IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF OKLAHOMA

STATE OF OKLAHOMA, ex rel, W. A. DREW EDMONDSON, in his capacity as ATTORNEY GENERAL OF THE STATE OF OKLAHOMA, and OKLAHOMA SECRETARY OF THE ENVIRONMENT C. MILES TOLBERT, in his capacity as the TRUSTEE FOR NATURAL RESOURCES FOR THE STATE OF OKLAHOMA, Plaintiffs, vs. TYSON FOODS, Inc., TYSON POULTRY, INC., TYSON CHICKEN, INC., COBB-VANTRESS, INC., AVIAGEN, INC., CAL-MAINE FOODS, INC., CAL-MAINE FARMS, INC., CARGILL, INC., CARGILL TURKEY PRODUCTION, LLC, GEORGE'S, INC., GEORGE'S FARMS, INC., PETERSON FARMS, INC., SIMMONS FOODS, Inc. WILLOWBROOK FOODS, INC. Defendants.	Case No. 4:05-cv-00329-GKF-SAJ

EXPERT REPORT OF VALERIE J. HARWOOD, Ph.D.

I. CREDENTIALS AND EXPERIENCE: VALERIE J. HARWOOD, Ph.D.

1. My education includes a Bachelor's degree in French from Iowa State University, a Bachelor's degree in Biology from the State University of New York at Plattsburgh, and a Ph.D. in Biomedical Sciences from Old Dominion University & Eastern Virginia Medical School in Norfolk, VA (1992).

2. From 1992 to 1995 I held a full-time postdoctoral research position at the University of Maryland Center of Marine Biotechnology. In 1995 I joined the Department of Natural Sciences at the University of North Florida as a tenure-track Assistant Professor, where I taught microbiology and related courses, and maintained a research laboratory until I joined USF in 1998. Since August, 1998 I have been employed by the University of South Florida (USF) in Tampa, FL in a full-time, tenure-track position. In 2004 I was promoted from Assistant Professor to Associate Professor, which is my current rank. My responsibilities at USF include teaching undergraduate and graduate courses in microbiology, mentoring undergraduate and graduate research students, university and community service, and maintaining an active research program. My research laboratory personnel currently include two technicians, seven Ph.D. students and one Master's student. My research focuses on microbial water quality, with particular emphasis on microbial source tracking (MST), a field of environmental microbiology that seeks to determine the source of fecal contamination in water by identifying specific molecular signatures in the DNA of fecal microorganisms.

3. I am the author of 28 peer-reviewed publications, over 30 technical reports, a book chapter, and have been an invited speaker on water quality research and MST over 50 times across the U.S., in the U.K. and in New Zealand. I also contributed substantially to the U.S. Environmental Protection Agency Microbial Source Tracking Guide Document. I am a reviewer for many scientific journals including Environmental Science & Technology, Microbiology and Journal of Applied Microbiology, and am a member of the editorial review board of Applied & Environmental Microbiology. I have served on state and federal grant panels including Sea Grant, National Oceanic and Atmospheric Administration (NOAA) and the United States Department of Agriculture (USDA), and have been awarded over $3 million in grant funding from various agencies including the National Science Foundation, NOAA, Sea Grant, USDA, United States Environmental Protection Agency (USEPA) and National Institutes of Health. My current funding for MST and related environmental microbiology research totals over one-half

million dollars from agencies including the Florida Department of Environmental Protection, the Florida Department of Health, NOAA, the USDA and the USEPA.

4. I was retained by the State of Oklahoma concerning its investigation of poultry waste disposal in the Illinois River Watershed (IRW). My experience and expertise was sought in the matter of microbial contamination of water bodies, its possible consequences to human health, and the major sources of microbial contamination to the IRW.

5. Compensation for my professional activities is at the rate of $250.00 per hour except when testifying under oath, in which case it is $375.00 per hour

II. WATERBORNE DISEASE

The Waterborne Route of Disease Transmission

6. One of the most common routes of disease transmission is the waterborne route, in which people ingest, inhale or encounter water that contains microbial pathogens. Many waterborne pathogens enter water primarily via fecal material from humans and animals, which can contain such diverse pathogens as viruses (e.g. noroviruses like Norwalk virus), bacteria (e.g. *Campylobacter, Salmonella*) and protozoa (e.g. *Cryptosporidium Giardia*). Humans can be exposed to contaminated water by drinking it, or through other activities such as swimming, floating, splashing, wading, fishing, or canoeing/kayaking. Human health risk is greatest from activities where full body contact occurs, because there is a greater risk of swallowing water than during activities where exposure to water is more limited (WHO, 2003). In Oklahoma, the Illinois River and its tributaries, i.e. Flint Creek and Baron Fork, are heavily used for “floating,” an activity that frequently involves full body contact (Caneday, 2008). Over 100,000 individuals spend almost half a million hours annually on this type of activity in the Illinois River watershed (IRW).

7. The most frequent result of exposure to waterborne pathogens is intestinal illness, technically known as enteric disease or gastroenteritis, which is characterized by symptoms such as nausea, vomiting, diarrhea, and fever (World Health Organization, 2003). Drinking or accidentally swallowing fecally contaminated water can lead to enteric disease. Acute febrile respiratory illness, which is more serious than gastroenteritis, has also been linked in epidemiology studies to elevated microbial pollution levels (Fleisher et al., 1998; World Health Organization, 2003). This type of illness is transmitted by inhaling water droplets (aerosols).

8. Enteric disease that is transmitted by the waterborne route is underreported, according to the World Health Organization (WHO) and many others (reviewed in (Leclerc et al, 2002). Underreporting leads to an underestimate of the economic and public health impact of specific diseases, including salmonellosis and campylobacteriosis (see below). Waterborne disease constitutes a serious burden on public health (Leclerc et al., 2002; World Health Organization, 2003) . As summarized by the WHO:

> "Infections and illness due to recreational water contact are generally mild and so difficult to detect through routine surveillance systems. Even where illness is more severe, it may still be difficult to attribute to water exposure. Targeted epidemiological studies, however, have shown a number of adverse health outcomes (including gastrointestinal and respiratory infections) to be associated with faecally polluted recreational water. This can result in a significant burden of disease and economic loss." (WHO, 2003).

9. Individuals differ in their susceptibility to infection by pathogens (Belanger & Shryock, 2007; U.S. Environmental Protection Agency, 2005a; World Health Organization, 2003), which in turn affects the minimum infectious dose. Infants and children, elderly and immunocompromised individuals have less robust immune systems than others, and are thus more susceptible to infection and more likely to suffer severe outcomes from an infection (Leclerc et al., 2002). According to the National Research Council (NRC) and the National Resources Defense Council (NRDC), the most vulnerable segments of the U.S. population to waterborne disease are infants, children, pregnant women, the elderly, and the immunocompromised (National Research Council, 2004; National Resource Defence Council, 2007). Young children have less developed immune systems than adults and are thus more susceptible to infection than healthy adults (World Health Organization, 2003). Children are also likely to play longer in the water and are more likely to swallow water than others (World Health Organization, 2003). Epidemiology studies have shown that children who swam are among the most likely to contract intestinal illness (Cabelli et al., 1979; Pruss, 1998; World Health Organization, 2003).

10. Many diseases, called zoonoses, are spread from animals that harbor human pathogens in their gastrointestinal tract. Campylobacteriosis and salmonellosis are important zoonoses in the U.S. which have major animal sources, or reservoirs (DuPont, 2007; Leclerc et al., 2002), and are transmitted in water contaminated by poultry feces and those of other animals (Leclerc et al., 2002; National Research Council, 2004). The 2007 U.S. EPA Report of the Experts Scientific Workshop on Critical Research Needs for the Development of New or Revised Recreational Water Quality Criteria recognized the importance of zoonoses to human

health risk, and placed the highest priority for further research on contamination from poultry and other agricultural animals (U.S. Environmental Protection Agency, 2007).

Pathogen Detection

11. Pathogens can be very difficult to detect in the environment, particularly in water samples where they are diluted (National Research Council, 2004). Furthermore, they may be in a physiologically stressed condition that makes standard, culture-based methods ineffective. As stated by the National Research Council (2004):

> 'Typical culture methods for pathogen and indicator bacteria in water and other environmental samples greatly underestimate the true concentrations of viable and potentially infectious cells—sometimes by as much as a thousandfold."

Conventional methods for detecting pathogens in food, fecal and water samples rely upon culturing, which means the organisms are grown in broth and/or on solid media that are designed to select for the desired target organism and to discourage the growth of non-target organisms. While these methods reliably detect pathogens that are healthy, such as those in clinical samples from infected patients, the conditions used to select for the target pathogen can inhibit the growth of stressed, but viable (living) pathogens. Once these organisms are excreted from their host they are subject to stress from a host of environmental factors including starvation, dessication, and exposure to ultraviolet light. The response of many bacterial pathogens to such stress is to enter a "viable but nonculturable" (VBNC) state (Oliver, 2005). In this state pathogens are metabolically active ("living"), but they cannot be cultured on media routinely used for their isolation. Many studies have indicated that pathogens which enter the VBNC state remain infectious (Baffone et al., 2003; Oliver & Bockian, 1995), including *Campylobacter jejuni* (Baffone et al., 2006) and *E. coli* O157:H7 (Makino et al., 2000). *Salmonella* is also known to become VBNC under environmental stress (Oliver, Dagher & Linden, 2005). Due to the ability of many pathogenic bacteria to become VBNC, testing for pathogens based on the use of culture-based methods alone is likely to yield false-negative results (negative test results when pathogens are actually present). The ability of VBNC pathogens to be revived (resuscitated) in a host means that infectious pathogens can be present in samples that test negative by culture methods alone.

Campylobacteriosis

12. Campylobacteriosis is caused mainly by *Campylobacter jejuni,* and secondarily by *C. coli* in the U.S. Campylobacteriosis is usually limited to mild to severe gastroenteritis, but can result in more serious outcomes such as Guillane Barré syndrome and Reiters syndrome (Friedman et al 2000). Worldwide, campylobacteriosis is among the most common forms of gastroenteritis (Friedman et al., 2004) and is associated with poultry feces and fecal-contaminated food and water throughout the world (Leclerc et al., 2002; Skovgaard, 2007) and in the U.S. (U.S. Environmental Protection Agency, 2005a). *Campylobacter* is a leading cause of waterborne gastroenteritis in the U.S. (Leclerc et al., 2002). Contaminated water is a known source of *Campylobacter* infection (Allos, 2001; Friedman et al., 2004; Leclerc et al., 2002; O'Reilly et al., 2007), and waterborne disease outbreaks from drinking untreated well and spring water have occurred (National Research Council, 2004). Drinking untreated water from a lake, river or stream is a known risk factor for contracting campylobacteriosis (Friedman et al., 2004).

