A COMPARATIVE ANALYSIS OF DETECTION TECHNIQUES FOR *SALMONELLA* IN SURFACE WATER

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ABSTRACT

A COMPARATIVE ANALYSIS OF DETECTION TECHNIQUES FOR *SALMONELLA* IN SURFACE WATERS

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Surface waters provide a challenge for pathogen detection due to changing environmental conditions and microbial populations that may affect method chemistry. Due to the importance of these pathogens with regard to human health, rapid detection and screening of water samples is necessary to determine the risks of potential pathogens present at any given time. The ANSR assay, using Nicking Enzyme Amplification Reaction (NEAR™) technology, originally developed for food safety, was tested for its effectiveness in detecting *Salmonella* in surface waters. The main objective of this study was to determine if the ANSR assay is appropriate for detection of *Salmonella* in surface waters and comparable to the current culture-based industry standard USEPA 1682 method. These methods were tested by comparing their ability to detect seeded and naturally-occurring *Salmonella* in surface waters from around Michigan and northern Ohio. The ANSR assay and the USEPA 1682 method found 84.4% and 31.4% of samples positive for naturally-occurring *Salmonella,* respectively. The ANSR assay has shown the ability to detect *Salmonella* in surface waters at least as well as the USEPA 1682 method, but because of its much higher potential for false positives and its low precision and accuracy rates, it should be used in conjunction with other detection methods to confirm positive results. This assay shows potential for use as a rapid screening tool for surface water samples in order to prioritize in depth testing at sites, however what appears to be false-positives will need to be reduced.

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KEY TO ABBREVIATIONS

ANSR	Amplified Nucleic Single Temperature Reaction
AOAC	The Association of Analytical Communities
AWWA	American Water Works Association
CFU	Colony Forming Unit
CDC	Centers for Disease Control and Prevention
DL	Detection Limit
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbant Assay
GFP	Green fluorescent protein
IAC	Internal Amplification Control
ISO	International Organization for Standardization
LIA	Lysine Iron Agar
MDR	Multiple Drug Resistant
MPN	Most Probable Number
MSRV	Modified Semisolid Rappaport-Vassiliadis Medium
NEAR™	Nicking Enzyme Amplification Reaction
ND	Non-detect
NSF Int.	National Sanitation Foundation International
OPR	Ongoing Precision Recovery
OTUs	Operational Taxonomic Units
PCR	Polymerase Chain Reaction

qPCR	Quantitative Polymerase Chain Reaction
rt-PCR	Real-time Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
UK	United Kingdom
US	United States of America
USEPA	US Environmental Protection Agency
VBNC	Viable but not culturable
WHO	World Health Organization
WRD	Water-related Disease
XLD	Xylose-lysine Desoxycholate Agar

CHAPTER 1. THE IMPORTANCE OF *SALMONELLA* IN WATER

1. General Information on *Salmonella*

1.1 *Salmonella* Taxonomy

Salmonella are Gram-negative, facultative anaerobic non-spore forming bacillus shaped bacteria, which belong to the family Enterobacteriaceae. The genus *Salmonella* consists of two recognized species, *Salmonella enterica* and *Salmonella bongori* (Table 1). *Salmonella enterica* is further divided into six subspecies: *enterica* (subsp. I), *salamae* (subsp. II), *arizonae* (subsp. IIIa), *diarizonae* (subsp. IIIb), *houtenae* (subsp. IV), and *indica* (subp. VI) (Table 1). These subspecies are further divided into over 2500 serovars based on their unique combinations of somatic O and flagellar H1 and H2 antigens (Table 1) (Popoff et al., 2004, Grimont and Weill, 2007, Su and Chiu, 2007, Chen et al. 2013). When referring to serovars it is not necessary to name the species and subspecies of *Salmonella*, instead only the genus and the non-italicized capitalized serovar name are required (Grimont and Weill, 2007). For example, *Salmonella enterica enterica* (subsp. I) Typhimurium would be *Salmonella* Typhimurium. This is accepted due to the uniqueness of each serovar name, which are not repeated within either species, or subspecies of *Salmonella*. The subspecies *Salmonella enterica* (subsp. I) contains most of these serovars (Grimont and Weill, 2007, Chen et al. 2013). Many *Salmonella* serovars are known to be enteric pathogens with most residing in the *Salmonella enterica* subspecies *enterica*. The most common of these serovars and arguably the most important in regards to human health are *S.* Enteritidis and *S.* Typhimurium with both combined being responsible for over 60% of all *Salmonella* outbreaks causing human illness and 58% of all reported cases of human illness caused by *Salmonella* (Figure 1, Figure 2) (Doyle et al., 2009, Bellido-Blasco and Arnedo-Pena, 2011). The *Salmonella* species *bongori* does not have any subspecies, but includes 22 serovars

(Grimont and Weill, 2007). Most serovars of *Salmonella* possess peritrichous flagella allowing for motility. Though motility is a crucial criterion for the identification of *Salmonella* spp. many serovars have been found to be non-motile (Bugarel et al., 2012).

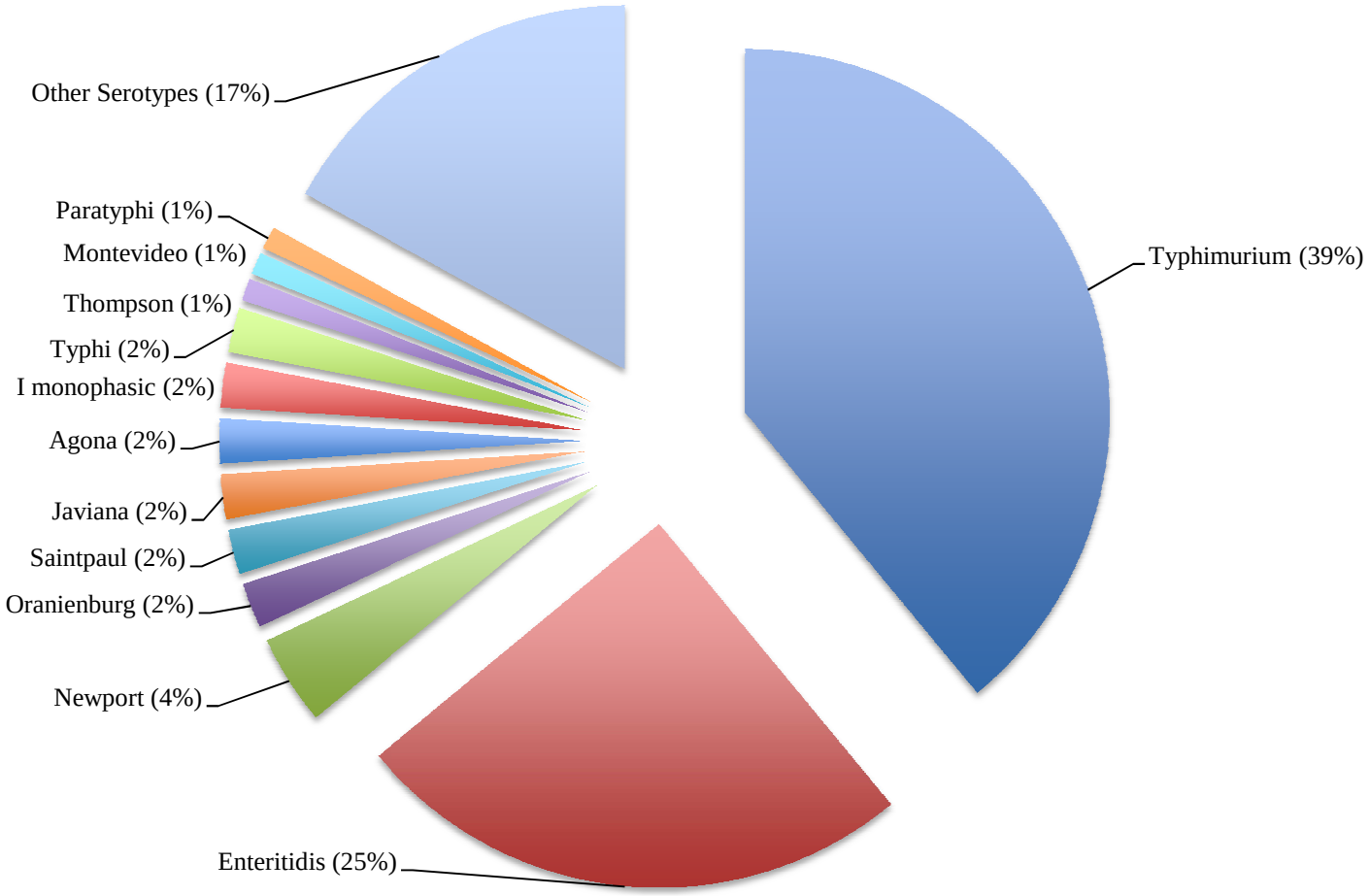
Table 1. *Salmonella* species and subspecies

Species	***S. enterica***						***S. bongori***
Subspecies	*enterica* (I)	*salamae* (II)	*arizonae* (IIIa)	*diarizonae* (IIIb)	*houtenae* (IV)	*indica* (VI)	
Number of Serovars	1,531	505	99	336	73	13	22
Human Health Significance	****	*	*	*	*	*	*
Preferred Host	Warm-blooded animals		Cold-blooded animals and the environment				
Characteristics							
Dulcitol	+	+	–	–	–	v	+
ONPG (2h)	–	–	+	+	–	v	+
Malonate	–	+	+	+	–	–	–
Gelatinase	–	+	+	+	+	+	–
Sorbitol	+	+	+	+	+	–	+
Grown with KCN	–	–	–	–	+	–	+
L(+)-tartrate[a]	+	–	–	–	–	–	–
Galacturonate	–	+	–	+	+	+	+
γ-glutamyltransferase	+[b]	+	–	+	+	+	+
β-glucuronidase	v	v	–	+	–	v	–
Mucate	+	+	+	– (70%)	–	+	+
Salicine	–	–	–	–	+	–	–
Lactose	–	–	– (75%)	+(75%)	–	v	–
Lysed by phage O1	+	+	–	+	–	+	v

a *d*-tartrate
b Typhimurium v, Dublin -
+ 90% or more positive reactions
- 90% or more negative reactions
v different reactions given by different

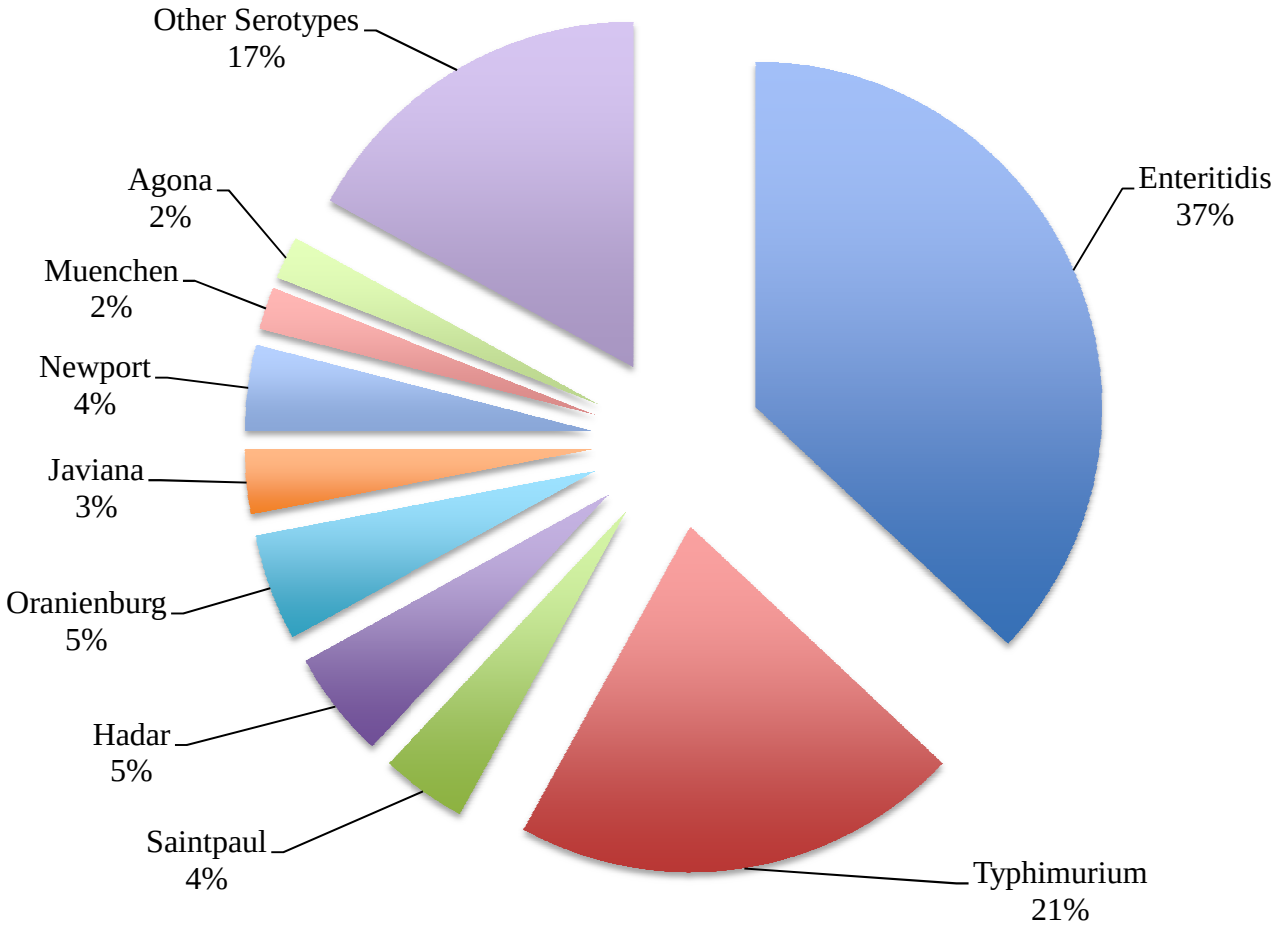
Combined and updated from: (Minor et al., 1982, Minor et al., 1986, Popoff et al., 2004, Grimont and Weill, 2007)

Figure 1. *Salmonella* serovars reported as causes of outbreaks of human illness around the world from 1992 - 2008



Total outbreaks N = 492
**Figure 1. reproduced from Doyle et al., 2009

Figure 2. *Salmonella* serovars reported as causes of cases of human illness around the world from 1992 - 2008



Total cases N = >55,739
**Figure 1 reproduced from Doyle et al., 2009

1.2 Clinical importance

Salmonella spp. are the disease-causing agents of salmonellosis in humans and, to this day, cause significant worldwide public health concerns. Salmonellosis has three clinically distinguishable forms: gastroenteritis, enteric fever, and septicemia. Serovars of salmonella that cause these illnesses are put into two categories or groups, typhoid and nontyphoid *Salmonella.* *Salmonella* Typhi and *S.* Paratyphi A, B, and C are referred to as typhoid *Salmonella* due to their roles as the causes of typhoid fever. All other *Salmonella* serovars being considered nontyphoid *Salmonella.* Both gastroenteritis and enteric fever caused by *Salmonella* spp. are still of concern today even in developed countries (Dale et al., 2007, Blasi et al., 2008, Berg, 2008, Kozlica et al., 2010, Levantesi et al., 2012, Chen et al. 2013). *Salmonella* infection is difficult to control due to the bacterium's widespread distribution, high tolerance to environmental stress, multiple drug resistance, and adaptability (Runkel et al., 2013, Chen et al. 2013). Both groups of *Salmonella*, typhoid and nontyphoid, are known to be transmitted through the fecal-oral route of infection. While the causes of typhoid and paratyphoid fever (*S.* Typhi and *S.* Paratyphi A, B, and C) are host-specific to humans, nontyphoid *Salmonella* serovars are known to be zoonotic pathogens and are of importance in both humans and animals.

Majowicz et al. (2010) reported that the global burden of nontyphoid *Salmonella* gastroenteritis had an average incidence of 1.14 episodes/100 person-years each year with a total of 93.8 million cases and 155,000 deaths. Incidences of disease were highest in Asia, which had an average of 4.72 episodes/100 person-years each year with a total of 83.4 million cases and 137,700 deaths (Majowicz et al., 2010). In the US, there are approximately 1 million cases of salmonellosis each year with approximately 19,000 of those cases requiring hospitalization (CDC, 2011). In the US, while there has been a 25% reduction in food-related illness caused by

pathogens such as *Escherichia coli* and *Campylobacter* in the last decade, there have been no significant changes in the incidence of salmonellosis (Osterholm, 2011).

Typhoid and enteric fevers caused by *S.* Typhi and *S.* Paratyphi, while not common in developed nations, are still a cause for concern in less developed countries around the world. Kindhuaser (2003) reported an annual incidence of 17 million cases of typhoid and paratyphoid fever worldwide. *Salmonella* serovars Typhi and Paratyphi are strictly human pathogens and human feces are the main source of typhoid fever transmission. Thus water that has been contaminated with sewage and feces can be a major source of infection (Levantesi et al., 2012).

1.3 Multiple Drug Resistance

Multiple drug resistant (MDR) *Salmonella* is an issue of emerging concern due to the increasing frequency of MDR strains in recent years (Lightfoot, 2004, Pond, 2005, Lynch et al., 2009). Antibiotic resistance is seen in both typhoid and nontyphoid *Salmonella* (Flor et al., 2011, Chen et al., 2013). Antimicrobial resistance among salmonellae rose from 20-30% in the early 1990s to 70% in various countries by the year 2000, with resistance rates varying among serovars (Su et al., 2004, Lauderdale et al., 2006, Parry and Threlfall, 2008, Lee et al., 2009). For example, two serovars that are both globally distributed but show different levels of resistance are *S.* Typhimurium and *S.* Enteritidis (Su et al., 2004., Helms et al., 2005). *Salmonella* Typhimurium has a much higher rate of resistance than *S.* Enteritidis (which is relatively more susceptible to antimicrobial agents), with *S.* Typhimurium definitive phage type (DT) 104 emerging as a global health problem since the 1980s (Su et al., 2004., Helms et al., 2005). Multidrug-resistant nontyphoid *Salmonella* strains are usually resistant to ampicillin, chloramphenicol/florfenicol, streptomycin/spectinomycin, sulfonamides, and tetracyclines (Helms et al., 2005). Resistance to extended spectrum cephalosporins, trimethoprim,

fluoroquinolones, ciprofloxacin, ceftriaxone and carbapenems have also been documented in nontyphoid *Salmonella* strains (Angulo et al., 2000, Guerra et al., 2002, Chiu et al., 2004, Jean et al. 2005, Lee et al., 2009, Hall, 2010, Su et al., 2011, Su et al., 2012). Similar patterns of resistance are prevalent in *S.* Typhi and *S.* Paratyphi, both of which exhibit resistance to chloramphenicol, beta-lactams, quinolones, azithromycin, ampicillin, trimethoprim-sulphamethoxazole, and ciproflaxin (Kumar et al., 2008, Chuang et al., 2009, Molloy et al., 2010, Zaki and Karande, 2011). As these MDR strains continue to disseminate there is an increased risk of nontyphoid and typhoid disease complications in both developed and underdeveloped nations.

2. *Salmonella* in Water

2.1 Survival in Water

Although reservoirs of *Salmonella* include both domestic and wild animals, survival and persistence in the environment have been shown to be important in the spread of *Salmonella*. While *S.* Typhi and *S.* Paratyphi are not as widely disseminated in nature as nontyphoid *Salmonella* serovars due to their restrictive host requirements (i.e., humans), water still plays a crucial role in the spread of typhoid and paratyphoid fever. Nontyphoid *Salmonella* is much more widely distributed in the environment perhaps because it is known to be a zoonotic disease (Percival and Williams, 2014). Water is often overlooked when it comes to *Salmonella* outbreaks, except for *S.* Typhi, which is known to spread through shared water sources. Most cases of salmonellosis (95%) are attributed to food contamination, although contaminated waters sources including drinking, surface, and groundwater have been implicated in human illness as well (Doyle et al., 2009, Percival and Williams, 2014).

2.2 Waterborne Outbreaks of *Salmonella*

Waterborne outbreaks of *Salmonella* are associated with poor-quality water due to inadequate or lack of treatment of a contaminated source or fecal contamination of the distribution system such as a sewage cross connection (Angulo et al., 1997). Infections by waterborne *Salmonella* can occur through drinking or swimming in contaminated water (O'Reilly et al., 2007, Hsu et al., 2011). In addition foodborne outbreaks may occur as a result of food that has been washed or come in contact with contaminated water (CDC, 2012a).

2.2.1 Waterborne outbreaks in developed countries

Present day waterborne outbreaks of *Salmonella* that occur in developed countries are mostly associated with nontyphoid *Salmonella* serovars (Levantesi et al., 2012). Historically, outbreaks caused by typhoid *Salmonella* serovars were present, but proper water treatment, through the use of municipal and wastewater treatment systems during the second half of the 20th century, has greatly contributed to reduction in waterborne outbreaks and the spread of typhoid fever in most industrialized countries, although in the US, 300-400 cases of typhoid fever are still reported each year (Smith et al. 2006, CDC, 2008, Lynch et al., 2009). In the US, sporadic cases of typhoid and paratyphoid fever still occur and are often a result of people becoming exposed during foreign travel in areas where the disease is more common. In Italy, the Indian subcontinent, and southeast Asia, typhoid fever remains endemic (DeRoeck et al., 2007, Rizzo et al., 2008, Lynch et al., 2009).

In the US, waterborne outbreaks have been most commonly associated with the *Salmonella* serovars Typhimurium, Enteritidis, Javiana, Newport, Bareilly, and Weltervreden (Covert, 1999, Craun et al., 2004). Between the years 1971 and 2000, 15 drinking-water-outbreaks of nontyphoid *Salmonella* occurred (6% of the total were considered to be zoonotic

pathogen waterborne outbreaks) (Table 2) (CDC, 1978, CDC, 1979, CDC, 1983, CDC, 1985, CDC, 1988, CDC, 1996, CDC, 1998, CDC, 2002, 2006, CDC, 2011). Eleven were associated with community water systems and groundwater (Craun et al., 2004). In 1993, a significant waterborne outbreak of *Salmonella* Typhimurium occurred in Gideon, Missouri, where 650 people were infected, 15 were hospitalized, and 7 died (Angulo et al., 1997). However, between 2000 and 2006, waterborne outbreaks of *Salmonella* were rare in the US (Yoder et al., 2004, CDC, 2006, Yoder et al., 2008). Two outbreaks occurred in 2008, demonstrating that waterborne outbreaks continue to occur in areas where there are inadequate water supply systems or deficiencies in water treatment (Berg, 2008, Kozlica et al., 2010). From March to April of 2008, a waterborne outbreak of *Salmonella* occurred in Alamosa, Colorado, where the drinking water was not chlorinated prior to the outbreak, but the city had been in compliance with all health-based drinking water standards (Berg, 2008). There were 442 reported illnesses (122 were laboratory-confirmed) and one death reported but the epidemiological estimates suggested up to 1,300 people fell ill (Berg, 2008). Four months later, in August of 2008, an outbreak of *Salmonella* I 4,[5],12:i- was identified in a rural community in Tennessee where an untreated private spring-fed water system was contaminated (five cases) (Kozlica et al., 2010). The use of private wells and septic systems have been identified as important risk factors for sporadic *Salmonella* infections in children (Denno et al., 2009).

Between 1995 and 2005, there was only one CDC-reported waterborne outbreak of *Salmonella*, which was linked to recreational water exposure in the United States (CDC, 1998, Pond, 2005). The scarcity of recreational waterborne *Salmonella* outbreaks seems to indicate that recreational waters are insignificant as the cause of *Salmonella* infections, but this is not always the case. Three Washington State county health departments have identified both aquatic and

marine recreational waters as important risk factors for sporadic *Salmonella* infections in children (Denno et al., 2009).

