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Facoltà di SCIENZE MM. FF. NN. – AGRARIA

TESI DI LAUREA SPECIALISTICA IN

SCIENZE E TECNOLOGIE PER L'AMBIENTE E IL TERRITORIO

Most Probable Number and Real-Time PCR Methodology for Simultaneous Detection and Evaluation of *Salmonella* Strains and *Escherichia coli* O157:H7 Presence in Irrigation Reservoirs

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ABSTRACT

In general, contamination of food by pathogens of animal origin is well established, but awareness is growing that fresh or minimally processed fruit and vegetables can also be sources of pathogenic bacteria. In particular *Escherichia coli* O157:H7 and *Salmonella* spp are known to be emerging human pathogens and are strongly associated with produce-related outbreaks. To better understand whether surface irrigation waters might be a source of these two pathogens, five irrigation ponds were studied. Water microbiological, environmental and chemical factors were studied to determine if there was a correlation between any of these factors and the presence of pathogens. The methodology for pathogens detection combined a Most Probable Number (MPN) method, a filtration technique for large volumes of surface water, a standard selective media and a real-time PCR TaqMan assay. *E. coli* O157:H7 concentrations ranged from not detectable to 0.98 MPN per L and from below detection limit to 2.07 MPN per L for *Salmonella* spp. With variables at disposal, it was found that *E. coli* presence seems to be related with water temperatures whereas Salmonella with nutrients in the water. We were able to explain about 65% of experiment variability of this system indicating there might be others variables governing the occurrence of the pathogens. In these systems more in depth study and more frequent sampling plan can be developed and correlated with the best agricultural and environmental management practices.

RIASSUNTO

In generale, la contaminazione di cibo da patogeni di origine animale è ben conosciuta ma sta aumentando la consapevolezza che frutta e verdura fresca o minimamente lavorata possa anche essere fonte di batteri patogeni. In particolare, l'*Escherichia coli* O157:H7 e le Salmonelle sono risaputi essere patogeni emergenti per l'uomo. Per meglio capire se le acque per irrigazione superficiali possano essere una sorgente di questi due patogeni, sono stati studiati 5 laghi usati come riserve per l'irrigazione considerando fattori microbiologici, ambientali e chimici delle acque per determinare se ci sia una correlazione tra questi fattori e la presenza di patogeni. La metodologia d'individuazione combina il metodo del numero di batteri più probabile (MPN), una tecnica di filtraggio per grandi volumi d'acqua con standard e selettivi mezzi di coltura e la real-time PCR quantitativa con la sonda TaqMan. Le concentrazione di *E. coli* O157:H7 trovate, vanno da non rilevabile a 0.98 MPN per L e da sotto il limite di detenzione a 2.07 MPN per L per le salmonelle. Con le variabili a disposizione fu

trovato che la presenza dell'E. coli sembra essere legata alle temperature mentre la Salmonella ai nutrienti. Dall'analisi multivariata dei dati sperimentali siamo stati in grado di spiegare circa il 65% della totale variabilità del sistema, indicando che ci potrebbero essere altre variabili a governare la presenza di patogeni. In questo tipo di sistemi, uno studio più accurato e una maggiore quantità di dati possono essere sviluppati e correlati a migliori pratiche di gestione agricola e ambientale.

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Abbreviations AND ACRONYMS

A/E	Attaching and Effacing
ATCC	American Type Culture Collection
CI	Confidence Interval
CL	Confidence Limit
CSTE	Council of State and Territorial Epidemiologists
Ct	Threshold Cycle
DAEC	Diffusely Adherent E. coli
Eae	E. coli Attaching and Effacing
EAEC	EnteroAggregative E. coli
EC	Eiken Chemical (broth)
EHEC	EnteroHaemorrhagic E. Coli
EIEC	EnteroInvasive E. Coli
EPEC	EnteroPathogenic E. Coli
ETEC	EnteroToxigenic E. Coli
FAM	6-carboxyfluorescein (dye for Salmonella)
FBDOs	Foodborne-disease Outbreaks
FC	Fecal Coliform
FDA	Food and Drug Administration
HC	Hemorrhagic Colitis
HEX	6-carboxyhexafluorescein (dye for E coli)
HUS	Hemolytic Uremic Syndrome
LEE	Locus of Enterocyte Effacement
LPS	Lipopolysaccharide
LTB	Laural Tryptose Broth
MCLG	Maximum Contaminant Level Goal
MMWR	Morbidity and Mortality Weekly Report
MOD	Matched Odds Ratio
MPN	Most Probable Number
MUG	4-MethylUmbelliferyl–ß-D-Glucuronide
NESPAL	National Environmentally Sound Production Agriculture Laboratory
NNDSS	National Notifiable Diseases Surveillance System

ONPG	Ortho-NitroPhenyl-ß-Galactoside
ORP	Oxidation / Reduction Potential
PBS	Phosfate Buffered Solution
PCA	Pricipal Conponents Analysis
PFGE	Pulsed-Field Gel Electrophoresis
Rn	Normalized Reporter (signal)
RV	Rappaport-Vassiliadis (broth)
SDWA	Safe Drinking Water Act
SFA	Segmented Flow Analysis
SIP	Salmonella invasion protein
TAMRA	Tetramethyl-6-Carboxyrhodamine (dye)
TB	Tetrathionate Broth
TN	Total Nitrogen
TP	Total Phosphorus
TSS	Total Suspended Solids
TC	Total Coliform
TT	Treatment Technique
TTP	Thrombotic Thrombocytopenic Purpura
USEPA	United State Environmetal Protection Agency
VT	VerocytoToxin
WBDO	Water Borne Disease Outbreak

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1 INTRODUCTION

1.1 Food safety

In recent decades, changes in food production and consumption have impacted the safety of food. The food industry has evolved from being local to global, where production and processing are centralized in different parts of the country and world. Large-scale “factory farms”, feedlots, and processors allow pathogens to be dispersed widely through fast-paced slaughterhouses and processing plants. Furthermore, some foodborne pathogens have become more virulent, while the U.S. population is aging and increasingly vulnerable to foodborne illness.

Unsafe foods cause an estimated 76 million illnesses and 5,000 deaths each year in the United States (Mead et al. 1999). Although anyone can develop a foodborne illness, those who are most at risk include the elderly, young children, pregnant women and their fetuses, and the immuno-compromised. While most illnesses occur as isolated cases, outbreaks of foodborne illness are clusters of cases that result from ingestion of a common contaminated food. A single outbreak can affect as few as two or as many as thousands of people.

Outbreaks are primarily investigated by state and local health departments who help the federal Centers for Disease Control and Prevention (CDC) investigate large or multi-state outbreaks. The CDC is also responsible for nationwide surveillance of outbreaks and for tracking new and emerging pathogens. The foodborne illness outbreaks are organized by CDC by pathogen, rather than emphasizing the food responsible for the outbreak, helping health officials and regulatory bodies to identify problems in the production of those foods (DeWaal et al. 2008).

In the United States, the primary agencies that inspect and regulate food are the United States Department of Agriculture (USDA), which oversees meat, poultry, and processed egg products and the Food and Drug Administration (FDA), which has regulatory responsibility for all other foods.

The Center for Science in the Public Interest (CSPI) has identified and categorized outbreaks linked to specific foods. Such data alert consumers to food-safety hazards, allow consumers to make informed risk decisions about the foods they eat, and provide better information to the government for setting priorities for food-safety resource allocation. Food attribution also enables identification of the food-pathogen combinations causing the most illnesses (DeWaal et al. 2008).

A total of 5,778 outbreaks, involving 168,898 cases of illness and occurring between 1990 and 2006, are included in the CSPI database. The five food categories, excluding multi-ingredient foods, linked to the most foodborne illness outbreaks are seafood, produce, poultry, beef, and eggs. These five food categories were responsible for 59% of all outbreaks and 54% of the illnesses. The produce category was linked to the largest number of foodborne illnesses associated with outbreaks, constituting 21% of all illnesses in CSPI's database. While outbreaks in most food categories increased between 2005 and 2006, the beef and dairy categories had a slightly lower number of outbreaks in 2006.

1.2 Major pathogens

Bacterial pathogens are responsible for 60% of outbreaks, while viruses cause 24%, chemicals/toxins cause 15%, and parasites cause 1%. The most prominent bacterial pathogens in the outbreak data are *Salmonella*, which accounts for 23% of all outbreaks, *Clostridium* (11%), and *Staphylococcus* (8%). Norovirus causes 90% of all virus outbreaks, accounting for 22% of outbreaks in the entire database. Both E. coli and Bacillus caused 5%, and Campylobacter caused 3% of all outbreaks, respectively (DeWaal et al. 2008).

Bacterial pathogens cause more than three-fourths of outbreaks linked to beef, dairy, eggs, luncheon and other meats, pork, and poultry. Outbreaks with multi-ingredient foods are more commonly caused by bacteria (58%), but are also often caused by viruses (40%).

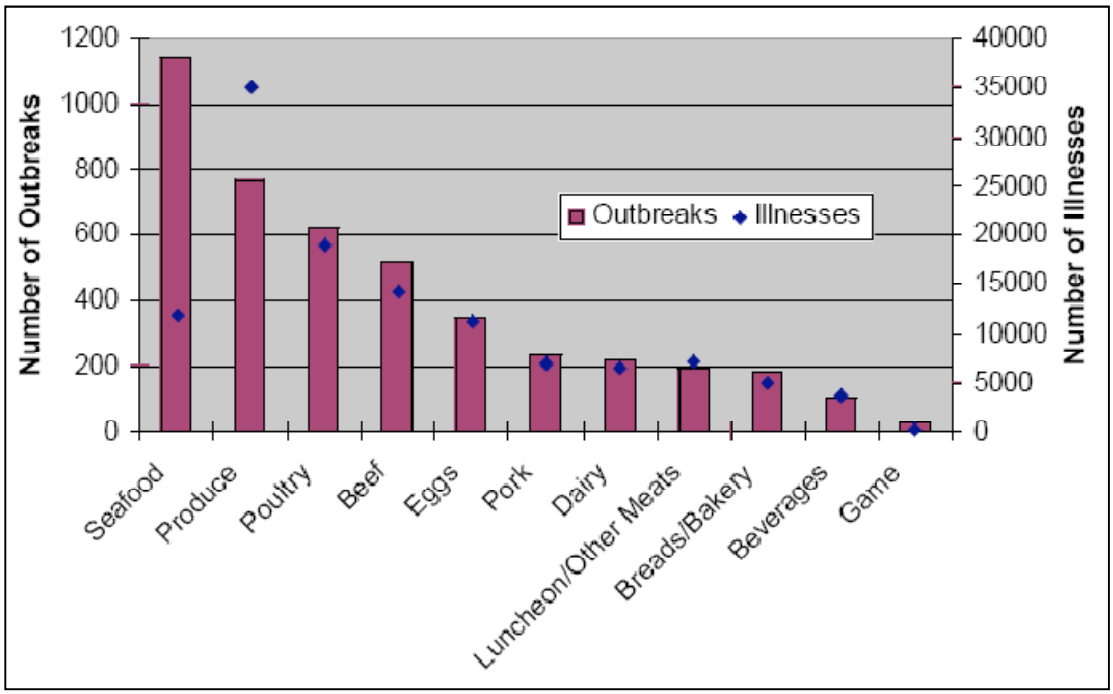

Figure 1.1 Outbreak and illnesses in food commodities 1990-2006 (DeWaal et al. 2008)

In outbreaks linked to produce and breads, bacteria and viruses each cause about half of the outbreaks. Game is the only commodity whose outbreaks are primarily caused by parasites. Chemicals and toxins are the most common cause of seafood and beverage- related outbreaks.

This thesis focuses on waterborne *Escherichia Coli* 0157:H7 and *Salmonella* spp. because of their status as emerging pathogens (Tauxe 2002) and their strong association with produce – related outbreaks (DeWaal et al. 2008). The major reservoir of these pathogens is farmed and wild animals. Traditionally, these pathogens have been associated with food products of animal origin like meat and eggs. However, recent outbreaks of foodborne disease associated with the consumption of fresh produce, have raised concerns that these products may be an increasing source of foodborne infections (DeWaal et al. 2008).

Produce is important to the health and well being of the American consumer. Americans enjoy one of the safest supplies of fresh produce in the world. However, over the last several years, the number of outbreaks of foodborne illness associated with both domestic and imported fresh fruits and vegetables has increased.

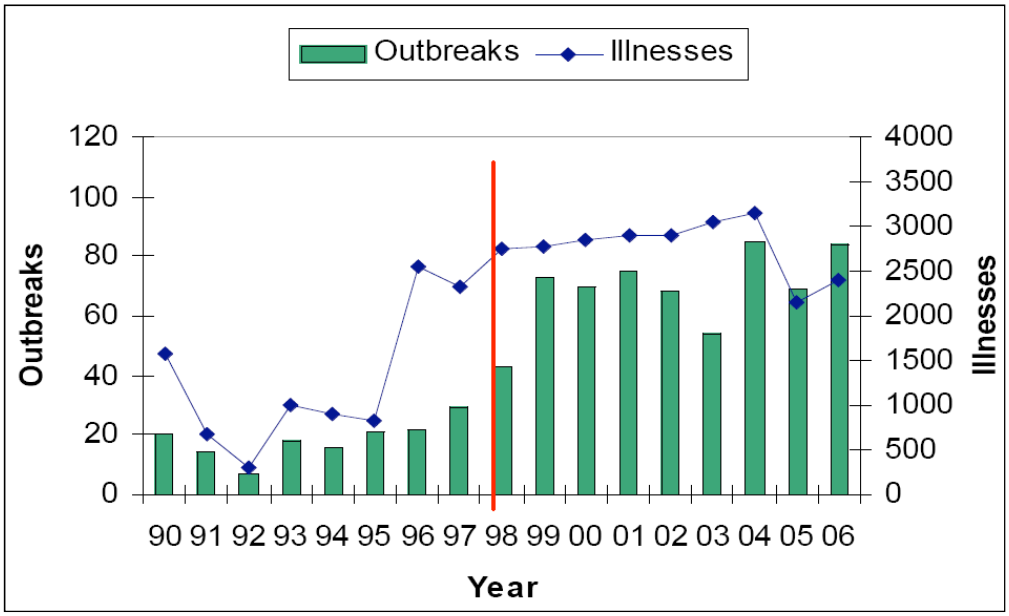

Figure 1.2 Produce outbreaks 1990 – 2006 Increasing trend both of illnesses and outbreaks. (DeWaal et al. 2008)

A total of 768 foodborne illness outbreaks involving 35,060 illnesses were linked to produce and produce dishes (DeWaal et al. 2008). The produce category had an average of 46 illnesses per outbreak. Vegetables were linked to 279 outbreaks with 14,743 illnesses, while fruits were

identified as the vehicle in 121 outbreaks with 7,802 illnesses. Produce dishes, including salads, were implicated in 368 outbreaks involving 12,515 illnesses.

More than 50% of produce outbreaks were attributed to food from restaurants and other food establishments, while private homes accounted for 13% of outbreaks. Norovirus was the major cause of produce outbreaks, accounting for 41% of all outbreaks. Salmonella was responsible for 18% of produce outbreaks, while E. coli caused 8%.