13. *C. jejuni* has a very low infectious dose (Leclerc et al., 2002; Skovgaard, 2007; U.S. Environmental Protection Agency, 2005a). As few as 500 *C. jejuni* can cause campylobacteriosis, and the 50% infectious dose of *C. jejuni* is reported at 800 cells (Black et al., 1988). One drop of blood from a poultry carcass contaminated by feces can contain up to 500 infectious cells (Hood, Pearson & Shahamat, 1988), and chicken intestines can contain up to one billion viable *Campylobacter* per gram (Belanger & Shryock, 2007; Berndtson, Tivemo & Engvall, 1992). *Campylobacter* concentrations in cattle and swine feces tend to be thousands-fold lower than in poultry (Belanger & Shryock, 2007; Jacobs-Reitsma, 2000), and the prevalence of *Campylobacter* is also lower in cattle compared to poultry (Hutchison et al., 2004).

14. The emergence of resistance to antibiotics in pathogenic *Campylobacter* species is an increasing concern in the U.S. (Belanger & Shryock, 2007; DuPont, 2007). Antibiotic-resistant pathogens make treatment of disease much more difficult, and even commensal (nonpathogenic) bacteria that carry antibiotic resistance genes can readily transfer these genes to pathogens. Recent estimates (2005) are that 1-2% of all broilers are treated with macrolide (e.g. erythromycin) or lincosamide (e.g. clindamycin) antibiotics for infections in the U.S. (Belanger & Shryock, 2007). Other antibiotics used frequently in the poultry industry, including the defendants in this case, include enrofloxacin, sarafloxacin, bacitracin, penicillin and its derivatives (e.g., ampicillin, amoxicillin, methicillin), gentamicin, and tetracyclines (see e.g., Bates #: TSN088218SOK, TSN088077SOK, TSN088197SOK, CM003570, SIM AG09496, CARTP109186). According to the World Health Organization, the use of antibiotics in food

animals contributes to the frequency of antibiotic resistance in both *Campylobacter* and *Salmonella* (World Health Organization, 2005). In 2007, a joint international meeting of experts from the Food and Agriculture Organization of the United Nations (FAO), the World Health Organization and the World Organisation for Animal Health (OIE) was held on the use of critically important antimicrobials used in food animal production (Food and Agriculture Organization of the United Nations, World Health Organization & Health, 2007). This group identified resistance of *Salmonella*, *Campylobacter* and *E. coli* in food animals, including poultry, to several antibiotic classes used widely in the U.S. (i.e. quinolones such as enrofloxacin and macrolides such as erythromycin) as the highest priority for risk management because of the critical uses of these antibiotics in treating human infections.

15. Campylobacteriosis in human populations frequently occurs in a sporadic pattern of infection (Friedman et al., 2004), meaning that unrelated individual cases tend to occur as opposed to related outbreaks affecting many individuals. Due to its sporadic pattern of occurrence and frequently self-limiting course (Belanger & Shryock, 2007), campylobacteriosis is greatly underreported to public health agencies because many individuals do not seek medical treatment. Furthermore, campylobacteriosis is infrequently reported, even when diagnosed (Allos, 2001). Only about 2.6% of diagnosed gastroenteritis cases caused by *Campylobacter* spp. are reported (Mead et al., 1999).

Salmonellosis

16. *Salmonella enteritis* causes about 40,000 reported cases of salmonellosis annually in the U.S. (CDC, http://www.cdc.gov/nczved/dfbmd/disease_listing/salmonellosis_gi.html#6). This figure is certainly an underestimate. Cases could occur at 30 times this rate since, like campylobacteriosis, salmonellosis is greatly underreported (Voetsch et al., 2004). The CDC estimates that a total of 1.4 million Americans are sickened by *Salmonella* each year (http://www.cdc.gov/nczved/dfbmd/disease_listing/salmonellosis_ti.html), and that up to 600 people per year die from salmonellosis in the U.S. The annual cost of salmonellosis in the U.S., including medical care and loss of productivity, is in the billions of dollars (Voetsch et al., 2004). Children, the elderly and immunocompromised are more susceptible to *Salmonella* infections (Voetsch et al., 2004) and other waterborne pathogens than the remainder of the population (U.S. Environmental Protection Agency, 2005a).The U.S. EPA estimates the minimum infectious dose of *Salmonella* at 100 – 1000 cells (U.S. Environmental Protection Agency, 2005a). As outlined above for *Campylobacter*, antibiotic use and the concomitant increase in antibiotic resistance is also a critical concern in *Salmonella.* As stated by the WHO (2005):

"there is clear evidence of adverse human health consequences due to resistant organisms resulting from non-human usage of antimicrobials: increased frequency of infections, increased frequency of treatment failures (in some cases death) and increased severity of infections, as documented for instance by fluoroquinolone-resistant human *Salmonella* infections."

17. *Salmonella* is frequently spread to carcasses from the gastrointestinal tract and feces of poultry during slaughter (Doyle & Erickson, 2006; Li et al., 2007; U.S. Environmental Protection Agency, 2005a); so much so that it has been used by the USDA Food Safety and Inspection Service to monitor food safety in processing plants (Federal Register, 2006). *Salmonella* is commonly isolated from poultry feces (Li et al., 2007; Santos et al., 2005), and fecally-contaminated poultry litter is known to be a reservoir for *Salmonella* (Payne et al., 2007). The prevalence of *Salmonella* is higher in poultry feces than in cattle feces (Hutchison et al., 2004).

18. *Salmonella* infections are frequently transmitted by the waterborne route. In 1993 over half of the population of a small town (1100 inhabitants) acquired salmonellosis from the unchlorinated public water system, and seven people died (Angulo et al., 1997). In 2004 an Ohio town was the site of an outbreak caused by contaminated drinking water that included salmonellosis and campylobacteriosis (O'Reilly et al., 2007), sickening 1450 people.

Pathogenic *E. coli*

19. Certain *E. coli* strains found in poultry are pathogenic, and cause disease in poultry (Trampel, Wannemuehler & Nolan, 2007). Strains that are pathogenic to humans, such as *E. coli* O157:H7 (Dipineto et al., 2006; Doane et al., 2007; Doyle & Schoeni, 1987) have been isolated from poultry. *E. coli* strains that are resistant to multiple antibiotics are common in poultry, and these strains can enter food and water supplies (Diarra et al., 2007). Individuals who work in the poultry industry are much more likely to carry antibiotic-resistant strains of *E. coli* than other community members (Price et al., 2007).

20. Summary of Waterborne Disease

- People can be sickened by waterborne pathogens through exposure via contaminated drinking water or recreational waters.
- The most vulnerable member of the population, both in terms of the frequency of contracting illness and the severity of illness, are immunocompromised individuals & children
- Animal feces (including poultry) contain human pathogens that are transmitted via the waterborne route.

- Pathogens, including *Campylobacter, Salmonella* and pathogenic *E. coli* strains, can enter a VBNC state in which they remain infectious but cannot be detected by culture methods.
- Campylobacteriosis and salmonellosis are both underreported diseases that cause a major disease burden in the U.S. and are transmitted via the waterborne route.
- *Campylobacter* and *Salmonella* are commonly found in high concentrations in poultry feces.
- The minimum infectious dose of *Campylobacter* is very low - around 500 cells.
- The minimum infectious dose of *Salmonella* is also very low – 100-1,000 cells.

III. WATER QUALITY TESTING AND RELATIONSHIP TO PUBLIC HEALTH

21. The goal of water quality testing in recreational waters is to protect the health of people who swim, play, or are otherwise exposed to the water. Fecal material from human and certain animals frequently contains bacterial, viral, and/or protozoan pathogens, which greatly increase the risk of waterborne disease in contaminated waters (U.S. Environmental Protection Agency, 2007). Due to limitations of time, expense, and methodology, it is virtually impossible for agencies or even research laboratories to test for all pathogens that could possibly come from a fecal source in a water sample. The general reliability and practicality of protecting public health by enumerating fecal indicator bacteria has led to the continued use of this practice worldwide for over 100 years. Fecal coliforms, *E. coli* and *Enterococcus* spp. (enterococci) are the most commonly used indicator bacteria for recreational water quality in the United States.

22. The U.S. Environmental Protection Agency (EPA) publishes recommended criteria for recreational water quality in the U.S. under section 304a of the Clean Water Act. States may adopt these standards or, with agreement from EPA, modify the standards or adopt scientifically defensible standards of their own (Federal Register, 2004). These criteria were developed for fecal indicator bacteria levels and are based on epidemiology studies conducted by EPA and published in 1986 (U.S. Environmental Protection Agency, 1986).

23. The link between indicator bacteria concentration and human illness from recreational water use has been demonstrated in many epidemiological studies over the course of more than 50 years, beginning with the U.S. Public Health Service studies in the late 1940s and early 1950s. In 1968 the National Technical Advisory Committee of the Department of the Interior proposed a geomean standard of 200 fecal coliforms/100 ml. This standard also stipulated that less than 10% of all samples should exceed 400 fecal coliforms/100 ml (U.S. Environmental Protection Agency, 1986). In 1972 the EPA began a series of epidemiology studies designed to determine the relationship between indicator bacteria and human health risk in marine and fresh waters contaminated by sewage. The marine water studies were conducted

in New York, Massachusetts and Lake Pontchartrain, LA, while the freshwater studies were conducted at Lake Erie, PA and Keystone Lake in Tulsa, OK. These studies concluded that a significant correlation between gastroenteritis frequency and enterococci concentrations existed at marine beaches, while gastroenteritis frequency was correlated with both *E. coli* and enterococci concentrations at freshwater beaches (U.S. Environmental Protection Agency, 1986).

