into water between high (weekly) and low (twice a month) tests' frequency(Moustafa & Havens, 2001). With regard to the potential for precipitation to influence the water quality, we found that more than 20mm of rainfall was significantly associated with water quality in the irrigation ditch. Both TC and *E. coli* counts in the

ditches was significantly elevated when rainfall amount was heavy, over 20mm. Previous studies monitoring surface water quality also indicated rainfall events deteriorated microbial water quality (Levy, Hubbard, Nelson, & Eisenberg, 2009; Brownell et al., 2007). The ditches may be more susceptible to changes in microbial quality following rainfall for several reasons. They may have been more likely to receive surface runoff, and, being shallow, water turbulence associated with increased flow could have suspended sediments. Haley *et al*. showed that there was a positive association between daily precipitation and *Salmonella* spp. concentration in a watershed. Post–rainfall sampling may reflect potential risk from microbial contamination of water sources higher than sampling during dry season (Haley, Cole, & Lipp, 2009).

There are several limitations to the described study and caution should be used when extrapolating the data. First, although the water quality parameters reported herein were specific of the geographic region and sampling timeframe outlined in the study; however, the principles and patterns of fluctuations are likely applicable to other surface water sources used for irrigation purpose,. Baseline of microbial water quality may depend on the existence of contaminants nearby including non-point and point source transmitted by surface runoff into the water sources. Given a type of water source (i.e. irrigation ditch or surface reservoir) and annual frequency of a heavy rainfall event in other agriculturally intensive areas, rough inference for fluctuation patterns of microbial quality in the water source could be possible.

A second limitation is the challenges in relating fecal indicator concentration with the presence of pathogenic agents. Indicator organisms should occur be deposited

concurrently and survive as long as pathogens in order to be of utility (Winfield & Groisman, 2003). Empirically this criterion is not always met and therefore the capacity of many fecal indicators to predict the presence of zoonotic pathogens is controversial (Lemarchand & Lebaron, 2003); (Wu, Long, Das, & Dorner, 2011)).

Finally, given the uncertainty of the significance of coliform and *E. coli* presented in irrigation water on the microbial safety of fruits and vegetables (Won and LeJeune, 2011), the benefit of intensive interval of testing may not be warranted. Therefore, there may be limited value for collecting sampling of irrigation waters without factoring in external events such as heavy rainfall have impacted the quality and the water is going to be used for irrigation shortly after the exposure to contamination.

3.5 Conclusion

We demonstrated empirically that that current irrigation water testing recommendation of taking a single sample once during or before an irrigation season failed to accurately predict water quality. This guideline lacks scientifically justification and does not account for important environmental permutations. Guidelines and recommendations for irrigation water quality thresholds and sampling frequency should consider the variety of different environmental factors and management approaches used: the sources of irrigation water and their potential for contamination with pathogenic or chemical agents, the amount of precipitation prior to sample collection, time to harvest, and the types of plants that are watered. Given the complexity of factors influencing water quality, testing strategies should be developed specifically for each particular situation. Relying on an

omnibus recommendation for testing frequency for all conditions may result in mischaracterization of water quality and misclassification of risks.

3.6 Acknowledgement

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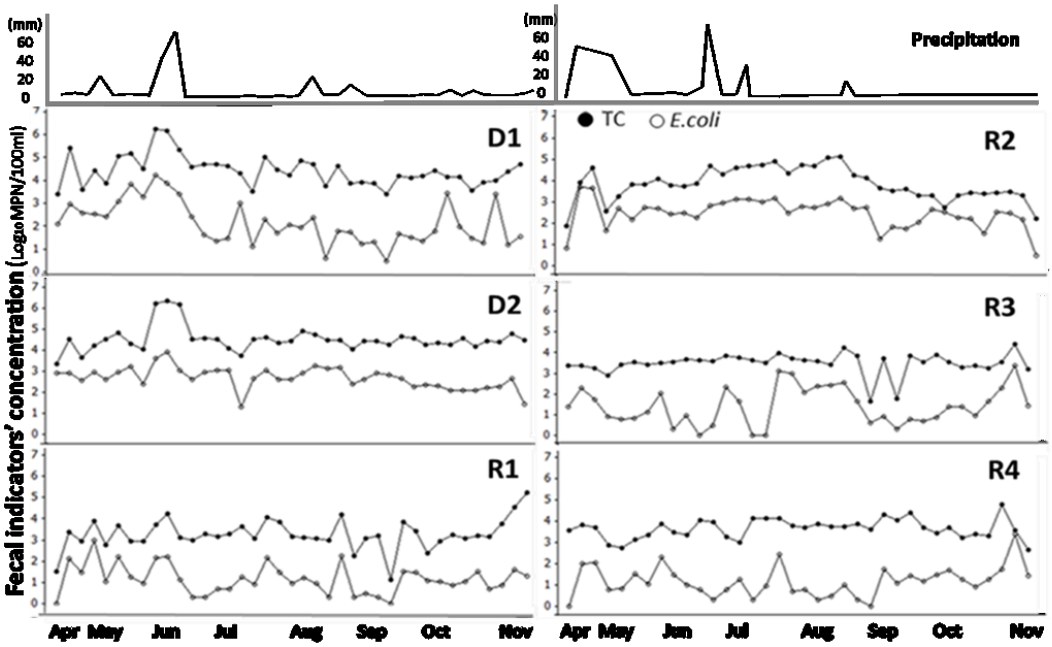

Figure 3.1 Monthly monitoring results of microbial irrigation water quality at reservoirs and irrigation ditches during 2010 irrigation season.