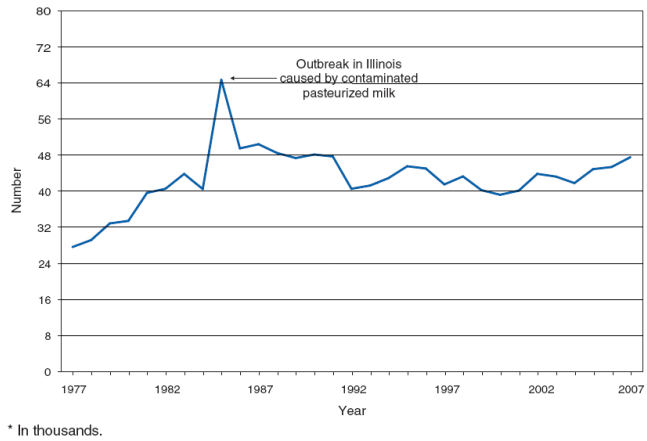

Figure 1.4 Salmonellosis. Number* of reported cases, by year – USA, 1976-2006 (Besser et al. 2009)

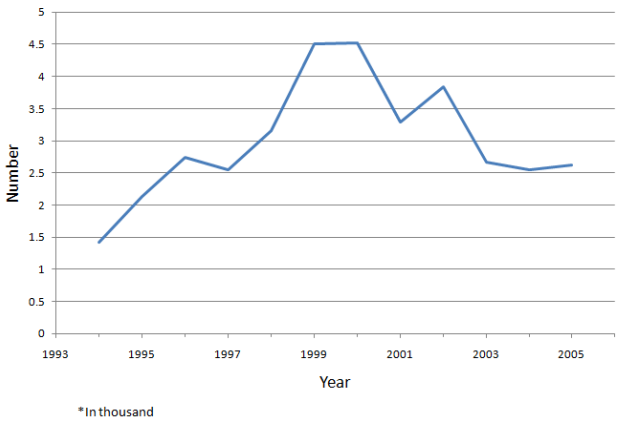

Figure 1.3 EHEC infection. Number* of reported cases, by year - USA, 1994-2006 (Besser et al. 2009)

Possible sources of the pathogenic contamination include improperly treated manure used as fertilizer in the fields and contaminated irrigation water (Solomon et al. 2002). This project focuses on irrigation water as a source of bacterial pathogens.

1.3 Irrigation Water Quality Concerns

Water is important in crop production because it is used in many activities such as irrigating, washing, and cooling. Irrigation water can spread pathogens, microorganisms that cause disease in humans, such as *Salmonella* spp., *E. coli* O157:H7, and *Cryptosporidium parvum* (also known as "Crypto"). Every time contaminated water comes in direct contact with fruit or vegetables, there is a risk that these pathogens may be transferred to the produce.

Soils fertilized with contaminated manure compost and the use of contaminated irrigation water in agriculture have been reported as transmission routes for pathogenic bacteria to the vegetables. In soils, reports suggest some pathogenic bacteria may persist more than 200 days (Islam et al. 2004; Muniesa et al. 2006). It has also been reported that pre-harvest crop contamination via contaminated irrigation water can occur through plant roots (Wachtel et al. 2002).

Microbial risk depends on:

- numbers and types of pathogens present and the susceptibility of the person consuming the product
- characteristics of the crop
- water source
- method of irrigation (sprinkler, drip, furrow, gravity)
- time of application in relation to harvest.

The diversity of cropping systems, scale of operation, use and design of equipment, regional and local practices, environmental influences, specifics of on-farm soil related factors, and many other production factors defy any attempt to develop an encompassing assignment of microbial risk to commodities or to crop management practices (Suslow et al. 2003).

1.3.1 Type of Pathogen

For pathogens, the illness contraction depend on the infection dose and on that pathogens ability to survive on the produce. The very young (less than 5 years of age), the elderly and those who are immune compromised are most susceptible to food-borne illness from low levels of pathogens. According to the latest statistics individuals undergoing cancer treatment or those with transplanted organs or autoimmune diseases such as rheumatoid arthritis, Crohn's disease, etc. have a weakened immune system that reduces the body's ability to resist infection by pathogens.

1.3.2 Crop Characteristics

Fruits and vegetables with large surface areas that can trap and hold moisture, such as lettuce, are at greater risk of containing living pathogens. Fruits or vegetables that are eaten raw or unpeeled present a higher risk. Processing or cooking produce reduces the potential for pathogens to survive (Sandra and Rebecca 2005).

1.3.3 Water Source

Irrigation water comes from 2 main sources: surface water or ground water. Surface water includes lakes, rivers, creeks, ponds and springs that come to the surface. Wells are ground water sources. Surface water is the most common source for irrigation. Generally, water from surface sources has a higher chance of contamination than water from ground water sources.

Surface water can be contaminated by:

- livestock or wildlife feces
- run-off from manure storages
- faulty septic systems
- storm runoff.

Surface water sources have the most variable levels of contamination because they may be subject to temporary or intermittent contamination sources. Rivers, streams and creeks have unpredictable water quality since activities upstream can rapidly change the levels of contamination entering the flowing water. Lakes tend to have better water quality; however, this may be compromised near an inflow from a river or creek or by activities causing contamination around or on the lake. The water quality in a pond depends on the source of the water for the pond and how well the pond is protected from further contamination. Ponds filled by a river, ditch or runoff will tend to have lower water quality than a pond filled by ground water, a spring or well.

When heavy rainfall occurs, the added volume can stir up the sediment at the bottom of a pond, creek or river. This sediment-laden water can contain higher levels of bacteria than calm water because bacteria tend to adhere to sediment particles.

Irrigation water taken directly from a well or a municipal source generally provides water of the most reliable quality. Ground water has gone through a natural filtration process as it travels through soil layers into the aquifer. There is still a potential for contamination to occur and well users must be vigilant in properly siting, constructing, maintaining and decommissioning their wells.

1.4 *Escherichia Coli O157*:H7 and *Salmonella* spp. produce-related outbreaks

The current system of surveillance for outbreaks of foodborne and waterborne diseases began in 1966, when reports of enteric disease outbreaks attributed to microbial or chemical contamination of food or water were incorporated into an annual summary. Since 1966, the quality of investigative reports has improved greatly, with more active participation by state and federal epidemiologists in outbreak investigations. Outbreaks of waterborne diseases and foodborne diseases have been reported in separate annual summaries since 1978 because of increased interest and activity in surveillance for waterborne diseases.

Outbreak surveillance has served three purposes:

- Disease prevention and control. The investigation of foodborne disease outbreaks leads to prevention and control measures in the food industry.
- Knowledge of disease causation. Outbreak investigations are a critical means of identifying new and emerging pathogens and maintaining awareness about ongoing problems.
- Administrative guidance. By analyzing several years of data on foodborne disease outbreaks, public health authorities can monitor trends over time in the prevalence of outbreaks caused by specific etiologic agents, the food that is the vehicle for the agent, and common errors in food handling. This information provides the basis for regulatory and other changes to improve food safety.

State, local, federal agencies, and territorial health departments, use a web-base standard form to report FBDOs and WBDOs to the National Notifiable Diseases Surveillance System (NNDSS), which is operated by CDC in collaboration with the Council of State and Territorial Epidemiologists (CSTE). An FBDO is defined as the occurrence of two or more cases of a similar illness resulting from the ingestion of a food in common.

The NNDSS has four limitations. First, several types of outbreaks are excluded from the FBDO Surveillance System, such as outbreaks that occur on cruise ships, outbreaks in which the food was eaten outside the United States, outbreaks that are traced to water intended for drinking, and indirect route of contamination. Second, the report does not include outbreaks where information on certain aspects of the outbreak are missing or incomplete. (Examples include etiology, the implicated food vehicle, or the factors that might have contributed to the outbreak). Third, the reported number of outbreaks attributed to one food vehicle category might not include all outbreaks attributable to a particular ingredient in that food. Finally, no standard criteria exist for classifying a death as being FBDO-related (Lynch et al. 2006).

Example of Salmonella outbreaks.

Between April and May 1998 there was a multistate outbreak of salmonella infections linked to toasted oats cereal (Samuel et al. 1998). The outbreak involved 11 states, a total of 209 cases were reported and at least 47 persons were hospitalized, representing an eightfold increase over the median number of cases reported in those states during 1993-1997.

Three multistate outbreaks of *Salmonella* infections associated with eating cantaloupe imported from Mexico occurred in the spring of consecutive years during 2000-2002 (Anderson et al. 2002). In total there were 155 confirmed cases in 16 states.

During 2005--2006, four large multistate outbreaks of Salmonella infections associated with eating raw tomatoes at restaurants occurred in the United States. The four outbreaks resulted in 459 culture-confirmed cases of salmonellosis in 21 states. The tomatoes had been supplied to restaurants either whole or precut from tomato fields in Florida, Ohio, and Virginia (Bidol et al. 2007).

In November 2006, public health officials at CDC and state health departments detected a substantial increase in the reported incidence of isolates of *Salmonella* .Illness was strongly associated with consumption of two brands of peanut butter produced at the same plant. Based on these findings, the plant ceased production and recalled both products on February 14, 2007. The outbreak strain of *Salmonella* subsequently was isolated from several jars of peanut butter and from two environmental samples obtained from the plant. As of May 22, 2007, a total of 628 persons infected with an outbreak strain of *Salmonella* had been reported from 47 states since August 1, 2006 (Julie et al. 2007).

Example of E. coli outbreaks.

There was an outbreak of *Escherichia coli* O157:H7 infections in Connecticut and Illinois during May 28 to June 27, 1996. It was associated with consumption of mesclun lettuce from a single producer. This is the first reported multistate outbreak of E coli O157:H7 infections associated with consumption of lettuce. This outbreak resulted in infection and illness of at least 61 persons, with 21 hospitalizations, and 3 cases of hemolytic-uremic syndrome (Hilborn et al. 1999).

A multistate outbreak of Escherichia coli O157:H7 infections occurred in the United States in June and July 1997. Two concurrent outbreaks were investigated through independent case-control studies in Michigan and Virginia. Isolates from 85 persons were indistinguishable by PFGE. Alfalfa sprouts were the only exposure associated with E. coli O157:H7 infection in both Michigan and Virginia (Breuer et al. 2001).

As of September 26, a total of 183 persons infected with the outbreak strain of E. coli O157:H7 had been reported to CDC from 26 states. Fresh spinach was identified as the source of the outbreak (Scott, J. N. M. et al. 2006).

1.5 Surface water characterization

Water quality measurements include physical, chemical and biological parameters.

1.5.1 Physical and chemical parameters

Physical water parameters encompass temperature which may affect the solubility of other parameters, pH which is affected by organic matter presence. Conductivity represents an indirect measure of total dissolved substances in the water whereas total suspended solids contribute to water's turbidity. Dissolved oxygen is an important requirement for the metabolism of aerobic organisms and also influences inorganic chemical reactions.
Most important chemical water parameters include nitrate (NO_3^-) as a major ingredient of farm fertilizer, ammonia (NH_4^+ or NH_3) as decomposition product of proteins and urea and used as fertilizer. Chlorides (Cl^-) in small amounts are required for normal cell functions in plant and animal life, whereas phosphate, in water, is an essential nutrient for the whole ecosystem.

1.5.2 Biological parameters

Fecal indicator bacteria (FIB) are used to assess the microbiological quality of water. Although these bacteria are not typically disease causing, they are associated with fecal contamination and the possible presence of waterborne pathogens. The density of indicator bacteria is a measure of water safety for body-contact recreation or for consumption. Fecal material from warm-blooded animals may contain a variety of intestinal microorganisms (viruses, bacteria, and protozoa) that are pathogenic to humans. Bacteriological tests for specific indicator bacteria are used to assess the sanitary quality of water and sediments and the potential public health risk from gastrointestinal pathogens carried by water. The suitability of indicator organisms for these purposes is ranked according to a specific set of criteria.

Table 1-1 Fecal indicator principal features and mains types of FIB

THE PREFERRED FECAL INDICATOR	FIVE MAINS TYPES OF FIB
can be tested for easily	Total coliform bacteria (TC)
is of human or other animal origin	Fecal coliform bacteria (FC)
survives as long as, or longer than, pathogens	Escherichia Coli (E. Coli)
is present at densities correlated with fecal contamination	Fecal streptococci (FS)
can be used as a surrogate for many different pathogens	Enterococci (EC)
is appropriate for fresh and saline aqueous environments	

1.5.2.1 Total and fecal coliform Bacteria

Total coliform bacteria are a group of digestive microorganisms found in high numbers in gut and feces of warm-blooded and cold-blooded animals and are therefore found in water that has been contaminated with fecal material. There are 16 species of TC.

Unfortunately, bacteria with the biochemical characteristics of TC are also found in non-contaminated water. Thus, in the absence of FC, the presence of TC may indicate older fecal contamination or the presence of decaying organic matter. Although the TC bacteria group is a less reliable indicator of sewage contamination, it is preferred as an indicator of treatment adequacy in drinking water supply systems because of its superior survival characteristics.

FC are a six species' sub-group of the TC that can grow at higher temperatures (>45 °C) and are considered to be present specifically in the intestines and feces of warm-blooded animals. The presence of FC in a water sample often indicates recent fecal contamination, meaning that there is a greater risk that pathogens are present than if only TC bacteria is detected.

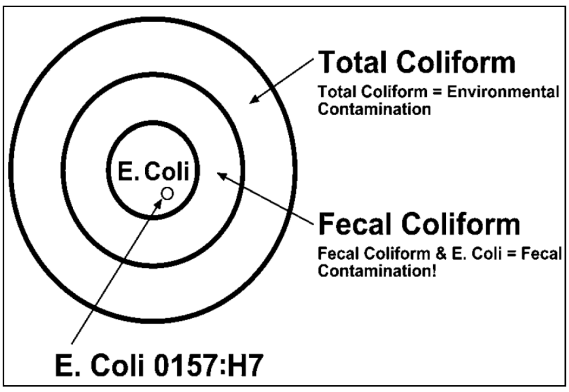

Figure 1.5 Coliforms classification grouping in Total and Fecal Coliform, E. coli and *E.coli* 0157:H7 (Selecky 2007)

Guidelines governing the microbial quality of irrigation water vary considerably between countries and between ground water, surface water, and human wastewater sources of water. The quality of water recommended for irrigation of crops likely to be consumed raw is often higher than that for processed or fodder crops.

1.5.2.2 Escherichia Coli (E. Coli)

Escherichia coli is the most numerous member of FC group (90% of FC are E. coli). It's a bacterium that is commonly found in the lower intestine of warm-blooded animals. Most E. coli strains are harmless, but some, such as serotype O157:H7 can cause serious food poisoning in humans. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2 (Bentley and Meganathan 1982) or by preventing

the establishment of pathogenic bacteria within the intestine (Reid et al. 2001). E. coli are not always confined to the intestine, and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination. The bacteria can also be grown easily and its genetics are comparatively simple and easily-manipulated, making it one of the best-studied prokaryotic model organisms.

1.5.2.3 Fecal streptococci and enterococci

The enterococci and many fecal streptococci are normal flora of the gastrointestinal tract, where they are present at high concentrations. Certain streptococcal species predominate in some animal species and not in others, but it is not possible to differentiate the source of fecal contamination based on the speciation of fecal streptococci. Fecal streptococci survive longer in the environment than FC, suggesting that they could be a useful indicator for the presence of long-lived excreted viruses. The ratio of FC to FS provides information about the source of contamination. A ratio greater than 4 was considered indicative of human fecal contamination, whereas a ratio of less than 0.7 was suggestive of contamination by nonhuman sources.

The enterococcus group is a subgroup of the fecal streptococci and includes two species that are common commensal organisms in the intestine of humans: *E. faecalis* (90-95%) and *E. faecium* (5-10%). *E. faecalis* is also found in cattle, dogs, horses, chickens, rabbits, rodents, sheep, swine and wild birds. The enterococci are differentiated from other streptococci by their ability to grow in 6.5% sodium chloride, at pH 9.6, and at 10 °C and 45 °C.