Table 2. Waterborne *Salmonella* outbreaks in the U.S. 1971 to 2008

Year	Etiological Agent	Outbreaks	Cases	Type of System[§]	Deficiency[a]	Location
1978	*Salmonella*	1	78	Com	-	-
1978	*Salmonella*	1	11	Ncom	-	-
1979	*Salmonella*	1	69	Ncom	-	-
1983	*Salmonella*	2	1150	Com	-	-
1985	*Salmonella* Typhi	1	60	Com	4	VI
1986	*Salmonella,* Mixed	1	9	Com	2	MS
1986	Salmonella	1	61	Com	4	UT
1993	*Salmonella* Typhimurium	1	625	Com	4	MO
1995	*Salmonella* Java	1	3	Rec	-	GA
1999	*Salmonella* Typhimurium	1	124	Com	3	MO
2000	*Salmonella* Bareilly	1	84	Ind	5	Multistate
2004	*Salmonella* Typhimurium	1	70	Ncom	3,4	MT
2007	*Salmonella* Newport	1	2	Ind	2	TN
2007	Mixed agents*	1	229	Ncom	2	WI
2008	*Salmonella* Typhimurium	1	1,300	Com	4	CO
2008	*Salmonella* serovar I 4,5,12:*i*:-	1	5	Ncom	2	TN

* Agents included *Salmonella*, Norovirus genogroup I, and *Campylobacter*

§ Community Water System, Ncom = Noncommunity Water System, Ind = Individual Water System, Rec = Recreational Waters

a Treatment deficiency codes: 2 - Untreated groundwater intended for drinking, 3 - Treatment deficiency, 4 - Distribution system deficiency, includes storage, 5 - *Legionella* in drinking water system

Australia has reported that of 10 observed drinking waterborne outbreaks, from 2001 to 2007, 50% were caused by *Salmonella* spp. making it the most common pathogen in drinking water outbreaks during this period (Dale et al., 2007). These outbreaks were associated with both typhoid and nontyphoid *Salmonella* spp. Unlike the community water outbreaks seen in the U.S., waterborne outbreaks of *Salmonella* in Australia were related to contaminated waters from bore holes, storage tanks, and roof-collected rain water (Taylor et al., 2000, Ashbolt and Kirk, 2006, Franklin et al., 2009).

European reporting of waterborne outbreaks is not as clearly defined as in the US, because drinking water is categorized as a food in Europe, precluding the definitive identification of specific waterborne outbreaks. In addition, country-specific reporting is limited. Two country -specific reviews have been conducted in recent years in Italy and in the UK (Smith et al., 2006, Blasi et al., 2008). Between 1998 and 2005 *Salmonella* spp. were identified in 21.5% of the total water-related disease (WRD) outbreaks in Italy (Blasi et al., 2008). This may be an underestimation of the true disease burden due to underreporting as seen in Table 3. While in England and Wales, between 1992 and 2003, *Salmonella* spp. were not associated with any waterborne outbreaks (Smith et al., 2006).

Table 3. *Salmonella* water-related disease (WRD) outbreaks in Italy 1998 - 2005

	Epidemic Focus			
	Drinking Water	***Agricultural***	***Shelfish***	***Total***
Total WRD Outbreaks	30	9	147	186
Total WRD Cases	1,017	32	1,497	2,546
***Salmonella* WRD Outbreaks**	11 (36.7%)*	2 (22.2%)*	27 (18.4%)*	40 (21.5%)
***Salmonella* WRD Cases**	63 (6.2%)*	6 (18.8%)*	232 (15.5%)*	301 (11.8%)
Unidentified WRD Outbreaks	1 (3.3%)*	3 (33.3%)*	80 (54.4%)*	84 (45.2%)
Unidentified WRD Cases	723 (71.1%)*	9 (28.1%)*	680 (45.4%)*	1,412 (55.5%)
% of Total WRD Outbreaks	5.9%	0.01%	14.5%	21.50%
% of Total Identified WRD Outbreak	37.9%	33.3%	40.3%	39.20%
% of Total Identified WRD Cases	21.4%	26.1%	28.4%	25.7%

* Percentages Reflect proportion of outbreaks/cases within each focues

Data obtained from: (Blasi et al., 2008)

According to the CDC, approximately 42,000 nontyphoid *Salmonella* infections and 400 deaths are reported each year in the US (CDC, 2012b). While many infections are mild in nature and thus left undiagnosed and unreported, waterborne *Salmonella* outbreaks are still of concern. It is estimated that the actual incidence of infection is at least 29 times higher than what is reported (CDC, 2012b). With the large number of *Salmonella* infections occurring in both humans and animals within the US, it is clear that human sewage and manure are sources of pathogens contaminating water supplies. As water infrastructures continue to age there is an increased probability of waterborne outbreaks occurring. Because poor and failing water infrastructure leads to increased waterborne outbreaks, significant investments in current water infrastructure will need to be made. For example, in the US the American Water Works Association (AWWA) has estimated that over the next 20 years more than $1 trillion will need to be invested to both repair and upgrade current water infrastructure (AWWA, 2012). The EPA has also expressed the need for substantial investment in water infrastructure over the next two decades (EPA, 2013).

2.2.2 Underdeveloped Nations

Underdeveloped nations face an even greater risk of waterborne *Salmonella* outbreaks, especially with regard to typhoid *Salmonella* serovars (Swaddiwudhipong & Kanlayanaphotporn, 2001, Srikantiah et al., 2006, Lynch et al., 2009, Kim, 2010). This risk is directly related to the absence of proper water infrastructure, with the World Health Organization (WHO), reporting 2.5 billion people still lacking access to improved sanitation and 768 million people lacking access to an improved drinking water source as of 2011 (WHO, 2013). The dissemination of MDR *S.* Typhi also poses an increased risk in underdeveloped nations, given that with MDR

typhoid serovars have been reported in multiple drinking water outbreaks in Asia (Mermin et al., 1999, Swaddiwudhipong & Kanlayanaphotporn, 2001, Lewis et al., 2005, Kim, 2010).

Typhoid *Salmonella* serovars remain major public health concerns around the world, but especially in both the Indian subcontinent and southeast Asia where they remain endemic allowing epidemics to continue to occur (DeRoeck et al., 2007). The main sources of typhoid and paratyphoid fever epidemics in Asia are contaminated drinking waters, which originate from a variety of sources including well water, unboiled spring water, and piped municipal water (Mermin et al., 1999, Swaddiwudhipong & Kanlayanaphotporn, 2001, Kim et al., 2003, Lewis et al., 2005, Bhunia et al., 2009, Farooqui et al., 2009). In 1997, a contaminated municipal water system in Dushanbe, Tajikistan led to an enormous epidemic of typhoid fever in which 8,901 cases were reported (Mermin et al., 1999). An overwhelming number (93%) of *Salmonella* Typhi isolates recovered from this outbreak showed resistance to seven different antibiotics (i.e. ampicillin, streptomycin, chloramphenicol, nalidixic acid, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole) (Mermin et al., 1999). Multiple drug resistance in *Salmonella* Typhi outbreaks is not limited to any one country in Asia. In 1999, a waterborne outbreak of MDR *S.* Typhi occurred in the Poppra District of the Tak Province in Thailand when the village's spring water became contaminated (Swaddiwudhipong & Kanlayanaphotporn, 2001). In 2000, an outbreak of MDR *S.* Typhi occurred in Mumbai, India when a well water source became contaminated (Misra et al., 2005). The largest single-point source outbreak of MDR *S.* Typhi occurred in 2002 in Bharatpur, Nepal when the only municipal water supply became contaminated, resulting in 5,963 cases of typhoid fever (Lewis et al., 2005). In 2004, a community water outbreak of MDR *S.* Typhi occurred in a small village near Karachi, Pakistan, in which over 300 people became ill, due to contamination of the village's well (Farooqui et al.,

2009). Most recently, Singla et al. (2013) reported an outbreak of MDR *S.* Typhi in a suburban area of the city of Chandigarh, India. While 90% of the known morbidity and mortality caused by typhoid fever occurs in Asia (Crump et al., 2004), it remains a major disease burden in Africa as well.

Salmonella Typhi is a major cause of enteric disease in Africa, but due to the absence of comprehensive surveillance studies and reliable measures of the incidence of disease occurring, the actual burden of typhoid fever is unknown (Crump et al., 2004). Much of Africa (e.g., the Venda region in South Africa) still lacks clean potable water and proper sanitization (Obi et al., 2004, Potgieter et al., 2005), which imposes an increased risk of waterborne infections and outbreaks. While little waterborne outbreak information is available for Africa, some studies have linked *Salmonella* to incidences of disease caused by contaminated water sources (Oguntoke et al., 2009, Bessong et al., 2009). Multiple Drug Resistant nontyphoid *Salmonella* serovars are reported in many African countries to be the most common cause of bacteraemia in children. Although the route of transmission is uncertain, clustering of cases during the rainy season suggests a possible waterborne/water-associated transmission route (Suresh & Smith, 2004, Gordon & Graham, 2008).

2.3 The role of water in foodborne outbreaks

The role of water in foodborne outbreaks is another confounding issue with the detection and reporting of waterborne *Salmonella* outbreaks. Contaminated water may play an important role in foodborne outbreaks (Berger et al., 2010), but the outbreaks are often attributed to the food product consumed and not the source of contamination itself. According to the CDC (2012a), the quality of the water that comes into contact with fresh fruits and vegetables is a critical factor influencing the potential for outbreaks to occur. This is particularly important since

large portions of surface waters, which are used for irrigation, are contaminated with effluent (e.g., 71% of irrigation waters in the UK) (Tyrrel et al., 2006). In the US, the CDC has reported 52 foodborne outbreaks of *Salmonella* since 2006, 13 of which were associated with fresh fruits or vegetables (CDC, 2014). According to the CDC (2012a), a number of foodborne outbreaks associated with fresh fruits and vegetables were linked to contaminated water. In 2002 and then again in 2005, during the investigation of two *Salmonella* outbreaks linked to tomatoes, after tracing the outbreaks back to Virginia, the outbreak serovar responsible in both outbreaks, *S.* Newport, was isolated from pond water used for irrigation (Greene et al., 2008). These two outbreaks and subsequent isolation of the *S.* Newport serovar from the same pond water three years apart shows that if the original source of contamination (e.g., the pond water) is not properly dealt with then the risk of outbreaks reoccurring increases substantially (Greene et al., 2008). This suggests that there may be an unknown number of *Salmonella* outbreaks that are due to contaminated water and are never reported because health officials cease investigations of outbreaks once a common food product has been identified, even though the food product itself may not be the original source of contamination. This represents an area of study that requires further investigation to fully understand the role of contaminated water in foodborne outbreaks.

3. Research Objectives

While many countries have established some guidelines for *Salmonella* in drinking water supplies, information and criteria for *Salmonella* in environmental waters are limited (Hsu et al., 2011). As incidences of waterborne and water-related *Salmonella* outbreaks increase, interest in pathogen detection and ecology in recreational, waste, and groundwater have grown. The current USEPA 1682 method for *Salmonella* detection (USEPA, 2006) for waste and surface waters in the US is based on most probable number (MPN) values obtained by enrichment of multiple

volumes/dilutions. This culture-based method is time consuming, labor intensive, and can be nonspecific for pathogenic *Salmonella* species, subtypes, and serovars without additional testing. Novel alternative methods that combine culture and genetic approaches that rapidly detect live organisms (results within 24 hours) are emerging with application to food but have not been evaluated for water. This study sought to determine whether a novel isothermal *Salmonella spp.* detection assay originally developed for food safety, which uses nicking enzyme amplification reaction (NEAR™) technology (Van Ness et al., 2003) allowing for rapid (<1 hr) total sample processing times and relatively low costs (~$10 /sample), is appropriate for the detection of *Salmonella spp.* in surface waters. This study also sought to determine whether this novel assay is comparable to the current culture-based USEPA 1682 method for *Salmonella* detection in surface and wastewaters. The causes of differences, if any, between the USEPA 1682 method and the novel isothermal *Salmonella spp.* detection assay are also of interest.

The objectives of this research were to:

- Compare the US EPA 1682 method and a novel isothermal method for *Salmonella spp.* detection in surface and wastewater.
    - Compare recovery efficiencies of the two methods in a paired set of sample matrices.
    - Determine the impact of the initial enrichment media being used in the USEPA 1682 method on detection of *Salmonella spp.* in water.
    - Determine the false positive and negative rates associated with the two methods based on genetic species identification in water samples.
- Determine the applicability of this new isothermal method in *Salmonella* detection in surface waters.

4. Methods of Detection

Salmonella detection methods are generally divided into two categories, culture-based and non-culture-based with many of these methods developed for the food industry first, and then later applied to environmental samples. While currently culture-based methods are preferred by regulatory agencies, due to their ability to show the presence of live possibly-infectious organisms, non-culture-based methods are of great interest to the water quality community due to their normally high specificity, rapid detection of *Salmonella* DNA targets, and their ability to process a much larger number of samples in a significantly smaller period of time. However, these non-culture-based methods do not allow for their users to claim any risk to human health based on their results because they cannot show any evidence that the *Salmonella* DNA that they are detecting is coming from live possibly-infectious organisms. It is precisely because of this that water quality regulatory agencies are unwilling to accept these methods results without evidence of live organisms. This need to show the presence of live organisms has led to the combination of culture-based and non-culture-based detection methods with an attempt to shorten the amount of time spent on culturing the organisms by testing suspected *Salmonella* isolates with molecular targets. For example, the ANSR™ assay for *Salmonella* detection developed by the Neogen Corp. utilizes a preenrichment step prior to analysis to allow for the growth of possible *Salmonella* isolates (Mozola et al., 2013). Nevertheless, if specific serovar identification is required, currently the only definitive way to identify all *Salmonella* serovars is through serotyping (Grimont and Weills, 2007).

4.1 Culture-based Detection of *Salmonella*

Culture-based detection methods for *Salmonella* all follow a general pattern for detection and identification. First samples are grown in a non-selective media (e.g., buffered peptone water

or trypticase soy broth). This is then followed by transferring the enrichment to one or more selective enrichment medias (e.g., selenite cysteine broth, RV10 broth, Hektoen enteric, *Salmonella-Shigella* agar, bismuth sulfite agar, phenol red-brilliant, Rappaport-Vassiliadis broth, modified semisolid Rappaport-Vassiliadis agar/broth, xylose lysine deoxycholate agar). After selection, isolates undergo biochemical screening (e.g., urea broth, lysine iron agar, triple sugar iron agar) and final typing using antisera to confirm specific serovars. If enumeration of samples is desired, a most probable number (MPN) approach is employed by running replicates of different sample volumes. The standard method for *Salmonella* detection in the US is the EPA 1682 method for *Salmonella* detection in biosolids (USEPA, 2006), which has recently been modified for use specifically in water, and is now the *EPA method 1200: Analytical Protocol for Non-Typhoidal Salmonella in Drinking and Surface Water* (USEPA, 2012). The other standard method for *Salmonella* detection, which is used in the food industry, but follows the same general protocol as the USEPA 1682 and 1200 methods is the ISO International Standard 6579 (2002). The USEPA 1682 method and the ISO International Standard 6579 are both regularly used for *Salmonella* detection in fresh and salt waters (Polo et al., 1999, Catalao Dionisio et al., 2000, Baudart et al., 2000, Martinez-Urtaza et al., 2004, Shellenbarger et al., 2008, Setti et al., 2009, Haley et al., 2009, Gorski et al., 2011). While these methods provide valuable information on the presence/absence, identities, and concentrations of viable and potentially infectious *Salmonella* spp., they also are both time-consuming and labor-intensive. Depending on the chosen selective medias and biochemical tests, running these methods can take up to a full 40 h week to achieve results. In the case of the USEPA 1682 method, specific species, subspecies and serovar identification is unachievable due to the use of only one polyvalent O antiserum as instructed in the method without the use of additional antisera or molecular identification. Even

so, these methods are often combined with molecular techniques to identify and characterize *Salmonella* isolates, such as in Walters et al.'s (2013) recent paper identifying *Salmonella enterica* diversity in central California coastal waterways and Palhares et al.'s (2014) paper examining *Salmonella* and antimicrobial resistance in an animal-based agricultural river system. When dealing with constantly changing environmental conditions, the amount of time required for running these methods is impractical if resampling is needed or risk-based decisions are being made.

4.2 Non-culture-based Detection of *Salmonella*

Non-culture-based detection methods for *Salmonella* present a much faster and less labor-intensive alternative to culture-based detection methods, but unless used in conjunction with culture-based methods, they are usually unable to determine if the organism(s) detected are alive or dead. Numerous non-culture-based methods for *Salmonella* detection are available with varying gene targets, and technologies with new methods continue to be investigated. There are endpoint PCR-based methods for a number of different gene targets including *invA*, which was utilized by Rahn et al. in 1992 for *Salmonella* detection in environmental samples (Ziemer and Steadham, 2003, Bonetta et al., 2011, Hsu et al., 2011). The 16s rDNA region is also being used as a gene target (Ziemer and Steadham, 2003, Lin et al., 2004) along with the *hilA gene* (Ziemer and Steadham, 2003), the enterotoxin gene *stx* (Ziemer and Steadham, 2003), repetitive DNA fragments (Ziemer and Steadham, 2003), a fur-regulated gene *iroB* (Ziemer and Steadham, 2003), and *Salmonella's* histidine transport operon (Ziemer and Steadham, 2003). Endpoint PCR is highly variable with its sensitivity to detect *Salmonella*, depending on the gene target chosen, the number of gene copies per cell, and whether or not a non-selective preenrichment step has been incorporated into the method's protocol. PCR with the *invA* gene has shown to have a

highly variable limit of detection, depending on the reaction conditions. After a non-selective preenrichment step is performed as low as 26 CFU/ml can be detected (Rahn et la., 1992, Fey et al., 2004, Mogandei et al., 2007).

Due to the complexity of environmental samples with diverse microbial communities endpoint PCR is often times supplemented by the incorporation of nested primer sets and multiplex PCR in order to target either multiple gene targets or multiple organisms of interest (Waage et al., 1999, Touron et al., 2005, Fang et al., 2012, Zhang et al., 2012, Xiao et al., 2013). Nested PCR has been shown to greatly increase the sensitivity of endpoint PCR allowing as low as 1 CFU/ml to be detected in environmental water samples with upwards of 10^3 CFU/ml of background microflora (Riyaz-Ul-Hassan et al., 2004).

Real-time quantitative PCR (qPCR) has been shown by Shannon et al. (2007) to detect as low as 100 fg of genomic DNA, or 22 gene copies based on a standard curve using *Aeromonas*. Klerks et al. (2004) also showed a quantifiable detection limit of 200 CFU/ml and a qualitative detection limit of 2 CFU/reaction using three different primer/probe sets for the genes *himA, invA, and spaQ* and an internal amplification control (IAC) targeting a green fluorescence protein (GFP)-coding sequence. Fey et al. (2004) also showed detection limits of 2 and 20 copies/reaction of DNA and RNA respectively, while using a SYBR Green rt-PCR from Qiagen (Hilden, Germany) using a specific 16s rRNA target and the RNA for *invA* as a control. More recently, rt-PCR has been used to targer the *bipA* gene with similar success to previous gene targets for *Salmonella* in water samples. Several commercial rt-PCR kits for *Salmonella* detection in food products have been released recently as well, and in 2012 were evaluated by Margot et al., who showed relatively consistent results with all seven kits which were evaluated with only four coming up with false- positive for some *Citrobacter* spp.

In 2003, Goodridge et al. compared a rapid MPN-based enzyme-linked immunosorbant assay (ELISA) method against a traditional MPN method and was able to show no significant difference between the two. In the last couple of years, reports of the possibility of using gold nanoparticles for electrochemical detection of *Salmonella* have been published, but as of yet have not been applied to water samples.

One of the most promising new non-culture-based technologies for environmental sample analysis and *Salmonella* detection specifically is next generation metagenomic sequencing. As these technologies continue to develop the analysis and identification of entire microbial communities down to species level or below will become more readily available. Li et al. (2012) have already used pyrosequencing with the gene targets *invA, iroB, hns, hisj, hila,* and *fimY* for the rapid identification of *Salmonella enterica.* Ong et al. (2013) recently used Illumina shotgun 16S rRNA sequencing on a diverse microbial community to identify twice as many species levels operational taxonomic units (OTUs) than previous protocols. As faster and more precise methods for *Salmonella* detection are developed, our ability to quickly identify potential risks to human health from the aquatic environment will also increase allowing for the reduction of illness from contaminated water sources.

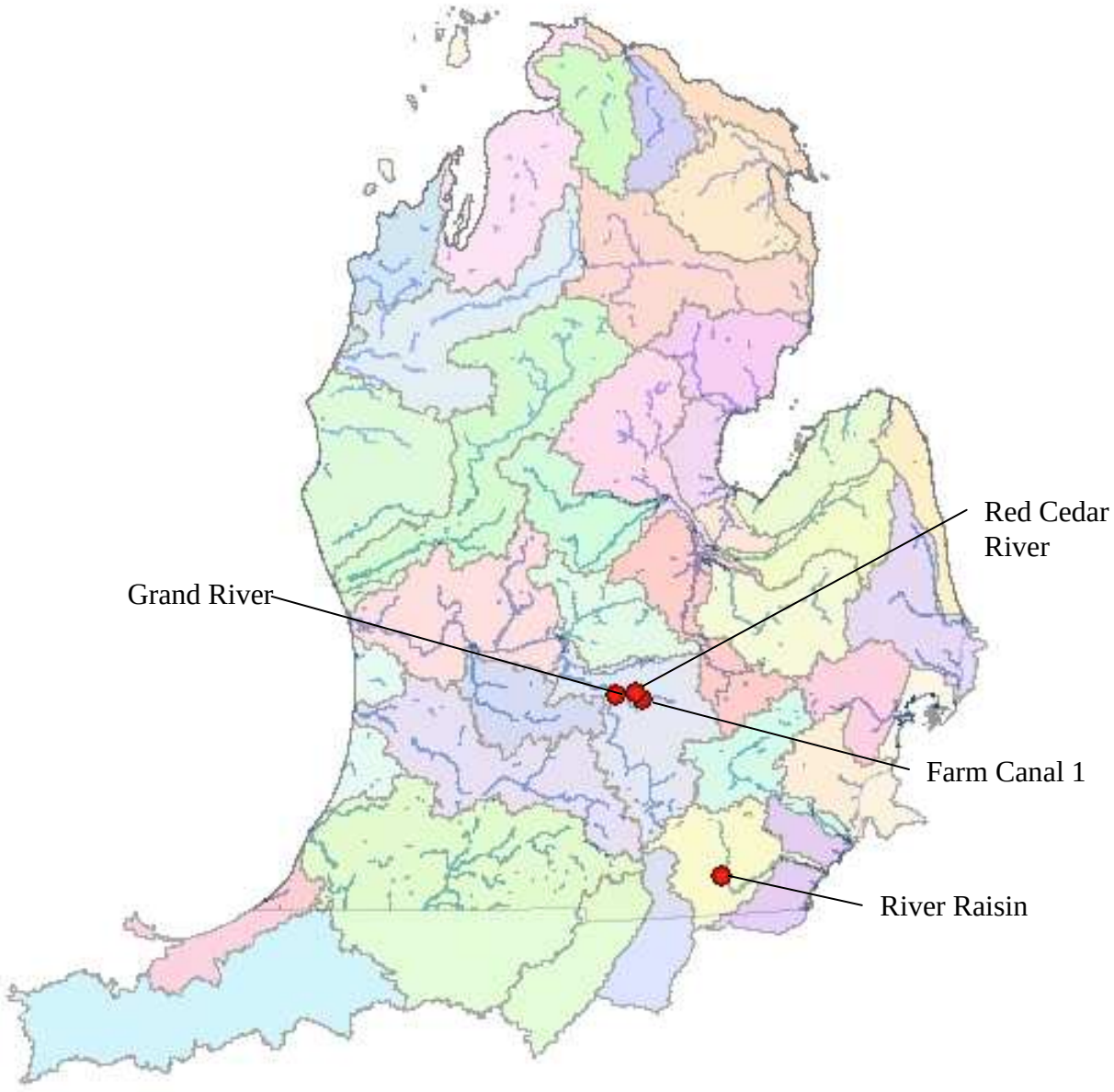
Chapter 2. Materials and Methods

1. Study Sites

1.1 Survey 1 Study Sites

The four surface water sites in this survey were chosen for their different characteristics including average flow rate, geographic locations within Michigan, watershed input, and the probable sources of fecal pollution input received. These sites included the Red Cedar River, the Grand River, the River Raisin, and a local farm canal. The Red Cedar River runs through urban and rural areas and eventually drains into the Grand River, which flows through a large city at the point of sample collection eventually emptying into Lake Michigan. The River Raisin flows through largely rural areas and empties into Lake Erie. The farm canal was isolated from any surface water input other than precipitation. Maps of the hydrology, major watersheds, and sampling locations were created using ArcGIS 9.3.1 (Figure 3).