24. The U.S. EPA relied on the previous fecal coliform standards to establish "acceptable risk" from recreational water use; that is it estimated that the 200 fecal coliform/100 ml geomean level would result in 8 cases of gastroenteritis per 1,000 swimmers at fresh water beaches, and 19 cases/1,000 at marine beaches. From this historically-based definition of acceptable risk and the results of its epidemiology studies conducted from 1972 – 1983, EPA developed the current water quality standards. These standards reflect the increased risk of gastroenteritis in swimmers compared to non-swimmers at beaches ("swimming-associated gastroenteritis rate per 1,000 swimmers). These standards are expressed in terms of a geomean value and a single sample maximum. The geomean (average) value is relatively low because it is intended to reflect risk from recreation and possible exposure to pathogens over an extended time period, and the single sample maximum ranges from low values for waters that receive intensive full body contact use to high values for waters that receive much lower use (U.S. Environmental Protection Agency, 1986). The geomean standards for enterococci are 33 colony forming units (CFU)/100 ml in fresh water or 35 CFU/100 ml in marine water, while the *E. coli* geomean (freshwater only) is 126 CFU/100 ml. Corresponding single sample maxima for areas that receive high full body contact use are 61, 104 and 235 CFU/100 ml respectively.

25. The State of Oklahoma utilizes the fecal coliform standard as well as the enterococci and *E. coli* standards to monitor recreational water quality (State of Oklahoma, 2006a). The State's statutes define primary body contact recreation as "direct body contact with the water where a possibility of ingestion exists." Floaters, canoers and rafters can be expected to experience primary body contact for at least a portion of their recreational activities in the IRW (Caneday, 2008; Teaf, 2008). In the case of primary body contact recreation, Oklahoma standards stipulate that the geomean fecal coliform concentration may not exceed 200 CFU/100 ml, and no more than 10% of samples may exceed 400 CFU/100. The last stipulation gives a *de facto* single sample maximum of 400 CFU/100 ml since most sites are not tested more than ten times per year. The *E. coli* and enterococci standards follow EPA's 1986 criteria.

26. Recreational water quality standards based on indicator bacteria concentrations have been supported since EPA's 1986 water quality criteria were published. In a review of 22 epidemiology studies, Pruss (1998) concluded that there is a "causal relationship" between gastroenteritis and recreational water quality as measured by indicator bacteria concentrations, particularly *E. coli* and enterococci (Pruss, 1998). More recently, a re-analysis of data from 27 epidemiology studies strongly supported the relationship between indicator bacteria (*E. coli* and enterococci) concentrations and gastroenteritis rates in recreational water users (Wade et al., 2003). Both the WHO (World Health Organization, 2003) and the European Union (EU) have adopted standards for recreational water quality that are based on indicator bacteria concentrations (enterococci and/or *E. coli*). In 2007 the U.S. EPA convened a group of experts to consider the impact of recent scientific and technical advances on recreational water quality criteria and implementation of standards (U.S. Environmental Protection Agency, 2007). The report recommended updating the criteria by incorporating new methods (e.g. quantitative PCR) and continuing to perform epidemiology studies in areas affected by various pollution sources, but also recommended the continuation of the current practice of enumerating indicator bacteria. Thus, indicator bacteria standards will doubtless be used to protect the health of recreational water users in the U.S. in the foreseeable future.

27. Summary of Water Quality and Public Health

- Indicator bacteria are used worldwide to monitor water quality because of the extreme difficulty entailed in monitoring all important pathogens.
- Numerous epidemiology studies have shown that indicator bacteria levels are correlated with the risk of gastroenteritis for recreational water users. These correlations are particularly significant and consistent for *E. coli* in fresh water, and for enterococci in both fresh and salt water.
- Recreational water quality standards are based on indicator bacteria levels in Oklahoma, the U.S. and the world.
- The Oklahoma recreational water standards are based on levels of indicator bacteria (fecal coliforms, *E. coli* and enterococci). These standards are based on increased risk of illness for swimmers when indicator bacteria levels are elevated above threshold criteria.

IV. WATER QUALITY IN THE ILLINOIS RIVER WATERSHED

28. The IRW in Oklahoma hosts an intricate network of tributaries to the Illinois River, including Sager Creek, Flint Creek, Peacheater Creek, Tyner Creek, Tahlequah Creek and the Baron Fork of the Illinois River. The State of Oklahoma defines impaired waters as those in which "…the water quality standard is not attained. The water body is impaired or threatened for one or more designated uses by a pollutant(s)…" (State of Oklahoma, 2006b). Indicator bacteria levels in each of these tributaries routinely exceed Oklahoma water quality standards, therefore these water bodies have been placed on the State's 303(d) list of impaired waters. This Oklahoma Scenic River is considered to be too polluted by fecal bacteria to support its designated use of primary body contact recreation. Dr. Teaf's Expert Report for this case describes the extent of impairment in the IRW; in summary over 75% of the Illinois River and its major tributaries are listed as impaired by high bacterial levels (Teaf, 2008).

29. The data collected by the State of Oklahoma for water quality assessment includes (but is not limited to) fecal coliform concentrations. Fecal coliforms are used by the State of Oklahoma to evaluate recreational water quality, but are not recommended by the U.S. EPA due to their lack of correlation with human illness in some locations (U.S. Environmental Protection Agency, 1986). However, *E. coli* is recommended for recreational water quality monitoring by the U.S. EPA. A comparison of *E. coli* and fecal coliform concentrations in water samples collected throughout the IRW shows that almost all of the fecal coliforms in these samples are *E. coli* (Figure 1). This relationship confirms the public health significance of elevated fecal coliform concentrations in IRW waters, i.e. they are nearly synonymous with *E. coli* concentrations, which are correlated with the risk of gastroenteritis for recreational water users.

30. Enterococci are responsible for many of the water quality exceedances throughout the IRW (Teaf, 2008). This group of fecal indicator bacteria is recognized as measure of recreational water quality by the U.S. EPA and the State of Oklahoma, and its levels are correlated with the risk of gastroenteritis in recreational water users in fresh and salt water (Teaf, 2008).

31. The State of Oklahoma recognizes the potential impact of poultry operations and other agriculture on water quality. Under the Oklahoma Registered Poultry Feeding Operation Act, it is required that "…there shall be no discharge to waters of the state." (Title 2; Registered Poultry Feeding Operation Act) Management of poultry litter/manure in the IRW is by land application, which is considered a passive waste management approach that can impact

surface and ground water quality as microorganisms move with surface and subsurface water flow (U.S. Environmental Protection Agency, 2005a). Broiler production generates large amounts of contaminated litter, i.e. up to 0.5 pounds of soiled litter per pound of meat produced, or 340 tons annually from a farm with only four houses (Dozier, Lacy & Vest, 2001). Used poultry litter is known to contain high levels of indicator bacteria. Contaminated poultry litter samples were collected by CDM from poultry houses in the IRW in 2006 (Camp Dresser & McKee (CDM), 2008). Ten samples, each from a different facility, were tested for indicator bacteria levels and for a poultry-specific biomarker (the biomarker is discussed in the Microbial Source Tracking Section below). The indicator bacteria concentrations in these samples were generally extremely high, with a geometric mean of ~1200 *E. coli* per gram of litter, and ~51,000 enterococci/g litter. The maximum levels for both indicator bacteria from any one location were over 100,000/g litter (Camp Dresser & McKee (CDM), 2008). *Salmonella* was detected in four of 24 contaminated poultry litter samples (16.7%), but *Campylobacter* was not detected by the culture-based methods used. More sensitive PCR methods that could detect viable but nonculturable pathogens would have been more suited to the detection of pathogens such as *Salmonella* and *Campylobacter* in poultry litter and environmental samples. Given the near-ubiquitous association of these pathogens with poultry feces, my opinion is that these pathogens were present, but that too few were present in a culturable state to be detected by the methods used, which were developed for the food industry and not for environmental samples where pathogens are physiologically stressed.

32. The anticipated pathway of surface water contamination from land-applied poultry litter would begin with runoff from the edges of fields on which litter had been spread. “Edge-of-field” samples collected by CDM in the IRW typically had very high levels of indicator bacteria (Camp Dresser & McKee (CDM), 2008). Some samples had *E. coli* levels of over 1 million/100 ml, which approaches the concentration found in raw sewage (Harwood et al., 2005). Soil samples collected from fields on which poultry litter had been land-applied as levels of up to 2,000 *E. coli* per gram of soil and 17,000 enterococci/g. As expected, IRW surface water samples had variable indicator bacteria levels; however, chronic exceedances of the primary body contact standard for bacteria levels were recorded throughout the IRW (detailed in Teaf, 2008). The data indicate that human exposure to fecal bacteria is occurring since the exceedances also occurred frequently at established “put-in” spots along the IRW, where people enter the water to swim, float, canoe or kayak.

33. Below the surface layer of soil in the IRW is a karst substratum that is riddled with cracks and fissures (Fisher, 2008). The effect of this karst terrain is that surface water and groundwater have a much greater physical connection than they do in other geological formations, and contaminants from the surface, including bacteria, can readily penetrate the shallow aquifer, and from there can find their way to deeper aquifers such as those used for drinking water (Davis, Hamilton & Van Brahana, 2005). Evidence for the widespread influence of surface contamination on groundwater quality is that indicator bacteria were isolated from springs, shallow wells and deep wells in the IRW (detailed in Teaf, 2008). Almost 1700 wells are registered for drinking water use in the Oklahoma portion of the IRW (Fisher, 2008). The owners of these wells generally do not disinfect or otherwise treat the water from the wells, therefore people can be exposed to pathogens that infiltrate the groundwater via runoff from fields on which poultry waste has been land-applied.

34. From 2000-2007 over one billion birds (chickens and turkeys) were produced by the defendants in the IRW (Fisher, 2008), or an average of over 141 million bird/year. In 2005-2006 there were over 1,900 active poultry houses in the IRW, generating an estimated 354,000 tons of waste (Fisher, 2008). Using the geometric mean values obtained from sampling poultry litter in the IRW shown above (and the knowledge that there are 907,184 g in a ton), the annual estimate of poultry litter-associated *E. coli* is 3.9×10^{14} cells (390 trillion), while for enterococci it is 1.6×10^{16} (16,000,000,000,000,000) cells. This material is spread on fields, generally within three to five miles of the area where it was produced, where it can leach into groundwater and run off into surface water (Fisher, 2008).

V. SPECIFIC EVIDENCE OF POULTRY FECAL CONTAMINATION IN THE IRW

35. Chemical/bacterial signal determined by principle components analysis.

Analysis of an array of chemical and bacterial parameters using the multivariate statistical method of principle components analysis has revealed a distinctive “signature” that is characteristic of soils and waters contaminated by poultry waste (Olsen, 2008). The measured parameters included metals, nutrients, physical measurements and indicator bacteria. A definitive poultry waste signature was derived from phosphorus, bacteria, organic carbon, potassium, copper, zinc, and nitrogen-containing compounds. The poultry waste signature was found in all sample types throughout the IRW, including edge-of-field, soils impacted by land application, rivers, streams, and their sediments, groundwater, and Lake Tenkiller. Olsen concluded that a significant source of bacterial contamination in the IRW was poultry waste, and that the signature was present at every leg of the transport pathway from litter to soil to edge-of-

field samples to surface water and ground water (Olsen, 2008). This finding is consistent with my own opinion that land application of poultry litter is a dominant source of bacterial contamination to IRW surface waters and groundwater.