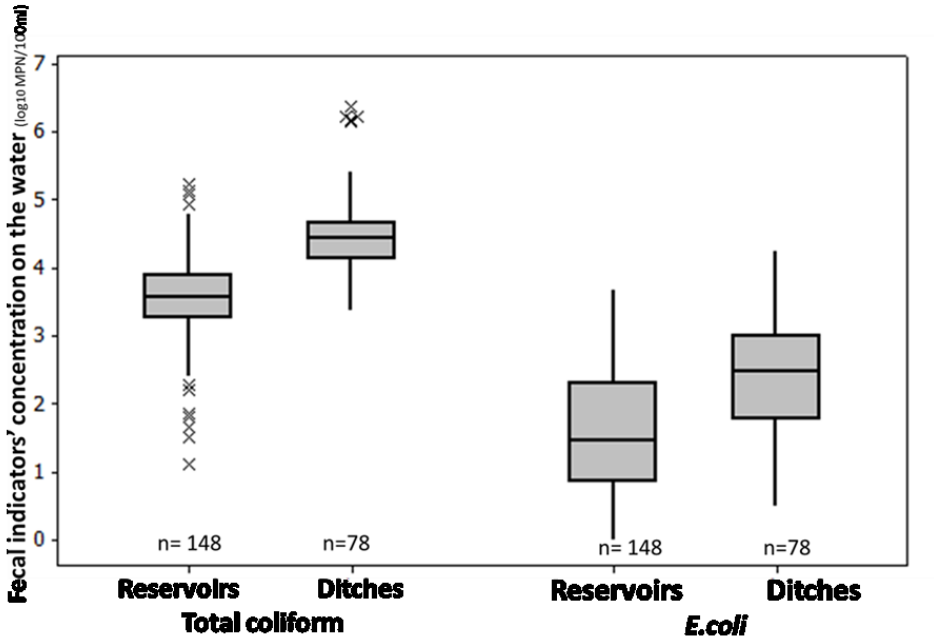

Figure 3.2 Difference of the concentration in fecal indicators between reservoirs and ditches. The concentrate on fecal indicators shows a significant difference between reservoirs and ditches ($P<0.0001$). Horizontal line is the median (the second quartile), boxes are the first to third quartile. Whiskers represent the range of data from the first and third quartiles to their respective extremes and points are the outliers

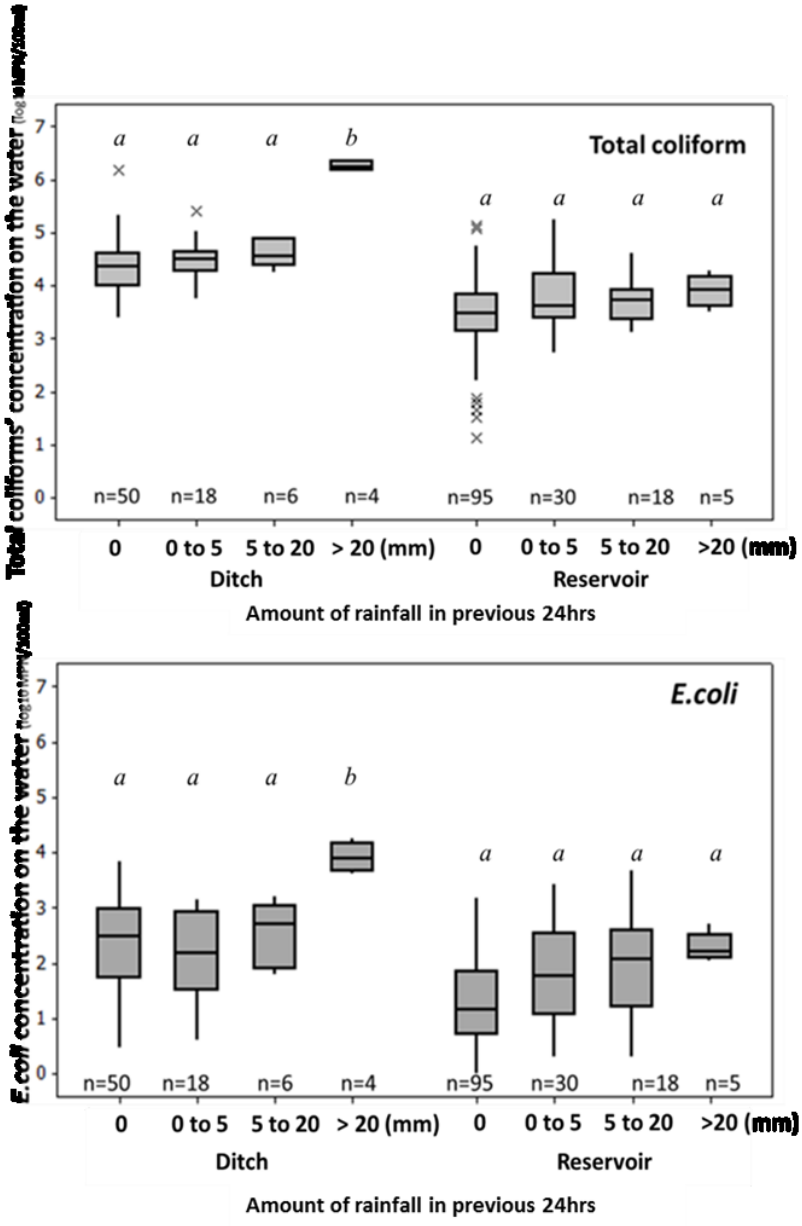

Figure3.3 Fecal indicators' concentration in the sources presented by categorized precipitation data.

Total coliform and *E. coli* counts in ditches after heavy precipitation(>20mm) was higher than the count after different categorized precipitation. Points with different letters are

significantly different ($P<0.01$). Horizontal line is the median (the second quartile), boxes are the first to third quartile. Whiskers represent the range of data from the first and third quartiles to their respective extremes and points are the outliers