Figure 3. Map of Survey 1 sampling sites



1.2 Survey 2 Study Sites

The 21 surface water sites in this survey were chosen from across Michigan and northern Ohio. These sites were chosen to represent the greatest diversity of flow rate, geographic location, watershed input, and probable sources of fecal pollution across the Michigan's Lower Peninsula and northern Ohio (Table 4). Maps of the hydrology, major watersheds, and sampling locations were created using ArcGIS 9.3.1 (Figure 4).

Figure 4. Map of Survey 2 sampling sites

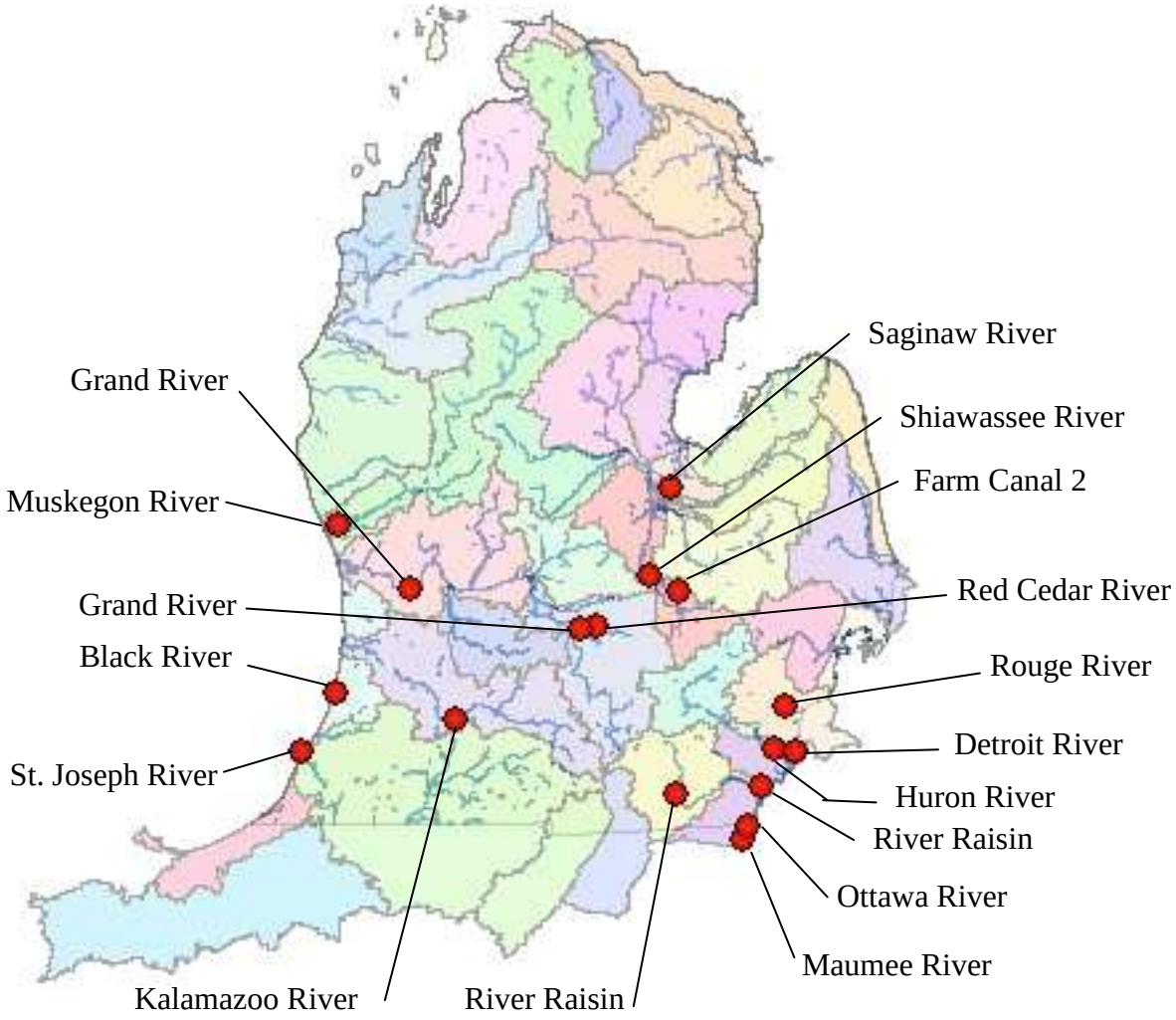


Table 4. Surface water sampling site conditions and watershed inputs

Sampling Site	Length (mi)	US Watersheds	Drainage area (mi^2)	Discharge (ft^3/s) on day of sampling	# of WWTPs discharging into surface water site	% of Rural land use in watershed	% Urban land use in Wateshed
Grand River (Lansing)	228	Upper Grand	780.24	1,150	5	87.81	8.80%
Grand River (Grand Rapids)	228	Lower Grand	5,096.44	10,100	5	84.55%	11.58%
Muskegon River	227.8	Muskegon	2,650.71	2,150	2	88.58%	11.58%
St. Joseph River	163.6	St. Joseph	4,710.38	3,960	3	89.93%	5.75%
Maumee River	137	Maumee	6,330.00	2,320	1	95.00%	3.00%
River Raisin (Adrian)	136.6	Raisin	442.11	367	3	90.26%	5.87%
River Raisin (Monroe)	136.6	Raisin	1,060.30	293	3	90.26%	5.87%
Kalamazoo River	128.1	Kalamazoo	1,021.44	1,200	1	88.44%	7.39%
Huron River	126.2	Huron	877	337	3	79.55%	11.71%
Shiawassee River	118.9	Shiawassee	508.04	429	4	90.01%	6.42%
Red Cedar River	50.4	Upper Grand	342.99	147	4	87.81	8.80%
Rouge River	47.4	Detroit	397.96	52	3	47.16%	48.79
Detroit River	28	Detroit, Lake St. Claire, Clinton River, Rouge River, Ecorse River	700	170,000	3	47.16%	48.79
Saginaw River	22	Saginaw	5,982.65	2,500	3	76.03%	19.18%
Black River	2.5	Black-Macatawa	283.61	103	2	85.76%	10.37%
Ottawa	20	Ottawa-Stony	153	15	N/A	87.58%	8.42%
Farm Canal 1	N/A	Upper Grand	N/A	N/A	0	87.81	8.80%
Farm Canal 2	N/A	Flint	N/A	N/A	0	84.00%	15.00%

Data Sources: (USGS, 2014, MI DEQ, 2014, OH EPA, 2014)

* This data was not available (N/A) for the specified

2. Samples

A total of 45 samples were collected from all study sites. The four sites for Survey 1 were each sampled six times over the course of four months during the fall of 2012. Samples for Survey 2 were collected only once from each site in order to facilitate the greatest diversity of samples available for analysis. Each sample consisted of 2, 1-L sample volumes collected by grab sampling at each site.

2.1 Sample Transport and Preparation

All samples were collected in sterile 1L sampling bottles and stored on ice during transport back to the laboratory. Once in the laboratory, samples were stored at 4°C until analysis, which began as soon as possible within 24 h after sample collection. Sample bottles were shaken thoroughly before analysis and 1L total was compiled from a mixture of the total 2L that was sampled. This was then composited and homogenized before analysis.

3. *Salmonella* Analysis

The two *Salmonella* detection protocols, using most probable number (MPN) enumeration, used in this study were a modified version of the USEPA 1682 method, and the ANSR™ Assay (USEPA, 2006, Mozola et al., 2013). Most probable number (MPN) enumeration is achieved through the use of qualitative presence/absence results from different dilutions or volumes of the sample in order to calculate out the most likely concentration of target organisms is present in the original sample volume. The USEPA 1682 method, consisting of primary enrichment in a non-selective broth followed by selective enrichment and characterization on selective indicator agar, was modified for the use in water by removing steps involved with biosolid dry weight analysis (USEPA, 2012). The ANSR™ Assay consisted of a single 24-h

nonselective enrichment step followed by cell lysis and immediate molecular analysis (Mozola et al., 2013). While most MPN protocols usually involve serial dilutions of a sample, our water samples were not diluted since *Salmonella* concentrations in environmental samples are very low. Instead, we analyzed five replicates of three undiluted sample volumes (20ml, 10ml, 1ml) for each sample resulting in 15 replicates for each sample. The theoretical detection range for our methods was 0.65 MPN/100ml to 161 MPN/100ml. Ongoing precision recovery samples, created using seeded laboratory reagent water, along with positive and negative controls were run through both protocols for each sample analysis batch.

3.1 USEPA 1682 method protocol

The three undiluted sample volumes (20ml, 10ml, 1ml) were transferred into sterile containers with Trypticase Soy Broth for primary enrichment. After incubation for 24h ± 2h at 36°C, six discrete drops of 30µl were applied equal distance apart on Modified Semisolid Rappaport-Vassiliadis (MSRV) medium. The inoculated MSRV plates were incubated for 18h ± 2h at 42°C, then two drops showing motility (a halo forming around the drop) were plated on separate xylose-lysine desoxycholate agar (XLD). After 24h ± 2h of incubation at 36°C, presumptive positive colonies were selected and transferred to lysine iron agar (LIA), triple sugar iron agar (TSI), and urea broth. After a final 24h ± 2h of incubation at 36°C, colonies from either LIA or TSI were tested against polyvalent O antiserum I and VI (BD, Franklin Lakes, New Jersey, USA), which detect the most common *Salmonella* serovars of concern, to confirm the presence of *Salmonella.*

3.2 ANSR™ Assay protocol

The three undiluted samples volumes (20ml, 10ml, 1ml) were transferred into sterile containers with Neogen's Enrichment Broth #3 (Neogen, Lansing, Michigan, USA) for primary

enrichment. All subsequent steps were performed per the manufacturer's directions. After incubation at 36°C for 24h ± 2h, 50µl of each replicate were placed in 1.2ml tubes for cell lysis. A total of 450µl of lysis buffer was added to each tube and then incubated at 80°C for 20 min. Prior to the addition of sample supernatant, tubes containing ANSR™ lyophilized reagent were preheated to 56°C for at least 3 min in the ANSR™ reader (Neogen, Lansing, Michigan, USA). After lysis, 50µl of the lysed sample's supernatant was transferred to tubes containing the ANSR™ lyophilized reagent, and homogenized. Reagent tubes were capped, the reader's lid closed, and assay run initiated. The presence/absence results for *Salmonella* were obtained for each replicate using fluorescent tags and enumerated using MPN methodology. Overall sampling handling time following enrichment for one sample consisting of the three undiluted sample volumes (20ml, 10ml, 1ml) and each of their five replicates was approximately 45 min compared to the approximately 40 hrs needed to run the USEPA 1682 method.

3.3 Recovery efficiency comparison

Volumes of 500ml were split from each sample during Survey 1 and were seeded with *Salmonella* Typhimurium ATCC#14028 for the determination of the recovery efficiencies of the USEPA 1682 method and the ANSR™ assay as described in USEPA 1682 method (USEPA, 2006). *Salmonella* Typhimurium ATCC#14028 cultures were grown overnight and enumerated on heart infusion agar (HIA). Samples were seeded with between $2.59x10^{-1}$ and $5.31x10^{-1}$ CFU/ml. Seeded samples were run through both protocols in order to compare the recovery efficiency of each. Laboratory reagent water was also seeded to determine the Ongoing Precision Recovery (OPR) for both the USEPA 1682 method and the ANSR™ assay in order to determine whether the two methods were performing correctly. Due to the lack of a defined acceptable

percent recovery for the ANSR™ assay, values defined by the USEPA for the USEPA 1682 method were used.

3.4 Initial nonselective enrichment media comparison

During Survey 2, a comparison of the effect, if any, of the initial nonselective enrichment media used with each assay was performed with naturally occurring bacteria. Samples cultured in each nonselective enrichment media (i.e. TSB and Enrichment Broth #3) were run through the USEPA 1682 method to determine if any statistically significant difference in the detection of *Salmonella* was occurring. A Fisher's Exact test for independence was performed to determine the statistical significance with a α-value of 0.05.

3.5 Independent molecular confirmation of Survey 2 samples

Since the ANSR™ assay is currently a novel technology for *Salmonella* detection and was not originally developed for environmental water analysis an independent molecular confirmation of its results was performed. This was necessary in order to strengthen our confidence in its results when detecting *Salmonella* in environmental samples in which we did not have a known concentration of our target organism.

3.5.1 Sample preparation and storage for independent molecular confirmation

After initial enrichment during Survey 2, 10ml of each sample replicate were aliquoted for later DNA extraction. The 10ml aliquots were centrifuged at 4500xg (4500rcf) for 15 minutes. The supernatant was then removed by aspiration. The remaining cell pellet for each replicate were then resuspended in 1ml of sterile laboratory reagent water and stored at -80°C until DNA extraction was performed.

3.5.2 DNA extraction

DNA extraction was performed using Qiagen's QIAmp DNA Mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. In a 1.5ml microcentrifuge tube, 20 µl of proteinase k, 200 µl of lysing buffer, and 200µl of sample were added. The microcentrifuge tube was then incubated at 56°C for 10 min in a waterbath. After incubation, the microcentrifuge tubes were centrifuged briefly and then 200 µl of 100% ethanol were added to the sample. This was then mixed by pulse-vortexing for 15 s and then briefly centrifuged to remove any remaining droplets of sample from the inside of the tube's lid. This mixture was then pipetted into a QIAmp spin column, and centrifuged at 6000 x g for 1 min. The column was then placed into a clean collection tube, while the filtrate was discarded. Next, 500µl of a wash buffer was added to the column and centrifuged at 6000 x g for 1 min. This filtrate was again discarded and the column was placed into a clean collection tube where 500 µl of a second wash buffer was added to the column and centrifuged at 20,000 x g for 3 min. Finally the column was placed into a sterile microcentrifuge tube, and was eluted with 200 µl of AE buffer by centrifugation at 6000 x g for 1 min after a 5 min incubation at room temperature. After extraction, DNA samples were stored at -20°C until analysis.

3.5.3 Molecular detection assay

DNA samples were analyzed for the presence of *Salmonella* using a proprietary custom microbial DNA qPCR array from Qiagen (Qiagen, Hilden, Germany). The qPCR array targeted the 16s rDNA specific for *Salmonella enterica* (NCBI Tax ID # 28901). The array also included a proprietary 16s rDNA target for general bacterial detection along with a positive PCR control, and a no template PCR control for confirmation of the presence of 16s rDNA, that the assay was working correctly, and the ct value cutoff for positive sample identification respectively. Samples were transported on ice to National Sanitation Foundation International's (NSF Int.)

world headquarters in Ann Arbor, Michigan for testing. Samples were run on the array as per the manufacturer's instructions. All preparation of the 96 well array plates was performed in the dark in order to avoid inactivating the mastermix. Array plates and Microbial qPCR mastermix (Qiagen, Hilden, Germany) were removed from -20°C freezer and allowed to thaw prior to the addition of reagents. The Microbial qPCR mastermix (Qiagen, Hilden, Germany) remained covered until its addition to the array plate. Each well received 14 µl of mastermix, 6 µl of DNAse-free water, and 5 µl of template. Wells were then sealed with optical thin wall 8-cap strips, and the array plate was placed into the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). Cycling protocol for qPCR is described in Table 5.

Table 5. Microbial DNA qPCR array cycling protocol for use with Qiagen's Microbial DNA qPCR custom array kit

Step	Time	Temperature	Cycles
Initial PCR activation	10 min	95°C	1
Denaturation	15 sec	95°C	40
	Slow ramp rate at 1.0°C per sec		
Annealing and Extension	2 min	60.0°C	
Plate Read			

A minimum threshold value of 10^2, and a cutoff ct value of 35 was used to determine whether a sample was considered positive.

3.6 Statistical Analysis

All statistical analysis was performed using R statistical software (R Core Team, 2012). For all descriptive statistics including log transformed data in figures, samples with no detectable levels of *Salmonella* were assigned a value of 0.325 MPN/100ml (half the lower detection limit) and samples, which contained levels of *Salmonella* above the upper detection limit, were assigned a value of 160.9 MPN/100ml in order to normalize and make figures more readable

with a y-intercept at 0. Following log transformed MPN/100ml, samples were plotted with 95% confidence intervals to examine any observable differences.

Fisher's Exact test for independence, which can be performed on smaller sample sizes, was chosen for analysis of the two methods' presence/absence data due to the restrictions for use of the Pearson's chi-squared test. Fisher's exact test is used to examine contingency between two categories. It assumes a null hypothesis of independence, so any p-value below $\alpha = 0.05$ indicates that the two categories' test results (e.g., presence/absence of *Salmonella*) are not significantly different from each other.

A Mann-Whitney U test was used to compare the concentrations of *Salmonella* detected by each method. This test allows for fewer assumptions to be made about the data, including normal distribution, and is an alternative to the paired Student's t-test with greater efficiency on non-normal distributions. This test allows for the assessment of paired populations by determining if their population mean ranks differ significantly. It assumes a null hypothesis that the two populations are the same. Thus any p-value that fails is above an alpha of 0.05 indicates that the two populations are not significantly different.

The USEPA 1682 method was used as our "gold standard" method in order to determine the sensitivity, specificity, precision, and accuracy of the ANSR™ assay, while the results obtained from qPCR were used to confirm the calculations based on the USEPA 1682 method and provide a measure to determine the USEPA 1682 method's sensitivity, specificity, etc. The following formulas were used:

$$Sensitivity\ (True\ Positive\ Rate) = \frac{True\ Positives\ (TP)}{\left(True\ Positives\ (TP) + False\ Negatives\ (FN)\right)}$$

$$Specificity\ (True\ Negative\ Rate) = \frac{True\ Negatives\ (TN)}{(False\ Positives\ (FP) + True\ Negatives(TN))}$$

$$False\ Positive\ Rate\ (FPR) = \frac{FP}{(FP + TN)}$$

$$False\ Negative\ Rate\ (FNR) = 1 - Sensitivity\ (True\ Positive\ Rate)$$

$$Accuracy = \frac{(TP + TN)}{(Positives + Negatives)}$$

$$Precision = \frac{TP}{(TP + FP)}$$

Chapter 3. Results

1. Survey 1 Results

1.1 Naturally occurring *Salmonella*

Salmonella was found in 21% (N = 5) of the total N = 24 samples with an average of 0.99 MPN/100ml using the USEPA 1682 assay (Table 6.). The ANSR™ assay detected *Salmonella* in 75% (N=18) of the total samples with an average of 3.77 MPN/100ml (Table 6). Sample MPN/100ml values ranged from < 0.65 to 2.90 MPN/100ml with the USEPA 1682 assay and < 0.65 to 15.29 MPN/100ml with the ANSR™ assay (Table 6). For the USEPA 1682 method, Table 7 shows each sampling site's contribution to the total number of samples collected, the number of positive samples per site, and percent of positive samples for each site. Using the USEPA 1682 assay, *Salmonella* was detected in 33.33% of the samples taken from the River Raisin during Survey 1. Only 16.67% of the samples taken from the Grand River using the USEPA 1682 assay had detectable levels of *Salmonella.* The Red Cedar River and Farm Canal 1 also detected *Salmonella* with the USEPA 1682 method in only 16.67% of each of their samples.

Table 6. Percent positive and MPN/100ml levels for naturally occurring *Salmonella* in surface water for the USEPA 1682 method and ANSR™ assay in Survey 1.

	EPA 1682	ANSR™ assay
Total % Positive (N =24)	21% (N=5)	75% (N=18)
Average (MPN/100ml)*	0.99	3.77
MPN Range (MPN/100ml)	<0.65§ – 2.90	<0.65§ – 15.29

* = Average MPN/100ml value calculated without the inclusion of Non-detect values
§ = <0.65 MPN/100ml Represents the lower detection limit for the MPN calculations

Table 7. Positive samples of naturally occurring *Salmonella* in surface water for the USEPA 1682 method in Survey 1.

	River Raisin	Grand River	Red Cedar River	Farm Canal 1
Samples	6	6	6	6
Positive Samples (N)	2	1	1	1
% Positive at site	33.33%	16.67%	16.67%	16.67%
Total % Positive (N=24)	8.33%	4.17%	4.17%	4.17%

The ANSR™ assay showed considerably higher levels of detection at each site, as seen in Table 8. *Salmonella* was detected in 83.33% of samples collected from the River Raisin and 100% of the samples collected from the Grand River using this assay. The Red Cedar River showed detectable levels of *Salmonella* from 50% of its samples, and Farm Canal 1 had 83.33% of its samples also showing detectable levels of *Salmonella.* The specific samples and their positive results for the naturally occurring *Salmonella* can be found in Table 9.

Table 8. Positive samples of naturally occurring *Salmonella* in surface water for the ANSR™ assay in Survey 1

	River Raisin	Grand River	Red Cedar River	Farm Canal 1
Samples	6	6	6	6
Positive Samples (N)	5	6	3	5
% Positive at site	83.33%	100.00%	50.00%	83.33%
Total % Positive (N=24)	20.83%	25.00%	12.50%	20.83%

Table 9. Naturally occurring and seeded samples found positive for *Salmonella* by the USEPA 1682 method and the ANSR™ assay during Survey 1.

	Naturally Occurring		Seeded**	
	USEPA 1682	**ANSR™**	**USEPA 1682**	**ANSR™**
River Raisin				
1*	—	+	+	+
2	—	—	+	+
3	+	+	+	+
4	—	—	+	+
5	—	+	+	+
6	+	+	+	+
Grand River				
1	+	+	+	+
2	—	+	+	+
3	—	+	+	+
4	—	+	+	+
5	—	+	+	+
6	—	+	+	+
Red Cedar River				
1	+	+	+	+
2	—	—	+	+
3	—	+	+	+
4	—	—	+	+
5	—	+	+	+
6	—	—	+	+
Farm Canal 1				
1	—	+	+	+
2	—	—	+	+
3	+	+	+	+
4	—	+	+	+
5	—	+	+	+
6	—	+	+	+

* Numbers correspond to sampling dates, 1 - 10/15/12, 2 - 10/22/12, 3 - 11/5/12, 4 - 11/12/12, 5 - 11/26/12, 6 - 12/17/12

** 100% of seeded samples were found positive by both methods. This was expected as high concentrations of the bacteria were seeded into the surface water samples for recovery analysis.

All samples found positive (N=5) for *Salmonella* using the USEPA 1682 assay were also found to be positive with the ANSR™ assay, however there were 13 samples negative by the USEPA 1682 method that were positive by the ANSR™ assay. Table 10 shows the five samples found positive with both assays along with their corresponding MPN/100ml values. With the exception of the River Raisin sample 6 (12/17/12), the concentrations were not noticeably different.

Table 10. MPN/100ml levels for *Salmonella* in water samples found to be positive by both the USEPA 1682 method and the ANSR™ assay during Survey 1.

	EPA 1682 method	ANSR™ assay
(1)* Grand River (MPN/100ml)	0.67	0.67
(1)* Red Cedar (MPN/100ml)	2.55	2.34
(3)* River Raisin (MPN/100ml)	0.72	1.44
(3)* Farm Canal (MPN/100ml)	2.9	0.72
(6)* River Raisin (MPN/100ml)	0.67	8.22

* Numbers correspond to sampling dates, 1 - 10/15/12, 2 - 10/22/12, 3 - 11/5/12, 4 - 11/12/12, 5 - 11/26/12, 6 - 12/17/12

Specific MPN/100ml values for each sample can be found in Table 11. Samples not found to be positive for *Salmonella* were listed as below the detection limit (DL) for both assays (<0.65 MPN/100ml).

Table 11. Survey 1. MPN/100ml values for naturally occuring *Salmonella* in surface water samples

	Sampling Date					
USEPA 1682 method	**1[a]**	**2**	**3**	**4**	**5**	**6**
Sampling Site						
River Raisin	<0.65*	<0.65	0.720	<0.65	<0.65	0.670
Grand River	0.670	<0.65	<0.65	<0.65	<0.65	<0.65
Red Cedar River	2.550	<0.65	<0.65	<0.65	<0.65	<0.65
Farm Canal 1	<0.65	<0.65	2.900	<0.65	<0.65	<0.65
ANSR™ assay	**1**	**2**	**3**	**4**	**5**	**6**
Sampling Site						
River Raisin	0.720	<0.65	1.440	<0.65	10.860	8.220
Grand River	0.670	0.720	0.650	6.370	2.260	15.290
Red Cedar River	2.340	<0.65	0.720	<0.65	3.300	<0.65
Farm Canal 1	0.650	<0.65	0.720	2.020	6.930	3.930

a Numbers correspond to sampling dates, 1 - 10/15/12, 2 - 10/22/12, 3 - 11/5/12, 4 - 11/12/12, 5 - 11/26/12, 6 - 12/17/12

* The lower detection limit of <0.65 MPN/100ml

Log transformed *Salmonella* MPN/100ml values for each site obtained with the USEPA 1682 method and with the ANSR™ assay can be found in Figures 5 – 8 and Figures 9 – 12, respectively. Average MPN/100ml values that have been log transformed for both detection methods can be seen in Figures 13 and 14. Note that all data were log transformed after adding 0.325 (half the lower detection limit) to samples without detectable levels of *Salmonella*.