36. Bacterial loading in the IRW.

An analysis of fecal coliform loading from various potential sources in the six counties that contribute to the IRW was conducted for this investigation (Teaf, 2008). Pets, deer and wildlife, and human sources (i.e. septic systems, wastewater treatment plant discharges) together accounted for an estimated 1.4% of total loading of fecal coliforms to the IRW, while livestock accounted for 98.6%. Poultry and cattle contributed an approximately equal, major load (estimated at 41% and 44% of all livestock contributions, respectively). Contaminated poultry litter and soil receiving land-applied poultry litter contains an even higher load of enterococci than fecal coliforms; thus poultry are doubtless a dominant source of fecal indicator bacteria to the IRW.

37. Microbial source tracking.

Fecal coliforms, *E. coli* and enterococci are broad, nonspecific indicators of fecal pollution because they are shed in the feces of almost all warm-blooded animals. Certain animals, such as poultry, frequently harbor human pathogens in addition to indicator bacteria in their gastrointestinal tract (U.S. Environmental Protection Agency, 2005a; U.S. Environmental Protection Agency, 2007). Because the detection of high-risk fecal contamination and its discrimination from other sources of indicator bacteria is needed to inform management decisions and risk assessment, source-specific testing methodologies have been developed and validated (Stoeckel & Harwood, 2007; U.S. Environmental Protection Agency, 2005b). A number of approaches, collectively termed microbial source tracking (MST) methods, have been the subject of investigation and research by many investigators across the country, including U.S. EPA scientists (Santo-Domingo & Sadowsky, 2007; Stoeckel & Harwood, 2007; U.S. Environmental Protection Agency, 2005b; U.S. Environmental Protection Agency, 2007).

38. MST methods can be roughly grouped into library-dependent and library-independent approaches. **Library-dependent** methods typically begin by culturing, or growing, indicator bacteria such as *E. coli* or enterococci from the feces or sewage of various host species (e.g. chickens, cattle, humans) that may impact water quality in the study area. The isolates are typed, or “fingerprinted” by highly discriminatory laboratory methods, and their fingerprints make up the known source library. Fingerprinting can be carried out by a variety of

phenotypic methods, including antibiotic resistance analysis (Hagedorn et al., 1999; Harwood, Whitlock & Withington, 2000; Wiggins, 1996) and carbon source utilization (Harwood et al., 2003). Genotypic fingerprinting, which detects differences among strains at the genetic level, can also be carried out by a number of methods, including ribotyping (Moore et al., 2005; Parveen et al., 1999), pulsed field gel electrophoresis (Stoeckel et al., 2004), and rep-PCR (Johnson et al., 2004).

39. Once the library has been validated for its ability to predict the source of bacteria that are not part of the sample set used to make the library, the fingerprints of isolates from water samples can be matched with their closest neighbors in the library. Because the source of the library isolates is known, the source of each isolate from the water can theoretically be inferred – either by direct matching or by a statistical routine. Interpreting the results of library-dependent MST methods is not usually straightforward, since (a) certain fingerprints in the library will generally be isolated from more than one host, leading to uncertainty about the source of isolates from water that match to these “cosmopolitan” strains, and (b) some water isolates may not match any of the library isolates, which indicates that the library is not comprehensive enough to be representative of the diversity of fecal bacteria in that environment (Harwood, 2007; U.S. Environmental Protection Agency, 2005b).

40. MST libraries are expensive and time-consuming to construct, and their applications across geographical distance or over time spans over one year has not been determined (Harwood, 2007; U.S. Environmental Protection Agency, 2005b; Wiggins et al., 2003). Comparisons among MST methods have been made in several studies (Griffith, Weisberg & McGee, 2003; Moore et al., 2005; Stoeckel & Harwood, 2007). Among the major drawbacks of library-dependent methods was their tendency to false-positive results (detection of contamination from a source when not actually present).

41. **Library-independent** MST methods are less subject to many of the concerns noted above, although careful method validation is still crucial (Griffith et al., 2003; Moore et al., 2005; Stoeckel & Harwood, 2007). Library-independent methods generally rely on detection of a specific gene found in a microorganism that is unique to a certain host species (e.g. cattle) or group of hosts (e.g. ruminants). Polymerase chain reaction (PCR), which is a highly reliable method for specifically detecting and replicating (amplifying) particular genetic sequences, is a generally used and widely accepted method to detect the source-specific microbe. PCR has been a valuable diagnostic tool in hospitals for the last 20 years (Murakawa et al., 1988). Within a few years of its first publication in 1986 (Mullis et al., 1986), PCR was becoming accepted as

a “gold standard” for certain clinical tests (e.g. (Barker, 1994; Wallet, Roussel-Delvallez & Courcol, 1996)). A PubMed search using the terms “diagnosis AND polymerase chain reaction” yields close to 12,000 citations, demonstrating the importance of PCR for identifying specific microbial species in modern disease diagnosis. PCR has also become a crucial forensic tool since the publication in 1988 of the ability to specifically amplify human DNA from a single hair (Higuchi et al., 1988).

42. Comparisons among MST methods have been made in several studies (Griffith et al., 2003; Moore et al., 2005; Stoeckel et al., 2004), which concluded that all of the methods had certain pros and cons. As stated above, one of the major drawbacks of library-dependent methods was their tendency to false-positive results (detection of contamination from a source when not actually present). Errors from library-independent methods tended more toward false-negative results, particularly in fecal samples from individual animals or humans. Since those reports were published, the field has advanced a great deal, particularly in terms of knowledge about how to validate (test the accuracy of) methods (Santo-Domingo & Sadowsky, 2007; Stoeckel & Harwood, 2007; U.S. Environmental Protection Agency, 2005b). Sensitivity (the frequency of positive results when the contaminating source is present) and specificity (the frequency of negative results when the contaminating source is absent) are among the most important attributes of a useful MST test. Therefore, the library-independent methods of MST are reliable tools for fecal source determination provided that the methods are properly validated for sensitivity & specificity.

43. *The poultry litter biomarker (PLB).* No published library-independent MST method was available in 2006 to specifically detect poultry fecal contamination. A study was therefore undertaken to determine if a library-independent MST method for specific detection of poultry feces and associated contaminated litter could be developed. As a result of these efforts a library-independent MST method for detecting and quantifying fecal contamination from poultry litter was developed for the IRW. This library-independent MST method will be referred to here as the poultry litter biomarker (PLB). The PLB method was initially validated for sensitivity using poultry litter contaminated with poultry feces, and for specificity using a variety of fecal samples from non-target hosts as described below. The PLB method was then utilized to detect and quantify the amount of poultry-specific contamination in environmental samples, including soil, edge of field, surface water and ground water samples collected in the IRW.

44. Figure 2 presents an overview of the PLB method development, which began with identification of candidate bacteria that were widespread (prevalent) in fecal-contaminated

poultry litter and also made up a substantial proportion (high concentration) of the bacterial population in the litter. Fecal-contaminated poultry litter rather than feces was used to develop the biomarker to ensure that the MST target could survive deposition on poultry litter and subsequent spreading on fields. The method development can be divided into the following stages: (I) target identification; (II) validation of target sequence for poultry litter –specific marker; and (III) quantification.

45. *Target identification.* A preliminary screening effort searched for bacterial DNA sequences that were common and represented a substantial fraction of the microbial population in fecal-contaminated turkey and chicken litter, as well as in soils impacted by land application of poultry litter. Because of the uncertainty about which bacterial group would yield the best poultry-specific target, DNA from three different groups was analyzed: *E. coli, Bacteroidales* (a bacterial family to which the genus *Bacteroides* belongs) and total bacteria. The 16S rRNA gene was chosen as the target for PCR. This gene is used as a "molecular chronometer" because it tends to be very stable and mutate at a very low rate, so that the rate of change is proportional to evolutionary distance and changes occur over geological time periods (generally thousands to millions of years) (Woese, Kandler & Wheelis, 1990). The use of this gene as a target reduces errors related to sensitivity and specificity because it is not prone to change. The 16S rRNA genes of each bacterial group were amplified by PCR, and terminal restriction fragment length polymorphism (TRFLP) was used to create DNA fragments that allowed identification of potential targets. Cloning and DNA sequencing of potential targets from each pool (*E. coli, Bacteroidales* or total bacterial DNA) was carried out to determine the precise sequence of the gene fragment. Each DNA sequence was compared to the worldwide NCBI (National Center for Biotechnology Information) database, which is a repository for gene sequences from all organisms. The DNA sequence comparison was used to screen out (discard) non-useful targets that had been identified in habitats or animals/humans other than the gastrointestinal tracts or feces of poultry. The DNA sequence screening process yielded four sequences that were ubiquitous in poultry litter and contaminated soil, and also contained unique sequences that allowed development of target-specific PCR primers. Three of these sequences were from the total bacteria DNA pool and one was from the *E. coli* DNA pool. The sequence derived from the *E. coli* pool was identified as the closely-related bacterium *Pantoea ananatis.* Following comparison with the NCBI database, none of the candidate sequences were from the *Bacteroidales* pool because all were found in other habitats or animals.

46. *Validation of target sequence.* A PCR primer set was developed for each of the four potential targets (three bacterial and one *E. coli*) (Table 1). To increase the sensitivity of detection, a nested PCR approach was employed in which DNA was first amplified using universal bacterial primers (or all-*E. coli* primers) followed by amplification of an internal fragment with the target primers. Assay sensitivity was tested against composite poultry litter samples and against soil samples on which poultry litter had been land-applied. Specificity of the assays was tested against fecal samples from beef and dairy cattle, swine, ducks, geese, and human sewage. The collection and handling of these fecal samples is detailed in Dr. Olsen's report (Olsen, 2008), but a brief description of the makeup of these samples is below.