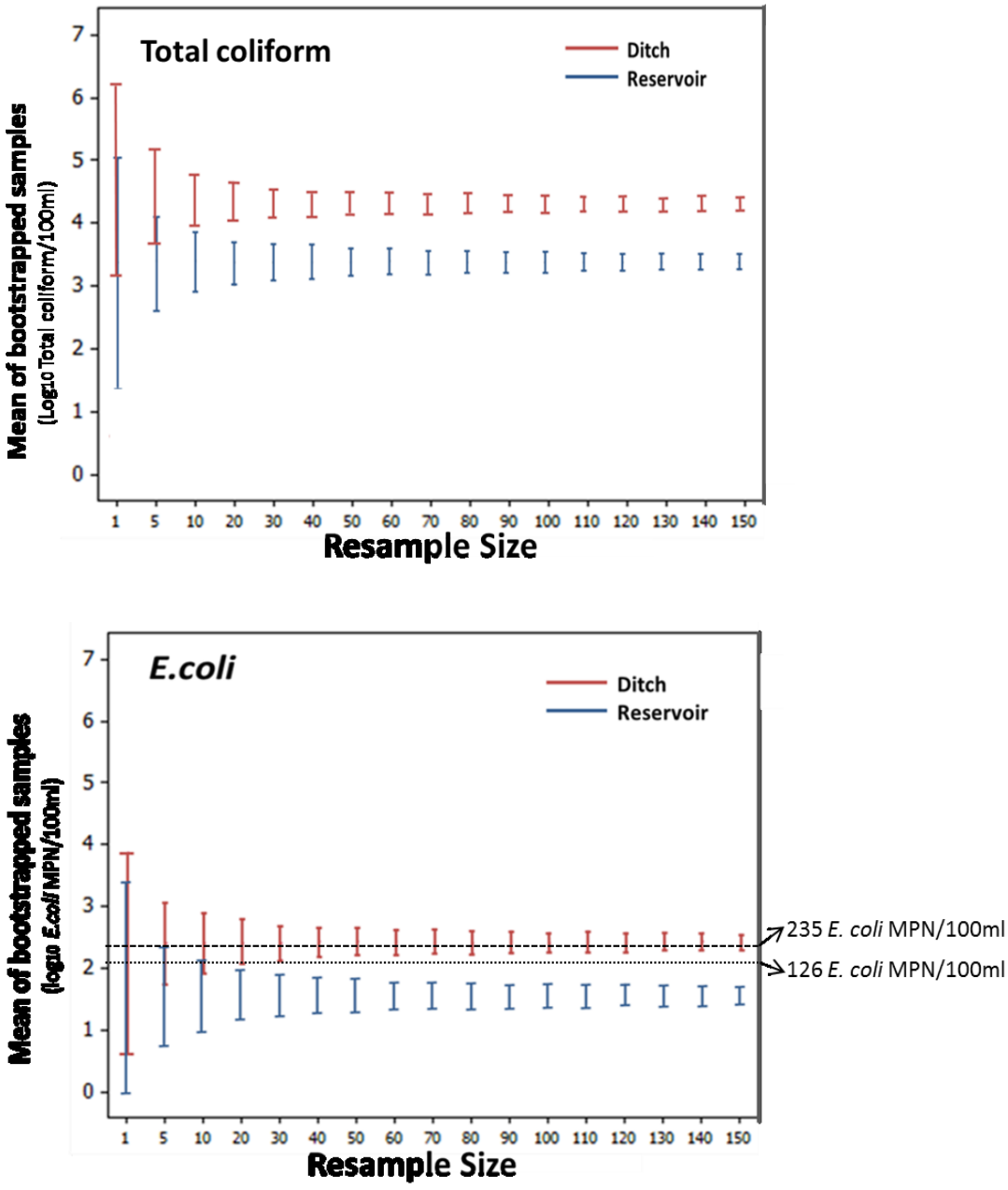

Figure 3.4 Bootstrapped confidences limits (95%) on the mean number with increasing sample size from 1 to 150. A dotted line and a dashed line indicate maximum guidelines

for a single sample recommended by FSLC (126 Generic *E. coli* MPN/100ml) and by LGMA (235 Generic *E. coli* MPN/100ml) respectively.

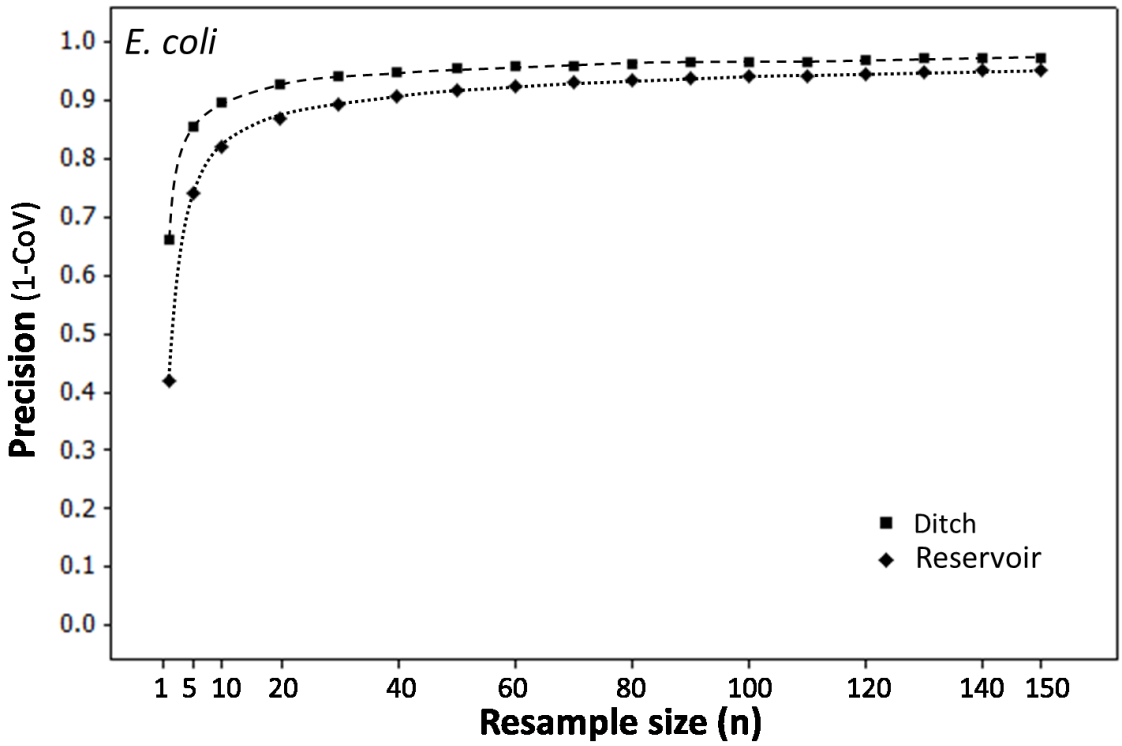

Figure 3.5 Level of precision to determine adequacy of sample sizes

Table 3.1 Proportion of fecal indicators' concentration over current guidelines

	LGMA* **(<235 *E.coli* MPN/100ml)**	**FSLC**** **(126 *E.coli* MPN/100ml)**
Reservoirs (n=148)	12.1% (18/148)	35 % (96/148)
Ditches (n=79)	45.6 % (36/79)	69.6% (55/79)

*California Leafy Green Products Handler Marketing Agreement (LGMA) has two Acceptance Criteria: ≤126 MPN (or CFU)/100 mL (rolling geometric mean n=5) and ≤235 MPN/100mL for any single sample for foliar application. So we chose the second criteria for this study.