1.6 *Escherichia coli* (E. coli)

Escherichia Coli is a Gram-negative, facultative anaerobic, enteric bacterium, prominent and essential resident of the gastrointestinal tract of warm-blooded animals and belong to the family of *Enterobacteriaceae.* Its niche is the mucous layer of the mammalian colon where it is thought to utilize gluconate more efficiently than other resident species, thereby occupying a highly specialized niche (Kaper et al. 2004). *E. coli* strains from mammals gastrointestinal systems usually do not cause disease (nonpathogenic) and are therefore called "generic *E. coli*" or "commensal *E. coli*. Nevertheless, some *E. coli* strains can cause serious infections in humans and other mammals (haemorrhagic colitis and haemolytic uremic syndrome). The pathogenic *E. coli* strains that cause diseases in the gastrointestinal tract are called "diarrheagenic" *E. coli*. Researchers noted that there were different types of these pathogenic *E. coli* (called "pathotypes") and therefore created six different categories of diarrheagenic *E. coli* based on the distinctive features of the various *E. coli* and the diseases they cause. Acronyms for these categories are the following: EPEC, EHEC, ETEC, EAEC, EIEC, and DAEC. The highly virulent *E. coli* O157:H7 belongs to the EHEC pathotype group (enterohaemorrhagic *E. coli*).

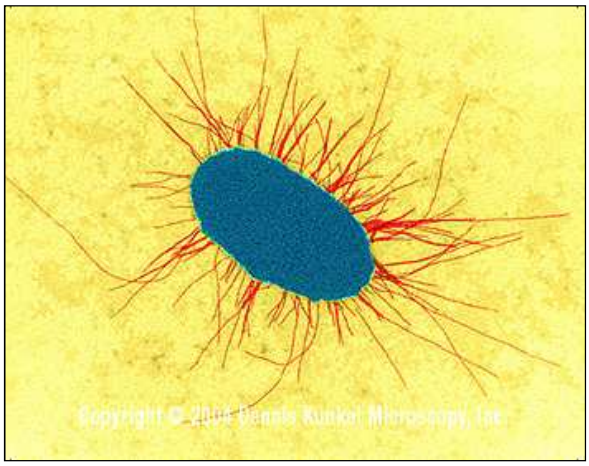

Figure 1.6 Transmission Electron Micrograph of *E. coli* with fimbriae on the cell surface. (Kim et al. 1994)

1.6.1 *E. coli* O157:H7 characterization and history

E. coli serotype O157:H7 is a gram-negative rod-shaped, sorbitol-negative bacterium. The letter "O" in the name refers to the somatic antigen (LPS located at the surface of the cell wall), whereas the "H" refers to the flagella antigen. *E. coli* O157:H7 strain has the 157th identified O antigen and the 7th identified H antigen.

The organism was first recognized as a human pathogen in 1982, when it was implicated in two outbreaks of haemorrhagic colitis. Undercooked hamburgers from the same fast food restaurant chain were identified as the vehicle. In 1983, Karmali and colleagues reported an association between infection with *E. coli* that produce Shiga toxins (including *E. coli* O157:H7) and post-diarrheal haemolytic uremic syndrome (HUS) (Karmali et al. 1983). In recognition of its distinct clinical manifestations, *E. coli* O157:H7 became the first of several strains referred to as enterohaemorrhagic *E. coli* or EHEC, which are now believed to account for over 90% of all cases of HUS in industrialized countries. Additional virulence traits and the O157 antigen are believed to have been acquired through horizontal transfer and recombination. *E. coli* O157 may be particularly adept at incorporating foreign DNA as a result of intrinsically high rates of defects in DNA repair mechanisms (LeClerc et al. 1996).

The incidence of HUS has fluctuated in some regions, but studies from three continents (Tarr et al. 1989) suggest an overall increase in this condition since the early 1950s. Overall the available evidence suggests that *E. coli* O157 emerged as an important human pathogen sometime during the past half century.

Figure 1.7 Scanning Electron Microscope Image of E. coli on the surface of the Small Intestine. (Kim et al. 1994)

1.6.2 Virulence factors and pathogenesis

E. coli O157:H7 causes disease by attaching to and colonizing mucosal cells of the large bowel, disrupting the brush border (attaching and effacing). The *eae* gene codes for a protein that is essential for this attachment. S*tx1* and *stx2* genes encode for Shiga-toxin production that are produced by the pathogen in the colon and besides causing local damage, they can travel via the bloodstream to the kidney where it is thought to play a role in causing HC and HUS (Kaper et al. 2004).

Production of Shiga toxins is not in itself sufficient to cause disease. Other factors thought to contribute to the virulence of *E. coli* O157:H7 include a 60 MDa virulence plasmid (pO157) (Law and Kelly 1995) and the Locus of Enterocyte Effacement (LEE) (Nataro and Kaper 1998). The ability of *E. coli* O157:H7 to produce A/E lesions is probably sufficient to cause non-bloody diarrhea but *stx* is essential for the development of bloody diarrhea, HC and HUS.

1.6.3 Clinicopathological features of EHEC disease

Although the symptoms of *E. coli* O157:H7 infections are distinctive, they may be confused at any single phase with other diseases or conditions. The initial symptoms of HC generally occur 1–2 days after eating contaminated food. Symptoms start with mild, non-bloody diarrhea that may be followed by a period of “crampy” abdominal pain and short-lived fever. The initial diarrhea increases in intensity during the next 24–48 hr to a 4- to 10- day phase of overtly bloody diarrhea accompanied by severe abdominal pain and moderate dehydration. In a proportion of patients, EHEC infection progresses to HUS, a life-threatening sequela characterized by a triad of acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia (Karmali et al. 1983). Some individuals with HUS experience neurological symptoms including lethargy, severe headache, convulsions, and encephalopathy (Tesh and O'Brien 1991). HUS incidence is higher in infants, young children, and the elderly (Karmali 1989).

1.6.4 Reservoirs and Sources of *E. coli* O157:H7

Cattle have long been regarded as the principal reservoir of STEC strains, including those belonging to serotype O157:H7. However, epidemiological surveys have revealed that EHEC strains are also prevalent in the gastrointestinal tracts of other domestic animals, including sheep, pigs, goats, dogs, horses, cats, frog, rodents, flies and birds (Wray et al. 1994; Kudva et al. 1996; Dale D. Hancock et al. 1998). Estimation of the incidence of carriage of EHEC is

complicated by the fact that fecal shedding may be transient and is almost certainly influenced by a range of factors including diet, stress, population density, geographical region, and season (Clarke et al. 1994). While many domestic animals carrying EHEC are asymptomatic (Pruimboom et al. 2000), certain EHEC strains are capable of causing diarrhea in cattle, particularly calves (Gyles 1992), in cats and dogs (Hammermueller et al. 1995).

EHEC can potentially enter the human food chain from a number of animal sources, most commonly by contamination of meat with feces or intestinal contents after slaughter. A Belgian survey found EHEC in samples of beef, lamb, deer, wild boar, ostrich, partridge, antelope, and reindeer (Pierard et al. 1994). One of the most common sources of human EHEC infection is hamburger patties made from ground beef (Karmali 1989).

Other proven food sources of EHEC infection include raw or inadequately pasteurized dairy products, fermented or dried meat products such as salami and jerky, and fruit and vegetable products which presumably had come into contact with domestic animal manure at some stage during cultivation or handling (Keene et al. 1997; Hilborn et al. 1999). One hypothesis states that the plant becomes contaminated when grown in fields fertilized with improperly treated manure and/or irrigated with water contaminated with cattle feces or contact with contaminated surface runoff (Solomon et al. 2002). The largest outbreak of EHEC disease yet reported occurred in Sakai, Japan, in 1996 and involved over 6,000 cases of HC and over 100 cases of HUS (Fukushima et al. 1997); the most likely source appears to have been radish sprouts in mass-prepared school lunches.

Other sources include accidental ingress of raw sewage into water distribution system. In fact, both of the first two adult cases of HUS reported in North America had drunk untreated surface water.

Since the infectious dose of EHEC is between 10 and 100 organism (Chalmers et al. 2000), contamination of water sources can be significant in its consequences even if the numbers are small.

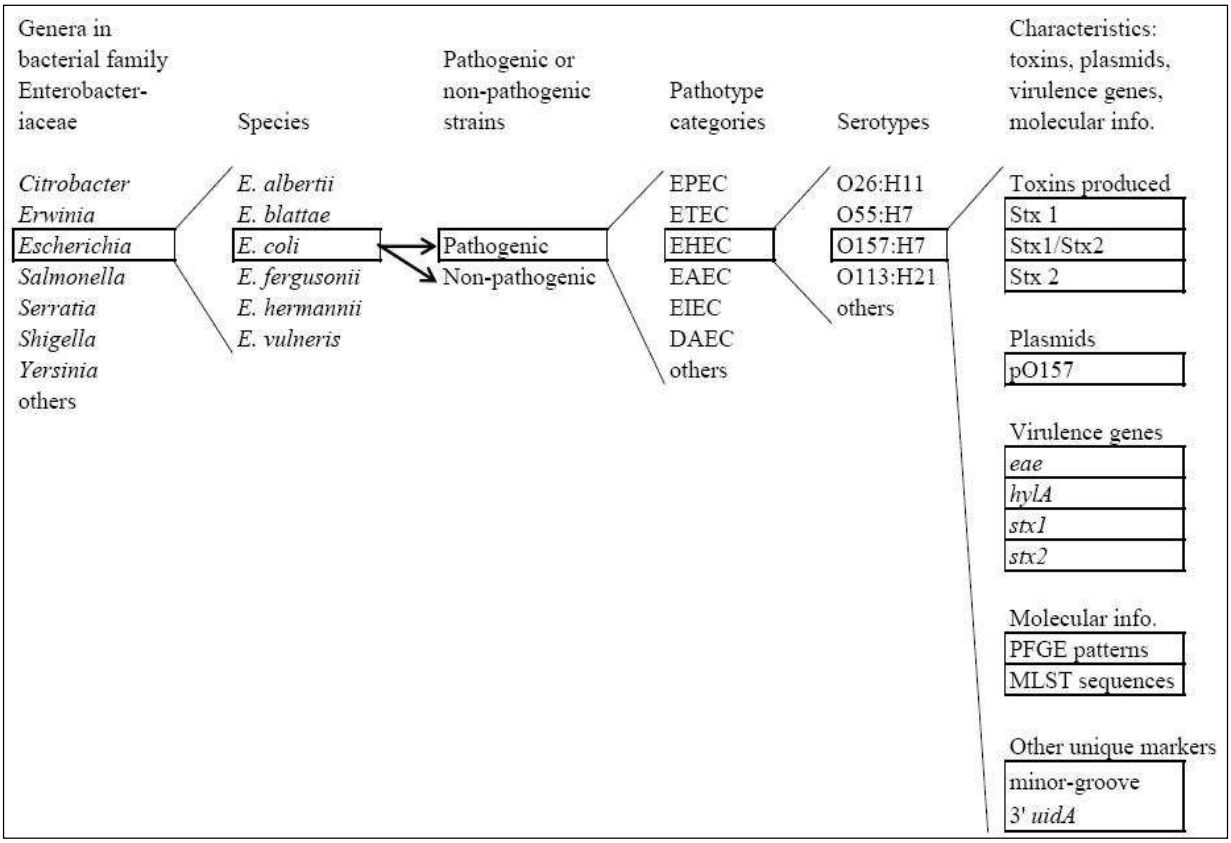

Figure 1.8 Organization of the different *E. coli* features used for identification, characterization and naming. (Steven et al. 2007)

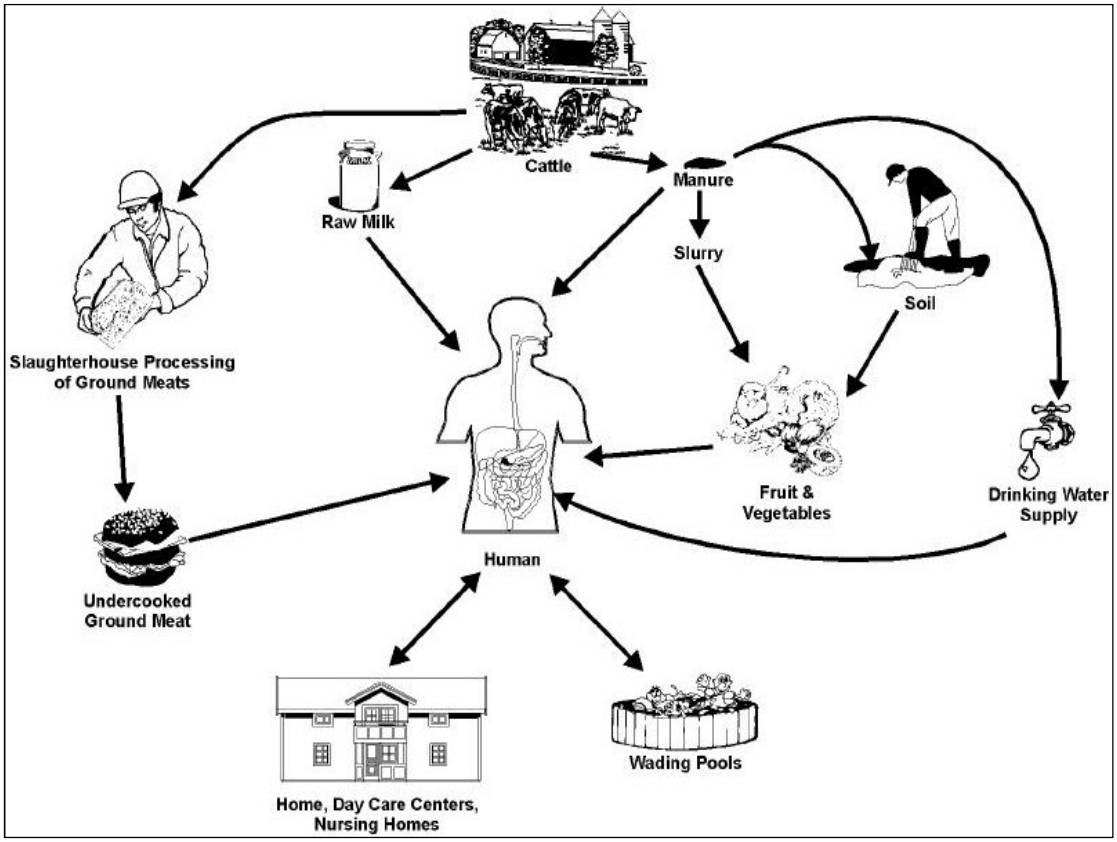

Figure 1.9 Sources of E. coli infection. (Petridis et al. 2002)

1.7 Salmonella spp

*S*almonella is a Gram-negative, facultative anaerobic, rod-shaped bacterium in the same proteobacterial family as *Escherichia coli*, the family Enterobacteriaceae, trivially known as "enteric" bacteria. Salmonellae live in the intestinal tracts of warm and cold blooded animals. Some species are ubiquitous. Other species are specifically adapted to a particular host. In humans, Salmonella are the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a foodborne infection/intoxication. Salmonella is also capable of causing bacteremia end focal infections.