47. Nontarget fecal samples (from animals other than poultry and human sewage) for specificity testing were collected as composites from groups of individuals (Table 2). Beef cattle fecal samples were collected from ten grazing fields, of which five were within the watershed and five were outside the watershed. Two independent duplicate samples were collected for each field, and each duplicate consisted of feces from ten scats (feces from ten scats = 1 composite). A total of 200 beef cattle scats were tested. Duck (5 composites) and goose (5 composites) fecal samples were collected in the same fashion, consisting of composites from ten individual scats, and independent duplicates were collected for each area (Table 2). For ducks, three landing areas inside the watershed and two outside the watershed were sampled, while for geese, two landing areas inside and three landing areas outside the watershed were sampled. Composite samples of fecal slurries were collected from swine facilities, one inside the watershed and one outside (2 duplicate samples/facility) and dairy cattle farms (one inside the watershed and two outside (2 duplicate samples per facility) human residential septic cleanout tanks (3 samples) and influent of three separate municipal wastewater treatment plants (3 samples). A total of 20 g of each fecal sample from each site was collected and was placed in a 20 ml, sterile, polystyrene tube containing 10 ml of 20% glycerol and shipped on dry ice to the laboratory.

48. The PCR assay with greatest sensitivity (consistently able to detect the target in contaminated samples and specificity (lack of detection in non-target samples) was produced by primer set LA35, which targets a 16S rRNA gene fragment of 571 base pairs that is 98% identical to the DNA of *Brevibacterium avium.* The sequence was detected in all litter samples, and in eight of ten contaminated soil samples. Among the non-target fecal samples, it was only detected in one composite goose and one composite duck sample, each of which was collected outside the IRW (Table 2). Furthermore, the PLB was detected in only one of two duplicate

samples from the cross-reactive duck and goose fecal composite, showing that it was present at low concentration in these samples.

49. PCR Validation Summary

- The nested PCR assay detected the PLB in all contaminated poultry litter samples, and in 80% of soils sampled from fields that received land-applied poultry. These tests indicated the method's sensitivity.

- The nested PCR assay did not detect the PLB in any of the nontarget fecal samples from the IRW, and found the target in low concentration (1 of 2 duplicates) from one duck and one goose sample collected outside the IRW. These tests indicated the method's specificity.

50. *Quantitative PCR.* A quantitative PCR (QPCR) assay was developed for the PLB using the LA35 primer set and Sybr green chemistry. This particular QPCR chemistry has the major advantage of allowing the production of a melting curve, which is determined by the temperature at which the double-stranded DNA of the PCR product melts and becomes single-stranded. Because the melting curve is particular to a given DNA sequence, this analysis allows a check of the purity and the identity of the QPCR product, which is particularly useful when analyzing environmental samples.

51. A QPCR assay should have a linear response to increasing concentrations of its target; in other words, the more copies of the gene are present, the more rapidly the signal rises. The precise quantitative nature of the PLB is demonstrated in Figure 3, which is a graph of crossing time (C_t) vs. gene copies of PLB. Crossing time is the time (generally in minutes) required until the fluorescent signal crosses a threshold above background levels, and is inversely proportional to gene copy number (the time required for the signal to rise above background levels is less as the concentration of target increases). The PLB gene fragment cloned into a plasmid was used as the template for the standard curve (Figure 3). The slope of the graph is negative (decreasing from left to right) because the C_t (time required to detect fluorescence) decreases with increasing concentrations of target DNA (in this case the PLB)

52. Although the same primers and annealing conditions (60º C) were used for both conventional nested PCR and QPCR, a number of fecal samples were re-tested by QPCR for specificity, including the goose and duck duplicate that were each found to be positive by the ultra-sensitive nested PCR. Table 3 contains results for previously tested samples (conventional

nested PCR) that were re-tested for specificity. Each of these samples was below detection limit, or negative by QPCR, including the duck and goose sample that were positive by conventional nested PCR. Seven newly-collected beef cattle samples (Camp Dresser & McKee (CDM), 2008) were assayed and three uncontaminated (clean) poultry litter samples were tested (Table 4). Each of these control (clean poultry litter) and non-target samples gave results of "below detection limit" (BDL). In other words, a QPCR signal was not present in non-target animal fecal samples and clean litter. These results confirm the specificity of the PLB QPCR assay.

53. The concentration of fecal indicator bacteria in used poultry litter was compared to the concentration of the PLB to establish the relationship between the indicator organisms of fecal contamination and the poultry-specific marker. Enterococci concentrations were strongly and very significantly correlated with the PLB ($r^2 = 0.7471$; $P = 0.013$) (Figure 4), and *E. coli* concentrations also had a positive relationship with PLB concentration ($r^2 = 0.3946$; $P = 0.052$). The correlation of the poultry-specific PLB with the general fecal bacteria indicators provides confidence that co-contamination of waters with both types of indicators is common, and that they indicate a substantial health threat to recreational water users due to the known association of pathogens such as *Campylobacter* and *Salmonella* with poultry feces.

54. The QPCR assay for the PLB was field-tested on litter, soil and water samples, including edge-of-field, surface water and ground water samples. A total of ten soiled litter samples, 187 water samples and 40 soil samples were tested. Three of the water samples (BS-REF; Table 4) were collected outside of the IRW where used poultry litter is not land-applied; therefore they represent reference water samples which should not contain the PLB. In fact, the PLB in each of these samples was not detected in the negative control (reference) samples (Table 4). All contaminated litter samples contained very high concentrations of the PLB, ranging from $2.2X10^7$ - $2.5X10^9$ (tens of millions to billions) gene copies/g (Table 5). The PLB was at high enough concentration to be quantified by QPCR in 34 water samples, including 16 edge-of-field samples (Table 5), one groundwater sample (56287-7-13-06) and one spring sample (LAL15SP2-7-11-06). Six soil samples had quantifiable levels of the PLB, with the greatest at $3.8 X 10^6$ gene copies/ml. Figures 5 and 6 show the results of QPCR testing for the PLB in water and soil samples, respectively. The level of quantified PLB for each site (location) is designated by a colored circle. Note that several sites were sampled more than once, so that the number of data points is fewer than the total number of samples in which the PLB was quantified.

55. *Nested Sybr green PCR.* When the PLB concentration was below detection limit in the QPCR assay, a nested variant of this assay (which is presence-absence, rather than quantitative) was used to determine if lower levels of the PLB were present. In this case DNA extracted from the environmental samples was first amplified by conventional PCR using universal bacterial (16S rRNA) primers. This primary amplification step was followed by a secondary amplification step with the PLB primers (the LA 35 set). The identity and purity of the PCR product was always checked by conducting a melting curve analysis. This nested Sybr green procedure allowed detection of the PLB in many samples in which the PLB was at too low a concentration to quantify. Of 40 total soil samples collected from fields that received land-applied poultry litter, 38 had detectable levels of the PLB. Of 187 water samples (including 3 reference unimpacted samples) 99 had PLB levels below the detection limit, but 88 water samples had detectable levels of the PLB, including 1 geoprobe (shallow groundwater) sample (GPGW-10-4-11-30-06). A total of 3 spring or groundwater samples had detectable or quantifiable concentrations of the PLB, demonstrating transport of poultry waste in the subsurface. Furthermore, two of the samples that contained quantifiable concentrations of the PLB (HFS16-BF2-03-8-27-05 and HFS22-BF2-01-8-1-06) were base flow samples, which consist mainly of groundwater. Figures 5 and 6 show the results of nested Sybr green PCR testing for the PLB in water and soil samples, respectively. Sites at which the PLB was detected, but was too low to quantify by QPCR are designated by black triangles.

VI. CONCLUSIONS

56. Testing of poultry litter, soils upon which poultry litter has been applied, and edge-of-field samples collected from ditches during runoff conditions all show high levels of fecal indicator bacteria, some of which approach the levels expected in raw sewage. When these bacteria reach the extensive network of IRW tributaries, they become dominant contributors to the fecal indicator bacteria loads that impair the use of the Illinois River and its tributaries as recreational waters. The fecal indicator bacteria concentrations observed in the IRW tributaries, including those that receive extensive recreational use, are not characteristic of those in rural areas that are unimpacted by fecal contamination; rather, they are similar to areas that are extensively impacted by sewage or large-scale animal farming. The pathogenic microorganisms that are excreted in poultry feces and land-applied on contaminated poultry litter can impact the health of those who use the river for recreation, and also penetrate into the groundwater and contaminate the area's rural drinking water source. Sampling of IRW surface

water, groundwater, soil and sediments has revealed a unique chemical and bacterial signature that indicates contamination by poultry; and this signature is not present in areas that are remote from poultry operations. The finding that a poultry litter-specific biomarker (PLB) is found in all environmental compartments tested in the IRW, from soil samples to edge-of-field samples to surface water and groundwater, firmly links a dominant portion of the indicator bacteria contamination to poultry waste, which is well known to contain important human pathogens such as *Salmonella* and *Campylobacter*. Thus, the disposal of poultry waste by land application in the IRW presents a substantial, serious and immediate threat to human health.

57. If land application of poultry litter continues in the IRW, the loading of bacteria and particulate matter, which contributes to water turbidity, will continue. Much of this particulate matter settles out in stream bottoms and forms a habitat where the microbial contaminants can survive for long time periods – on the order of months or longer. The quality of surface water and groundwater in the IRW will continue to decline and the threat to human health will remain or increase. If land application of poultry litter ceases a major source of microbial contamination to the IRW will be removed. Once land application ceases and rain events over a season scour the contaminated soils and sediments, microbial water quality should substantially improve and the threat to human health will substantially decrease.

58. My opinions in this matter are my own, and do not reflect an official view of the University of South Florida.

Valerie J. Harwood, Ph.D.
Associate Professor
Department of Biology
University of South Florida

Table 1. Nucleotide sequences and targets of primers used in this study.

Primer	Target	Sequence (5'-3')	Position	Tm (^{o}C)	T-RF
LA35F	*Brevibacterium*	ACCGGATACGACCATCTGC	166-184	57	147.3
LA35R	clone LA35	TCCCCAGTGTCAGTCACAGC	717-736	58	
SA19F	*Kineococcus*	TACGACTCACCTCGGCATC	163-181	56	158.9
SA19R	*spp.*	ACTCTAGTGTGCCCGTACCC	602-621	55	
SB37F	*Rhodoplanes*	AACGTGCCTTTTGGTTCG	143-160	56	142.9
SB37R	*spp.*	GCTCCTCAGTATCAAAGGCAG	616-626	55	
SA15F	*Pantoea*	CGATGTGGTTAATAACCGCAT	490-510	56	500.8
SA15R	*ananatis*	AAGCCTGCCAGTTTCAAATAC	668-688	55	

Table 2. Specificity of the nested PCR assay for PLB against nontarget fecal samples from within and outside the watershed.