**The standard of Food Security Learning Center (FSLC) includes*E. coli*: ≥ 126 MPN/100 mL is unacceptable water quality that shall not be used for irrigation

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Chapter 4: Absence of direct association between coliforms and Escherichia coli in irrigation water and on produce

Abstract

Irrigation water is considered a potential source of pre-harvest pathogen contamination of vegetables. Hence, a number of organizations have recommended microbiological standards for water used to irrigate edible plants. The purpose of this study was to determine the strength of association between microbial quality indicators (coliforms and *Escherichia coli*) in irrigation water and on irrigated vegetables. Data analyzed included original results from a cross-sectional study conducted in the Midwest USA during the summer 2009 and information presented in two previously published studies. In the cross-sectional study, Repetitive-PCR (Rep-PCR) was performed on *E. coli* from water and vegetables isolated on the same farm in the cross-sectional study to determine genetic relatedness. There were no significant correlations between fecal indicators on leafy greens (lettuce and parsley, n=100) or fruit (tomatoes and green peppers, n=30) and irrigation water used ($P>0.40$) in the cross-sectional study or in the previously published dataset (lettuce and waste irrigation water, n=15, $P>0.40$ Dataset1; lettuce and irrigation water, n=32; $P=0.06$ Dataset 2). Rep-PCR banding patterns of *E. coli* strains were all distinguishable among the pairs of *E. coli* recovered from produce and irrigation water on the same farm. From the available data, the concentration of indicator organisms from a single measure of irrigation water quality was not associated with their presence on

produce. In the absence of additional information, the use of a single microbial water quality parameter as an indicator of produce safety is of limited value to predict the safety of the produce.

4.1 Introduction

In the US, consumption of raw and minimally processed fruit and vegetables has significantly increased during the last two decades (Putnam, Kantor, & Allshouse, 2000). Concurrently, the number of reported outbreaks of foodborne diseases related to contaminated produce has also grown. A total of 190 produce-associated outbreaks, 16,058 illnesses, 598 hospitalizations and eight deaths were reported to the Centers for Disease Control and Prevention (CDC) from 1973 to 1997 (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). Moreover, produce-associated outbreaks account for an increasing proportion of all reported foodborne outbreaks with known food items. For example, only 0.7% of reported foodborne disease outbreaks were attributed to produce in the 1970s, that fraction rose to 6% in the 1990s (Sivapalasingam et al., 2004). In a more recent analysis of foodborne outbreaks (1990-2005), produce was responsible for 13% of outbreaks and 21% of foodborne illnesses in the US (DeWaal & Bhuiya, 2007). Despite the magnitude and increasing nature of this public health problem, the primary routes of produce contamination have yet to be clearly identified. Irrigation water unintentionally contaminated with animal feces and human waste is considered as one possible route of vegetable contamination in the field (21,34). Moreover, in light of the scarcity of fresh water in some regions of the world, the desire to use reclaimed waste

water in a safe way further highlights the need to assess the impact of water quality on the microbial contamination.

From a historical perspective in the US, microbial standards for use of reclaimed irrigation water quality were initially established by the state of California in 1918 as a fixed threshold limit (Crook, 1985). These guidelines remained in place for almost 70 years before the State of California, in 1987, revised this standard to a threshold of less than 2.2 total coliforms MPN per 100ml,a level based on drinking water criteria at the time(Page et al., 1996). This standard was broadly accepted by many states in US (Crook & Surampalli, 1996). In the 1980s, regulatory agencies and National Research Council (NRC) started using 'quantitative risk assessment approach' to evaluate the risks, cost and benefit of public health in the context of environmental decision making (Hammond & Coppock, 1990). This quantitative risk assessment method was used to estimate the risk of human infection associated with ingested pathogens coming from produce consumed raw irrigated with microbiologically contaminated water (Shuval, Lampert, & Fattal, 1997). This risk assessment model was also applied to develop the guideline of microbial reclaimed irrigation water quality in world health organization (WHO) based on achieving less than an annual risk of 10^4 illnesses per person per year (pppy) among individual consuming these products (Blumenthal, Mara, Peasey, Ruiz-Palacios, & Stott, 2000; California leafy green marketing agreement. 2010).

Despite the availability of QMRA tools, they are not uniformly applied to develop the unified standard of irrigation water quality. Current microbial standards for irrigation water have varied significantly among agencies and states(U.S. Environmental Protection

Agency, 2004). For instance, The California leafy green marketing agreement (LGMA) requires irrigation water to contain less than 126 MPN generic *E. coli* per 100ml for irrigation water quality(California leafy green marketing agreement. 2010)whereas only irrigation water containing less that 1.1 MPN generic *E. coli* per 100ml is accepted by Food Security Learning Center (FSLC) standards (An Initiative of The Pew Charitable Trusts at Georgetown University,). Despite the knowledge that pathogen occurrence and concentration plays an important role in determining final exposure risk (Hamilton, Stagnitti, Premier, Boland, & Hale, 2006), there is a limited empirical data on the association between microbial contamination of water and vegetables under different growing conditions. The role that contaminated irrigation water plays in the contamination of fresh produces and on the incidence of foodborne disease still remains poorly understood. The goal of our study was to determine the association between the magnitude of microbial indicators in irrigation water and their presence on watered produce.

4.2 Materials and methods

4.2.1 Data extraction from published literature

A recent scoping review of the literature inclusive of literature published between January1990 to April2010 on the subject of microbial contamination of leafy vegetables (Ilic et al., 2011)was used to identify papers reporting both irrigation water quality and

microbial counts on leafy green vegetables. Additional publications reporting temporally matched microbial counts in irrigation water and on vegetables were identified through an electronic bibliographic search using the search of the PubMed, AGRICOLA and BIOSIS, CAB Direct database using the search terms TOMATO and IRRIGATION. Raw data of microbial counts representing microbial contamination in water and on produce collected on the same day were extracted and used in the analyses described below.