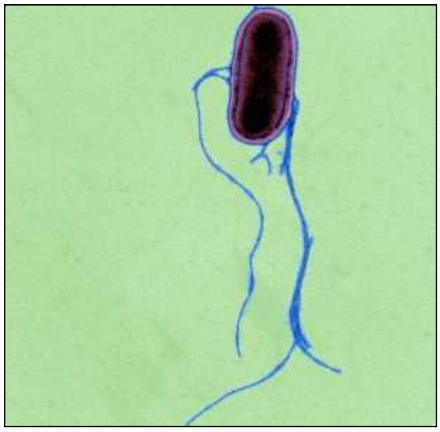

Figure 1.10 Transmission Electron micrograph of *Salmonella typhi* with fimbriae on the cell surface. (Dougan 2006)

1.7.1 *Salmonella* spp characterization and history

Salmonella nomenclature has been controversial since the original taxonomy of the genus. At first names were given according to clinical considerations, e.g., *Salmonella typhi*, *Salmonella cholerae-suis, Salmonella abortus-ovis*, and so on. When serological analysis was adopted into the Kauffmann-White (Kauffmann 1966) scheme in 1946, the nomenclature for *Salmonella* genus evolved in one serotype-one species concept. Each serotype was considered a separate species (for example, *S. paratyphi A, S. newport,* and *S. enteritidis*). This concept, if used today, would result in 2,463 species of *Salmonella*. Names derived from the geographical origin of the first isolated strain of the newly discovered serovars were next chosen, e.g., *S. london, S. panama, S. stanleyville*. The defining development in Salmonella taxonomy occurred in 1973 when Crosa et al. (Crosa et al. 1973) demonstrated by DNA-DNA hybridization that all serotypes form two DNA hybridization group (two species): *S. bongori* and *S. enterica* (Reeves

et al. 1989). There are over 2500 serovars within both species, which are found in a disparate variety of environments and which are associated with many different diseases. The vast majority of human (>99.5%) are subspecies *S. enterica*.

Salmonella serovars can be found predominantly in one particular host, can be ubiquitous, or can have an unknown habitat. Typhi and Paratyphi A are strictly human serovars that may cause primarily enteric fever often associated with invasion of the bloodstream. Salmonellosis in these cases is transmitted through fecal contamination of water or food. Gallinarum, Abortusovis, and Typhisuis are, respectively, avian, ovine, and porcine Salmonella serovars. While approximately 2000 serotypes of Salmonella have been associated with enterocolitis, at a given time it is a smaller set of about 10 serotypes that accounts for the majority of infections. Ubiquitous *Salmonella* serovars (e.g., Typhimurium) cause very diverse clinical symptoms, from asymptomatic infection to serious typhoid-like syndromes in infants or certain highly susceptible animals (mice). In human adults, ubiquitous Salmonella organisms are mostly responsible for foodborne toxic infections.

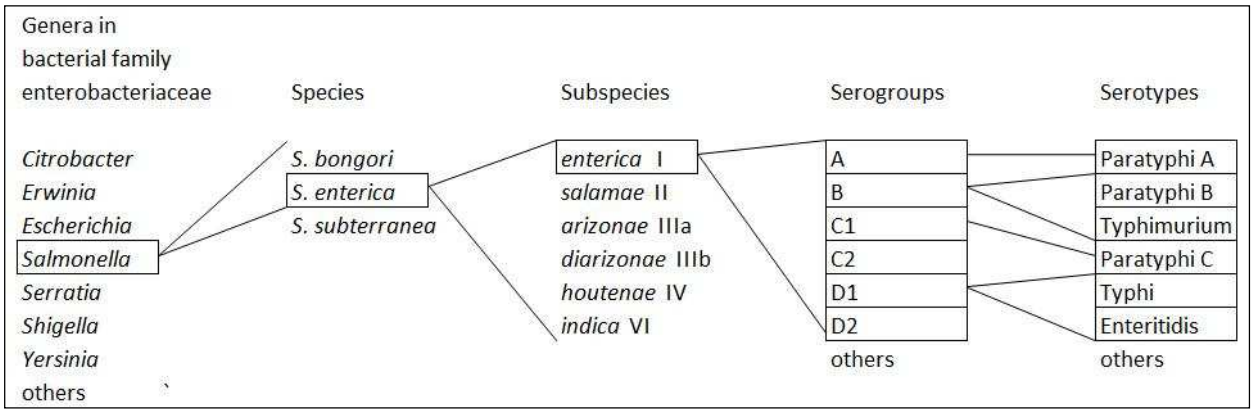

Figure 1.11 Organization of the different *Salmonella* features used for identification, characterization, and naming

1.7.2 Virulence factors and pathogenesis

Salmonella infections in humans vary with the serovar, the strain, the infectious dose, the nature of the contaminated food, and the host status. Certain serovars are highly pathogenic for humans. Strains of the same serovar are also known to differ in their pathogenicity. An oral dose of at least 10^5 *Salmonella Typhi* cells are needed to cause typhoid in 50% of human volunteers, whereas at least 10^9 *S. Typhimurium* cells (oral dose) are needed to cause symptoms of a toxic infection (Darwin and Miller 1999). Infants, immunosuppressed patients, and those affected with blood disease are more susceptible to *Salmonella* infection than healthy adults.

S. typhi and the paratyphoid bacteria cause typhoid or typhoid-like fever in humans. Other forms of salmonellosis generally produce milder symptoms, in particular gastroenteritis involving nausea, vomiting, abdominal cramps, diarrhea, fever, and headache.

In the pathogenesis of nontyphoidal salmonellosis, after the bacteria enter the human digestive tract, it is clear that an early manifestation of the host-pathogen interaction is attachment to and invasion of the intestinal epithelium where nontyphoidal bacteria are phagocytosed by macrophages in the lamina propria. Macrophages and intestinal epithelial cells then attract T cells and neutrophils with interleukin causing inflammation and suppressing the infection.

In contrast, typhoidal salmonellae, *S. typhi* and a few related *Salmonella* species, specifically target grossly visible clusters of lymphatic tissue called Peyer patches. These clusters are the primary pathway for the gut to present antigens to the immune system. *Salmonella* pathogenicity island 1 (SPI1) contains the genes for the type III secretion system such as *invJ*, spaO, *sicAP*, *sipABCD.* They encode the injection of virulence proteins into the epithelial cell. The proteins disrupt the normal brush border and force the cell to form membrane ruffles, which engulf the bacilli and create vesicles. These carry the bacteria across the epithelial cell cytoplasm and the basolateral membrane, where they are presented to macrophages. Within the macrophage, *S. typhi* travels undetected by the immune system. *S. typhi* resists digestion because the SPI2 encodes for virulence factors that prevent or alter fusion of the vacuole with other intracellular compartments. Afterward, the bacteria induce macrophage apoptosis, breaking out into the bloodstream to invade the rest of the body (Parry et al. 2002).

1.7.3 Clinicopathological features of *Salmonella* disease

Typhoid fever is an infectious feverish disease up to 40°C with coughing headache severe and diarrhea in the first phase. After about 3 weeks, in the second phase of the illness, symptoms are abdominal pain and faces that may also contain blood. Intestinal perforation or profuse bleeding from the intestinal mucosa may occur if typhoid fever is left untreated (Easmon 2009). It can be life-threatening, but antibiotics are an effective treatment. The disease lasts several weeks and convalescence takes some time. The incubation period is 10 to 20 days and depends on, among other things, how large a dose of bacteria has been taken in. In the mild disease, the bacterium is eliminated very early in the course of the disease and there are perhaps only mild symptoms. It is possible to become a healthy carrier of infection.

1.7.4 Reservoirs and Sources of *Salmonella*

Salmonellae are disseminated in the natural environment (water, soil, and plants used as food) through human or animal excretion. Humans and animals (either wild or domesticated) can excrete *Salmonella* either when clinically diseased or after having had salmonellosis, if they remain carriers. *Salmonella* organisms do not seem to multiply significantly in the natural environment (out of digestive tracts), but they can survive several weeks in water and several years in soil if conditions of temperature, humidity, and pH are favorable.

Transmission of *Salmonella* to humans is usually by consumption of contaminated food, but human-to-human transmission and direct animal-to-human transmission can occur (Prost and Riemann 1967). The most common sources of *Salmonella* are beef, poultry, and eggs (Tauxe 1991). Eggs can be contaminated through cracks in the shell or transovarally from an infected ovary or oviduct to the yolk prior to deposition of the shell (Snoeyenbos et al. 1969); internally infected eggs left at room temperature can rapidly achieve *Salmonella* concentrations of 10^{11} cells per yolk. This mode of transmission can be especially difficult to control because the egg-laying hens are usually asymptomatic (St. Louis et al. 1988). *S. enteritidis* now frequently rivals *S. typhimurium* as the most common cause of salmonellosis in the United States, and there has been a similar increase in many other parts of the world as well (Mishu et al. 1994).

Salmonella may be associated with contaminated meat (cattle, pigs, goats, chicken, etc.) that may originate from animal salmonellosis, but most often it results from contamination of muscles with the intestinal contents during evisceration of animals, washing, and transportation of carcasses. Surface contamination of meat is usually of little consequence, as proper cooking will sterilize it (although handling of contaminated meat may result in contamination of hands, tables, kitchenware, towels, other foods, etc.). However, superficial cooking of contaminated ground meat is more likely to produce a Salmonella infection. Infection may follow ingestion of any food that supports multiplication of Salmonella such as eggs, cream, mayonnaise, creamed foods, etc. Prevention of *Salmonella* toxic infection relies on avoiding contamination (improvement of hygiene), preventing multiplication of *Salmonella* in food (constant storage of food at 4°C), and use of pasteurized and sterilized milk and milk products. Vegetables and fruits may carry Salmonella when contaminated with fertilizers of fecal origin, or when washed with polluted water (Todar 2005). Salmonellae can also survive sewage treatments if suitable germicides are not used in sewage processing.

The incidence of foodborne Salmonella infection/toxication remains reletavely high in developed countries because of commercially prepared food or ingredients for food. Any contamination of commercially prepared food will result in a large-scale infection. In underdeveloped countries, foodborne *Salmonella* intoxications are less spectacular because of the smaller number of individuals simultaneously infected, but also because the bacteriological diagnosis of *Salmonella* toxic infection may not be available. However, the incidence of *Salmonella* carriage in underdeveloped countries is known to be high (Todar 2005).

1.8 Mechanism of vegetable contamination

Vegetables can become contaminated at any point in the production chain: during growth, harvesting, processing, distribution and final preparation. Preharvest contamination of vegetables can occur directly or indirectly via wild and domestic animals, insects, water, soil, dusts, water used to apply fungicides and insecticides, dirty equipment002C and human handling. Potential postharvest sources include feces, human handling, transport containers, rinse water, ice and processing equipment (Beuchat and Ryu 1997). However, the most important considerations are the application of manure or compost as fertilizer to fields where crops are grown and the fecal contamination of irrigation water.

Cattle are considered to be the primary but transient reservoir of EHEC, carry the pathogens usually asymptomatic and shed them in their feces. Animal manure is intensively used worldwide as a crop fertilizer, especially in areas where intensive livestock farming co-occur with arable farming. This includes both conventional and organic production. A proportion of this manure will contain human pathogenic bacteria which will have the potential to enter the food chain when they are applied to fields used for the production of fresh produce. The prevalence of EHEC O157 in cattle varies considerably within and between countries, depending on season, sampling strategy, detection method, and geographic location (1-87% positive herds and 0-68% positive animals within the herd) (Schouten 2005). Concentrations of EHEC O157 in EHEC-positive manure varied widely between 10 CFU and 106 CFU g^{-1} (Fegan et al. 2004). Contamination of vegetables grown in soils enriched with contaminated manure will largely depend on the survival capabilities of the pathogen in manure and manure-amended soils. The conditions for survival of enteric pathogens are considered to be unfavourable once excreted from the animal gut (Unc and Goss 2004). However, pathogens like EHEC O157 and *Salmonella enterica* are able to survive for extended periods (up to months) in manure (Scott,

L. et al. 2006) and manure-amended soil (Gagliardi and Karns 2002) and can become associated with vegetables grown in soils enriched with contaminated manure (Islam et al. 2004).

1.8.1 Survival and multiplication of pathogens in raw produce

The survival and/or growth of pathogens on fresh produce is influenced by the organism, produce item, and environmental conditions in the field and thereafter, including storage conditions. In general, pathogens will survive but not grow on the uninjured outer surface of fresh fruits or vegetables, due in part to the protective character of the plant's natural barriers (for example, cell walls and wax layers). In some cases pathogen levels will decline on the outer surface (Burnett and Beuchat 2001).

In the field, the physical environment of leaf surfaces is considered to be inhospitable for the growth and survival of bacteria (for example, lack of nutrients and free moisture, temperature and humidity fluctuations, and ultraviolet light) (Dickinson CH 1986). Environmental conditions, however, can greatly influence bacterial populations; the presence of free moisture on leaves from precipitation, dew, or irrigation may promote survival and growth of bacterial populations (Beattie and Lindow 1999). Certain conditions, such as sunlight, particularly the shorter ultraviolet wavelengths, can damage bacterial cells (Sundin and Jacobs 1999).

Growth on intact surfaces is not common because foodborne pathogens do not produce the enzymes necessary to break down the protective outer barriers on most produce. This restricts the availability of nutrients and moisture. One exception is the reported growth of *E. coli* O157:H7 on the surface of watermelon and cantaloupe rinds. Survival of foodborne pathogens on produce is significantly enhanced once the protective epidermal barrier has been broken either by physical damage, such as punctures or bruising, or by degradation by plant pathogens (bacteria or fungi).

In case of fresh-cut produce, they have been injured through peeling, cutting, slicing, or shredding. These same operations can transfer pathogenic microorganisms, if present, from the surface of the intact fruit or vegetable to the internal tissues. Injured cells and released cell fluids provide a nourishing environment for microbial growth (Harris et al. 2003).

1.8.2 Attachment and infiltration

Bacterial attachment on the surface of sound produce is limited in contrast to attachment on processed meat tissues. However, attachment and infiltration of microbial cells do occur via aerial and/or root part. Aerial internalization is facilitated by the stomata, lenticels, broken trichomes, and bruises and cracks in the skin surface of fruits and vegetables (Dingman 2000). Within the soil it is likely that the exudates released from roots sustained bacterial growth during the early stages of the sprouting process. Exudates from germinating seeds are known to enable bacterial growth within the vicinity of the plant (Warriner et al. 2003; Bernstein et al. 2007). Access into the inner vascular system is restricted by protective border cells on the root surface. However, bacteria can gain entry into the plant via cracks in the epidermis and fissures created during emergence of lateral roots (Barraquio et al. 1997). It has been reported that *E. coli* O157:H7 can become established on the surface and internal root structures of lettuce during plant development. Therefore, it is possible that during the early stages of sprouting *E. coli* and *Salmonella* became internalized within sprouts and subsequently become distributed throughout the plant (Warriner et al. 2003).

SCOPE OF THE THESIS

In the proposed research we will determine the presence of two major food borne pathogenic organisms in irrigation water sources (*E. coli* O157:H7 and *Salmonella*) and examine the correlation of these pathogenic organisms to surrounding land use practices, seasonal trends, and temporal factors. Results of this research will provide produce growers with information to be used in documenting the quality of irrigation water sources and identifying land use practices which might be increasing potential for pathogens to be present in irrigation water sources. Identification of these practices will in turn serve as a vector for determining preventative measures. The results of this research will enhance protection and safety of the nation's agriculture and food supply.