Figure 5. Log transformed MPN/100ml values for River Raisin that were obtained from the USEPA 1682 method in Survey 1

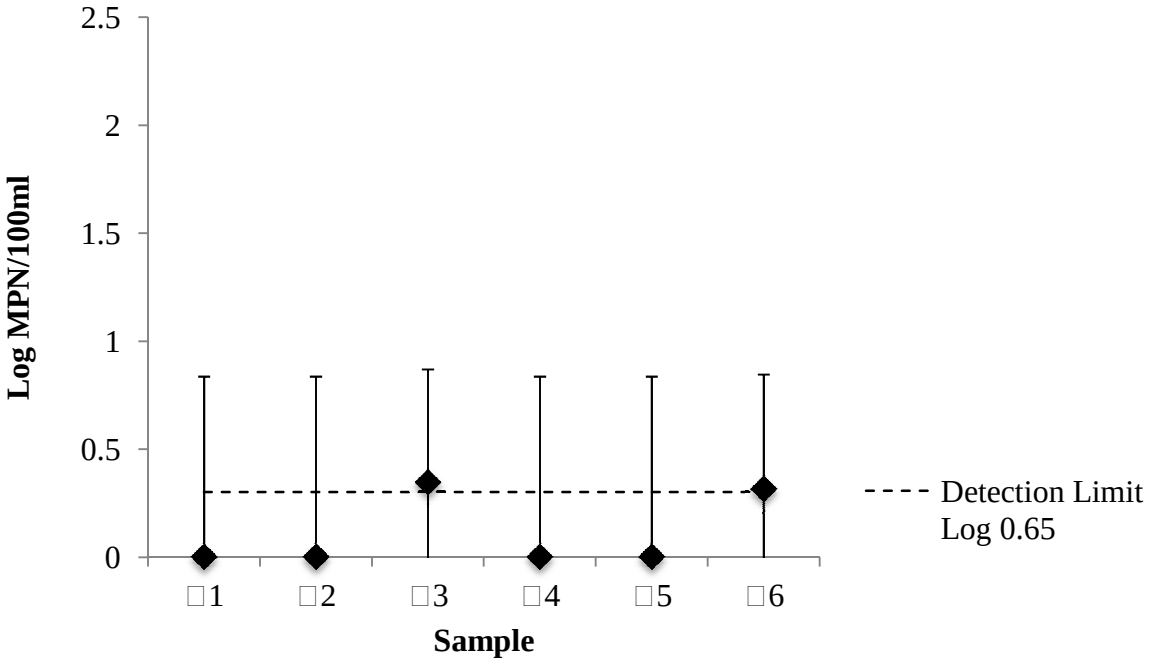


Figure 6. Log transformed MPN/100ml values for Grand River that were obtained from the USEPA 1682 method in Survey 1

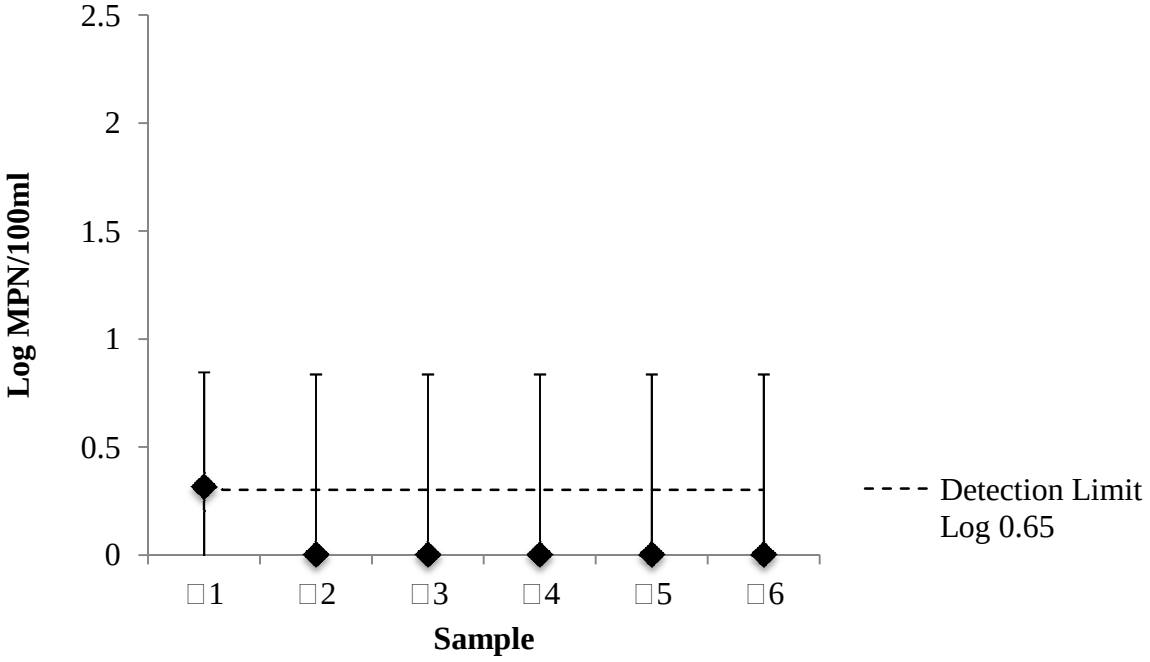


Figure 7. Log transformed MPN/100ml values for Red Cedar River that were obtained from the USEPA 1682 method in Survey 1

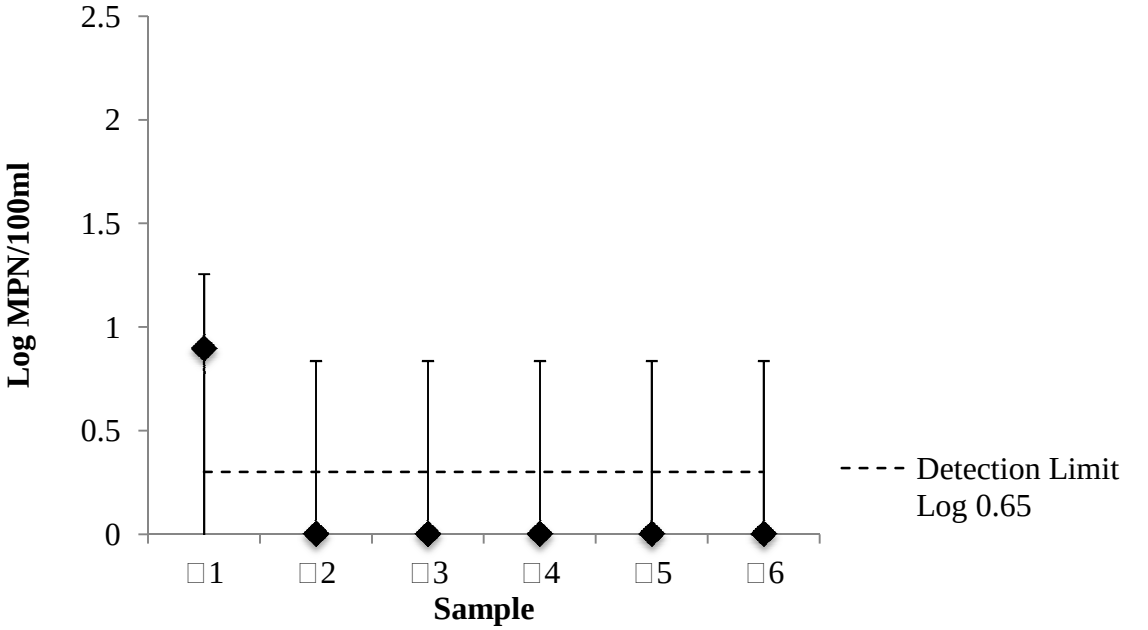


Figure 8. Log transformed MPN/100ml values for Farm Canal 1. that were obtained from the USEPA 1682 method in Survey 1

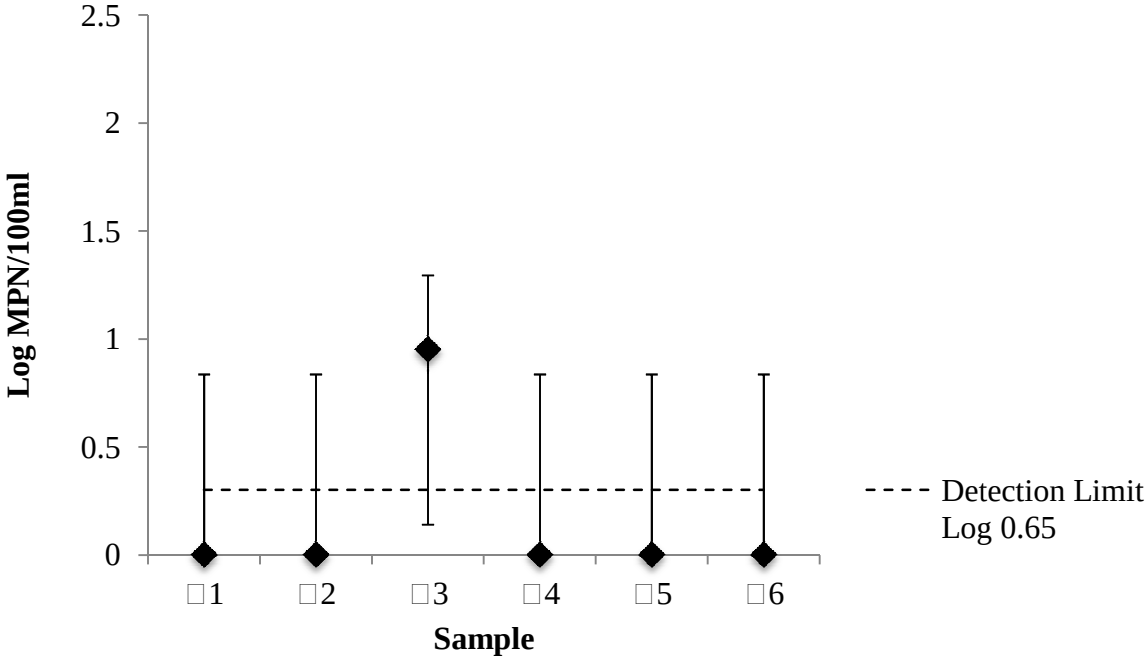


Figure 9. Log transformed MPN/100ml values for River Raisin that were obtained from the ANSR™ assay in Survey 1

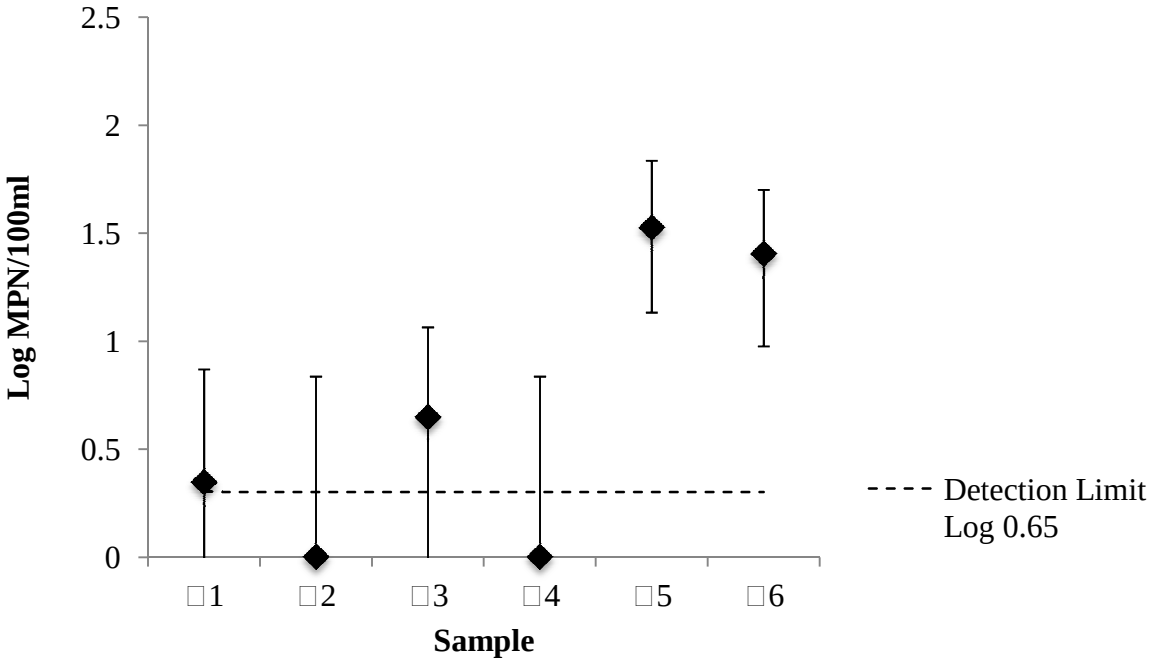


Figure 10. Log transformed MPN/100ml values for Grand River that were obtained from the ANSR™ assay in Survey 1

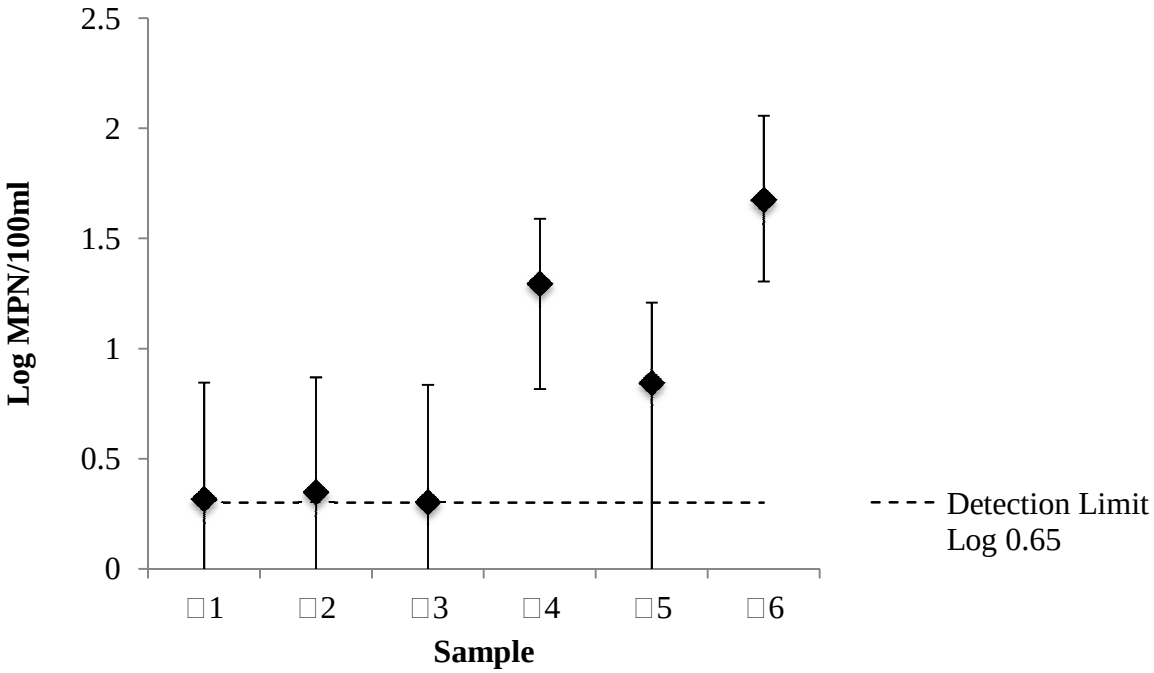


Figure 11. Log transformed MPN/100ml values for Red Cedar River that were obtained from the ANSR™ assay in Survey 1

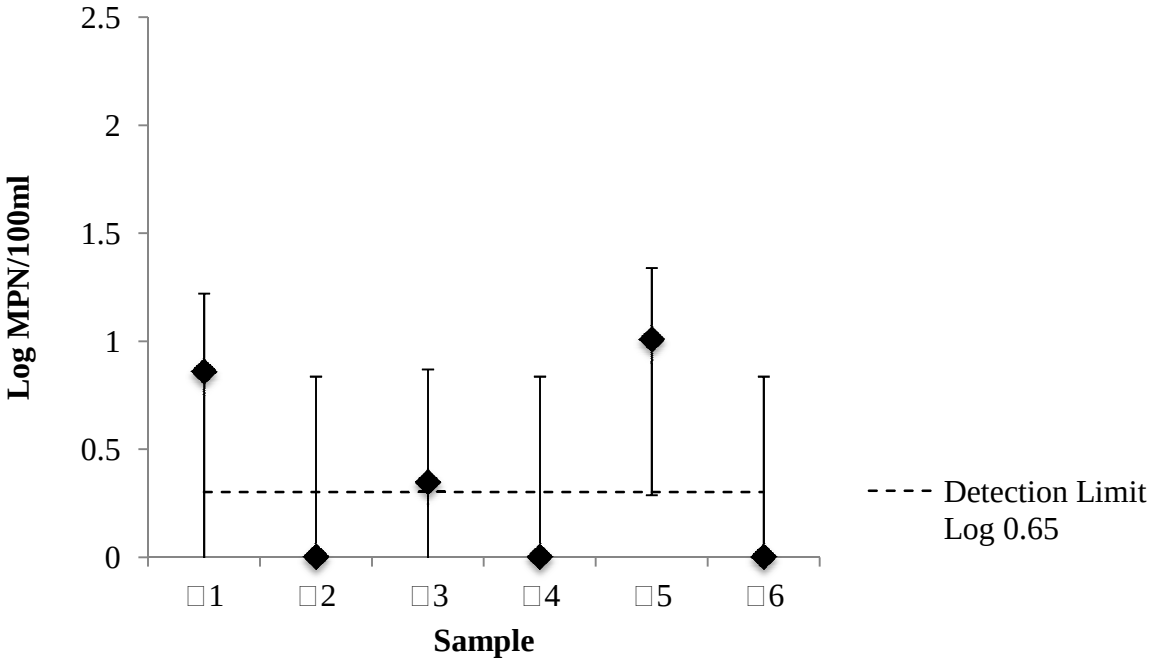


Figure 12. Log transformed MPN/100ml values for Farm Canal 1 that were obtained from the ANSR™ assay in Survey 1

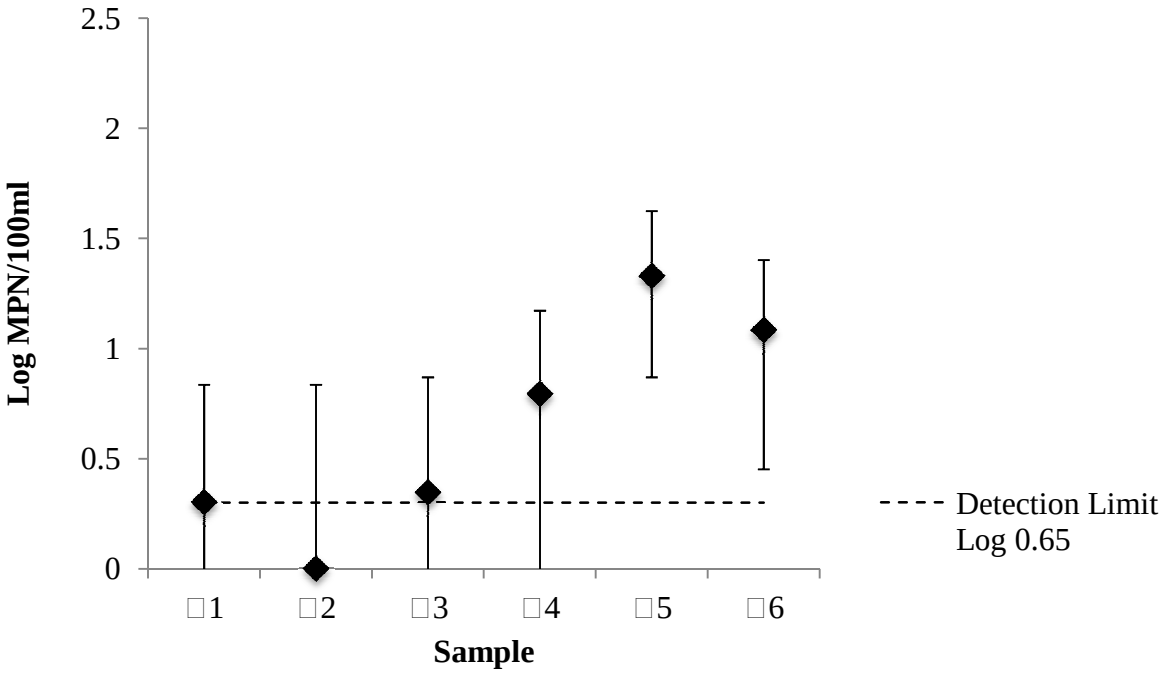


Figure 13. Log transformed average MPN/100ml values for each sampling site obtained from the USEPA 1682 method in Survey 1

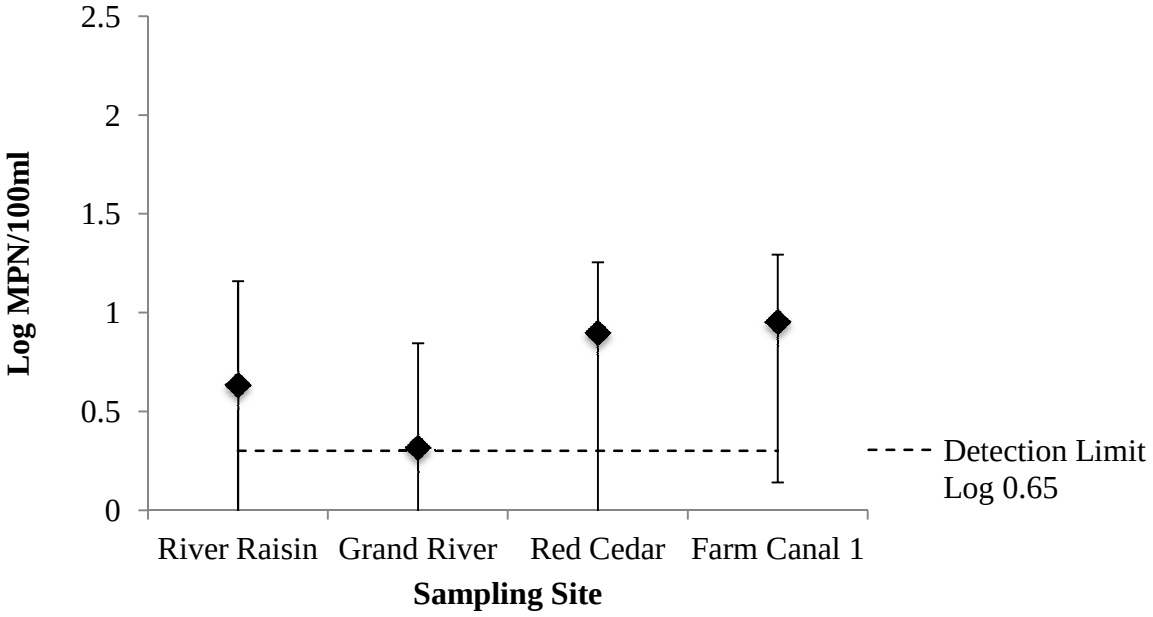


Figure 14. Log transformed average MPN/100ml values for each sampling site obtained from the ANSR™ assay in Survey 1

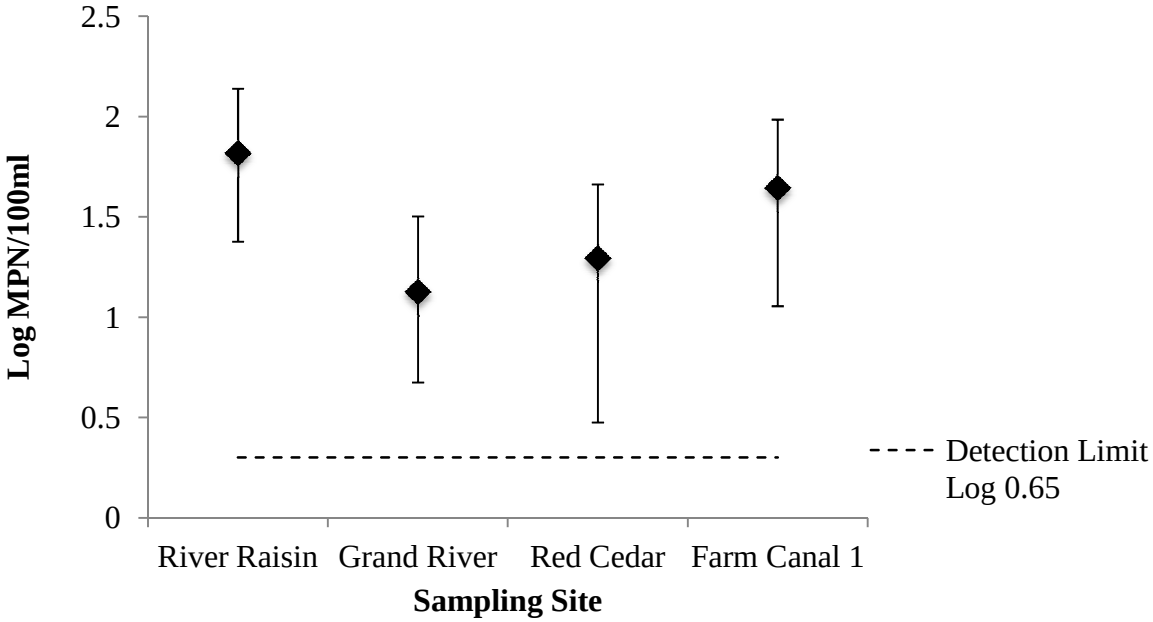


Figure 15 shows a comparison of the average log transformed MPN/100ml values for *Salmonella* using both the USEPA 1682 method and the ANSR™ assay in Survey 1 where six samples were collected from each of the four surface water site for a total of N = 24 samples. *Salmonella* concentrations in MPN/100ml were log transformed in the same manner as mentioned above. Figures 16 – 19 show a comparison of the log transformed concentrations of *Salmonella* detected by both methods at each site, with non-overlapping 95% confidence intervals. The two methods were found to be significantly different by a Mann-Whitney U test (p-value 0.003, α=0.05).