4.2.1.1 Dataset 1

The source of Dataset 1 was a paper published by Senouci et al (1994), 'Contamination bactérienne de végétauxirrigués avec des eauxusées (SENOUCI, HASSAR, & SCHWARTZBROD, 1993)' (Bacterial contamination of vegetables irrigated with wastewater). In that study, over the course of an 8 week period (22th of June to 12th of August, 1992), lettuce (*Lactuca sativa*) and water samples were collected and analyzed quantitatively for total coliforms (TC), fecal coliforms (FC) and fecal streptococci(FS). Twenty-six vegetable-water pairs corresponding to same sampling date were reported and available for analysis.

4.2.2.2 Dataset 2

In 2008, Biscaro et al. fromPortugal, published the paper titled by 'Aspectossanitários do cultivo da alfaceamericana, irrigada com águasrecptoras de efluentesurbanos (Biscaro, Guimarães-Tomazela, Cruz, & Lopes, 2008)' ('Sanitary aspects of the lettuce culture irrigated with urban effluent'). These investigators reported the microbiological quality of lettuce (*Lactuca sativa*) when vegetables were irrigated with polluted surface water.

Thirty-two temporally matched pairs of data containing fecal coliform concentrations on vegetables and in water used for irrigation were reported. Similar to Dataset 1, these data were collected over an 8-week period (Summer, 2002).

4.2.2 Cross-sectional study

Irrigation water samples and in-field vegetables (lettuce, parsley, tomatoes, and green peppers) were collected from 120 farms located in Ohio, Kentucky and Indiana, USA during summer (June to September, 2009). Irrigation water samples were collected from a final discharge point of irrigation sources on each farm(pond, stream, well and ditch) closest to produce contact on the same day the vegetable samples were collected. Vegetables were aseptically harvested from a single field in each farm using a systematic sample collection approach at predetermined points (Laboski, Peters, & Bundy, 2006). Vegetable samples were categorized as either 'leafy greens' including lettuce and parsley, or 'fruit' including tomatoes and green peppers. Approximately 100 gram of leafy greens and three or four fruits were collected in each farm. All the samples were transported to the laboratory chilled and analyzed within 24 hours post-collection.

Vegetable quality analysis Leafy green samples were weighed and phosphate buffered saline (PBS) was added to make a one fifth dilution (i.e. 10 gram leafy green to 40ml PBS). Each sample was shaken for 2 minutes on an orbital shaker (250 rpm) and subsequently the rinsates serially diluted. Rinsates were spiral-plated (WASP Spiral Plater, Microbiology International, Frederick, MD) in duplicate onto tryptone bile X-glucuronide agar (TBX) plates(Merck, Darmstadt, Germany). The TBX plates were incubated at 35℃ for 24 hours and blue/green colored colonies and colorless colonies

were counted as *E. coli* and total coliforms respectively. Surface area (square.cm) of fruit samples was estimated using average circumferences of the fruit assuming a spherical conformation. Sufficient PBS was added to each fruit sample to cover the specimens in the sample bags (typically 500ml/ 3 fruits). The volume of PBS used in each sample was recorded to calculate a final amount of *E. coli* and total coliforms released into the buffer per square cm of surface area.

Water quality Analysis Total coliform and *E. coli* counts in 100ml aliquots of the water samples were determined by an MPN method using the Quanti-tray test (Idexx Laboratories, Westbrook, ME). Enrichment broth from *E. coli*-positive wells was struck onto TBX agar plates and incubated overnight. Up to five blue/green colonies from each plate were amplified by enrichment in brain heart infusion broth (BHI) overnight at 37C, and then mixed with 20% buffered glycerol before storage at -70C.

4.2.3 Repetitive-PCR

Banked *E. coli* isolates were recovered from -70C by culture on TBX plates overnight at 37 ºC. Five *E. coli*-suspect colonies in each plate were selected and subsequently DNA was extracted using the Ultraclean™ Microbial DNA Isolation Kit (MO BIO Laboratories, CA, USA) according to the manufacturer's instructions. Repetitive-PCR was performed with primerBOX-A1R (5´-CTACGGCAAGGCGACGCTGACG-3´)(Versalovic, Schneider, De Bruijn, & Lupski, 1994) as described by Dombeck et al(Dombek, Johnson, Zimmerley, & Sadowsky, 2000). Amplicons were separated by gel-electrophoresis in 2 percent agarose gels and visualized under ultraviolet light

illumination after staining with ethidium bromide. Cluster analysis of banding patterns was performed using an unweighted pair group using mathematical averages (UPGMA) tree method (BioNumerics 2.5, Applied Maths, Kortrijk, Belgium).

4.2.4Data analyses

Microbial counts reported in the literature and present in rinsates and water were$\log_{10}$ transformed. Spearman's correlation analysis was used to evaluate the association between microbiological quality of irrigation water and vegetables for all datasets using Minitab 15 statistical software (Minitab Inc., State College, Pa).

4. 3 Results

4.3.1 Dataset 1

Frequency and magnitude of water and vegetable contamination in Dataset 1 (France, Senouci et al., 1994) and Dataset 2 (Portugal, Biscaro et al., 2006) were previously reported. Total coliforms (TC), fecal coliforms (FC) and fecal streptococci (FS) were detected in all irrigation water samples and all vegetable samples (n=15, respectively). Concentration of these three fecal indicators in wastewater used for irrigation ranged between 10^6 and 10^8 CFU per 100ml. Concentration of TC and FC in vegetable ranged from 10^4 to 10^6 CFU per a gram. The concentration of FS was 10^5 to 10^6 CFU per gram on plants. In correlation analysis, indicators of wastewater microbial quality and contamination of vegetable were not statistically associated or negatively associated in Dataset 1 (TC: ρ=0.033*P*=0.91FC: ρ=-0.235*P*=0.398FS: ρ=-0.609 *P*=0.015) (Fig4.1).

4.3.2 Dataset2

In Dataset 2, all vegetable and water samples were positive for fecal coliforms. Fecal coliform in water was observed in the range between 10^4 and 10^7 MPN per ml. Counts of fecal coliforms on vegetable ranged from 6 to 10^4 MPN per ml. In Dataset 2, indicators of irrigation water microbial quality and contamination of vegetable were not statistically associated (ρ=-0.332 P= 0.063) (Fig 4.2).