2 MATERIALS AND METHODS

2.1 Experimental sites

The study was conducted on five irrigation ponds located in South Georgia (USA) within the Atlantic Coastal Plain, a flat, sandy/clay area with many draining streams and well adapted to a wide variety of agricultural product. The ponds are in South central/western part of the state (Fig. 2.1).

The climate is humid subtropical. Weather data recorded at Tifton Experimental Station from 1911 and 2008 indicate a mean annul temperature of 18.9° C with a mean winter temperature of 10.6° C and a mean summer temperature of 26.7° C. The mean annual precipitation is 1625 mm and the mean number of rainy days $year^{-1}$ is 101. The rainier month is July with a mean of 138 mm of rain and the less rainy is October with 57.7 mm. From August 2008 to January 2009 the precipitation was 629 mm and the mean temperature was 17.6° C with a mean minimum of 11.6° C and a mean maximum of 18.1° C. (Southeast Regional Climate Center, 2009 - University of North Carolina at Chapel Hill).

The irrigation ponds are artificial relatively small lakes made for the purpose of irrigating surrounding crops. The pond's water balance weighs, besides irrigation water (70%), upon water lost to various sources such as evaporation and seepage through the bottom of the pond and storage space for sediment. (Thomas 2003). Ponds are always fed by runoff water and, have a continuous inflow/outflow, and the ponds are sometimes supplemented with pumped groundwater.

The ponds under study are all surrounded by cropped fields such as pepper, broccoli, tomatoes, squashes, cucumbers and they may also have also an adjacent wooded riparian zone where deer, rodents and others several forms of wildlife may live and be a potential input of fecal contamination along with avian communities. There are no cattle-grazing next to the ponds.

Physical ponds characteristic are synthesized in table 2-1.

Table 2-1 Ponds ID, dimensions and surface slope nearby the ponds.

ID	AREA (m^2)	PERIMETER (m)	Slope nearby(%)
NP	43700	1040	5.5
SC	84100	1340	5
GF	40500	930	4.2
LV	5650	300	3
WF	24000	605	3.7

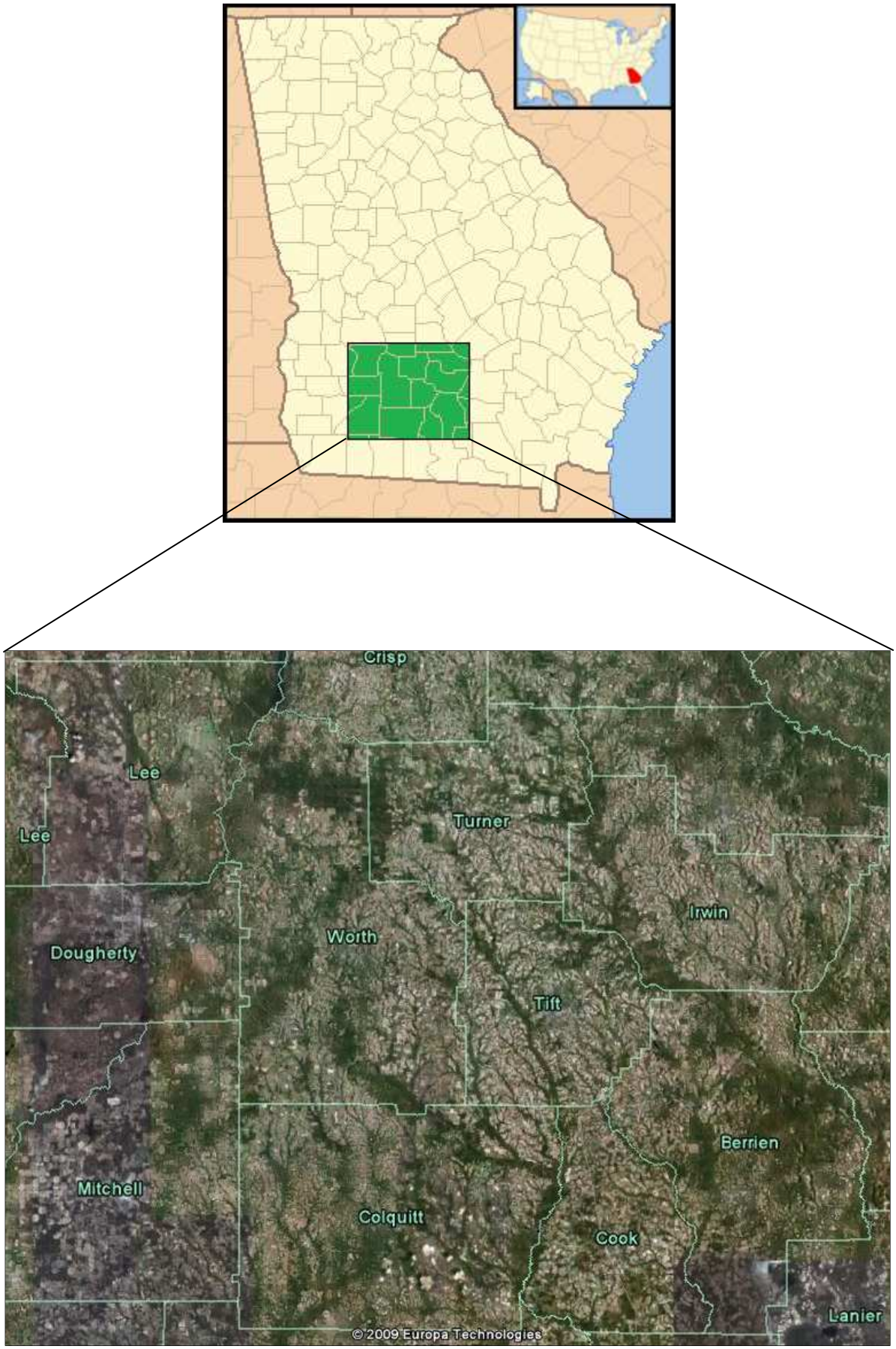

Figure 2.1 Sampling location sites in South central/western part of Georgia, USA.

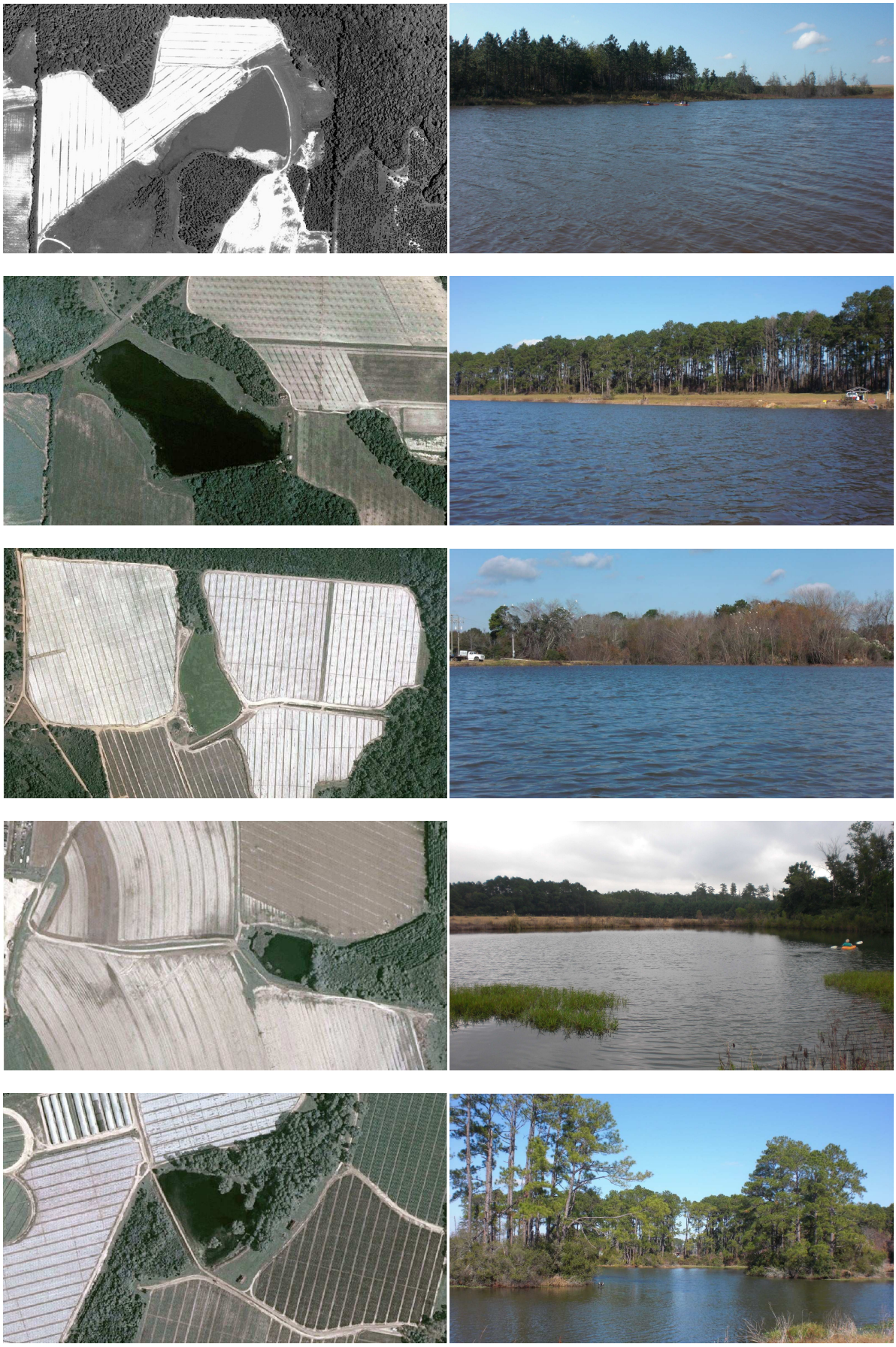

Figure 2.2 Sampling sites. From top to bottom NP, LV, GF, SC and WF. Aerial photographs and edge view.

2.2 Water sampling

Between July, 2008, and January, 2009, water samples were collected from 5 irrigation ponds. Three aliquots per ponds were taken cross a transect from a kayak. Water was sampled just below the surface. Because of the low concentration of *E. coli* O157:H7 and *Salmonella*, the first sample was composite and collected in a 10-L sterilized container for pathogens analysis. The second one was for indicator bacteria, collected in a 1-L plastic sterile bag. The last 1.5-L sample was collected in a plastic sterilized bottle for total suspended solids (TSS) and nutrients data collection. Samples were placed in coolers with ice and taken to the water chemistry laboratory in NESPAL building within a few hours.

After water sampling, others water measurements were taken with YSI 650 MDS, a multi-parameter environmental monitoring system. It's a hand held microcomputer based instrument that allows the user to gather water quality data by means of a 6-series multiple-probe sonde placed in the water. These parameters include temperature, dissolved oxygen (DO), conductivity, pH, oxidation/reduction potential (ORP), and turbidity (TRB).

Samples and measurements were taken about twice monthly, and in particular after a big rain or storm. Heavy rain events cause more surface water runoff to the ponds and therefore may represent a worst case scenario.

Figure 2.3 Water sampling (A), Multi-parameter Display System (B), 6-Series Multi-parameter Water Quality Sonde (C). (YSI Environmentalt)

2.3 Samples preparation

2.3.1 Nutrients

In order to measure the nutrient concentration in the ponds' water, a TrAAcs 800 Autoanalyzer was used. The TrAAcs system is a continuous flow wet chemistry analyzer that determines analyte concentrations using a colorimeter to detect changes in color produced by the presence of the analytes. It is typically used for automated testing of water, waste water and sea water (Colella 2009).

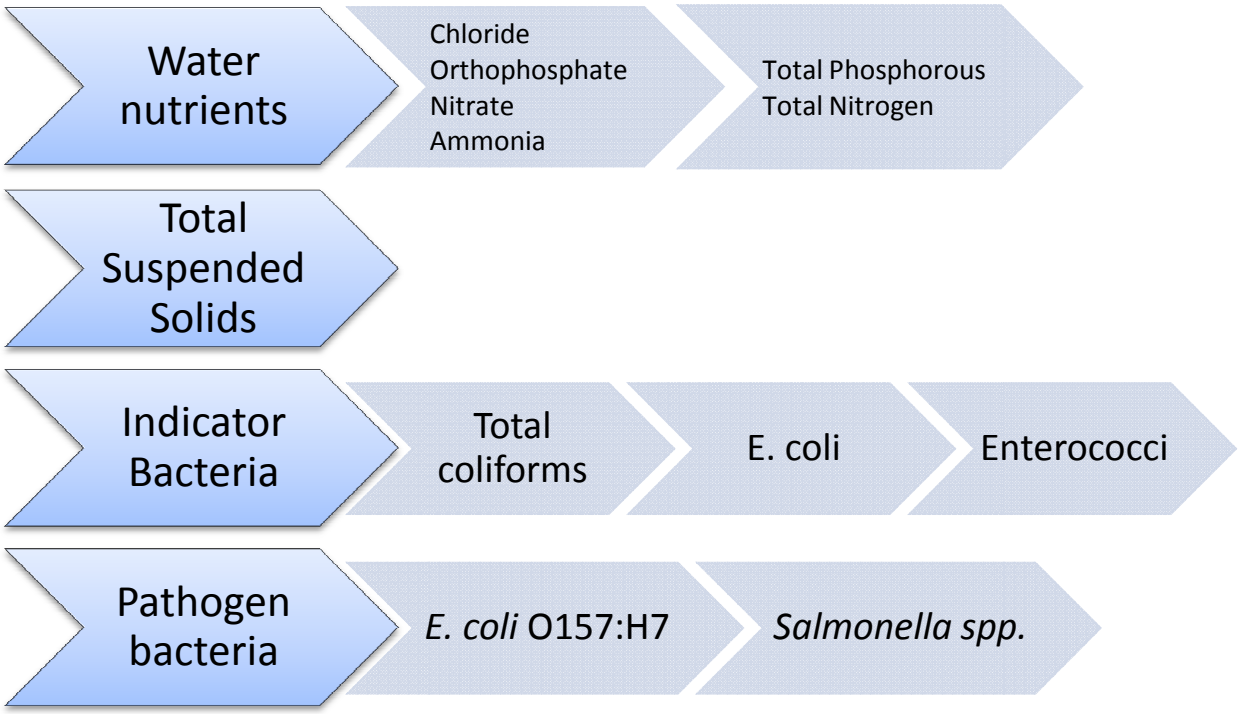

Figure 2.4 Laboratory analysis diagram

In Segmented Flow Analysis (SFA) a continuous stream of material is divided by air bubbles into discrete segments in which chemical reactions occur. The continuous stream of liquid samples and reagents are combined and transported in tubing and mixing coils. The tubing passes the samples from one apparatus to the other and each apparatus may perform different functions, such as distillation, dialysis, extraction, ion exchange, heating, incubation, and subsequent recording of a signal. The air bubbles segment each sample into discrete packets and act as a barrier between packets to prevent cross contamination as they travel down the length of the tubing. The air bubbles also assist mixing by creating turbulent flow, and provide operators with a quick and easy check of the flow characteristics of the liquid. Samples and standards are treated in an exactly identical manner as they travel the length of the tubing, eliminating the necessity of a steady state signal. However, since the presence of

bubbles create an almost square wave profile, bringing the system to steady state does not significantly decrease throughput and is desirable in that steady state signals (chemical equilibrium) are more accurate and reproducible (Coakly and William 1981).

Figure 2.5 TrAAcs 800 Autoanalyzer for nitrate, orthophosphate, chloride and ammonia measurement in water.