Figure 15. Comparison of log transformed average MPN/100ml values for *Salmonella* using the USEPA 1682 method and the ANSR™ assay in Survey 1

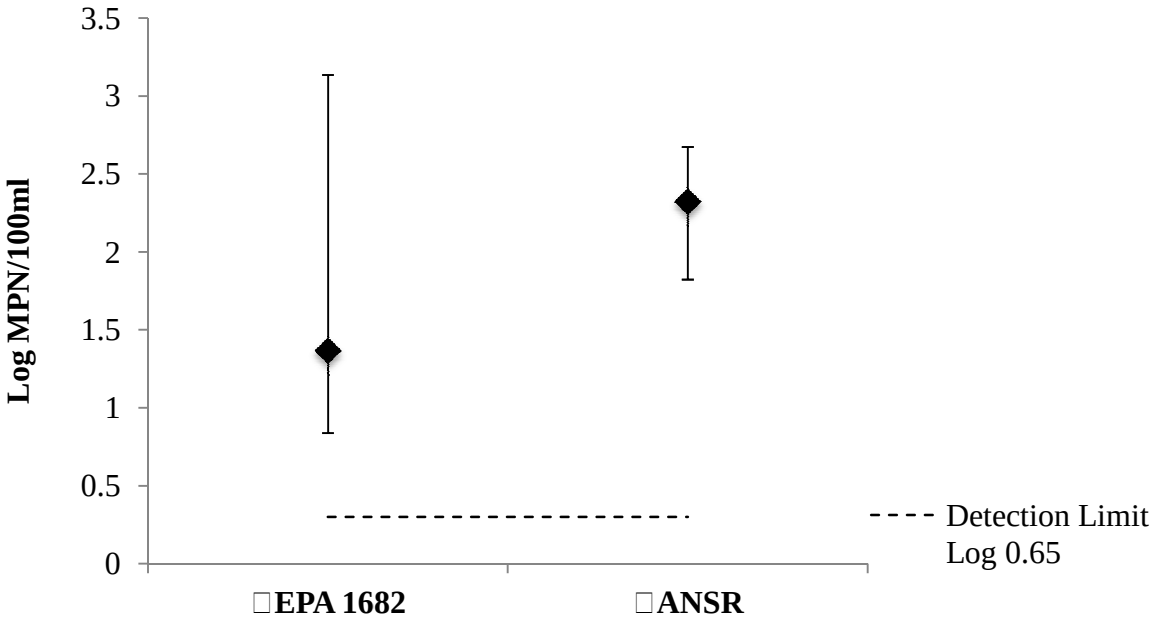


Figure 16. Comparison of Log transformed MPN/100ml values of *Salmonella* for River Raisin using the USEPA 1682 method and the ANSR™ assay in Survey 1

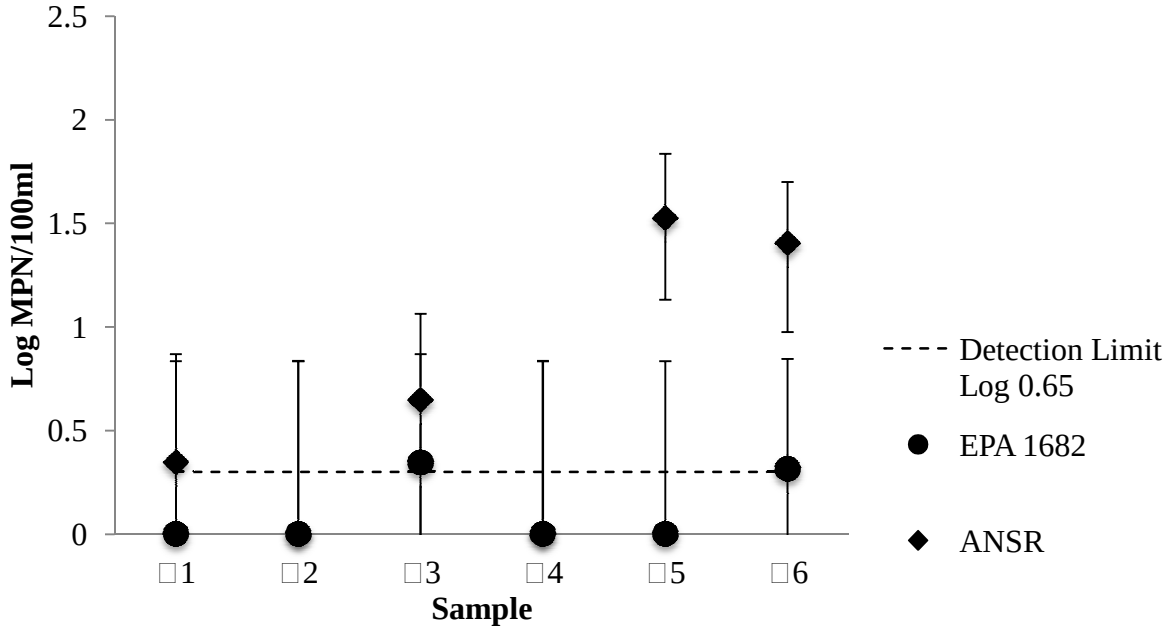


Figure 17. Comparison of Log transformed MPN/100ml values of *Salmonella* for Grand River using the USEPA 1682 method and the ANSR™ assay in Survey 1

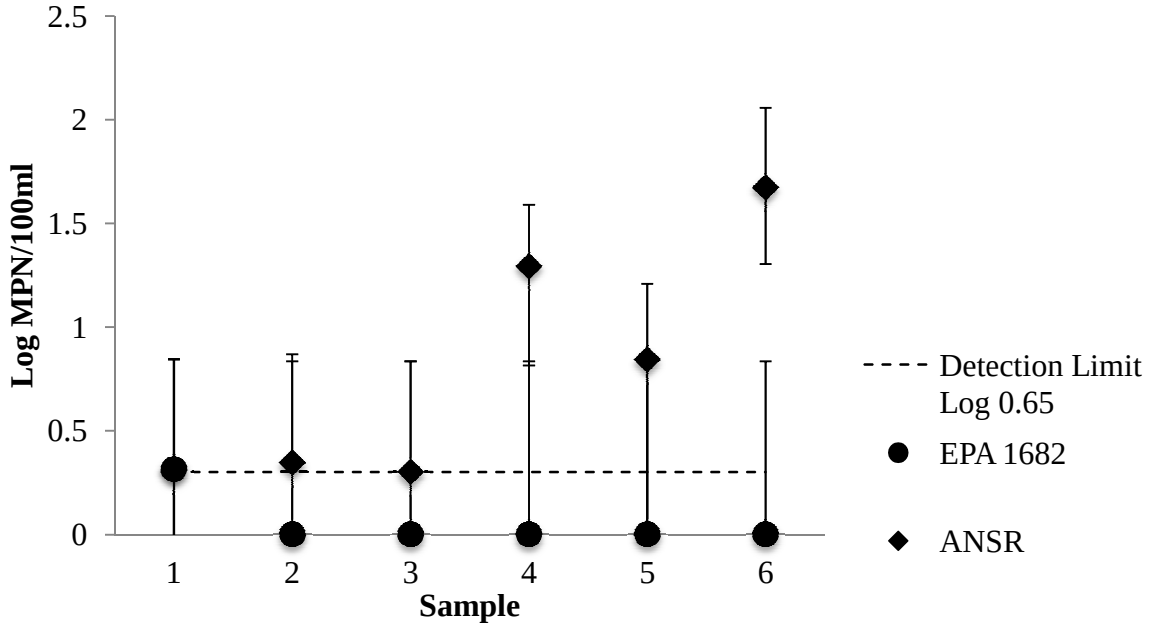


Figure 18. Comparison of Log transformed MPN/100ml values of *Salmonella* for Red Cedar River using the USEPA 1682 method and the ANSR™ assay in Survey 1

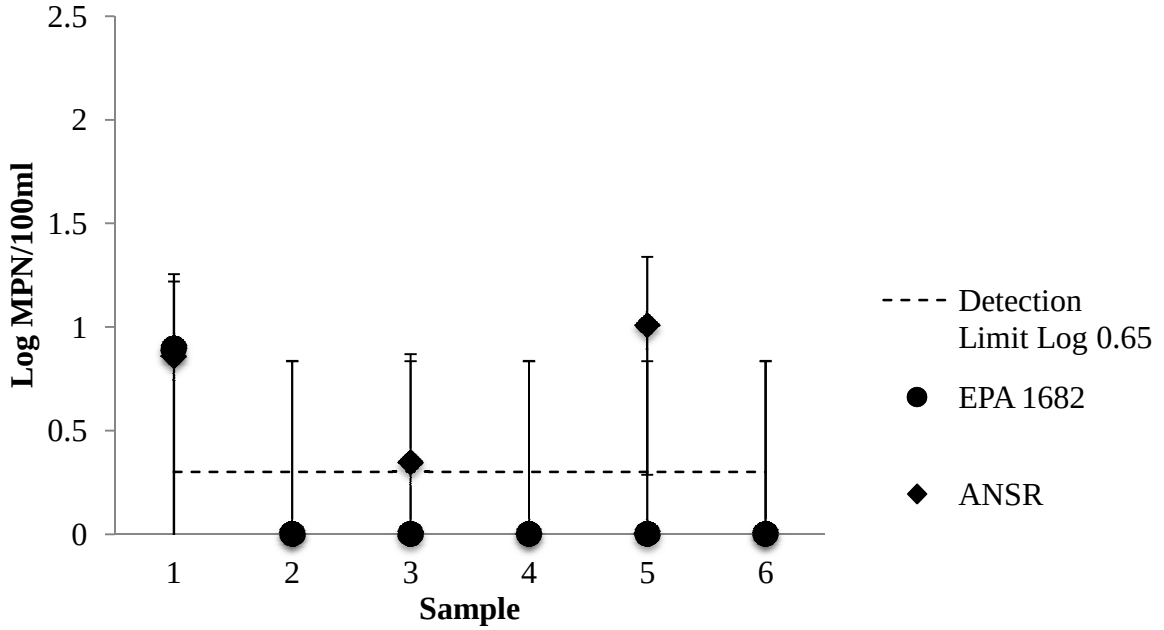
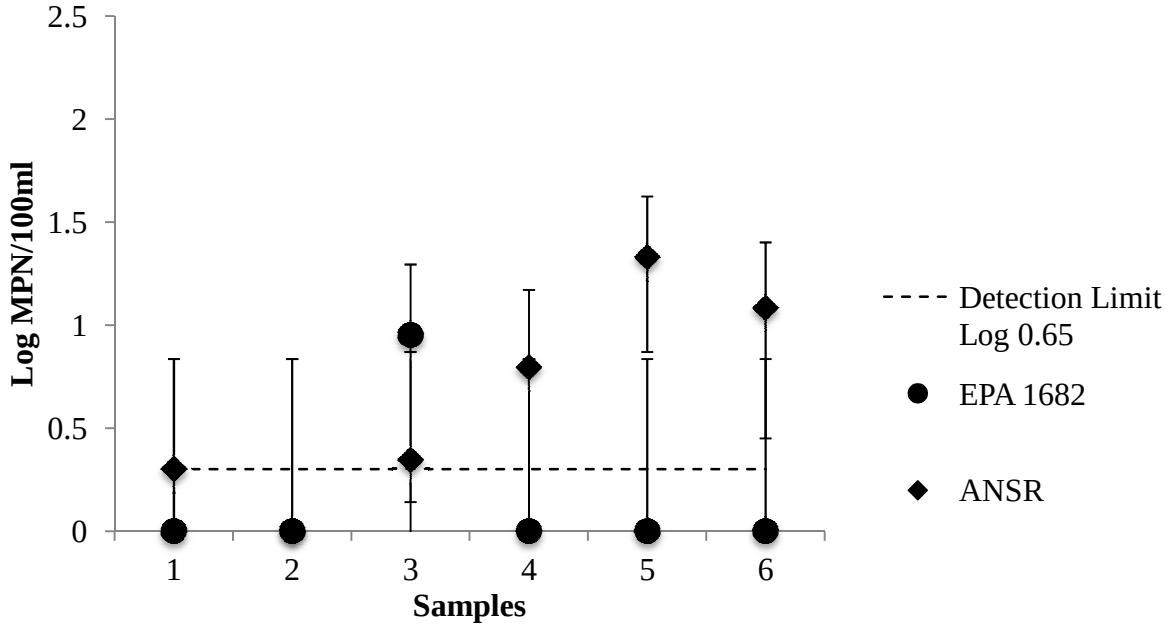
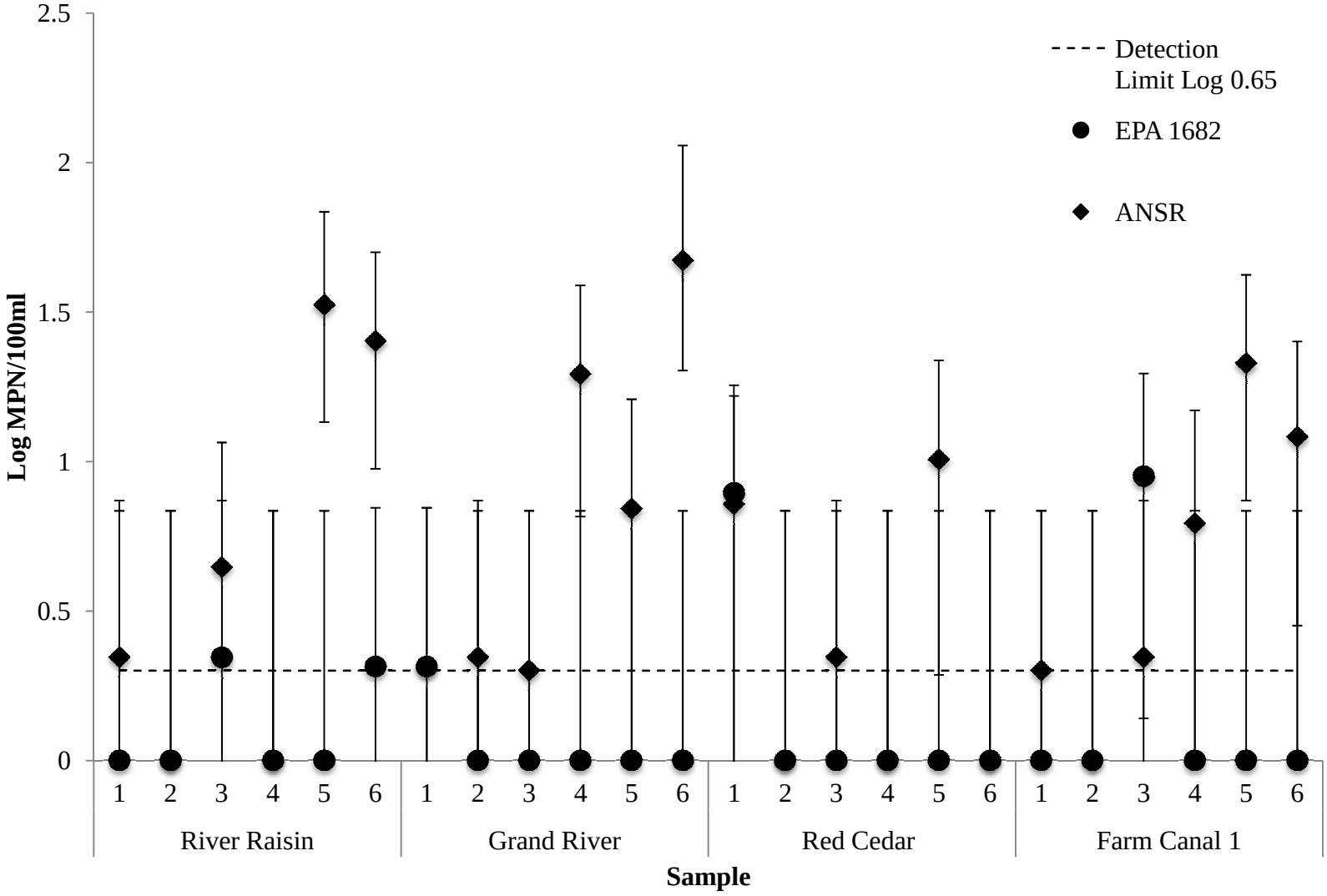


Figure 19. Comparison of Log transformed MPN/100ml values of *Salmonella* for Farm Canal 1. using the USEPA 1682 method and the ANSR™ assay in Survey 1



The combined results of all samples tested during Survey 1 can be seen in Figure 20, which shows only four (River Raisin samples 5 and 6, Grand River sample 6, and Farm Canal sample 5) out of the 24 samples taken having notable differences in the *Salmonella* concentrations when comparing the USEPA 1682 method and the ANSR™ assays.

Figure 20. Log transformed MPN/100ml values for naturally occuring *Salmonella* for all samples examined during Survey 1



1.2 Recovery efficiency comparison

Samples, which were seeded with *Salmonella* Typhimurium (ATCC#14028) in order to determine the recovery efficiency for each of the N = 24 samples collected during Survey 1, had MPN/100ml values ranging from 4.13 to 160.90 MPN/100ml for both the USEPA 1682 method and the ANSR™ assay (Table 12). Using a Mann-Whitney U test with seeded *Salmonella* sample concentrations the USEPA 1682 method and the ANSR™ assay were found to be not significantly different (p-value of 0.2467, $\alpha = 0.05$). The USEPA 1682 method's maximum and minimum percent recoveries recorded during the Survey 1 study were 251.21% and 11.73% respectively and for the ANSR™ assay they were 620.78% and 0% respectively. The average percent recovery for the USEPA 1682 method for each sampling site ranged from 53.16% at the Red Cedar River to 87.65% at the River Raisin during Survey 1 (Table 12). The average percent recovery for the ANSR™ assay for each sampling site ranged from 47.57% at the Red Cedar River to 288.88% at Farm Canal 1 (Table 12). Of the total N=24 samples taken during this survey, only one sample from the USEPA 1682 method had a percent recovery outside of the acceptable range (0 – 246%) as defined by the USEPA 1682 method, while the ANSR™ assay had seven samples out of this range (Table 12). Since the Ongoing Precision Recovery samples (OPR) (recovery efficiency samples using seeded laboratory reagent water) for both the USEPA 1682 method and the ANSR™ assay had percent recoveries all within the USEPA 1682 method's acceptable range (0 – 246%) it is suggested that the samples whose percent recoveries fall outside of the acceptable range are yielding poor results due to a matrix interference effect (e.g., environmental contamination inhibiting the growth and recovery of *Salmonella*).

Table 12. Recovery efficiency results for the USEPA 1682 method and the ANSR™ assay during Survey 1.

Site	Sample #*	Seeded Concentration (CFU/100ml)	USEPA 1682 method			ANSR™ assay		
			Final Concentration (MPN/100ml)	% Recovery**	Average % Recovery at each site	Final Concentration (MPN/100ml)	% Recovery**	Average % Recovery at each site
River Raisin								
	1	45.60	91.78	76.25		34.77	74.67	
	2	28.50	11.81	40.25		91.78	320.52 §	
	3	29.10	4.13	11.73	87.65	10.12	29.86	270.26
	4	25.90	23.98	91.45		160.90	620.78 §	
	5	36.40	91.78	251.25 §		160.90	412.20 §	
	6	28.10	16.14	54.99		54.22	163.51	
Grand River								
	1	45.60	34.77	74.78		91.78	199.80	
	2	28.50	19.07	65.70		15.71	52.54	
	3	29.10	19.07	64.49	73.72	11.81	38.39	99.74
	4	25.90	12.27	46.18		6.36	0.00	
	5	36.40	54.22	148.06		15.29	35.80	
	6	28.10	12.45	43.10		91.78	271.88 §	
Red Cedar River								
	1	45.60	34.77	70.66		12.27	21.78	
	2	28.50	34.77	120.72		19.07	65.70	
	3	29.10	11.51	38.48	53.16	13.04	42.39	47.57
	4	25.90	7.97	29.56		5.01	18.11	
	5	36.40	15.24	40.98		17.07	37.83	
	6	28.10	5.55	18.57		28.34	99.58	

Table 12. (cont'd)

Farm Canal 1.							
	1	45.60	34.77	75.54		91.78	199.85
	2	28.50	12.68	43.30		91.78	320.52 §
	3	29.10	34.77	109.64	86.73	10.12	32.34
	4	25.90	54.22	208.36		160.90	614.23 §
	5	36.40	23.98	64.99		9.98	8.38
	6	28.10	5.55	18.57		160.90	557.95 §
OPR Samples ¶							
	1	45.6	91.78	201.27		34.77	76.25
	2	28.5	34.77	121.86		23.19	81.27
	3	29.10	6.81	23.43		34.77	119.62
	4	25.90	9.98	38.58		54.22	209.61
	5	36.40	34.77	95.52		9.98	27.42
	6	28.10	28.34	100.73		16.14	57.37

* Numbers correspond to sampling dates, 1 - 10/15/12, 2 - 10/22/12, 3 - 11/5/12, 4 - 11/12/12, 5 - 11/26/12, 6 - 12/17/12

** Acceptable percent recovery range is from 0 to 246% as defined by the USEPA 1682 method. The ANSR™ assay acceptable percent recovery range is currently undefined

§ Percent recoveries that are outside of the acceptable range as defined by the USEPA 1682 method

¶ Ongoing Precision Recovery samples

The average percent recovery for the USEPA 1682 method during Survey 1 was 75.32%, with a maximum percent recovery of 251.5% and a minimum percent recovery of 11.73%, which falls with the USEPA 1682 method's defined acceptable percent recovery range of 0 to 246% (Table 13). The average percent recovery for the ANSR™ assay during Survey 1 was 176.61% with a maximum percent recovery of 620.78% and a minimum percent recovery of 0% (Table 13).

Table 13. Overal recovery efficiency comparison for N=24 samples in Survey 1.