4.3.3 Cross- sectional Study

4. 3.2.1 Association of microbial quality between water and vegetables

Total coliforms (TC) were present in 50% (65/130) of all water samples. *E. coli* was detected in 19% (33 /130) of irrigation water samples. There were seven farms from which both the irrigation water and the produce yielded positive results for *E. coli*. Counts of TC and *E. coli* on irrigation water analyzed in this study ranged between undetectable levels to10^6 per 100ml. Ninety-nine percent (99/100) and 21% (21/100) of fruit samples were positive for TC and *E. coli*, respectively. Counts of TC on fruit samples ranged from undetectable levels to 10^7 percm^2. *E. coli* counts on the fruit samples ranged between undetectable levels to 10^2 per cm^2. For leafy green samples, 93 % (28/30) of specimens tested positive for TC and 33% (10/30) of samples were *E. coli* -positive. Concentrations on TC and *E. coli* on leafy green samples were as high as 10^7 per gram, and 10^5 per gram, respectively. Notably, coliforms were present on all the

fruit samples (50/50) and 93% (14/15) of leafy green samples irrigated with water that had no detectable total coliforms.

Coliform and *E. coli* counts from individual samples were correlated (irrigation water, r^2=0.612 P<0.001; vegetables, r^2=0.339 P<0.001) (fig 4.3). However, *E. coli* was not detected in 32 irrigation water samples (49.2%, 32/65) that were positive for total coliforms. Only 15.4% of irrigation water samples (20/130) had both of total coliforms and *E. coli*. For leafy green vegetables, both *E. coli* and total coliforms were present in 33% of samples.

4.3.2.2 Differentiation by DNA fingerprinting of *E. coli* strains

The rep-PCR banding patterns of the five *E. coli* isolates tested from each independent source were indistinguishable. However, the rep-PCR patterns of isolates recovered from water were clearly distinguishable from those found on produce from the same farms. Dice similarity coefficients between isolates recovered from water and produce ranged from 75 to 90 % (Figure 4.4). It was not possible to recover frozen *E. coli* isolates from both the water and the produce of four of the seven enrichments previously identified as positive for this organism.

4.4 Discussion

In this study, analysis of data from three continents collected by multiple investigators yielded similar consistent finding: The microbial quality of irrigated vegetable was not significantly correlated with the microbial quality of irrigation water used. In terms of

assumptive statistical power (1-β) for correlation analysis (Zar, 1999), the sample size employed for these analyses (tomato: water association, n=100; and the leafy green: water association, n=30) was determined to be sufficient to detect, with 80% power and 95% of confidence interval, associations between fruit and water and leafy greens and water if the correlation coefficient (r) was more than 0.277 or 0.479, respectively.
In the cross-sectional study, 8% (8/100) of water sample used for fruit irrigation exceed LGMA standards and Florida Tomatoes guidelines for fresh irrigation water quality (less than 126 generic *E. coli*/ 100 ml) while all the water sample used for leafy green irrigation was in the range of the guideline. This LGMA guideline was similar to the EPA standards for recreational water based on human exposure directly from contaminated swimming beaches (U.S. Environmental Protection Agency, 2002).

Nevertheless, thirty-two percent of leafy green samples (8/25) which were irrigated with water in which *E. coli* was not detectable were contaminated with *E. coli*. In the case of fruit samples on same dataset, *E. coli* was present for 24% of fruit samples (17/72) irrigated with water containing undetectable numbers of *E. coli*. This result was similar to previous studies: Takayanagui et al (2001, 2007) reported the presence of *Salmonella* spp. and parasites on in-field leafy greens despite the absence of these organisms in the waste water used for irrigation (Takayanagui et al., 2001; Takayanagui et al., 2007) . We did however identify seven pairs of water and vegetable sample where *E. coli* was detected from both of the paired specimens. However, Rep-PCR DNA fingerprint of the generic *E. coli* strains failed to provide evidence that the *E. coli* present in the irrigation water was the source of the *E. coli* on the vegetables. Söderström et al

(2008) also reported a similar result that none of identical strains of *E. coli* O157 were recovered from the leafy greens irrigated with water in which *E. coli* O157 was detected (Söderström et al., 2008).

Collectively, the data reported herein indicated that when considered in isolation, a single irrigation water sample, even those collected near the time of vegetable testing, provide little insight into the likelihood of microbial contamination of the produce and water used for irrigation. Moreover, several other studies have reported that the abundance and distribution of pathogen and fecal indicators vary temporally in water and are influenced by climatic conditions such as rainfall events, temperature and seasonality (Haley, Cole, & Lipp, 2009; Jofre, Blanch, & Lucena, 2010; Shelton et al., 2011). Albeit that a recently published systematic review of the literature concluded that *E. coli* was the most consistent predictor of gastrointestinal (GI) illness following recreational fresh water contact(Wade, Pai, Eisenberg, & Colford Jr, 2003). The utility of generic *E. coli* as an indicator organism for microbial food safety of vegetables is controversial (Doyle & Erickson, 2006). Hence, unconditional use of fecal indicators, without any consideration of environmental contexts, in microbial irrigation water standards may need to be approached with caution.

In the socio-economic context, irrigation water quality is still important since supplying safe and clean water for agricultural use directly associated to cost. EPA report showed that irrigators paid an average of $42 per acre for accessing fresh irrigation water source from off-farm suppliers in 2003 in the US (Gollehon & Quinby, 2006). Shuval et al (1997) estimated that an additional cost of $3-30million per case of disease prevented

would be required to meet USEPA guideline (zero FC/100ml) as opposed to using WHO guideline for waste irrigation water quality (1000FC/100ml)(Shuval et al., 1997). Higher irrigation water standards would increase farm production costs without reciprocal increases in food safety. Research in developing countries introduced agricultural practices for reducing risk and treatment cost such as changing an irrigation method and irrigation cessation before harvesting time for cost-efficient achievement (Keraita, Konradsen, Drechsel, & Abaidoo, 2007a; Keraita, Konradsen, Drechsel, & Abaidoo, 2007b).

Additional empirical data that address the relation between microbial quality of irrigation water and fresh produce may be required for regulatory agencies to establish standards for microbial quality of irrigation water with a scientific justification.