TrAAcs 800 Autoanalyzer is capable of measuring a wide range of elements, given the appropriate flow cell, filter and manifold set-up. In this research NH_4^+, Cl^-, NO_3^- and PO_4^{3-} were measured but the system can also analyze Nitrite (NO_2^-), total Nitrogen (TN), total Phosphorus (TP), Silicates and Sulfite. The instrument is equipped with an autosampler for multiple sample analysis. Resolution, reproducibility and accuracy are all dependent upon the method in use (Bran 1987).

Varying levels of sample preparation were necessary. All samples must be free of undissolved material and have a volume of at least 5 ml (Colella 2009). The filtrate from the TSS analyses was used for TrAAcs analyses. Standard operating procedures were followed for preparing standard solutions for use as calibrants and spiked samples to ensure accurate data (Coker 2008).

Ea ch sample was analyzed in duplicate and the average reported.

Another instrument was used to measure TP and TN in the water. In this case the autoanalyzer was used an Autoanalyzer 3 (AA3). The AA3 is a fully automated system for analyzing nutrients in environmental waters and many other applications. The system is a continuous flow analyzer which works under the same principles as the TrAAcs. Standard operating procedures were followed for preparing standard solutions, calibrants and spiked samples to ensure accurate data (Ghimire 2008).

For samples running, samples and standards need to be digested and autoclaved. To ensure that reagents are prepared properly, Bran - Lubbe Autoanalyzer application method # G-175-96 for TP and method # G-172-96 for Nitrogen were adopted.

Figure 2.6 Autoanalyzer 3 for Total nitrogen and Total Phosphorus measurement in water

2.4.2 Total suspended solids (TSS)

Total Suspended Solids are defined as those solids which are retained by a glass fiber filter and dried to constant weight at 105°C (Clesceri et al. 1998). Standard operating procedures for determining the mass concentration of suspended solids were adopted.

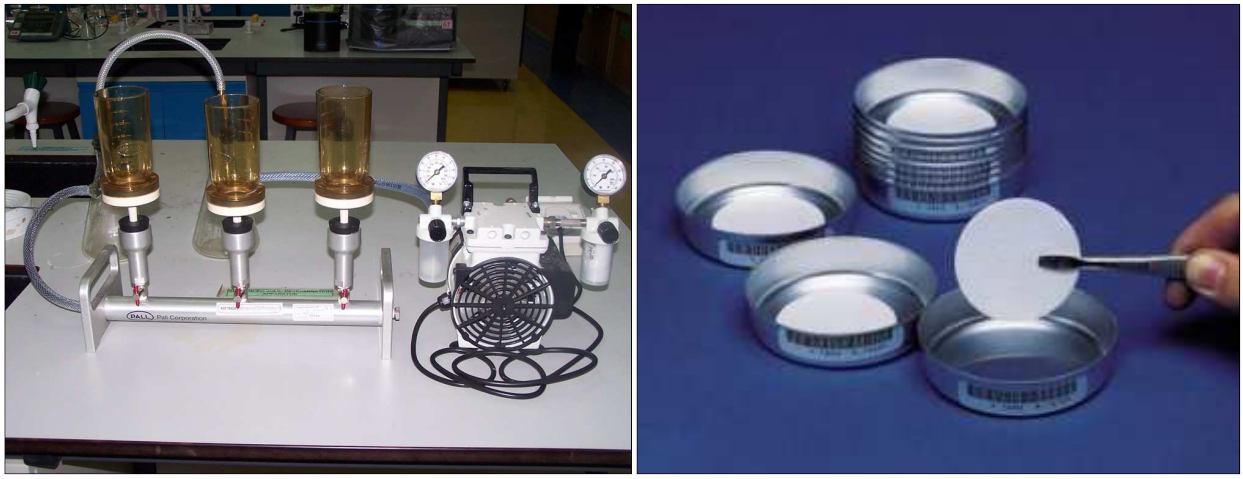

Figure 2.7 1. Total Suspended Solids filtration apparatus and vacuum pump. 2.Glass microfiber filters discs in disposable aluminum dishes.

A well-mixed subsample was filtered through a weighed standard 5.5 cm glass-fiber filter with a vacuum pump and the residue retained on the filter was dried to a constant weight at 105°C and then cooled in a desiccator to balance temperature and weigh. The increase in weight of the filter represents the total suspended solids. The results were expressed in PPM (mg/L) as follow:

$$TSS = (A-B) \times 1000/C$$

Where: A = weight of filter and dish + residue in mg

B = weight of filter and dish in mg

C = volume of sample filtered in mL

2.4.3 Fecal indicator bacteria analysis

In order to identify indicator bacteria in water sample, IDEXX Colilert reagent was used for the detection of TC and *E. coli*. Colilert is a patented Defined Substrate Technology® (DST®), is used in over 90% of all US State labs. Colilert® is, in fact, used more than all other methods combined and it's US-EPA approved. DTS® simultaneously detects TC and *E. coli*. Two nutrient-indicators, ONPG and MUG, are the major sources of carbon in Colilert and can be metabolized by the coliform enzyme β-galactosidase and the *E. coli* enzyme β-glucuronidase, respectively (Fig. 2.8). As coliforms grow in Colilert®, they use β-galactosidase to metabolize ONPG and change it from colorless to yellow. E. coli use β-glucuronidase to metabolize MUG and create fluorescence. Since most non-coliforms do not have these enzymes, they are unable to grow and interfere. Colilert® can simultaneously detect these bacteria at 1 cfu/100 ml within 24 hours even with as many as 2 million heterotrophic bacteria per 100 ml present. The few non-coliforms that do have these enzymes are selectively suppressed by Colilert's specifically formulated matrix (IDEXX_Laboratories 2009).

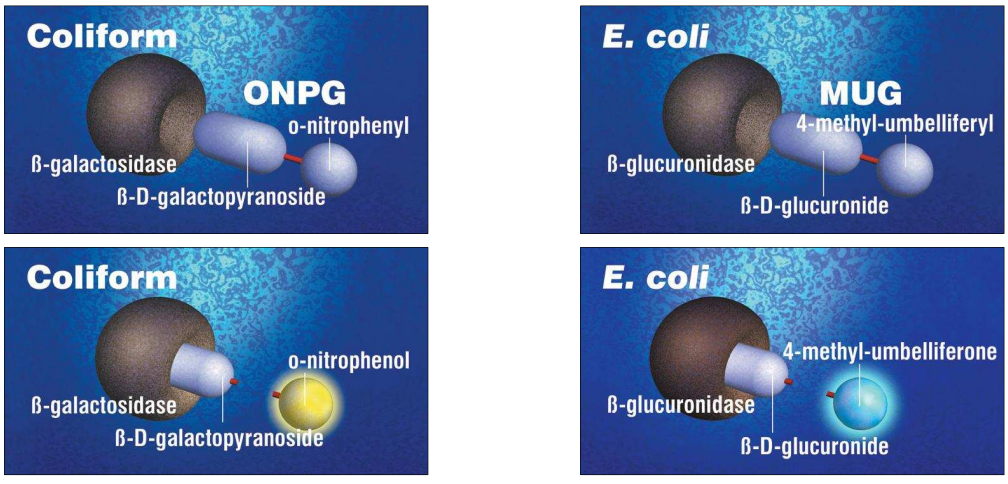

Figure 2.8 Colilert® mechanism for total coliforms and E. coli detection in water

For enterococci detection IDEXX Enterolert™ was used. It uses a Defined Substrate Technology® (DST®) nutrient-indicator for detection. This nutrient-indicator fluoresces when

metabolized by enterococci. DST improves accuracy and avoids the need for hazardous sodium azide suppressants used in traditional media.

When enterococci utilize their ß-glucosidase enzyme to metabolize Enterolert™'s nutrient-indicator, 4-methyl-umbelliferyl ß-D-glucoside, the sample fluoresces (Fig. 2.9). Enterolert™ detects enterococci at 1 cfu per 100 mL sample within 24 hours (IDEXX_Laboratories 2009).

Figure 2.9 Enterolert™ mechanism for Enterococci detection in water

For each pond sample, appropriate dilutions of the subsamples were prepared in sterile polystyrene 100 mL vessels: one set for Colilert® and one for Enterolert™. One package of powdered Colilert® and Enterolert™ reagents were then added to the vessels and the sample-reagent combination was mixed and then poured into a Quanti-Tray, a sterile plastic disposable panel containing 51 wells. The tray was then mechanically sealed and incubated for 24 h at 35.5°C for Colilert® and 24 h at 41°C for Enterolert™. With each set of samples a blank sterile water sample was run. The results were read under bright light for coliform and the number of positive wells was counted. Any yellow well was considered a positive reaction and indicative of the presence of coliform. On the other hand, for *E. Coli* and Enterococci, the results were read in a dark environment by placing the Quanti-Tray under and within 12 cm of a 365-nm-wavelenght UV light. In this case any fluorescence in a well was considered positive for that well and indicated the presence of E. coli or enterococci. On the basis of the number of positive wells, MPN tables and a dilution factors were used to determine the coliform, E. coli and enterococci density per 100 ml of sample. Quanti-Tray wells showing colorless and no fluorescence were considered negative (Budnick et al. 1996).

2.5 Pathogens' detection

2.5.1 Most Probable Number method

Most Probable Number (MPN) is a procedure to estimate the population density of viable microorganisms in a test sample. The essence of the MPN method is to dilute the sample to such a degree that inocula in the tubes will sometimes but not always contain viable organisms (point of extinction). The "outcome", i.e., the number of tubes with growth at each dilution, will imply an estimate of the original, undiluted concentration of bacteria in the sample (Blodgett 2006). The method is based upon the application of the theory of probability to the numbers of observed positive growth responses to a standard dilution series of sample inoculum placed into a set number of culture media tubes. Positive growth response after incubation may be indicated by such observations as gas production in fermentation tubes or visible turbidity in broth tubes, depending upon the type of media employed. The sample should be diluted in such a manner that higher dilutions of the sample will result in fewer positive culture tubes in the series. The number of sample dilutions to be prepared is generally based on the expected population contained within the sample. If particularly high microbial populations are expected, the sample must be diluted to a range where the MPN can be obtained. Most reliable results occur when all tubes at the lower dilution are positive and all tubes at the higher dilution are negative. Generally tenfold serial dilutions are used in either a 3, 5 or 10 tube MPN series. The pattern to tubes that show growth and those that do not are compared with a statistical table to calculate MPN (Blodgett 2006). When a higher number of tubes are inoculated in the series, the confidence limits of the MPN are narrowed. For particularly high microbial populations, the values obtained by MPN are generally not considered to be as precise as population numbers derived from direct plating methods; however, it should be emphasized that MPN values are only estimates while plate counts are direct counts of living organisms expressed in cfu/ml. MPN values are, however, particularly useful when low concentrations of organisms (<100 cfu/g) are encountered in such materials as milk, food, water and soil where particulate matter of the matrix may interfere with obtaining accurate colony counts (USDA 2008).

In application of probability theory to the determination of MPN values, it should be kept in mind that the following assumptions are generally considered to be accepted: (a) the organisms are randomly and evenly distributed throughout the sample, (b) the organisms exist as single entities, not as chains, pairs or clusters and they do not repel one another, (c) the

proper growth medium, temperature and incubation conditions have been selected to allow even a single viable cell in an inoculum to produce detectable growth and (d) the population does not contain viable, sub-lethally injured organisms that are incapable of growth in the culture medium used (USDA 2008).

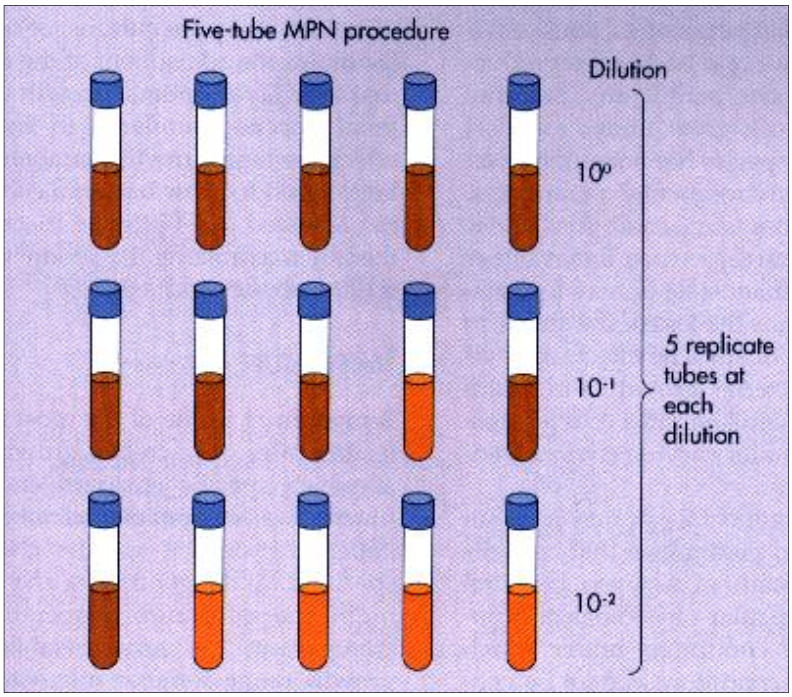

Figure 2.10 Example of 5-tube 3-fold dilution Most Probable Number procedure

The MPN is the number which makes the observed outcome most probable. It is the solution for λ, concentration, in the following equation

$$\sum_{j=1}^{k} \frac{g_j m_j}{1-\exp(-\lambda m_j)} = \sum_{j=1}^{k} t_j m_j \qquad \text{(Equation 1)}$$

where exp(x) means e^x, and

K denotes the number of dilutions,

g_j denotes the number of positive (or growth) tubes in the *j*th dilution,

m_j denotes the amount of the original sample put in each tube in the *j*th dilution,

t_j denotes the number of tubes in the *j*th dilution.

In general, this equation can be solved by iteration (USDA 2008).

MPN technique has following advantages:

1. Interpretation of the results requires minimal experience and training as results can be got by simply observing for the presence of gas or no gas.
2. Water samples with high turbidity can be analyzed, since there is no apparent deleterious effect.
3. Because of the dilutions used in the range of 1:1 or 1:100, toxic substances present in the sample can be diluted out.
4. MPN technique is the effective method for analyzing samples such as muds, sludges, sediments etc.

MPN's disadvantages are:

1. The MPN procedure takes a very long time for the confirmed test result.
2. In MPN, the results are probability calculations and can not be accurate.
3. MPN requires more glass wares and media.
4. False positive results are of common occurrence (Hagedorn 2009).

We used 95% confidence limit MPN table for a 3 tube test series using 1, .1 and .01 ml of inoculum quantities. 95% confidence intervals have the following meaning: before the tubes are inoculated, the chance is at least 95 percent that the confidence interval associated with the eventual result will enclose the actual concentration.

2.5.2 Real-time quantitative PCR TaqMan assay

Polymerase chain reaction (PCR) is a method that allows exponential amplification of short DNA sequences (usually 100 to 600 bases) within a longer double stranded DNA molecule. PCR entails the use of a pair of primers, each about 20 nucleotides in length, that are complementary to a defined sequence on each of the two strands of the DNA. These primers are extended by a DNA polymerase so that a copy is made of the designated sequence. After making this copy, the same primers can be used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round of synthesis. This leads to logarithmic amplification. Since it is necessary to raise the temperature to separate the two strands of the double strand DNA in each round of the amplification process, a thermo-stable DNA polymerase (Taq polymerase) isolated from *Thermus aquaticus*, a bacterium that grows in hot pools is used; it is not then necessary to add new polymerase in every round of amplification. After several (often about 40) thermal cycles of amplification, the PCR product is

analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain (Hunt 2009). This type of PCR is a qualitative tool for detecting the presence or absence of a particular DNA (Dorak 2009).