	USEPA 1682 method	**ANSR™ assay**
Average % Recovery	75.32%	176.61%
Max % Recovery	251.25%	620.78%
Min % Recovery	11.73%	0%
Average OPR % Recovery**	96.90%	95.26%
Acceptable % Recovery	0 - 246%	UD*

* Undefined by the literature
** Ongoing Precision Recovery

Figures 21 – 24 compare the methods by showing the log transformed MPN/100ml values of recovered seeded *Salmonella* from each sample from the various sites. Overlapping 95% confidence intervals are seen for most experiments. The different water samples from the various locations did not influence any difference in recoveries between the two methods.

Figure 21. Comparison of Log transformed MPN/100ml values of seeded *Salmonella* for River Raisin using the USEPA 1682 method and the ANSR™ assay in Survey 1

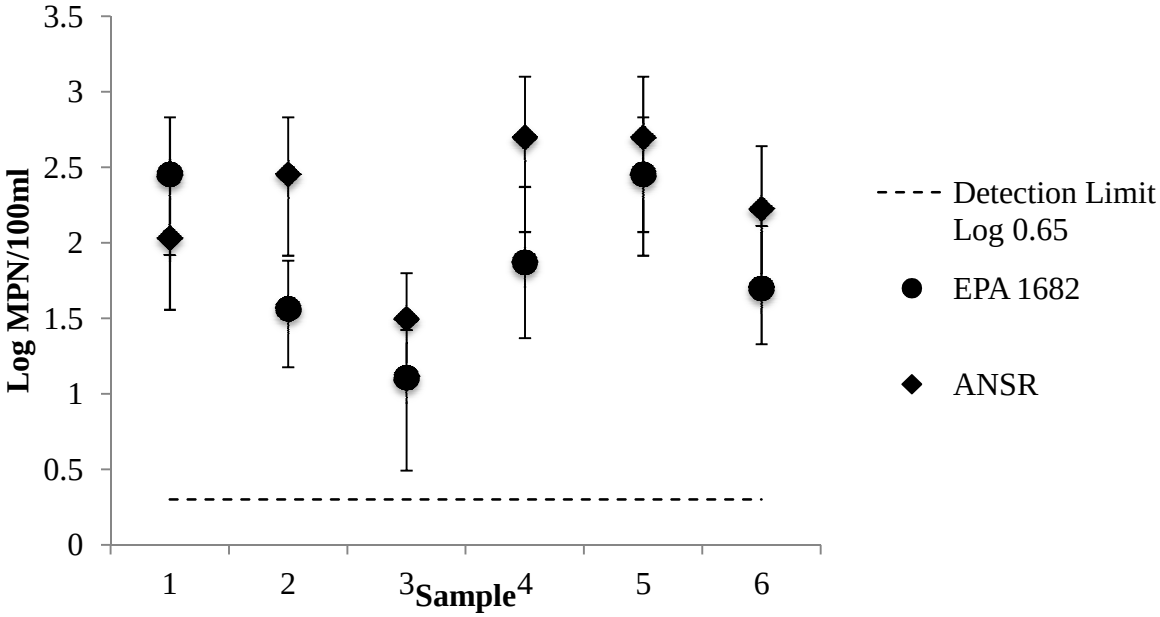


Figure 22. Comparison of Log transformed MPN/100ml values of seeded *Salmonella* for Grand River using the USEPA 1682 method and the ANSR™ assay in Survey 1

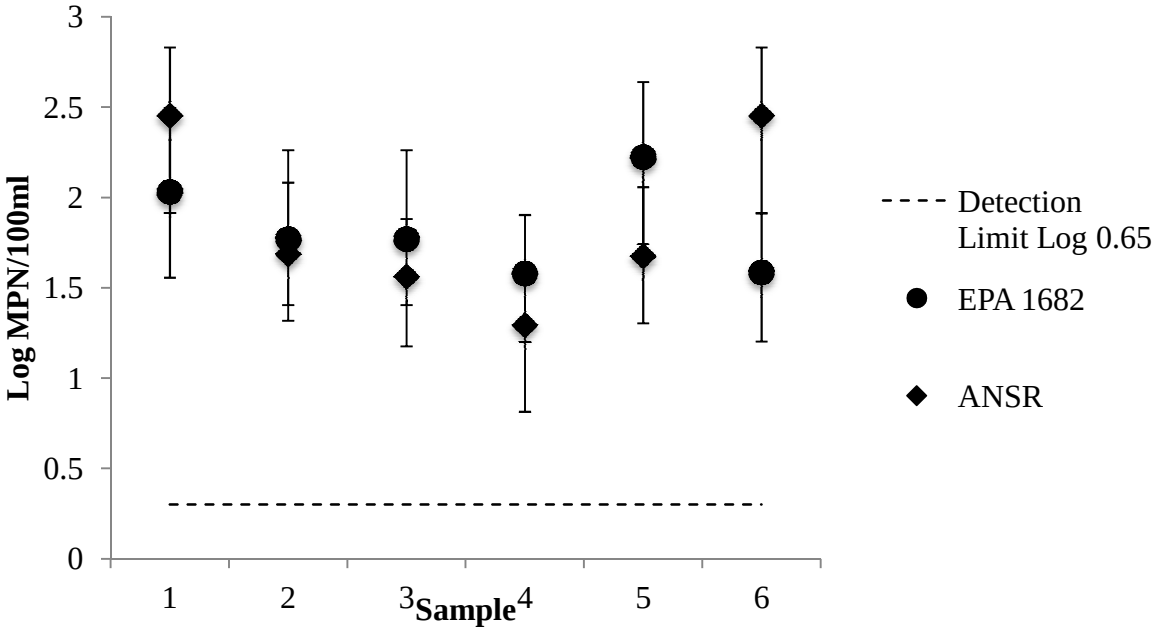


Figure 23. Comparison of Log transformed MPN/100ml values of seeded *Salmonella* for Red Cedar River using the USEPA 1682 method and the ANSR™ assay in Survey 1

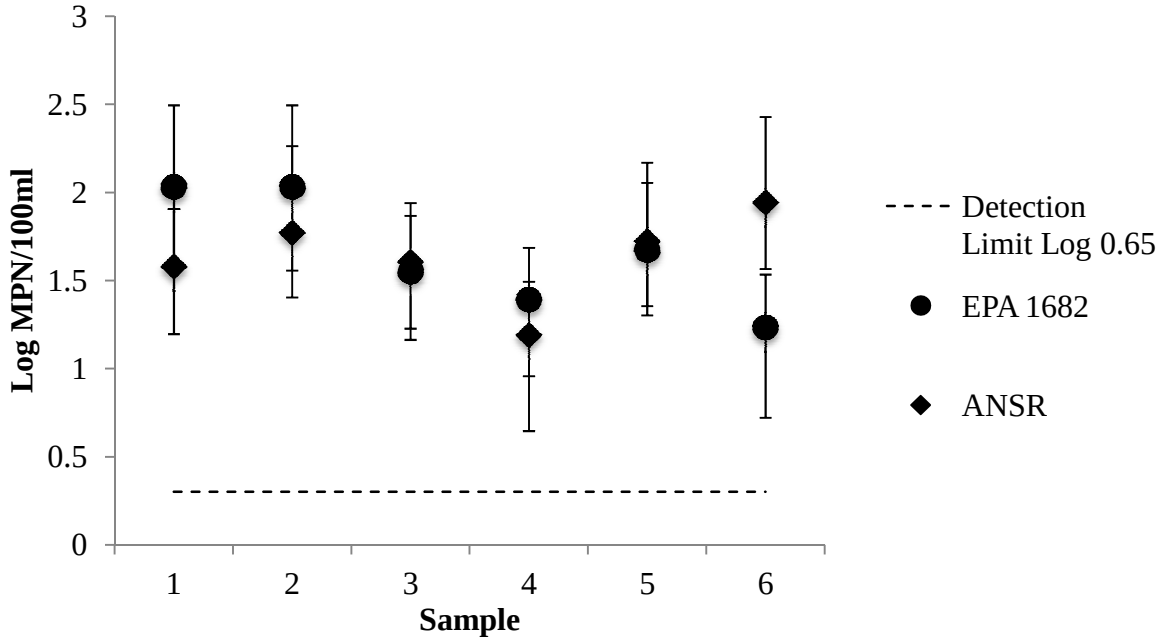
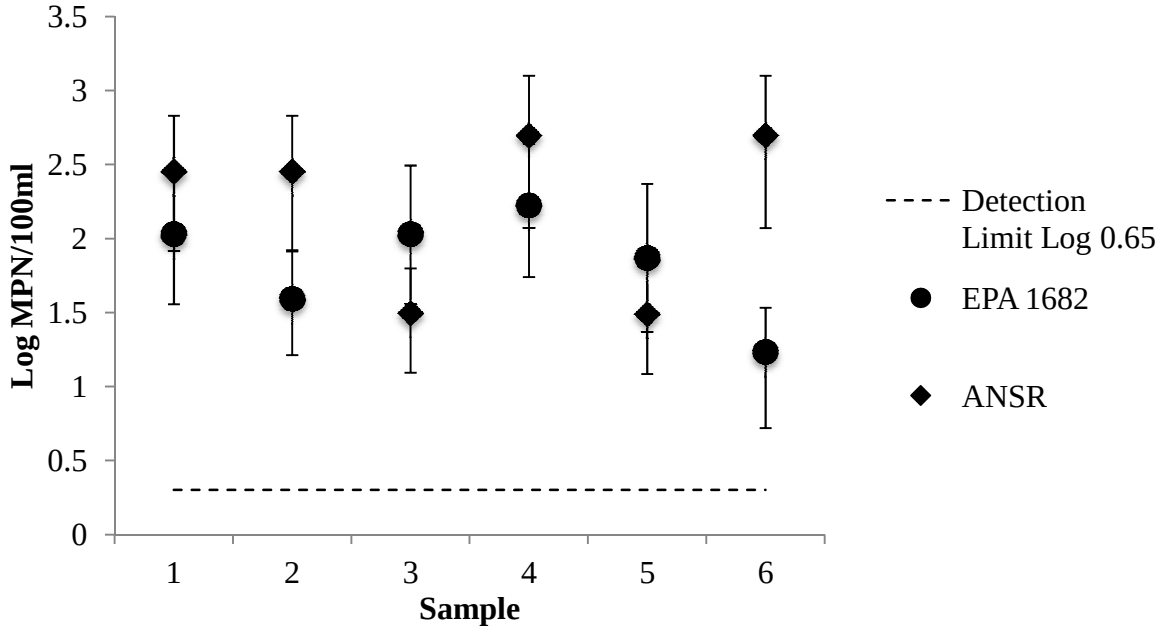


Figure 24. Comparison of Log transformed MPN/100ml values of seeded *Salmonella* for Farm Canal 1 using the USEPA 1682 method and the ANSR™ assay in Survey 1



2. Survey 2 Results

2.1 Initial nonselective enrichment comparison

A comparison of the initial nonselective enrichment media (TSB used in USEPA 1682 method and Enrichment media #3 used in the ANSR™ assay) was undertaken to ascertain whether this step influenced the growth of *Salmonella* and the subsequent lower detection by the USEPA 1682 method. Both were compared for naturally occurring *Salmonella* with the USEPA 1682 method. Samples that were found positive for naturally occurring *Salmonella* using TSB and/or Enrichment media #3 during this comparison can be seen in Table 14. Overall, 42.9% or 9 out of 21 samples were found positive for naturally occurring *Salmonella* when enriched with TSB. With the use of the Enrichment Media #3, 28.6% or 6 out of 21 samples were found positive for naturally occurring *Salmonella* (Table 14). Samples found positive with the use of TSB and Enrichment media #3 had very similar average MPN/100ml values with 1.38 and 1.11 MPN/100ml respectively. Both initial nonselective enrichment media had very similar ranges with samples found positive with TSB ranging from the lower limit of detection <0.65 to 2.09 MPN/100ml, and with samples found positive with Enrichment media #3 ranging from the lower limit of detection <0.65 to 1.34 MPN/100ml (Table 14). The two media types were not statistically different (p-values 0.0095, α=0.05) using a Fisher's exact test for contingency. A Mann-Whitney U test found no significant difference between the two media types results (p-value 0.2821, α=0.05).

Table 14. MPN/100ml values for naturally occuring *Salmonella* using the USEPA 1682 method with two different initial enrichment medias (TSB¶ & Enrichment Media #3) during Survey 2.

	MPN/100ml	
	TSB¶	**Enrichment Media #3**
Farm Canal 2	<0.65§	<0.65
Shiawasse River	<0.65	<0.65
Grand River 1	<0.65	<0.65
Raisin River 1	<0.65	<0.65
Saginaw River	<0.65	<0.65
Red Cedar River	1.30	<0.65
Detroit River	<0.65	<0.65
Rouge River	<0.65	<0.65
Grand River 2	1.39	<0.65
Muskegon River	2.09	1.34
Black River	0.65	1.30
St. Joseph River	<0.65	<0.65
Kalamazoo River	1.44	1.34
Ottawa River	2.90	1.34
Maumee River	0.67	0.67
River Raisin 2	<0.65	<0.65
Huron River	<0.65	<0.65
AS (A)*	<0.65	<0.65
AS (B)*	1.30	<0.65
HRC (A)*	0.67	<0.65
HRC (B)*	<0.65	0.65
Total % Positive (N =21)	42.9% (N=9)	28.6% (N=6)
Average (MPN/100ml)[a]	1.38	1.11
MPN Range (MPN/100ml)	<0.65 - 2.09	<0.65 - 1.34

¶ Tryptic Soy Broth

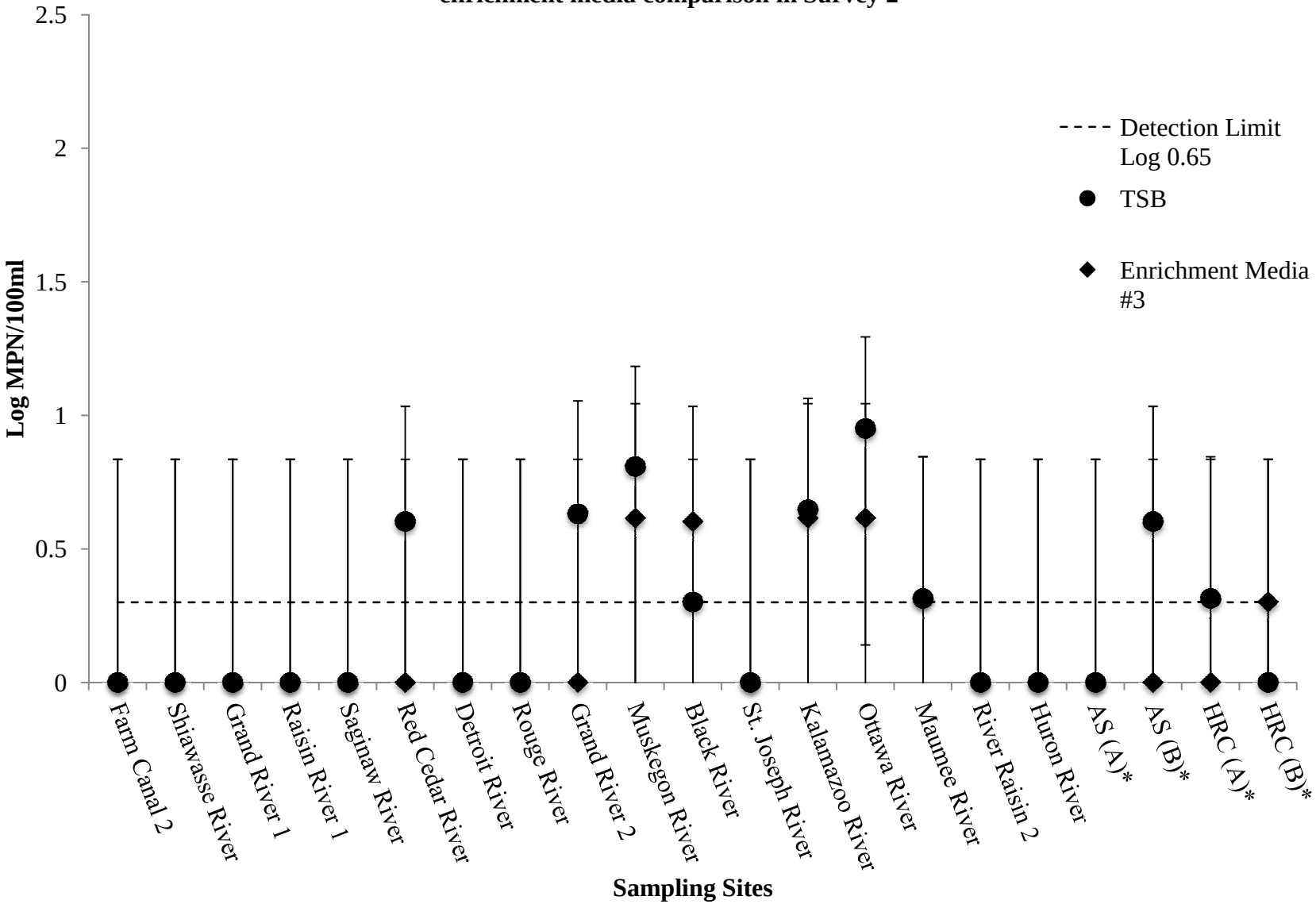
§ <0.65 MPN/100ml Represents the lower detection limit for the MPN calculations

* Wastewater Treatment samples are given individual codes for their location in the treatment train AS (A) & HRC (A) - Influent, AS (B) & HRC (B) -

* Average MPN/100ml value calculated without the inclusion of values that fell below the detection limit of <0.65 MPN/100ml

Figure 25 shows the log transformed MPN/100ml values for naturally occurring *Salmonella* during the initial nonselective enrichment media comparison for all samples taken during Survey 2. All of the 95% confidence intervals show an overlap. However, TSB appears to improve the detection and more samples would need to be collected and tested to show statistical significance.

Figure 25. Log transformed MPN/100ml values for naturally occuring *Salmonella* during initial enrichment media comparison in Survey 2



2.2 Survey 2 detection method comparison

During Survey 2, 21 samples were tested with 42.9% (N = 9) and 95.2% (N = 20) found positive by the USEPA 1682 method and the ANSR™ assay, respectively (Table 15). Samples found positive by the USEPA 1682 method had concentrations ranging from the lower detection limit of the MPN method of <0.65 MPN/100ml to 2.09 MPN/100ml (Table 15). Samples found positive by the ANSR™ assay had concentrations ranging from the lower detection limit (<0.65 MPN/100ml) of the MPN method to the upper detection limit (>160.90 MPN/100ml) of the MPN method (Table 15). The average concentration of *Salmonella* detected by the USEPA 1682 method and the ANSR™ assay were 1.38 and 24.37 MPN/100ml, respectively. The two methods' results from Survey 2 were found to be significantly different (p-value 0.000004, $\alpha = 0.05$) by a Mann-Whitney U test.

Table 15. MPN/100ml values and total percent of samples found positive for naturally occuring *Salmonella* by the USEPA 1682 method and the ANSR™ assay during Survey 2

	USEPA 1682 method	ANSR™ assay
Farm Canal 2	<0.65§	2.62
Shiawasse River	<0.65	12.99
Grand River 1	<0.65	17.67
Raisin River 1	<0.65	17.07
Saginaw River	<0.65	5.17
Red Cedar River	1.3	6.57
Detroit River	<0.65	15.63
Rouge River	<0.65	1.95
Grand River 2	1.39	2.62
Muskegon River	2.09	27.08
Black River	0.65	3.28
St. Joseph River	<0.65	11.73
Kalamazoo River	1.44	17.07
Ottawa River	2.9	160.9
Maumee River	0.67	8.55
River Raisin 2	<0.65	12.68
Huron River	<0.65	160.9
AS (A)*	<0.65	0.72
AS (B)*	1.3	0.65
HRC (A)*	0.67	1.55
HRC (B)*	<0.65	<0.65
Total % Positive (N =21)	42.9% (N=9)	95.2% (N=20)
Average (MPN/100ml)**	1.38	24.37
MPN Range (MPN/100ml)	<0.65 – 2.09	<0.65 – >160.90¶

§ <0.65 MPN/100ml Represents the lower detection limit for the MPN

* Wastewater Treatment samples are given individual codes for their location in the treatment train AS (A) & HRC (A) - Influent, AS (B) & HRC (B) -

* Average MPN/100ml value calculated without the inclusion of values below the detection limit of <0.65 MPN/100ml

¶ >160.90 MPN/100ml represents the upper detection limit for MPN

Although there were considerably more samples found positive by the ANSR™ assay (N = 20) than with the USEPA 1682 method (N = 9), all of the samples that were found positive by the USEPA 1682 method were also found positive by the ANSR™ assay. These samples along with their MPN/100ml values can be seen in Table 16.

Table 16. MPN/100ml values for naturally occuring *Salmonella* in water samples found to be positive by both the USEPA 1682 method and ANSR™ assay during Survey 2.

	EPA 1682 assay	ANSR™ assay
Red Cedar River	1.30	6.57
Grand River 2	1.39	2.62
Muskegon River	2.09	27.08
Black River	0.65	3.28
Kalamazoo River	1.44	17.07
Ottawa River	2.90	160.90
Maumee River	0.67	8.55
AS (B)*	1.30	0.65
HRC (A)*	0.67	1.55

* Wastewater Treatment samples are given individual codes for their location in the treatment train AS (A) & HRC (A) - Influent, AS (B) & HRC (B) - Nondisinfected effluent

As seen in Figure 26, Survey 2 had considerably more samples (N = 11) with different observable concentrations between the two detection methods than were seen in Survey 1 (see Figure 20). This included the samples from the Shiawassee River, the Grand River, River Raisin, the Detroit River, the Muskegon River, the St. Joseph River, the Kalamazoo River, the Ottawa River, the Maumee River, and the Huron River.