	Number of samples tested (Number of samples containing potential biomarker)			
Fecal sample (inside or outside watershed)	*Brevibacterium* clone LA35	*Rhodoplanes* clone SB37	*Kineococcus* clone SA19	*Pantoea ananatis* clone SA15
Beef cattle (outside)	5 (0)	5 (2)	5 (1)	5 (0)
Beef cattle (inside)	5 (0)	5 (3)	5 (5)	5 (1)
Dairy cattle (outside)	2 (0)	2 (1)	2 (1)	2 (1)
Dairy cattle (inside)	1 (0)	1 (1)	1 (0)	1 (0)
Swine (outside)	1 (0)	1 (1)	1 (1)	1 (0)
Swine (inside)	1 (0)	1 (0)	1 (0)	1 (0)
Duck (outside)	2 (1)*	2 (2)	2 (2)	2 (2)
Duck (inside)	3 (0)	3 (1)	3 (1)	3 (2)
Goose (outside)	3 (1)*	3 (3)	3 (2)	3 (2)
Goose (inside)	2 (0)	2 (2)	2 (1)	2 (1)
Human (outside)	2 (0)	2 (2)	2 (2)	2 (1)
Human (inside)	4 (0)	4 (3)	4 (1)	4 (1)

Table 3. QPCR results for testing of feces from beef cattle (MAN-BC), duck (MAN-DK), goose (MAN-GS), swine (MAN-SW) and human sewage (MAN-HM) that were previously used in specificity testing by the nested PCR method.

Sample ID	Matrix	DNA (mg/L or mg/g)[a]	qPCR Poultry Specific Biomarker (copies/g fecal material) [b]	qPCR Matrix Spike Amplified?[c]	Biomarker Melt Peak Identified?	Other Melt Peaks Observed?
MAN-BC-9a	Fecal material	11.7	BDL	Yes	NA	NA
MAN-DC-3a	Fecal material	13.9	BDL	Yes	NA	NA
MAN-DK-1a	Fecal material	13.6	BDL	Yes	NA	NA
MAN-DK-2a	Fecal material	1.0	BDL	Yes	NA	NA
MAN-DK-3a	Fecal material	3.5	BDL	Yes	NA	NA
MAN-DK-4a	Fecal material	24.9	BDL	Yes	NA	NA
MAN-DK-5a	Fecal material	2.3	BDL	Yes	NA	NA
MAN-GS-1a	Fecal material	1.6	BDL	Yes	NA	NA
MAN-GS-2a	Fecal material	1.7	BDL	Yes	NA	NA
MAN-GS-3a	Fecal material	1.3	BDL	Yes	NA	NA
MAN-GS-4a	Fecal material	2.0	BDL	Yes	NA	NA
MAN-GS-5a	Fecal material	0.7	BDL	Yes	NA	NA
MAN-SW-2	Fecal material	12.9	BDL	Yes	NA	NA
MAN-HM-2	Fecal material	0.4	BDL	Yes	NA	NA
MAN-HM-5	Fecal material	0.4	BDL	Yes	NA	NA
MAN-BC-9a	Fecal material	11.7	BDL	Yes	NA	NA

[a] "0" indicates that the DNA concentration was less than the detection limit.
[b] "Present" indicates that the biomarker was amplified, but was not quantifiable. "BDL" indicates below detection limits
[c] if "no" indicates that sample did not amplify with qPCR even after a sepharose cleanup was performed and the sample was diluted to a lower DNA concentration indicative of inhibition.

Table 4. QPCR results for testing of clean litter (BS-bedding material), seven additional beef cattle fecal samples collected in 2008 (BC-) and reference water samples (collected outside the watershed in an area thought to be free of poultry impact).

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Sample ID	Matrix	DNA (mg/L or mg/g)[a]	qPCR Poultry Specific Biomarker (copies/L water or g soil or g litter) [b]	qPCR Matrix Spike Amplified? [c]	Nested qPCR Amplified? [d]	Biomarker Melt Peak Identified? [d]	Other Melt Peaks Observed?
BS-REF3-SW-9-01-05	Water	0.7	BDL	Yes	No	NA	NA
BS-REF2-SW-8-30-05	Water	1.8	BDL	Yes	No	NA	NA
BS-REF1-SW-8-30-05	Water	1.1	BDL	Yes	No	NA	NA
BC-20F 1-7	Fecal material	26.1	BDL	Yes	No	NA	NA
BC-20F 8-10	Fecal material	5.0	BDL	Yes	No	NA	NA
BC-21F	Fecal material	17.2	BDL	Yes	No	NA	NA
BC-22F	Fecal material	19.2	BDL	Yes	No	NA	NA
BC-23F	Fecal material	15.8	BDL	Yes	No	NA	NA
BC-24F	Fecal material	15.7	BDL	Yes	No	NA	NA
BC-24F-02	Fecal material	0.2	BDL	Yes	No	NA	NA
BM-WS1	Bedding material	20.7	BDL	Yes	No	NA	NA
BM-WS2	Bedding material	2.7	BDL	Yes	No	NA	NA
BM-RH1	Bedding material	2.6	BDL	Yes	No	NA	NA

[a] "0" indicates that the DNA concentration was less than the detection limit.
[b] "Present" indicates that the biomarker was amplified, but was not quantifiable. "BDL" indicates below detection limits
[c] if "no" indicates that sample did not amplify with qPCR even after a sepharose cleanup was performed and the sample was diluted to a lower DNA concentration indicative of inhibition.
[d] N/A, not applicable. The sample was not run with the nested qPCR assay and/or the biomarker melt peak was not identified because the biomarker did not amplify in the qPCR sample run.

Table 5. QPCR results for litter, soil and water samples with quantifiable concentrations of the poultry litter biomarker (PLB).

Sample ID	Matrix	Gene copies/mL water or /g soil or litter		
FAC-010-9-22-06	Litter	2.04E+09	±	4.14E+08
FAC02-6-21-06	Litter	4.13E+08	±	1.78E+07
FAC-03-7-6-06	Litter	1.03E+09	±	8.00E+07
FAC-04-7-12-06	Litter	1.67E+08	±	2.98E+07
FAC-05-7-13-06	Litter	1.47E+09	±	1.93E+08
FAC-06-7-20-06	Litter	4.46E+08	±	7.34E+07
FAC-07-8-3-06	Litter	2.49E+09	±	9.54E+07
FAC-08-8-15-06	Litter	1.47E+09	±	2.25E+08
FAC-09-8-31-06	Litter	7.57E+08	±	1.55E+08
FAC1-6-20-06	Litter	2.15E+07	±	7.07E+06
LAL-16C-2-7-18-06	Soil	1.42E+04	±	1.97E+03
LAL6-A-2-6-14-06	Soil	1.55E+04	±	2.57E+03
LAL6-D-2-6-15-06	Soil	4.98E+03	±	1.88E+02
LAL8-A-2-6-19-06	Soil	7.00E+03	±	4.43E+02
LAL12-A-2-Q-7-6-06	Soil	3.56E+05	±	1.56E+05
LAL5-A-2-6-13-06	Soil	3.75E+06	±	1.33E+06
EOF-1-6-17-06	Water	1.15E+05	±	1.80E+04
EOF-222-4-13-07	Water	1.32E+05	±	2.71E+04
EOF-SPREAD-010-5-9-06	Water	1.05E+07	±	1.70E+06
EOF-SPREAD-023-6-18-06	Water	1.11E+05	±	2.49E+03
EOF-SPREAD-064-5-4-06	Water	1.89E+06	±	7.63E+04
EOF-SPREAD-065-5-4-06	Water	3.45E+04	±	1.64E+03
EOF-SPREAD-071-5-9-06	Water	3.63E+04	±	8.25E+03
EOF-SPREAD-073B-6-18-06	Water	5.56E+07	±	5.25E+06
EOF-SPREAD-17A-01-5-1-06	Water	2.48E+06	±	4.71E+05
EOF-SPREAD-60-01-4-29-06	Water	3.90E+07	±	8.26E+06
SPREAD-023-4-25-06	Water	1.25E+06	±	2.35E+05
SPREAD-036-4-25-06	Water	1.48E+05	±	4.04E+04
56287-7-13-06	Water	2.58E+04	±	9.58E+03
EOF27-6-8-05	Water	3.48E+05	±	2.30E+04
EOF28-6-8-05	Water	2.61E+03	±	1.21E+02
EOF-SPREAD-007-5-4-06	Water	2.08E+05	±	2.66E+04
EOF-SPREAD-053B-5-4-06	Water	9.66E+05	±	3.86E+05
EOF-SPREAD-071-5-9-06	Water	8.78E+03	±	1.81E+03
HFS-14-PEAK-4-25-06	Water	1.75E+05	±	6.06E+04
HFS-20-EVENTB-5-10-06	Water	2.77E+04	±	5.61E+03
HFS-22EVENTB-5-31-06	Water	2.37E+02	±	7.14E+01

RS-349-BIO-8-11-06	Water	2.86E+03	±	8.60E+02
Spread-029-4-25-06	Water	1.33E+04	±	2.67E+03
Spread30-01-3-31-06	Water	8.51E+05	±	2.64E+05
EOF-SPRD-26-4-25-06	Water	1.81E+06	±	5.46E+05
HFS02Libby-6-15-05	Water	1.13E+05	±	4.65E+04
HFS16-BF2-03-8-27-05	Water	6.61E+04	±	8.11E+03
HFS22-BF2--01-8-1-06	Water	1.26E+05	±	5.76E+04
HFS23-7-16-05	Water	6.87E+03	±	2.96E+03
LAL15SP2-7-11-06	Water	2.19E+03	±	5.94E+02
RS-233-5-21-07	Water	3.29E+05	±	1.09E+05
RS-399A-5-2-07	Water	1.01E+04	±	2.88E+03
HFS-20-9-16-05	Water	4.83E+04	±	1.53E+04
HFS-20-EvA-5-9-06	Water	1.13E+06	±	5.70E+04

Figure 1. Fecal coliform concentrations vs. *E. coli* concentrations in IRW samples.