4.5. Conclusion

In this study, the concentration of indicator organisms on produce was not associated with a single measure of irrigation water quality. This subsequently brings into question for the utility of indicators to predict pathogens on fruit and vegetables and food safety. In the absence of additional information, such as the rate of pathogen population decline on specific plants under defined environmental conditions, method of water application, and the irrigation-to-harvest interval, the use of a single microbial water quality parameter as an indicator of produce safety should be reconsidered. The presence of coliforms and *E. coli* on produce irrigated with microbiologically "clean" water and the diversity of *E. coli* subtypes identified on vegetable surfaces indicate that irrigation water

may not be a primary source of vegetable contamination with bacteria. Other routes of contamination, such as soil splash, animal intrusion, dust, *etc*. may be more important sources of vegetable contamination. The low correlation coefficient between microbial quality of vegetable and water in this study may indicate that application of strict current standards for irrigation water quality may not yield the desired decrease in microbial contamination and increases in food safety.

4.6. Acknowledgements

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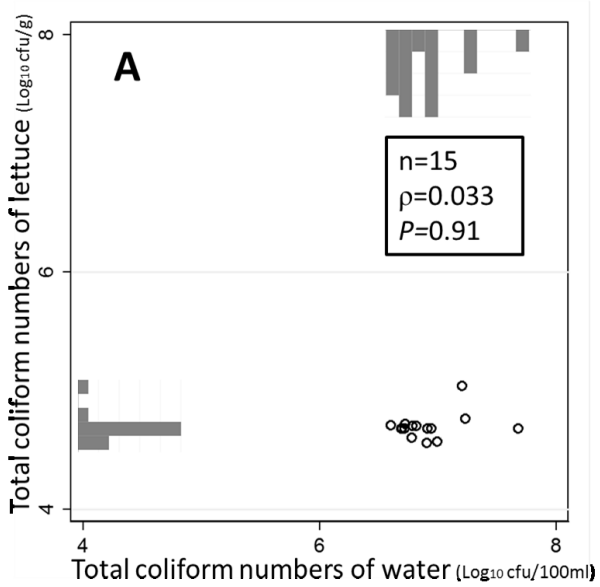
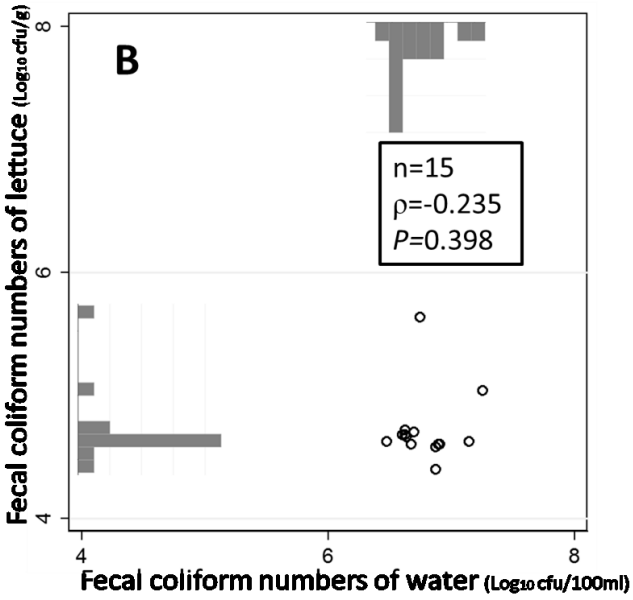
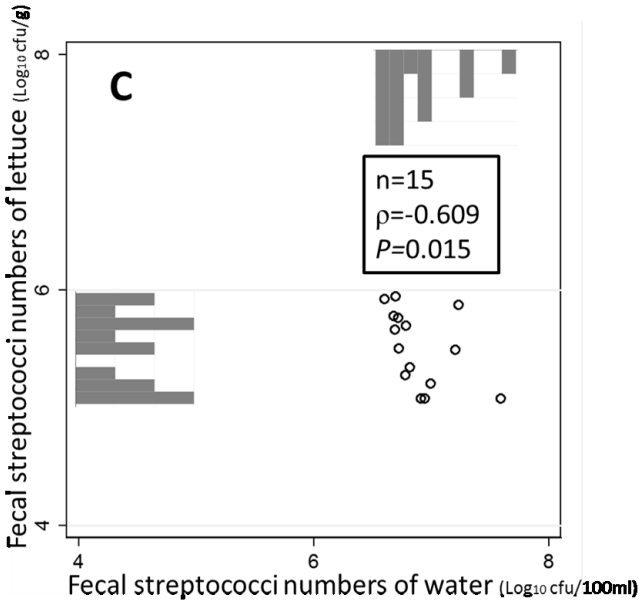




continued

Figure 4.1 Relationship between microbial quality of waste irrigation water and lettuce, Dataset 1; A: Total coliform B: Fecal coliform C: Fecal streptococci (point, raw data; bars, frequency distribution)

Figure 4.1 continued



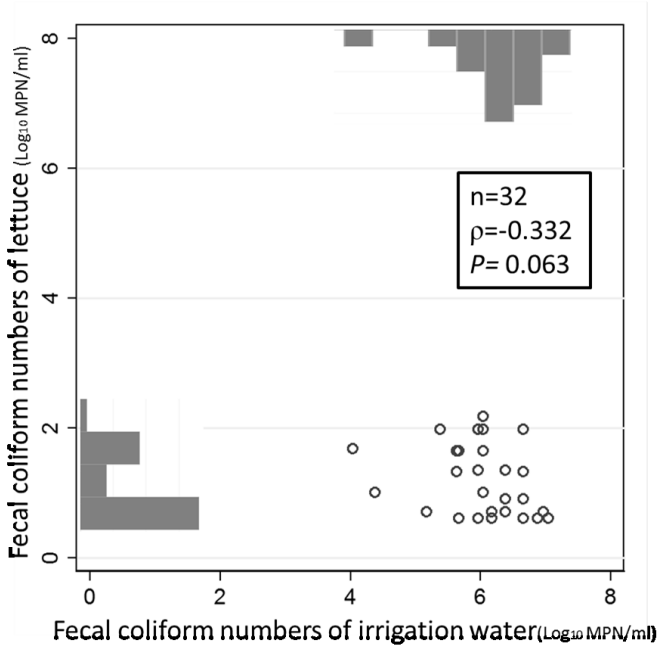


Figure 4.2 Relationship between microbial quality of waste irrigation water and lettuce in dataset 2 (point, raw data; bars, frequency distribution)