Figure 26. Survey 2 Log transformed MPN/100ml values for naturally occuring *Salmonella* using the USEPA 1682 method and the ANSR™ assay

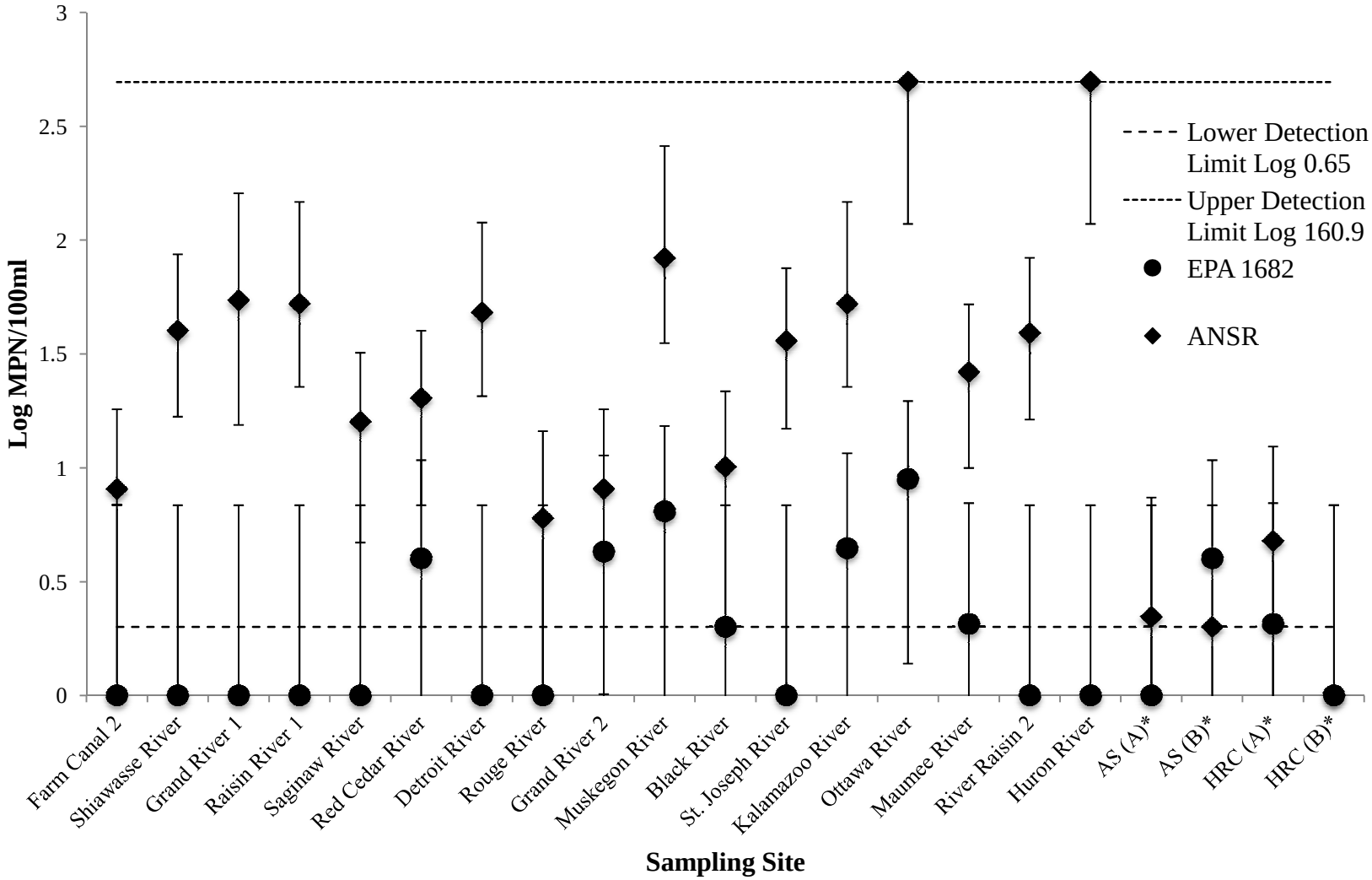
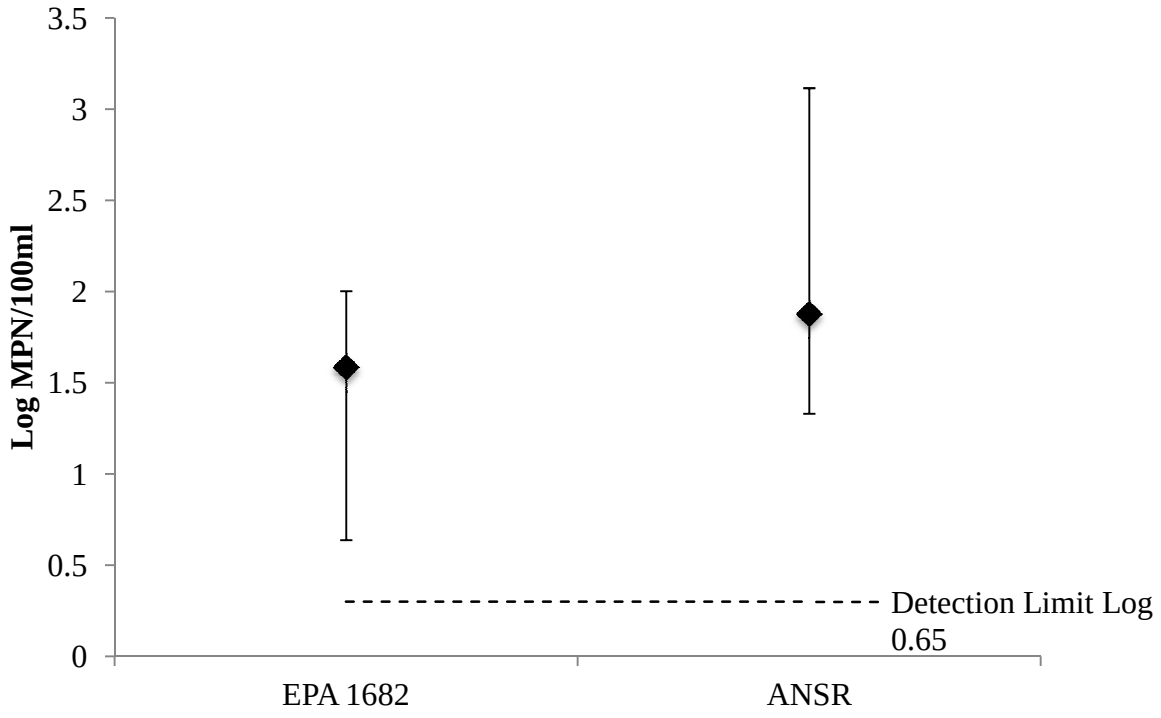


Figure 27, seen below, shows the average log transformed MPN/100ml values for both the USEPA 1682 method and the ANSR™ assay with the 95% confidence intervals for both overlapping.

Figure 27. Survey 2 average Log MPN/100ml values for the USEPA 1682 method and the ANSR™ assay



3. Independent Genetic Confirmation and Combined Survey Results

All 21 samples tested during Survey 2 by the USEPA 1682 method and ANSR™ assay were also analyzed by qPCR as an independent test in order to determine if the discrepancies between the two were false positives or negatives. Table 17 shows the results of the qPCR analysis for both the USEPA 1682 method samples and the ANSR™ assay. Of the 21 samples 42.9% (N = 9) were positive by USEPA 1682 method whereas 47.62% (N = 10) were found positive by qPCR with a 90.48% (N = 19) overall agreement between the two (Table 17). Of the 21 samples tested with the ANSR™ assay (with 95.2% positive, N=20), 71.43% (N = 15) were found positive by qPCR with a 76.19% (N = 16) overall agreement between the two (Table 17).

Table 17. Samples found positive for naturally occuring *Salmonella* by the USEPA 1682 method and the ANSR™ assay and confirmed with qPCR during Survey 2.

	USEPA 1682	qPCR	ANSR™ assay	qPCR
Farm Canal 2	—	—	+	+
Shiawasse River	—	—	+	+
Grand River 1	—	—	+	+
Raisin River 1	—	—	+	+
Saginaw River	—	+	+	—
Red Cedar River	+	+	+	+
Detroit River	—	—	+	—
Rouge River	—	—	+	+
Grand River 2	+	+	+	+
Muskegon River	+	+	+	+
Black River	+	+	+	+
St. Joseph River	—	—	+	+
Kalamazoo River	+	+	+	+
Ottawa River	+	+	+	+
Maumee River	+	—	+	+
River Raisin 2	—	—	+	+
Huron River	—	—	+	+
AS (A)*	—	—	+	—
AS (B)*	+	+	+	—
HRC (A)*	+	+	+	—
HRC (B)*	—	+	—	—
Total % Positive (N =21)	42.9% (N=9)	47.62% (N=10)	95.2% (N=20)	71.43% (N=15)
% Agreement with qPCR	90.48% (N=19)		76.19% (N=16)	

* Wastewater Treatment samples are given individual codes for their location in the treatment train AS (A) & HRC (A) - Influent, AS (B) & HRC (B) - Nondisinfected effluent

Twelve ANSR™ assay samples from Survey 1 were also analyzed by qPCR in order to increase the confidence of the statistical analysis. The results for these samples can be seen in Table 18. Using the statistical software, R, Fisher Exact Test's for contingency was run for each combination of sample results from the USEPA 1682 method, the ANSR™ assay, and the qPCR assay. The null hypothesis with this test is that the positive and negative results obtained by each of the two methods are independent of the results obtained by the other, thus should reveal different results. The alternative hypothesis is that the results of one method are dependent (or

congruent) on the results obtained by the other. The comparison of the USEPA 1682 method against the ANSR™ assay at 95% confidence was not significant (p-value 0.08059) and thus does not allow for the rejection of the null hypothesis that these two method's results are independent of each other. The comparison of the USEPA 1682 method against the qPCR assay at 95% confidence was found to be significant (p-value 0.001905) allowing for the rejection of the null hypothesis that these two tests are independent. The comparison of the ANSR™ assay and the qPCR assay at 95% confidence was significant (p-value 0.04448) and thus allows for the rejection of the null hypothesis that these two tests are independent.

Table 18. Survey 1. ANSR™ assay samples analyzed by qPCR

Site	Sample*	ANSR™ assay	qPCR**
River Raisin			
	4	—	—
	5	+	—
	6	+	+
Grand River			
	4	+	—
	5	+	—
	6	+	—
Red Cedar River			
	4	—	—
	5	+	—
	6	—	—
Farm Canal 1.			
	4	+	—
	5	+	—
	6	+	—

* Numbers correspond to sampling dates, 1 - 10/15/12, 2 - 10/22/12, 3 - 11/5/12, 4 - 11/12/12, 5 - 11/26/12, 6 - 12/17/12

** Samples from Survey 1. were not concentrated as previously described for samples saved for genetic testing during Survey 2.

When Survey 1 and Survey 2's results are combined, the total percent positive for the USEPA 1682 method and ANSR™ assay were 31.1% (N = 14) and 84.4% (N = 38), respectively (Table 19). The average MPN/100ml for the USEPA 1682 method for both surveys was 0.81 MPN/100ml (Table 19). The average MPN/100ml for the ANSR™ assay for both surveys was 12.34 MPN/100ml. The USEPA 1682 method showed a range of MPN/100ml values from <0.65 to 2.90 over the course of both assays, while the ANSR™ assay showed a much wider range of values from <0.65 to >160.90 MPN/100ml (Table 19).

Table 19. Survey 1 and Survey 2 combined results for method comparison

	USEPA 1682 method	**ANSR™ assay**
Survey 1. (N = 24)		
Total % Positive	20.8% (N = 5)	75.0% (N = 18)
Average (MPN/100ml)*	0.31	2.83
MPN Range (MPN/100ml)	<0.65 - 2.90	<0.65 - 15.29
Survey 2. (N = 21)		
Total % Positive	42.9% (N = 9)	95.2% (N = 20)
Average (MPN/100ml)*	1.38	23.21
MPN Range (MPN/100ml)	<0.65 - 2.90	<0.65 - >160.90**
Survey 1 and 2 Combined		
Total % Positive	31.1% (N = 14)	84.4% (N = 38)
Average (MPN/100ml)*	0.81	12.34
MPN Range (MPN/100ml)	<0.65 - 2.90	<0.65 - >160.90

* Average MPN/100ml calculated with only the inclusion of samples found to be positive

** <0.65 MPN/100ml and >160.90 MPN/100ml represent the lower and upper limits of detection for the MPN method, repectively

The ANSR™ assay had a calculated sensitivity of 100%, specificity of 22.58%, a false positive rate of 77.4%, and a false negative rate of 0% based on the USEPA 1682 method. When these values were calculated based on the results obtained from qPCR assay for samples positive by the ANSR™ assay in part of Survey 1 (Table 18) and Survey 2, the ANSR™ assay's sensitivity and false negative rate were identical and only small variations were seen in its calculated

specificity and false positive rate (25% and 75%, respectively). The ANSR™ assay also had a precision and accuracy of 36.8% and 46.67%, respectively. Using the qPCR as a reference, the USEPA 1682 method had a sensitivity and specificity of 80% and 90.9%, respectively. The USEPA 1682 method also had a false positive and negative rate of 9.1% and 20% respectively. The USEPA 1682 method's precision and accuracy were higher than the ANSR™ assay as well with 80% and 85.7%, respectively.

Chapter 4. Discussion

The 54% (N = 13/24) discrepancy of positive samples between the methods seen in Survey 1 (USEPA 1682 method 5/24 positive, ANSR™ assay 18/24 positive) was initially concerning. Due to the USEPA 1682 method's recognition as the "gold standard" for *Salmonella* detection this discrepancy was hypothesized to have been caused by false positive results from the ANSR™ assay and possibly some false negatives from the USEPA 1682 method. While the ANSR™ assay provided similar MPN/100ml values (Table 10) the high percentage of samples could not be confirmed as "true positives" using the qPCR assay. These initial results show a difference in the sensitivity and specificity of the two methods even while each method's recovery efficiency with seeded surface water samples, when using a Mann-Whitney U test, were found to not significantly differ (p-value 0.2467, $\alpha = 0.05$) in their ability to detect *Salmonella* when present in concentrations above both methods' limits of detection.

Due to the discrepancies seen between the two methods in Survey 1, a second survey was performed, but instead of only examining water samples from four different sites (three of which were within relatively close proximity as seen in Figure 3), Survey 2 looked at 17 different surface water sites (Figure 4) from a variety of different areas around Michigan and Northern Ohio, along with four wastewater samples. This was done in order to analyze the performance of the two methods in a more diverse set of water samples with varying drainage areas, major watersheds, flows, and wastewater inputs (Table 4). Sampling was limited to the lower half of Michigan's Lower Peninsula and Northern Ohio, in order to increase the chances of finding *Salmonella* in surface water samples due to the known input from wastewater treatment plants and nonpoint source pollution which are more abundant than in Northern Michigan.

Survey 2 showed a similar pattern of detection as in Survey 1, with a 52.3% (N=11/21) discrepancy between the two methods (USEPA 1682 method 9/21 positive and the ANSR™ assay 20/21 positive). Two (Ottawa River and Farm Canal 2) out of the 21 surface waters chosen for this survey did not receive discharges from wastewater treatment plants (Table 4). While the Ottawa River sample was found to be positive by both methods, only the ANSR™ assay found Farm Canal 2 positive. The majority of surface waters where sampling took place also had large proportions of rural land use cover contributing to each respective watershed (Table 4).

The exclusion of the use of different enrichment media between the two methods as a factor in the difference in detection seen in this study, was achieved after comparing the samples (N = 21) enriched by TSB (42.9% positive) and Enrichment media #3 (28.6% positive) using a Fisher Exact test (p-value 0.0095, $\alpha = 0.05$). This suggests that the initial enrichment media does not contribute to any significant differences in detection seen using the USEPA 1682 method. In order to attempt to confirm these results, the two media would have to be tested using the USEPA 1682 method, the ANSR™ assay, and qPCR. It is still not clear whether the amplification reaction is responsible for the increased detection seen in the ANSR™ assay. That being said, the use of TSB with the ANSR™ assay was not advisable due to the assay's sensitivity to matrix effects caused by variability of composition of other enrichment media. Thus more work in this area is needed in order to determine if any significant differences would occur if alternative enrichment or selective media were used prior to sample analysis with the ANSR™ assay. For example, sodium pyruvate, which has been recognized as a key molecule for the resuscitation of *Salmonella* from a viable-but-not-culturable (VBNC) or sub-lethally injured state back to a growing and colony forming state, is present in the ANSR™ assay enrichment media but not in the TSB (Gurtler and Kornacki, 2009, Morishige et al., 2013). This may have contributed to

considerable disparity between the two methods if high enough levels of possibly VBNC *Salmonella* cells were present in the sample. The ANSR™ assay as well as the qPCR should be able to detect those populations along with other healthy organisms but the qPCR did not confirm this hypothesis.

Although 84.4% of samples positive for all surface water samples (N = 45) during this study using the ANSR™ assay is high, other studies have reported similar rates in surface waters (e.g., rivers and streams) (e.g., Haley et al., 2009) (Table A1). In surface waters that regularly receive fecal pollution through agricultural runoff and wastewater effluent discharges, Haley et al. (2009) reported that 79.2% (N = 57) of their 72 samples collected from surface waters in a rural watershed were found positive for *Salmonella,* while Thomas et al. (2012) found 78.4% of samples were also positive (Table A1). The majority of land use (>75%) within each of the watersheds where surface water sampling took place for this study was rural, with the exception of the Detroit watershed, which had approximately 50% rural and 50% urban land use, but has one of the largest areas of treated and untreated sewage input in the state (i.e. the city of Detroit) (Table 4).

As seen in Table A1, *Salmonella* detection in surface water varies widely in both percent detected and concentration. *Salmonella* prevalence can range anywhere from 2.55% (Martinez-Urtaza et al. 2010) to 100% (Lemarchand and Lebaron, 2003, Bonadonna et al. 2006) with concentrations of 0.006 CFU/100ml to 10^6 CFU/100ml (Joyti et al., 2010, Lemarchand and Lebaron, 2003). One of the most important factors in the relative presence and concentration of *Salmonella* in any given surface water is the pollution input being received, whether it be raw human sewage as in the case of Jyoti et al. (2010) with *Salmonella* concentrations reaching up to

10^6 CFU/100ml or in the case of Patchanee et al. 2010 where *Salmonella* prevalence was high in a variety of watersheds with different nonpoint sources of pollution.

All methods have advantages and disadvantages, however PCR methods are now gaining acceptance in regard to both their specificity and sensitivity. The samples from Survey 2 were tested using a proprietary qPCR 16s rDNA gene target array, which has a detection limit of 100 gene copies for *S. enterica* in metagenomic samples (Fosbrink et al., 2013, Qiagen, 2014). This qPCR array allowed for the confirmation of 90.48% (N = 19) and 76.19% (N = 16) of the samples from the USEPA 1682 method and ANSR™ assay, respectively (Table 17).

This use of an independent genetic confirmation of our results, allowed us to determine that while the ANSR™ assay has 100% sensitivity it also has a high false positive rate (77.4%), and low specificity (22.58%). In contrast, the USEPA 1682 method had a lower sensitivity (80%), but had a higher specificity (90.9%), and a considerably lower false positive rate (9.1%). That being said, use of the ANSR™ assay may still be applicable to water quality testing as a screening method, but currently cannot be used as a replacement for standard culture-based methods. Perhaps it is suited more as a tool for rapid screening of sites suspected of receiving fecal pollution that may pose a human health risk or used in conjunction with multiple pathogen detection studies. One would need to understand which microbes are causing the false positives and perhaps redesign the primers.

While most water quality is still monitored through the use of indicator organisms (e.g., *E. coli*), a growing interest in pathogen detection and monitoring has arisen. This is in part due to molecular methods, which have been undergoing a general increase in specificity, sensitivity, and decrease in assay time and cost, becoming more readily available for widespread use. Interest in the detection and monitoring of individual pathogens has also arisen from the

recognition that current fecal indicators may be unable to accurately predict the presence and levels of enteric pathogens, but this is still debated within the scientific community (Lemarchand and Lebaron, 2003, Arvanitidou et al., 2005, Harwood et al., 2005, Savichtcheva et al., 2007, Wu et al., 2011, Payment and Locas, 2011). However, without the incorporation of a growth and possibly selection step, molecular techniques for pathogen detection cannot differentiate between live and dead organisms, which is crucial if an accurate assessment of potential risk to human health is to be determined. The ANSR™ assay represents a potentially valuable resource for water quality testing due to its inclusion of both molecular detection and culture techniques. While one can also run culture and PCR together, currently setting up an MPN would be more time consuming. In addition, results are available within minutes with ANSR™ while PCR would take 2 to 3 more hours.

Since the Nicking Enzyme Amplification Reaction (NEAR™) technology developed by Van Ness et al. (2003), was only recently licensed by Neogen for use in the development of the ANSR™ assay for the detection of *Salmonella* in food matrices, there are currently no previous publications on its potential application and efficacy in water matrices. The only current publication available is Neogen's own validation study for approval by the association of analytical communities (AOAC) in 2013 (Mozola et al., 2013). This technology, and specific assay require more study in order to determine whether they have widespread applicability in water quality testing. It may be that new primers are needed for use with water samples, or additional purification or dilution steps may need to be taken in order to prevent any matrix interference effects from influencing detection results.

Because this assay incorporates both molecular detection and growth in a non-selective enrichment media, this assay should be able to incorporate the speed and accuracy of this

molecular based detection, and the increased confidence of having potentially live infectious organisms present through the use of an enrichment step. However, since an enrichment step is used by this assay and the results are presence/absence, a MPN enumeration method must also be incorporated in order to determine concentrations of the target organism. The ANSR™ assay is reported to have a detection limit of 10^4 CFU/ml after enrichment with 99.1% inclusivity for all *Salmonella* genetic subgroups and 100% exclusivity based on the 38 non-salmonellae tested (Mozola et al., 2013). However, these values are based on detection in specific food matrices (e.g., ground beef, milk, etc.), and as we have seen here water matrices can produce considerably different results.

In comparison to more traditional culture-based methods (USEPA 1682, ISO 6579) for *Salmonella* detection, the ANSR™ assay is considerably faster, less labor intensive, and has been shown to be inclusive for all genetic subgroups of *S. enterica* and *S. bongori*, unlike the USEPA 1682 method which has the potential for excluding less common *Salmonella* serovars at several different steps. For example, in the USEPA 1682 method, non-motile or monophasic (*Salmonella* which can only express one "H" flagular antigen) would fail to show motility on MSRV (Bugarel et al., 2012). Also, almost 300 environmental serovars, which are known to have variable lactose fermentation capacity, would cause false negative results on XLD and TSI media (Table 1), which are critical in the USEPA 1682 method for distinguishing potential positive *Salmonella* samples from non-target organisms, with Park et al. (2012) showing a specificity of only 73.0% for *Salmonella* on XLD media. Marita et al. (2006) reported the isolation of 10 strains of *S.* Enteritidis, which is one of the most common serovars associated with human illness (Figures 1 and 2), produced negative results for lysine-decarboxylase activity (i.e. the lysine iron agar slants used in the USEPA 1682 method). There is also the limitations of

the use of the polyvalent O antiserum (a-I & vi), used in the USEPA 1682 method, which while targeting the *Salmonella* serovars most commonly associated with human illness only covers about 23.73% (N=612) of all of the 2,579 recognized *Salmonella* serovars, without the inclusion of testing with additional antiserums (Grimont and Weill, 2007). These deficiencies in ability of the current USEPA 1682 method in detecting less common *Salmonella* serovars are often addressed through modification of method procedures through the inclusion of additional selective media and biochemical tests, along with the possible addition of molecular methods, and confirmatory steps (e.g., pulsed field gel electrophoresis or additional serotyping).

The USEPA 1682 method is considered the "gold standard" for *Salmonella* detection in water, but it is limited by the ability of each of its tests to include or exclude potential *Salmonella* isolates based on selection criteria that are not consistently shared among serovars of *Salmonella* (e.g., lactose fermenters, non-motile strains, and serovars without O antigens recognized by the polyvalent O antisera used by the method). The ANSR™ assay is also limited by its own detection limit and its sensitivity to matrix effects, which can inhibit its PCR reaction. While the limitations of traditional culture-based methods seem to be significant, culture-based detection is still used more often than molecular methods for *Salmonella* detection (Table A1). Looking at the studies found in Table A1, 75% of *Salmonella* detection studies were conducted using culture-based methods. While molecular methods were used less often, they had almost equivalent average detection rates with culture-based methods having an average of 33.37% samples positive for *Salmonella*, and molecular methods having 31.74% positive. Both detection methods showed a very wide range of detection with 4 – 79.17% positive and 3.13 – 87.5% positive for culture-based and molecular methods, respectively (Table A1).

It is also important to consider that if the *Salmonella* were clumped together or attached to suspended particles in the water samples they may not have been distributed evenly throughout the samples, thus allowing for some samples to receive greater concentrations of *Salmonella* than others as seen in Droppo et al. (2009) where the resuspension of sediment particles greatly affected *Salmonella* detection. The even distribution of organisms throughout the sample is one of the basic assumptions made in order to perform MPN enumeration. If this assumption is violated then the estimated concentration of samples may be incorrect. Although this is a potential flaw with MPN calculations, the use of sample homogenization and filtration may allow for the reduction of any influence this may have on samples. This area requires more research to determine what significance suspended solids have in differences between paired samples. However, only five out the 20 studies examined in Table A1 since 2009 reported on *Salmonella* concentrations.

In order to determine any further applicability of the ANSR™ assay for water quality testing, a series of experiments should look at the detection limit for various *Salmonella* serovars, the effects of sodium pyruvate on the regrowth of VBNC cells and the ability of the ANSR™ assay to detect those cells. Also, determining if reducing the non-selective enrichment time for the ANSR™ assay and incorporating a selective media before performing the assay, will help to increase the confidence that *Salmonella* is being detected rather than a false positive caused by an interference effect from the water matrix. It is also crucial to determine the effects, if any, that different water types and quality (e.g., fresh, marine, brackish) have on the ability of the assay to accurately detect *Salmonella.* In addition, more study is needed in determining the cause of false positives between the PCR and ANSR™ assay. This could be accomplished by enriching samples initially in TSB media, testing them with PCR, and then transferring them to Enrichment media

#3 and retesting by PCR and also by the ANSR™ assay. This could also be followed by isolation and identification through the use of selective media and serotyping, allowing us to determine if any specific serovars are not detected by specific methods.