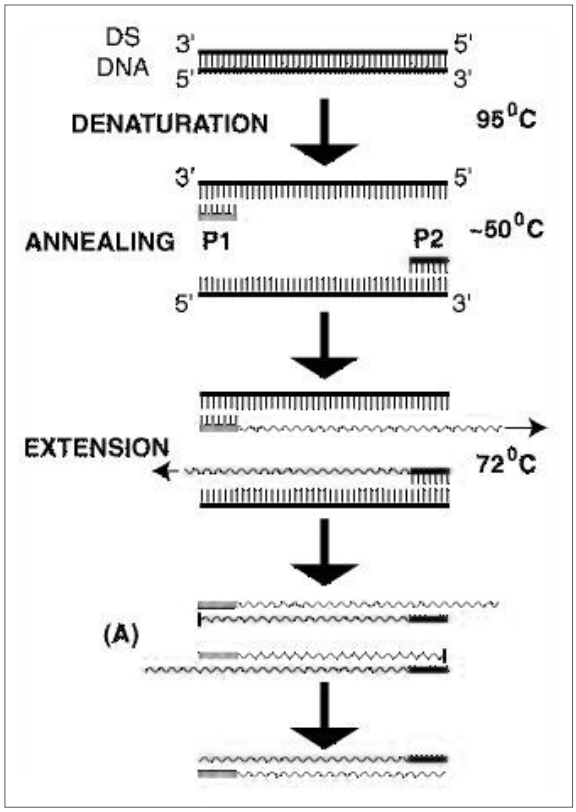

Figure 2.11 Polymerase chain reaction diagram: strand's denaturation, primers annealing and strand's extinction (Meyer 2005)

In order to obtain a quantitative PCR (qPCR) the progress of the reaction is monitored by a detector in "real-time". There are several methods of quantification but the most reliable is the fluorescent reporter probe methods. Fluorescent probes are pieces of DNA complimentary to your gene of interest that are labeled with a fluorescent dye. The simplest and most commonly used type of probe is the Taqman-type probe. These probes are labeled with a fluorescent reporter dye (FAM) at one end (5' base) and a quencher dye (capable of quenching the fluorescence of the reporter, usually TAMRA) at the other (3' base). Hence under normal circumstances the fluorescent emission from the probe is low. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Förster 1948). During PCR, if the target of interest is present,

the probe specifically anneals between the forward and reverse primer sites. The 5'-3' nucleolytic activity of the Taq polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3' end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle and doesn't interfere with the exponential accumulation of product.

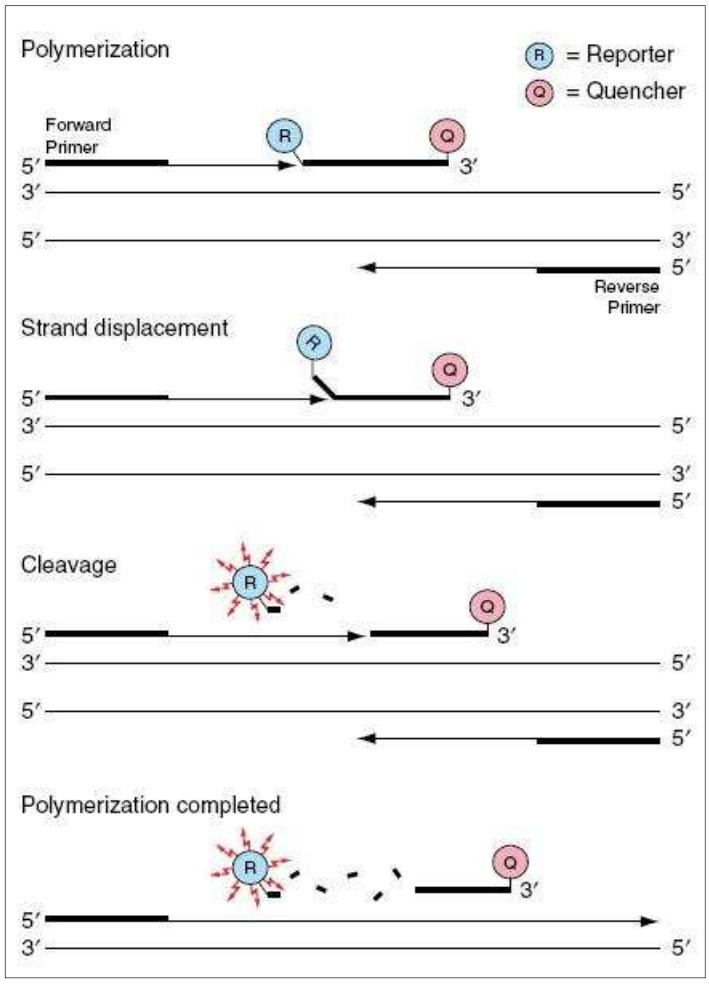

Figure 2.12 TaqMan fluorescent probe for real-time target DNA detection (Meyer 2005)

The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, any non specific amplification is not detected (AppliedBiosystems 2002).

Quantitative PCR amplifications are typically represented in the form of a graph known as amplification plot, where the measured fluorescence ΔRn (normalized reporter signal minus the baseline signal) is represented as a function of the PCR cycle number. The plot displays the amplification curve for each cell selected within the plate grid. The semi-logarithmic plot is

useful when determining threshold and baseline settings for the run. An assumption that is common to all qPCR methods is that the fluorescence is directly correlated to the amount of double stranded DNA present in the amplification reaction (Higuchi R. et al. 1992). The amplification curves are sigmoid shaped and can be split into three phases. The geometric phase is the earliest segment in the PCR, in which product increases exponentially since the reagents are not limited. It's characterized by high and constant amplification efficiency. It occurs between the first detectable rise in fluorescence and before the beginning of the linear phase. When plotted on a log scale of DNA vs. cycle number, the curve generated by the geometric phase should approximate a straight line with a slope. This phase is also used to evaluate the baseline fluorescent "noise" (Karlen et al. 2007). The linear phase is characterized by a linear increase in product as PCR reagents become limited and by a leveling effect, where the slope of the amplification curve decreases steadily. At this point, one or more components have decreased below a critical concentration, and the amplification efficiency has begun to decrease. This phase is called linear because amplification approximates an arithmetic progression, rather than a geometric increase (Yuan et al. 2006). Because the amplification efficiency is continually decreasing during the linear phase, it exhibits low precision. In the plateau phase one or more of the reactants become limiting or the amplification is inhibited by the accumulation of the PCR product itself (Wittwer et al. 1997): the PCR stops and the Delta Rn signal remains relatively constant.

C_T value is the cycle at which a significant increase in Delta Rn is first detected. The Threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification for the most accurate reading.

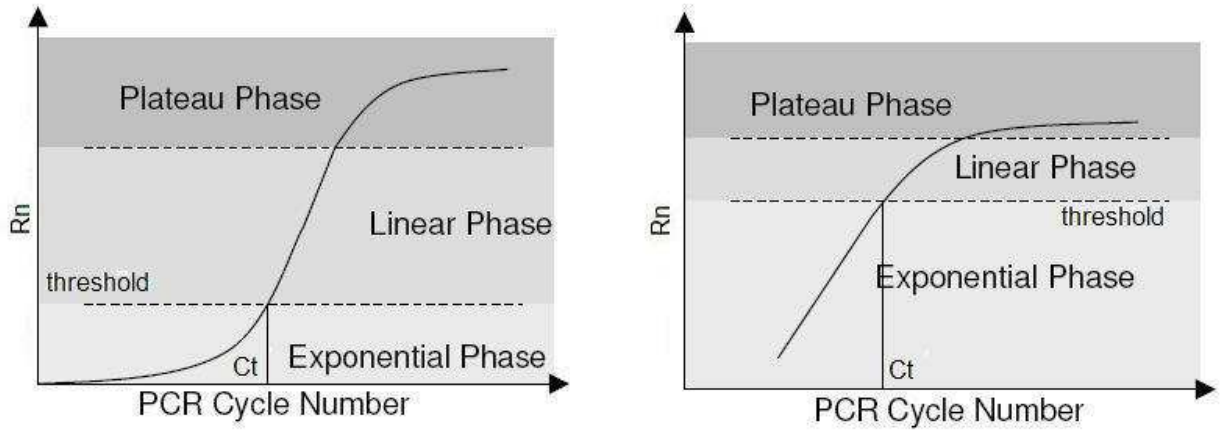

Figure 2.13 Representations of real-time PCR amplification curves. Linear and logarithmic view (Yuan et al. 2006)

2.5.3 Pathogens' biochemical confirmation

Latex agglutination test is a rapid detection method to identify a specific bacteria serotype. The latex test was found to be a simple, highly efficient and reliable test in detecting *E. coli* 0157:H7 with 100% sensitivity and specificity (March and Ratnam 1989). Colorless non-sorbitol fermenting colonies are tested with RIM™ *E. coli* O157:H7 Latex Test to determine if they are members of serogroups O157 and/or H7 and, therefore, potential verocytotoxin-producer.

Each latex reagent is coated with a different antibody: one with an antibody against *E. coli* O157, another with an antibody against *E. coli* serotype H7, and the third with a normal rabbit globulin as control latex. When the sensitized latex particles are mixed on a test slide with fresh colonies of O157 and/or H7 strains of *E. coli*, an immunochemical reaction takes place resulting in agglutination of the finely dispersed particles. With no agglutination, a negative result indicates that the test organism is not *E. coli* O157:H7. The control latex reagent identifies non-specific agglutination (Remel 2009).

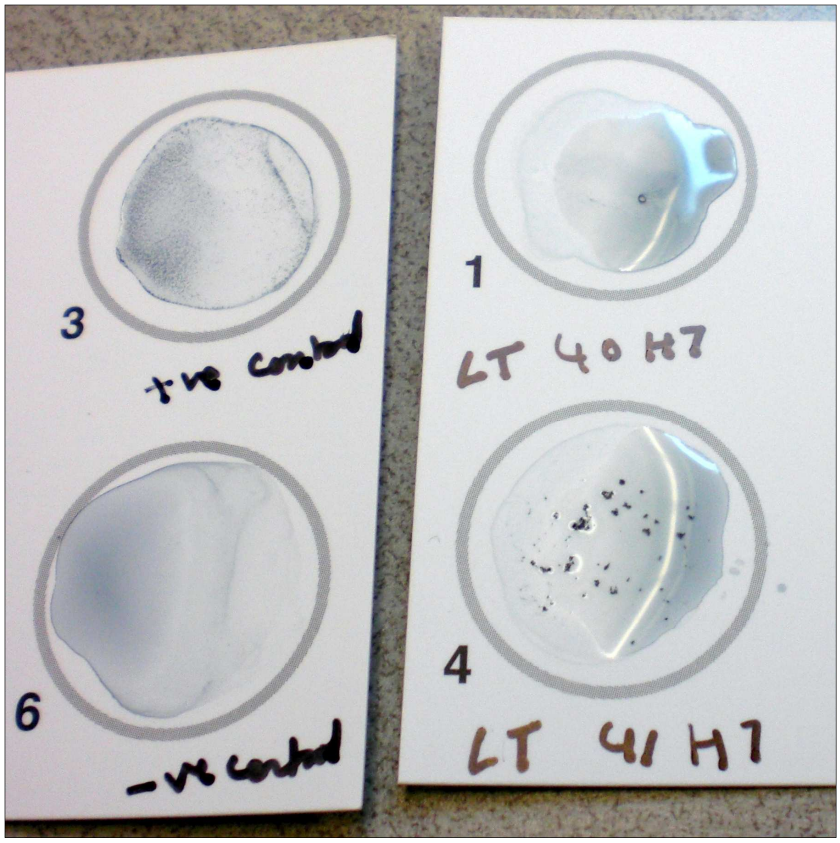

Figure 2.14 RIM™ E. coli O157:H7 Latex Test for E. coli O157:H7 serological confirmation: positive and negative control to the left, samples to the right.

2.6 *Escherichia coli* O157:H7 and *Salmonella* spp determination

Escherichia coli O157:H7 and *Salmonella* spp. were quantified by combining a concentration method with standard cultural methods and a TaqMan confirmation step (Loge et al. 2002; Jenkins et al. 2009). A 293 mm, 1 µm pore size FALP filter (Millipore, Bedford, MA, USA) was prewetted in methanol to make it hydrophilic, and sealed in a custom-made filter holder that was attached to a vacuum pump (Fig. 2.15). Methanol (500 ml of 50%) and then 1 L of distilled water were pumped through the filter before the 10 L of sample were pulled through it. The whole 10 L were filtered or until the filter became clogged. The filter was dried and eluted with 100 ml of PBS in a plastic container by scrubbing with a sterile stiff corn brush for 10 min. Eluted extract was collected in two 50 ml centrifuge tubes, centrifuged at 10 thousand RPM for 15 min, resuspended in 25 ml of PBS to consolidate the extracted material, and centrifuged again for 15 min. The pellet was resuspended in 10 ml of PBS, and 1 ml aliquots were used to inoculate the first five tubes containing 9 ml of Laural Tryptose broth for E. coli detection and tetrathionate broth (TB; Becton, Dickinson and company, Franklin Lakes, NJ, USA) for Salmonella detection and followed by two 10-fold dilutions to complete a five-tube, tree by 10-fold dilution scheme. The inoculated LTB and TB were incubated at 35°C for 24 h.

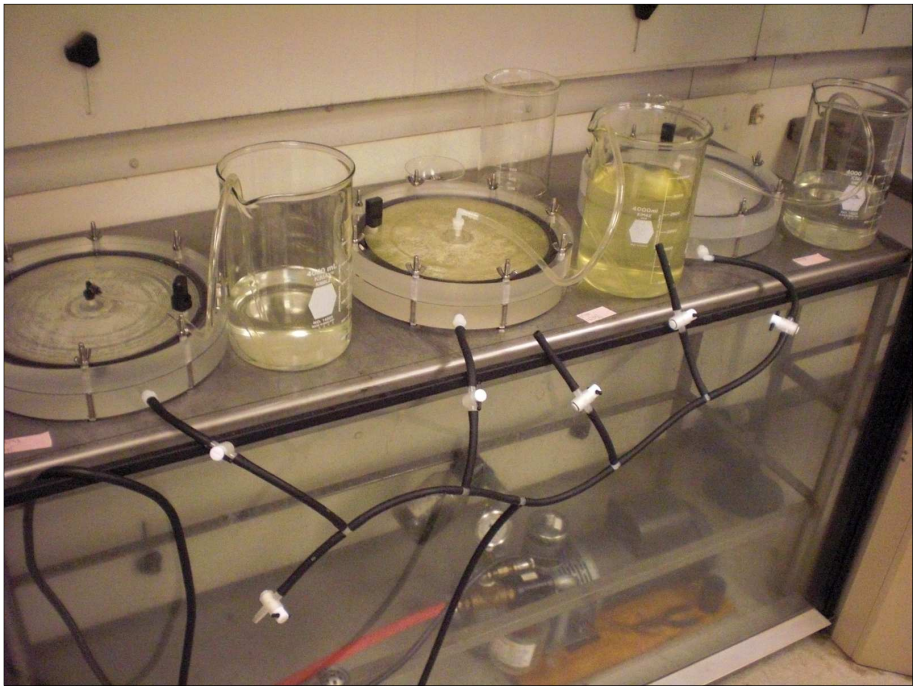

Figure 2.15 Samples filtration apparatus for pathogens bacteria isolation: vacuum pump and 1 µm pore size FALP filter.