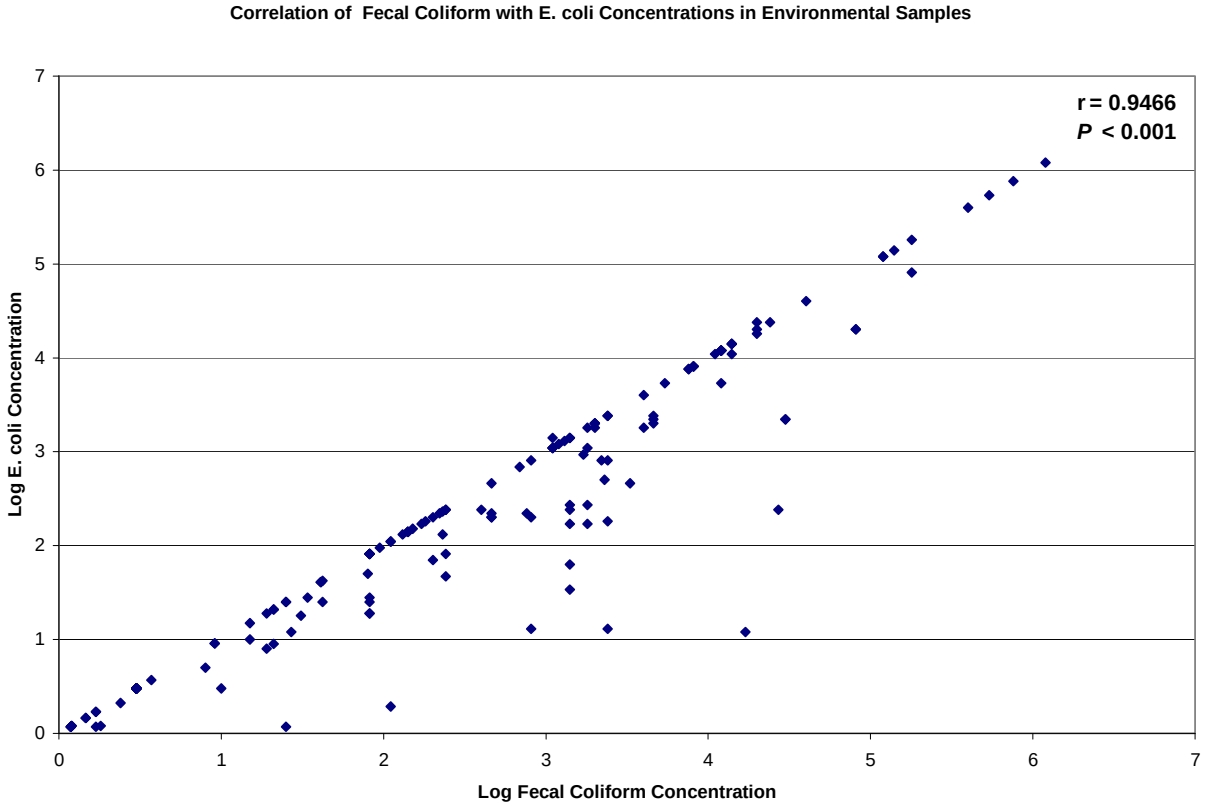


Figure 2. Overview of poultry litter biomarker (PLB) development and validation.

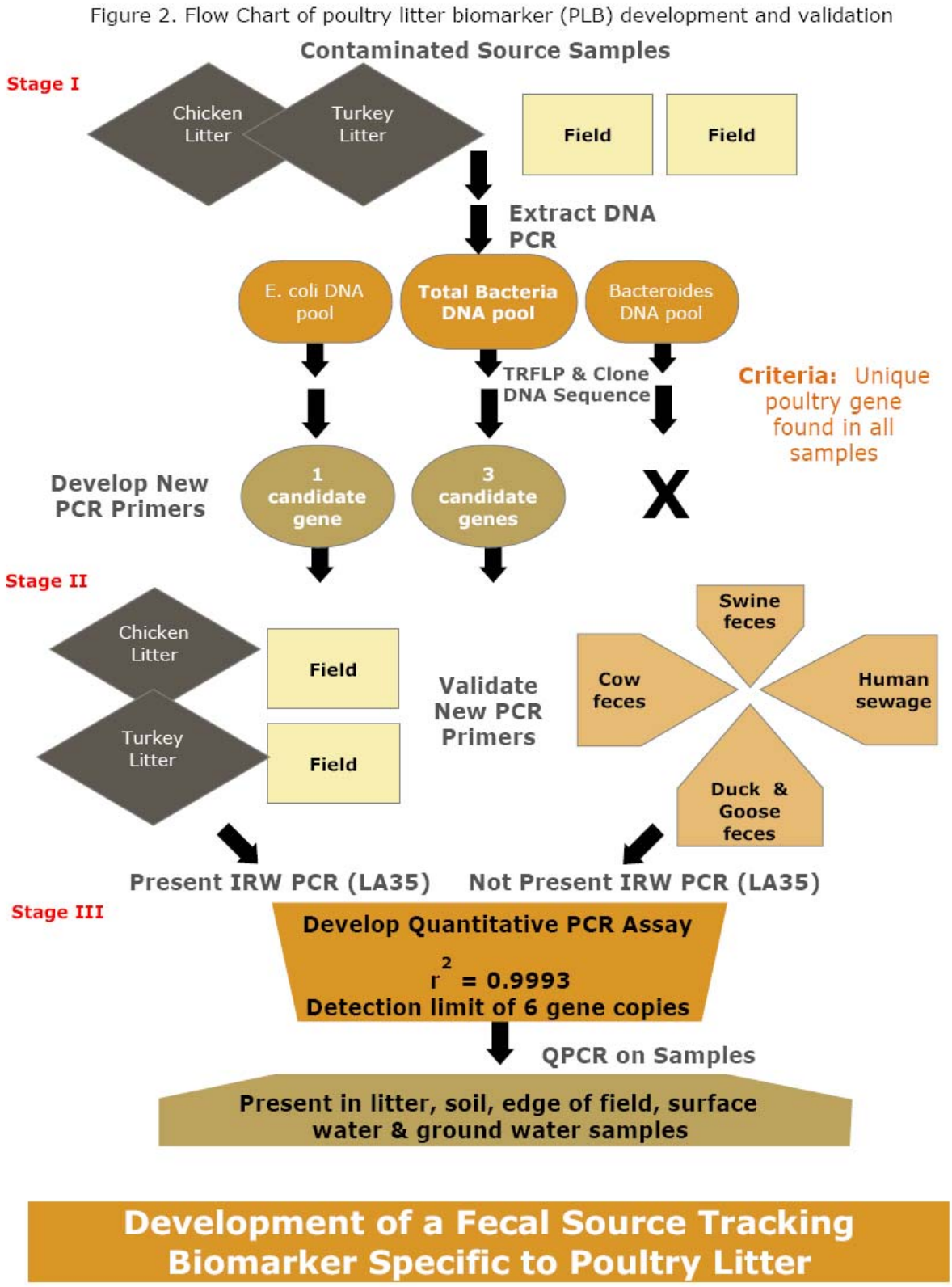


Figure 3. Standard curve showing the linear relationship between fluorescent signal (Ct value) and increasing poultry litter biomarker (PLB) gene copy number.

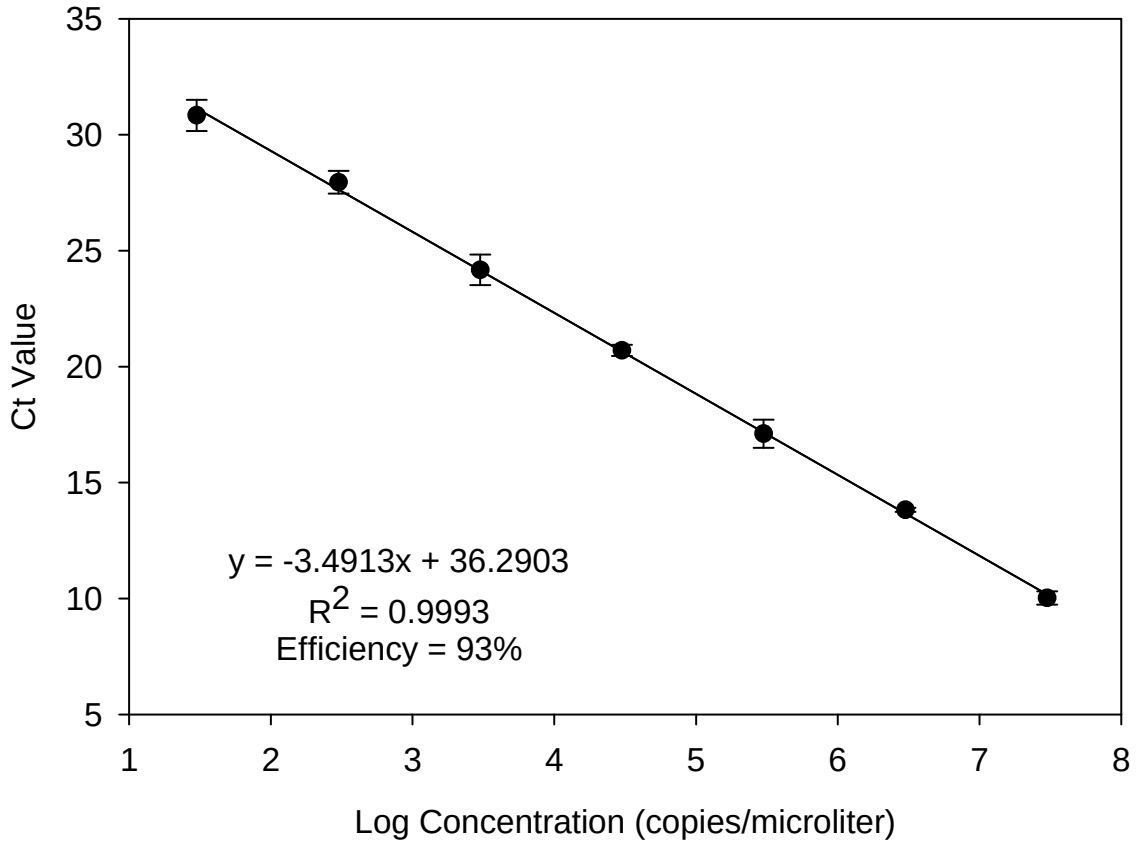


Figure 4. Correlation of enterococci concentrations with the poultry litter biomarker (QPCR) concentration.