APPENDIX

Table A1. Studies on *Salmonella* occurance in surface waters

Frequency positive samples %(or # of isolates*)	Total samples	Values	Surface water matrix	Survey extent	Country	Reference
62.7% (N=241)	384	ND**	Freshwater	8 sampling sites, 4 years	Brazil	Palhares et al. (2014)
7.20% (N=19)	264	ND	Freshwater	12 sampling sites, 22 months	USA	Liang et al. (2013)
31.11% (N=42)	135	ND	Freshwater	12 sampling site, 4 months	USA	Haack et al. (2013)
29.84%(N=57)	191	<0.03 - 0.418 MPN/100ml	Freshwater	10 sampling sites, 2 years	China	Xiao et al. (2013)
78.4% (N=91)	116	ND	Freshwater	3 sampling sites, 2 years	Canada	Thomas et al. (2012)
30.71% (N=74)	241	<0.075 - 0.725 MPN/100ml	Freshwater	14 sampling sites, 2 years	USA	Walters et al. (2013, 2011)
49.4%(N=154)	312	ND	Freshwater	13 sampling sites, 12 months	Cameroon	Henriette et al. (2012)
7.14% (N=18)	252	ND	Freshwater	20 sampling sites, 14months	USA	Gorski et al. (2011)
8.47% (N=29)	342	ND	Freshwater	9 sampling sites, 2 years	Canada	Jokinen et al. (2011)
12.90% (N=24)	186	ND	Freshwater	4 sampling sites, 2 years	Canada	Jokinen et al. (2010)
54.65% (N=47)	86	ND	Freshwater	$ sampling sites, 2 years	USA	Patchanee et al. (2010)
87.5% (N=7)	8	10^4 - 10^6 CFU/100ml	Freshwater	8 sampling sites, 1 day	India	Jyoti et al. (2010)
6.99% (N=10)	143	ND	Freshwater	10 sampling sites, 10 months	USA	Schriewer et al. (2010)
3.13% (N=1)	32	ND	Freshwater	8 sampling sites, 14 months	Australia	Ahmed et al. (2009)
62.5% (N=20)	32	ND	Freshwater	1 sampling site	Canada	Droppo et al. (2009)

Table A1. (cont'd)

4.12% (N=10)	243	ND	Seawater	6 sampling sites, 4 years	Morocco	Setti et al. (2009)
79.17% (N=57)	72	0.25 - 3.63 MPN/100ml	Freshwater	6 sampling sites 1 year	USA	Haley et al. (2009)
42.10% (N=8)	19	0.2 - 1.7 MPN/100ml	Freshwater	Numerous sampling sites, 2 years	USA	Byappanahalli et al. (2009)
15.66% (N=13)	87	ND	Freshwater	2 sampling sites, 1 year	Czech Republic	Dolejská et al. (2009)
4-15%[a]	1600	ND	Freshwater	24 sampling sites, 3 years	Canada	Wilkes et al. (2009)
14.3% (N=7)	49	ND	Freshwater	10 sampling sites, 5 days	Netherlands	Heuvelink et al. (2008)
20.0% (N=7)	35	ND	Wetland water	25 sampling sites, 2 months	Canada	Shellenbarger et al. (2008)
74.70% (N=62)	83	ND	Freshwater	82 sampling sites, 1 day	USA	Meinersmann et al. (2008)
11.16% (N=26)	233	ND	Freshwater	Random samples, 2 years	Mexico	Simental and Martinez-Urtaza (2008)
3.80% (N=3)	79	ND	Freshwater	8 sampling sites, 1 year	Netherlands	Schets et al. (2008)
9.93% (N=72)	725	ND	Freshwater	25 sampling sites, 15 months	New Zealand	Till et al. (2008)
53.33% (N=30)	30	ND	Freshwater	5 sampling sites, 3 months	Japan	Svichtcheva et al. (2007)
92.59% (N=25)	27	1.51×10^3 - 4.15 $\times 10^3$ CFU/100ml	Seawater	Random samples, 6 months	Lebanon	Harakeh et al. (2006)
94.44% (N=17)	18	3.73×10^2 - 1.10×10^5 CFU/100ml	Freshwater	Random samples, 6 months	Lebanon	Harakeh et al. (2006)
100%[b]	>300	10 - 10^4 MPN/100ml	Freshwater[b]	8 sampling sites, 2 years	Italy	Bonadonna et al. (2006)
2.55% (N=18)	707	ND	Seawater	4 sampling sites, 4 years	Spain	Martinez-Urtaza et al. (2004)

Table A1. (cont'd)

5.36% (N=43)	802	ND	Freshwater	16-21 sampling sites, 2 years	Canada	Gannon et al. (2004)
74.93% (N=281)	375	ND	Freshwater	8 sampling sites	South Africa	Obi et al. (2004)
100% (N=10)	10	10^2 - 10^5 MPN/100ml	Freshwater	1 sampling site, 1 year	Italy	Bonadonna et al. (2003)
6.23% (N=89)	1429	ND	Freshwater	135 sampling sites, 2 years	Canada	Johnson et al. (2003)
57.14% (N=8)	14	ND	Freshwater	Weekly sampling, 5 months	Africa	Obi et al. (2003)
100% (N=8)	8	0.006 - 4.24 CFU/100ml	Freshwater		France	Lemarchand and Lebaron (2003)
230 isolates + 183 isolates from flood events	76	ND	Freshwater	4 sampling sites, 19 months	France	Baudart et al. (2000)
48.39% (N=135)	279	1.3 - 74 CFU/100ml	Seawater and estuarine water	6 sampling sites, 2 years	Portugal	Catalo Dionisio et al. (2000)
7.02 (N = 222)	3164	N/A	Seawater and freshwater	236 sampling sites, 5 years	USA	Polo et al. (1999)
136 isolates	48	18.8 - 204.3 MPN/100ml	Freshwater	2 sampling sites, 2 years	India	Sharma and Rajput (1995)
31.1% (N=14)[C]	45	<0.65 - 2.90 MPN/100ml[c]	Freshwater	21 sampling sites, 2 years	USA	This thesis
84.4% (N=38)[d]	45	<0.65 - >160.90 MPN/100ml[d]	Freshwater	21 sampling sites, 2 years	USA	This thesis

* Number of isolates used when frequency of positive samples not reported

** Concentration of *Salmonella* was Not determined (ND)

a different percentages in different seasons

b Highly contaminated area with direct sewage discharge occuring

c *Salmonella* detected using the USEPA 1682 method

d *Salmonella* detected using the ANSR™ assay

Figure A1. River Raisin MPN/100ml values for Survey 1 obtained from the USEPA 1682 method

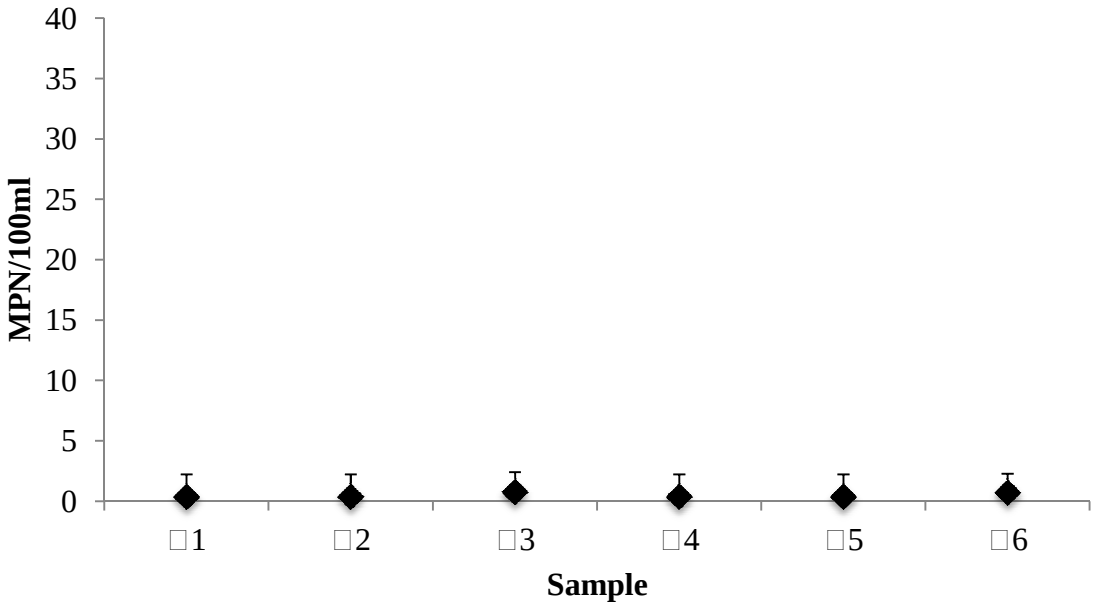


Figure A2. Grand River MPN/100ml values for Survey 1 obtained from the USEPA 1682 method

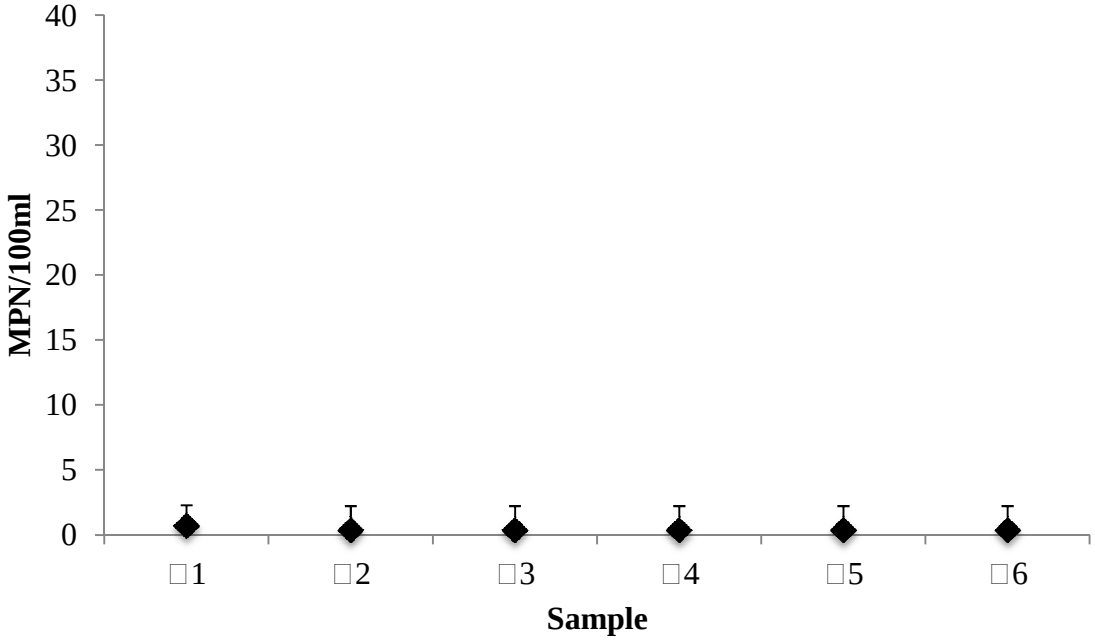


Figure A3. Red Cedar River MPN/100ml values for Survey 1 obtained from the USEPA 1682 method

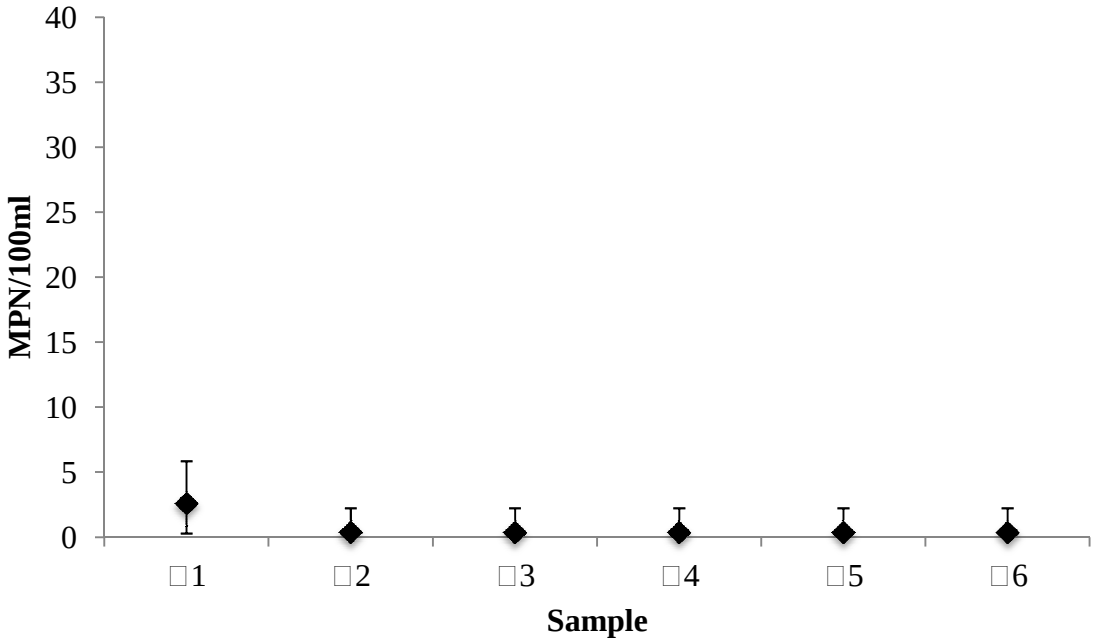


Figure A4. Farm Canal 1 MPN/100ml values for Survey 1 obtained from the USEPA 1682 method

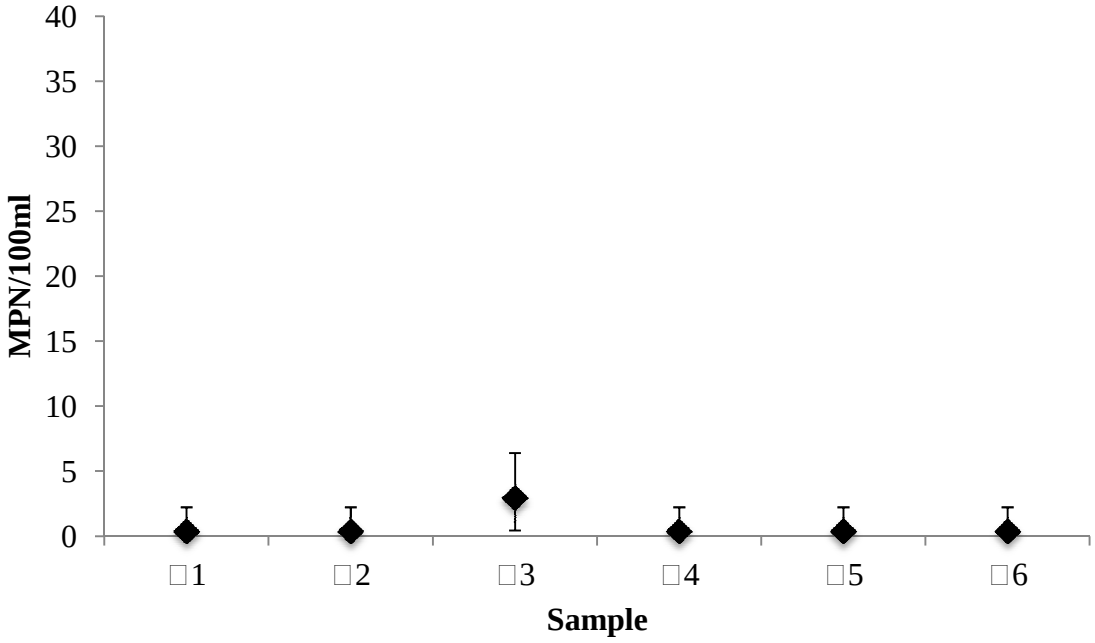


Figure A5. River Raisin MPN/100ml values for Survey 1 obtained from the ANSR™ assay

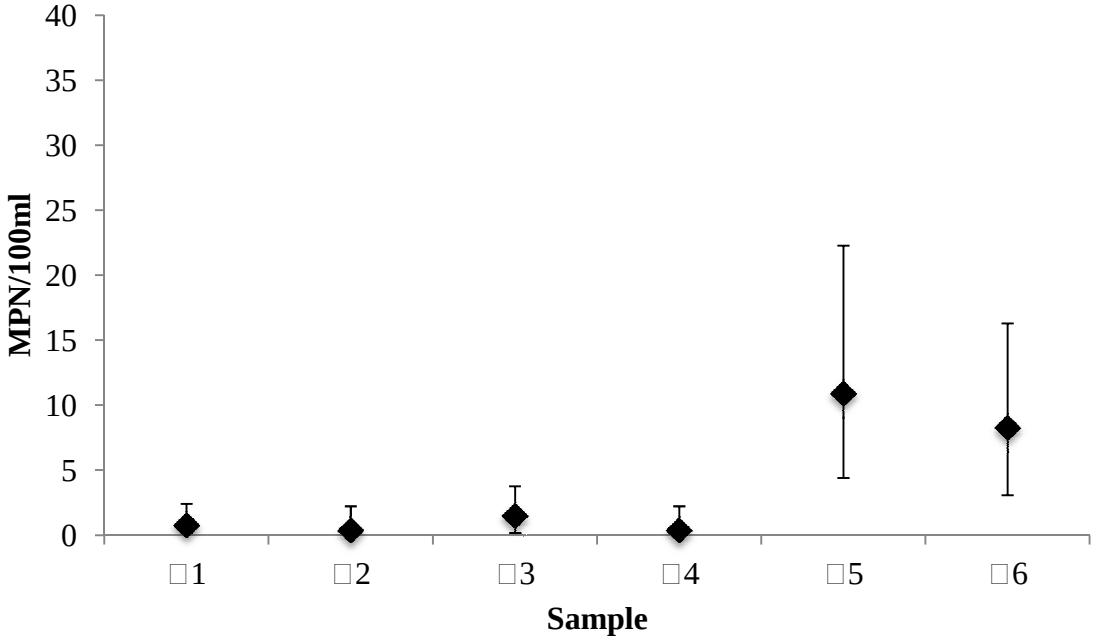


Figure A6. Grand River MPN/100ml values for Survey 1 obtained from the ANSR™ assay

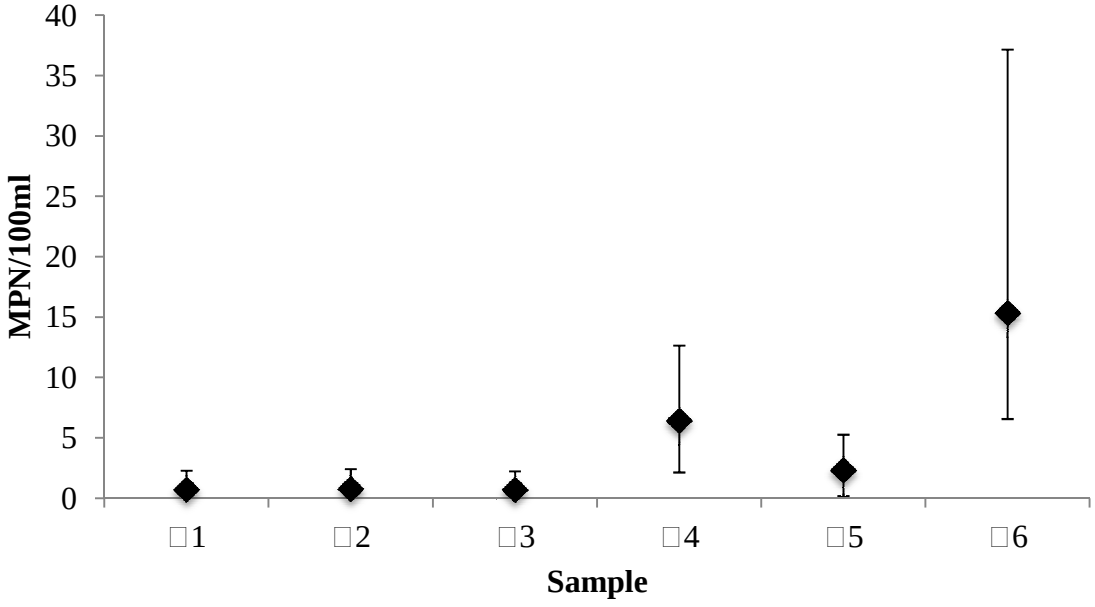


Figure A7. Red Cedar River MPN/100ml values for Survey 1 obtained from the ANSR™ assay

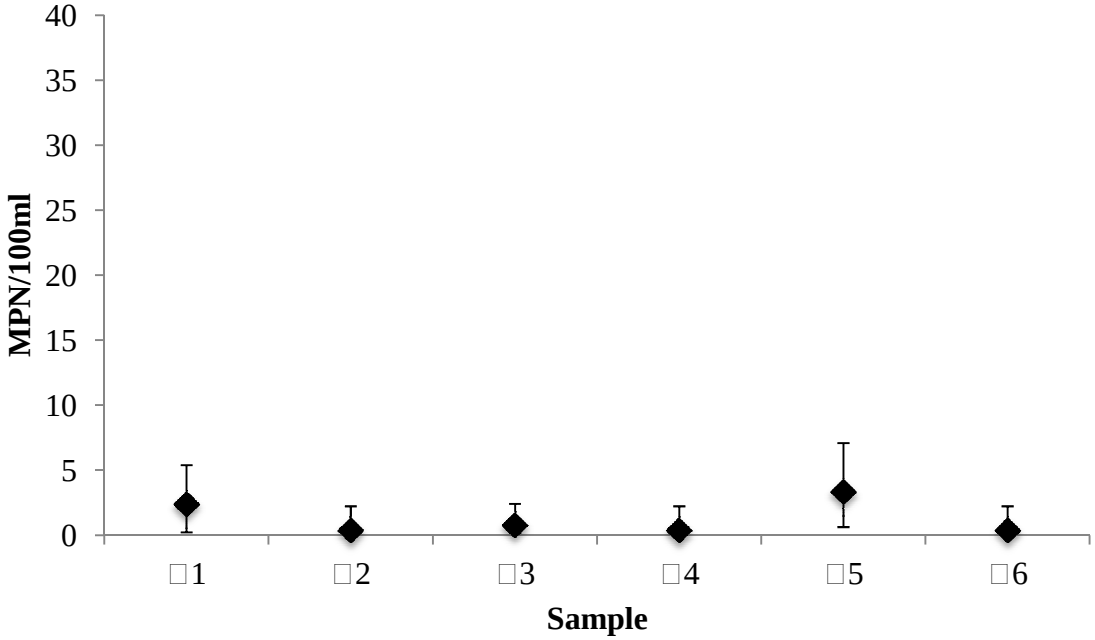
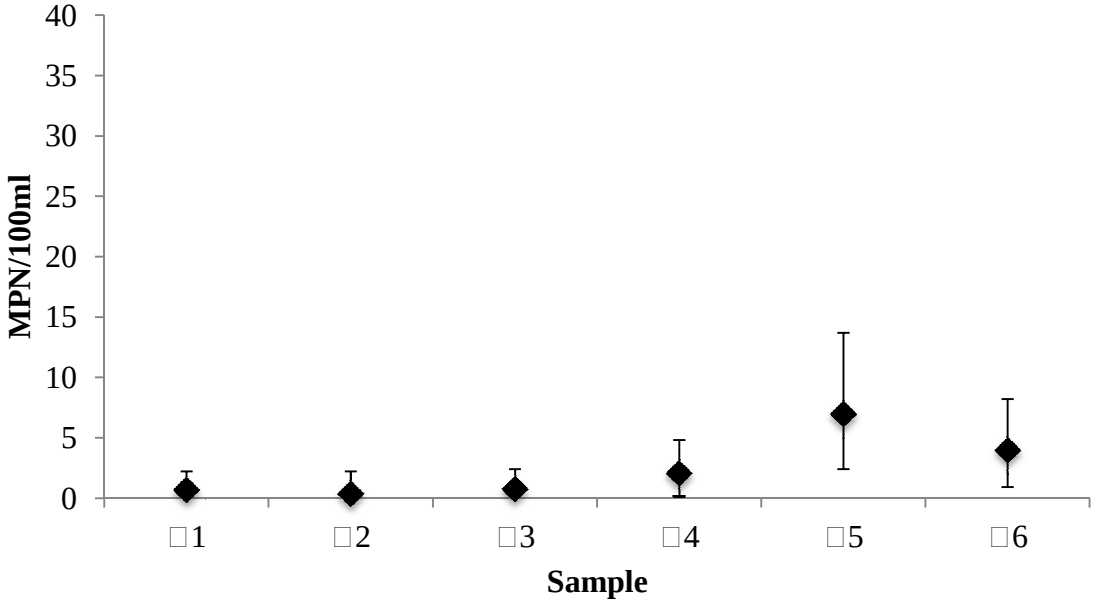


Figure A8. Farm Canal 1. MPN/100ml values for Survey 1 obtained from the ANSR™ assay



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