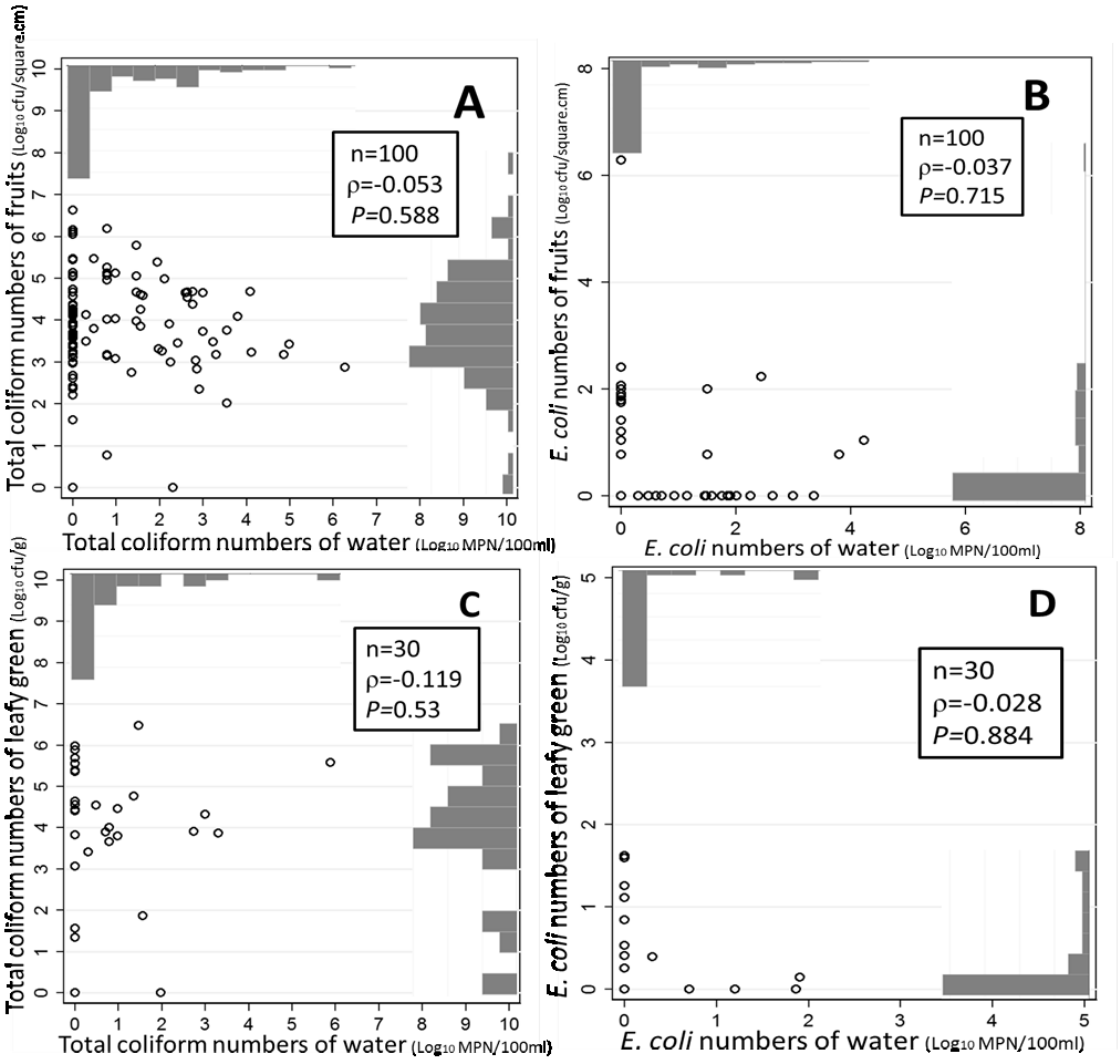


Figure 4.3 Relationship between irrigation water quality and fruit (A,C n=100 pairs), leafy greens (B,D n=30 pairs)(point, raw data; bars, frequency distribution), Cross-sectional study.

Figure 4.4 Box AR1-PCR banding patterns and similarity indices (UPGMA) of *E. coli* detected in vegetable and irrigation water.

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Chapter 5: Conclusions

Fresh water sources used for drinking and irrigation in agricultural intensive areas, Ohio was contaminated with fecal sources. For the first, approximately 47 percent of samples collected from private wells used for drinking purposes were positive for total coliform in rural areas in Ohio. This founding indicated that this sources may be exposed to environmental contamination. Generic *E .coli* and *E. coli* O157 was found in approximately 9 % and 3.8% of water samples respectively. The drinking water may play a role of transmission of *E .coli* O157 which can cause AGI to residents who obtained drinking water from the wells. However, we did not find any significant associations between the occurrence of fecal indicators and *E .coli* O157 and the potential risk factors including the density of dairy farm concentration and ground water pollution potentials. Notwithstanding the failure of contamination source identification, strict risk management strategies are still needed to enhance microbial quality of drinking well water in these regions. In addition to that, the developments of risk communication plans are also required to inform the potential risk of contaminated water to consumers who use the water without treatments.

For the second, we found that current guidelines for irrigation water did not reflect actual contamination levels of irrigation water in the fields in terms of water test frequencies

and threshold levels of fecal indicators. Empirical distributions was developed based on the concentration of total coliforms and generic *E coli* collected from six different surface water sources by using bootstrap analysis. Current guidelines often recommend a single test prior to water use or periodical sampling (i.e. monthly and quarterly) without considering the environmental factors affecting the increase of concentrations of fecal indicators in the water. However, our results showed that the test frequency was dependent on temporal (i.e. precipitation or normal climate) and spatial variances (i.e. ditches or reservoirs) of fecal indicators' concentrations in the bootstrap analysis. Current guidelines for irrigation water tests provide limited assurance of food safety. Given the complexity of factors influencing water quality, testing strategies should be developed specifically for each particular situation with scientific justifications.
For the third , fecal indicators in irrigation water did not have significant associations with those in raw vegetable irrigated by the water during pre-harvest periods. The founding indicated that fecal indicators on plants may be influenced by other environmental factors and the microbial quality of fresh produce cannot be solely predicted by the quality of irrigation water.

Collectively, risk associated with water contamination depends upon intended use. Prevention approaches are needed to be focused on the influence of water deterioration on human health. Regulatory agencies are also required to protect drinking well water and develop the relevant guidelines to mitigate the risk of AGI. For irrigation water, there is a need to investigate the role of the water as transmission routes of zoonotic

pathogens to human based on scientific and empirical evidences, including additional or alternative strategies for water sampling and testing.

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