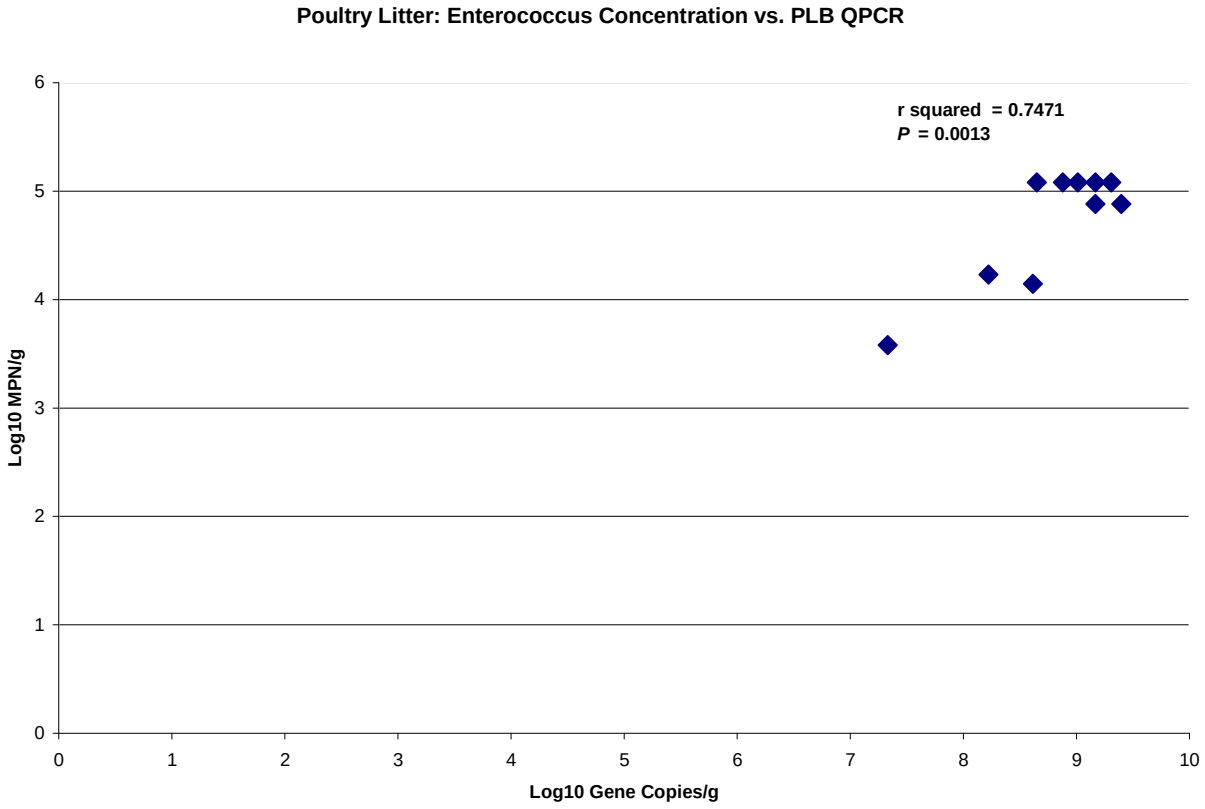


Figure 5. Water sample locations that were positive for the poultry litter biomarker (PLB) in the IRW. Colored circles mark quantifiable levels determined by QPCR. Black triangles mark detectable levels by nested Sybr green PCR.

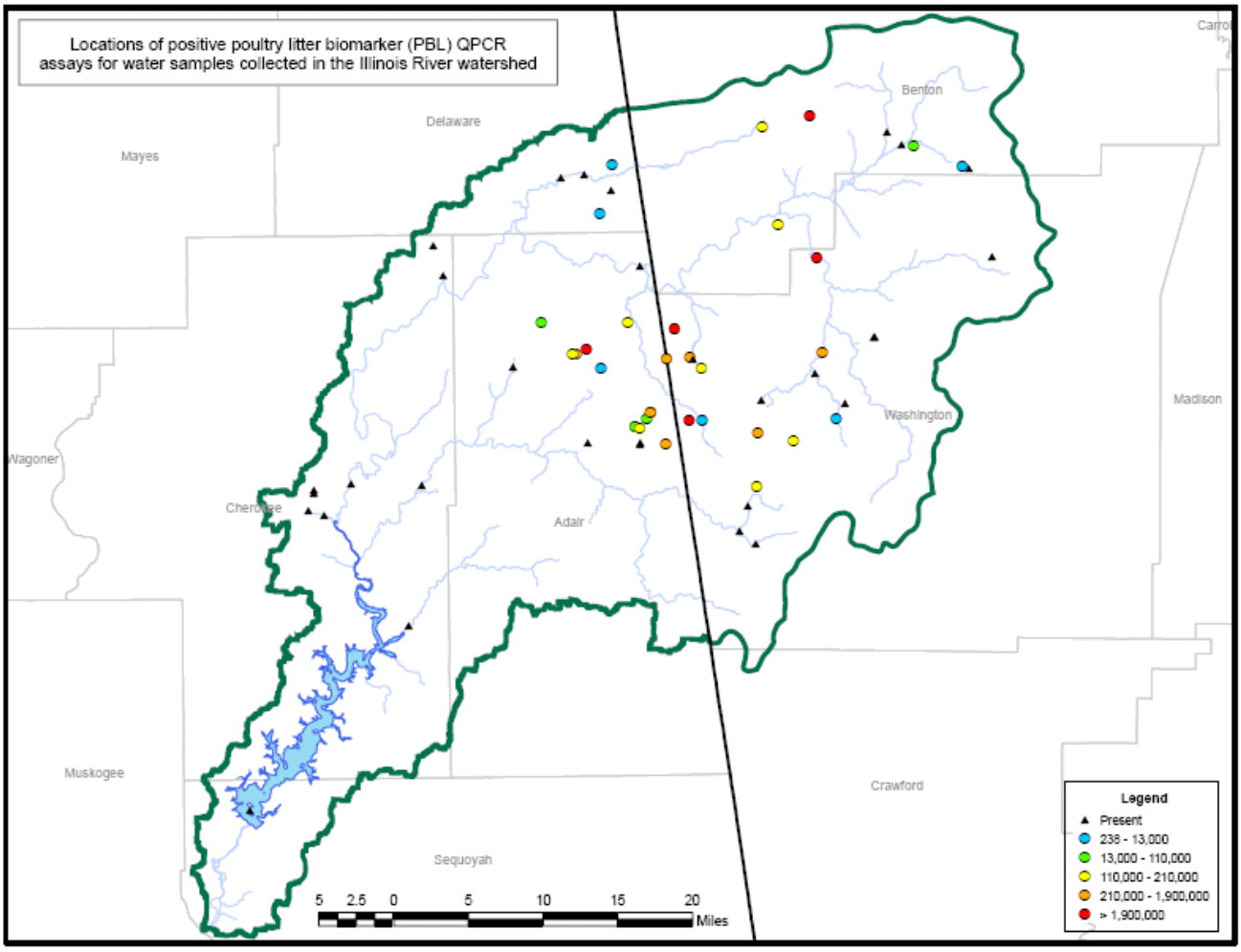
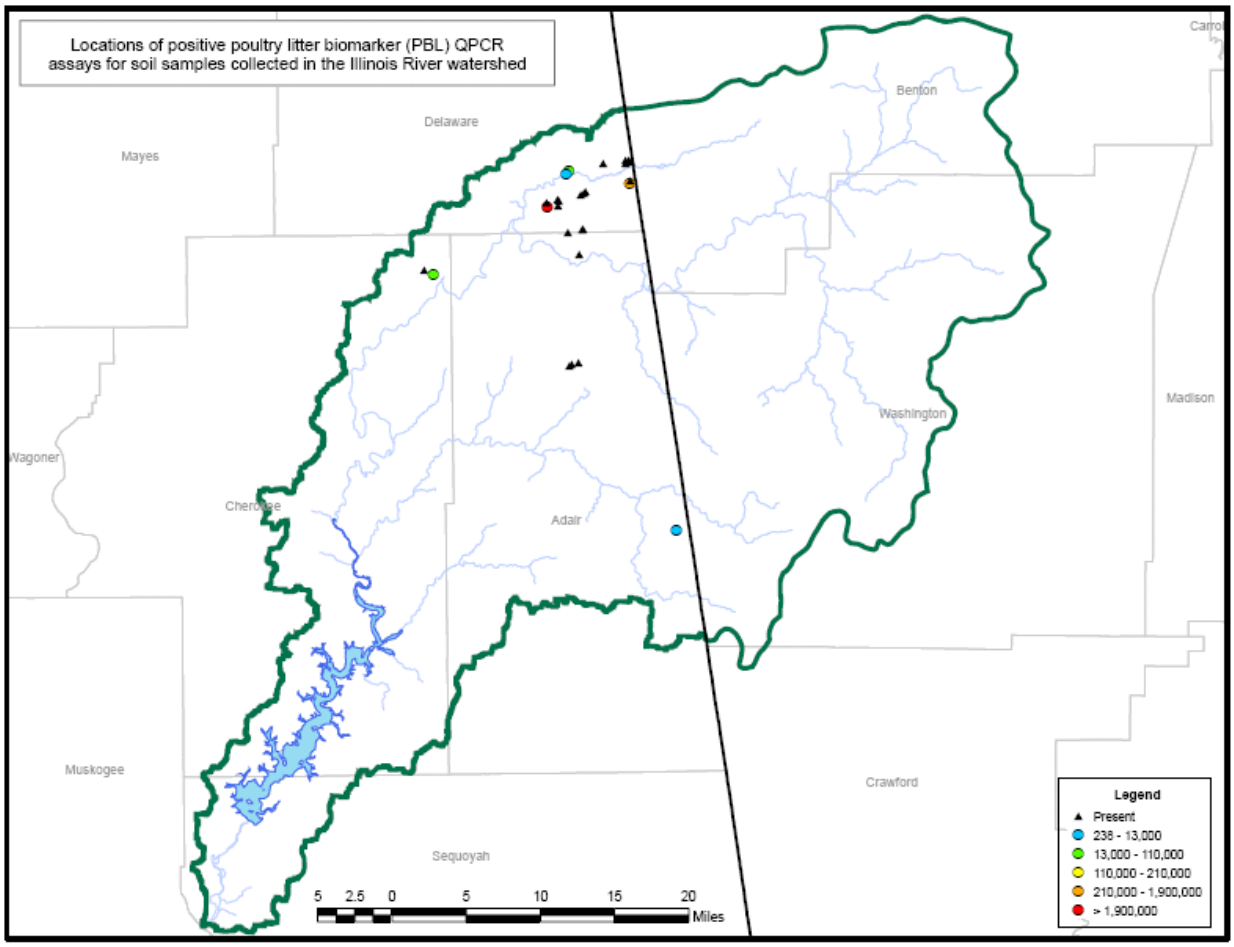


Figure 6. Soil sample locations that were positive for the poultry litter biomarker (PLB) in the IRW. Colored circles mark quantifiable levels determined by QPCR. Black triangles mark detectable levels by nested Sybr green PCR.



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