Enrichment broth (9 ml) was inoculated with 1 ml of one LTB tube showing growth. A Sorbitol MacConkey [SMAC (Becton, Dickinson and Company, Franklin Lakes, NJ, USA)] agar plate was inoculated with 10 – 100 µl of one LTB tube showing growth and streaked for E. coli colony isolation and purity. Both agar plate and EC broth tube were incubated at 35°C for 24 h. The same procedure was followed with XLT4 agar plates and RV enrichment broth for *Salmonella* spp isolation and purity.

After 24 h, Eppendorf microcentrifuge tubes were filled with 1 ml of each LTB and TB and centrifuged at maximum speed for 3 minutes. Then pellets, constituted by bacteria, were resuspended in 1 ml of PBS, centrifuged again and the supernatant was poured off. Eppendorf tubes were so frozen in -80°C freezer.

Once prepared, pellets of each microcentrifuge tubes were resuspended with 1 ml of PBS, centrifuged at 13 thousand RPM for 5 min, 100 µl of ultra-pure water was added and the tubes were heated at 100°C for 5 min to extract the DNA before the PCR reaction.

Results from filtering pond water spiked with known numbers of pathogens bacteria through the FALP filter indicated a range of recovery between 50% and 61% for *E. coli O157:H7* and between 50% and 70% for *Salmonella* (Jenkins et al. 2008).

2.6.1 DNA amplification and detection

Real-Time PCR was performed on extracted DNA using primers targeting genes associated with 150 pb-*eaeA* gene for *E. coli* O157:H7 and 250 pb-*SipB/C* gene for *Salmonella* spp. TaqMan *eaeA* probe and TaqMan *SipB/C* probe with reporter dye FAM and quencher dye TAMRA were used respectively for *E. coli* and *Salmonella* (Tab. 2-2). Primers, probes and Master Mix were synthesized by Applied Biosystems (Foster City, CA, USA).

PCR reagents were placed in a 96-wells plate, each well corresponded to a LTB or TB tube and the Real-Time PCR was performed in a 26 µl volume containing:

- 1 µl of each forward and reverse primer;
- 12.5 µl of TaqMan® Universal PCR Master Mix;
- 0.5 µl of TaqMan® probe (Applied Biosystems, Foster City, CA, USA);
- 2 µl of DNA sample;
- 9 µl of ultra pure water.

All PCR reactions were performed using a ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) under conditions of 1 cycle at 50°C for 2 min, 1

cycle at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1min (Sharma 2002). For each PCR assay, positive (*E. coli* O157:H7 ATCC #35150, *Salmonella enterica* serovar Typhimurium ATCC #13311) and negative controls (ultra pure water) were included in order to exclude PCR inhibitors presence during the reaction (Bessetti 2007).

	***Escherichia coli* O157:H7**	***Salmonella* spp**
Primers	*aeaA* forward 5'-GTA AGT TAC ACT ATA AAA GCA CCG TC-3' *aeaA* reverse 5'-TCT GTG TGG ATG GTA ATA AAT TTT TG'3'	*SipB/C* forward 5'-ACA GCA AAA TGC GGA AGC TT-3' *SipB/C* reverse 5'-GCG CGC TCA GTG TAG GAC TC-3'
TaqMan® Probe	*eaeA* gene 5'-AAA TGG ACA TAG CAT - CAG CAT AAT AGG CTT GCT-3'	SipB/C gene 5'-CCA GGT CAC TGA CTT - TAC TGC TGC TAA TAC CAA-3'

Table 2-2 Salmonella and E. coli O157:H7 TaqMan® Probes nitrogenous bases sequences.

A replicate LTB or TB tube of a particular dilution was considered positive for *E. coli* O157:H7 or *Salmonella* spp, if at least one isolate from that tube had a positive fluorescence threshold cycle (C_T) in the TaqMan assay (Briones and Reichardt 1999).

2.7 Data analysis

Since data were not normally distributed, the nonparametric statistic test (Kruskal-Wallis) was used to compare sampling sites for each parameter, in order to have descriptive statistics. Kruskal-Wallis ANOVA by ranks is a between-group one-way analysis of variance test that assumes that the variable under consideration is continuous and that it was measured on at least an ordinal (rank order) scale. The test assesses the hypothesis that the different samples in the comparison were drawn from the same distribution or from distributions with the same median. Thus, the interpretation of the Kruskal-Wallis test is basically identical to that of the parametric one-way ANOVA, except that it is based on ranks rather than means. The p-level for highlighting was 0.05.

In order to have a more accurate analysis of the results the PCA (Principal Component Analysis) method was adopted. The aim of PCA is to reduce the dimensionality of a set of variables while retaining the maximum variability in terms of the variance-covariance structure. In other words PCA tries to explain the variance-covariance structure of a data set using a new set of coordinate systems that is lesser in dimension than the number of original variables. Given a set variables, a principal component (PC) model transforms these variables into a new set of uncorrelated variables, lesser in dimension, and yet can capture most of the variability in the original data set. Each coordinate in the new transformed system is known as a principal component. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible.

Mathematically, given $X_1, \ldots, X_p$ with variance and covariance matrix, PCA method seeks r new variables $Z_1, \ldots, Z_r$ with:

$$Z_i = a_{i1}X_1 + a_{i2}X_2 + \ldots + a_{ip}X_p \qquad \text{(Equation 2)}$$

so that Z_1 synthesize as much total variability as possible, Z_2 synthesize as much as total variability as possible after Z_1, so on and so forth. However, the number of PC thus extracted will never exceed the number of original variables. Coefficients a_{ij} of equation 2 are called loadings. The loadings can be understood as the weights for each original variable when calculating the principal component.

Data were analyzed with STATISTICA data analysis software system (Version 8.0) (Stat Soft, Inc. 2007).

3 RESULTS

During the sampling period the rainiest month was August followed by October, November and December 2008. All four months were above the monthly average total precipitation (Fig. 3.1), on the contrary, rainfall in September, 2008, which was below the average.

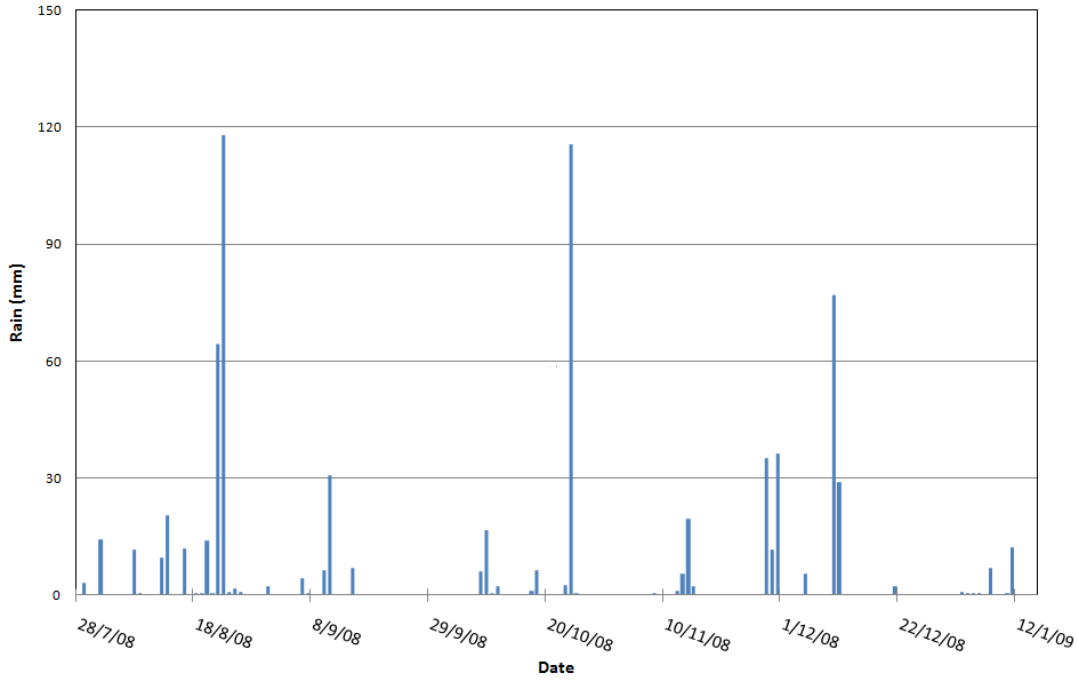

Figure 3.1 Daily rainfalls in whole sampling period.

Temperatures had a decreasing trend according to the season but it can be observed that both maximum and minimum temperatures have an increment of about 8°C from the second half of November through the end of the year.

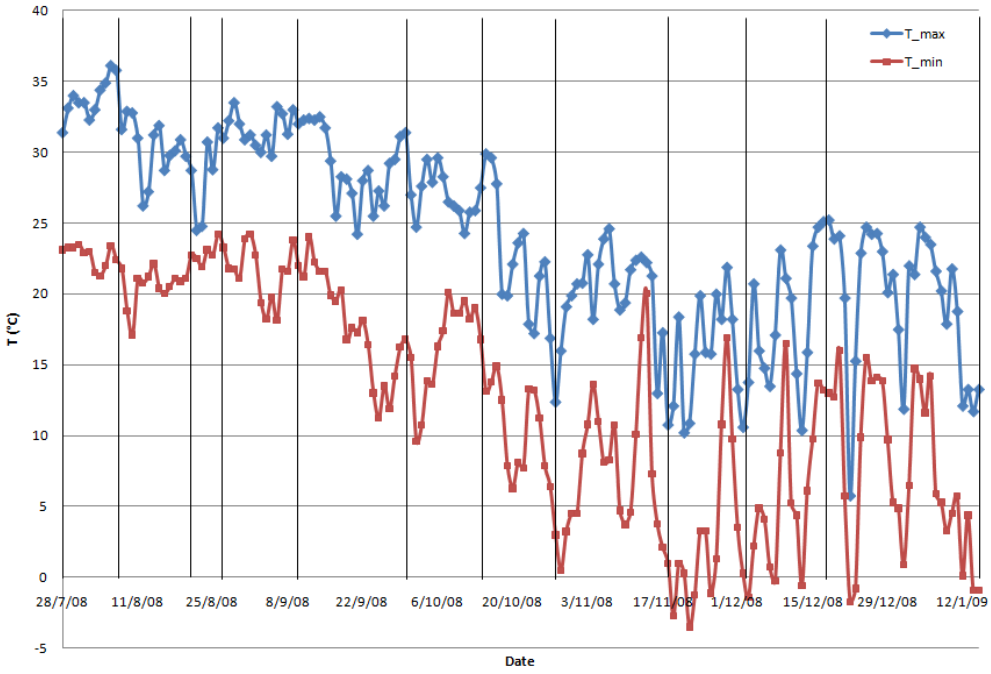

Figure 3.2 Maximum and minimum air temperature and sampling date operations.

Water nutrient concentrations didn't reflect seasonal rainfall trend except for TN: in fact TN was significantly correlated to precipitations (R=0.313). A significant correlation was observed also between TSS and rainfall (R = 0.333).

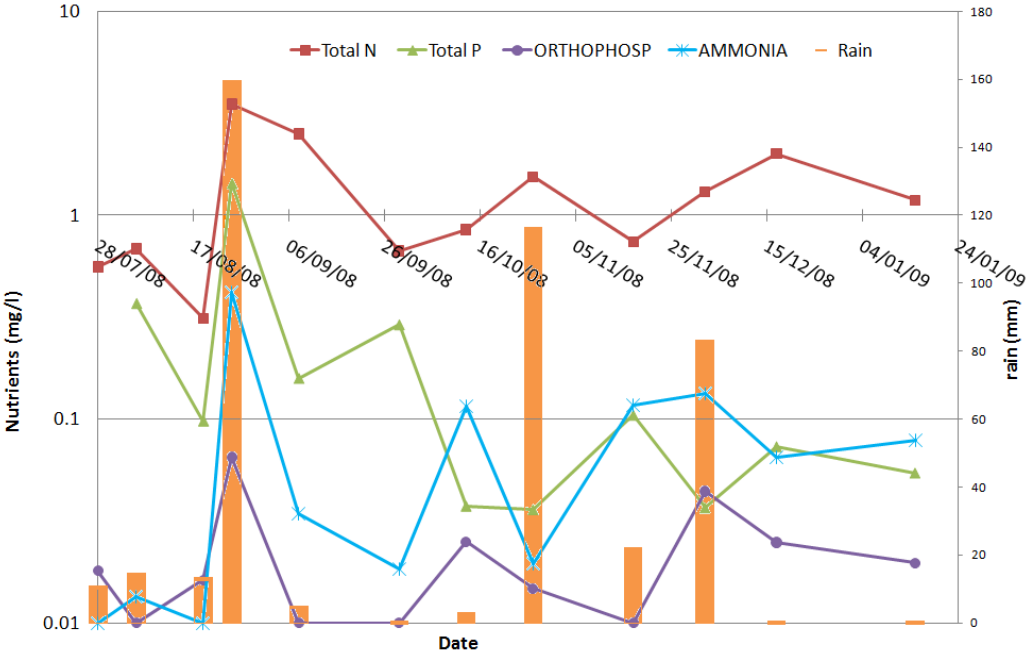

Figure 3.3 Nutrients concentration and rainfalls by experimental period

On the contrary, the other nutrients were not correlated with the rainfall (Fig. 3.3), even if the nutrients highest concentrations (Cl^- apart) were reached in coincidence with the massive rainfall event (August 25th and 26th). During that time PO_4^{3-}, NH_4^+, NO_3^-, TP and TN concentrations were 0.18, 1.34, 6.88, 2.85 and 7.34 g/L respectively (Tab. 3-1).

	Average	Median	Minimum	Maximum	23rd Percentile	75th Percentile	Dev. Std.
T max °C	23.60	25.500	10.61	34.89	16.89	31.11	8.02
T min °C	12.46	14.722	-0.89	24.22	2.11	21.61	9.17
Rain (mm)	41.10	11.684	0.00	184.66	0.00	82.80	56.46
ECw (mS/cm)	0.29	0.222	0.06	2.87	0.11	0.25	0.54
NO_3^- (mg/L)	0.85	0.316	0.00	6.88	0.01	1.11	1.39
Cl^- (mg/L)	8.16	7.307	3.06	14.92	4.53	11.37	3.73
PO_4^{3-}(mg/L)	0.02	0.011	0.00	0.18	0.00	0.02	0.04
NH_4^+ (mg/L)	0.09	0.018	0.00	1.34	0.01	0.10	0.20
TP (mg/L)	0.23	0.064	0.00	2.85	0.03	0.15	0.49
TN (mg/L)	1.46	0.867	0.13	7.34	0.73	1.61	1.54
TTS (ppm)	11.69	11.009	0.40	32.12	4.64	16.53	8.36
TC	3322.26	4405.450	204.60	4838.40	1841.60	4838.40	1688.05
E. coli	45.65	8.000	0.00	551.00	2.50	34.10	99.24
Enterococci	550.02	49.900	1.00	4838.40	12.80	175.45	1308.85
Salmonella	0.20	0.000	0.00	2.07	0.00	0.19	0.45
E. coli O157:H7	0.09	0.000	0.00	0.98	0.00	0.00	0.25

Table 3-1 Variables descriptive statistics.

The chemical water quality was significantly different among the 5 ponds, in particular for ECw, NO_3^-, Cl^- and TSS (Fig. 3.4, 3.5). In detail ECw in NP and SC ponds (medians of 0.07 and 0.1 mS/cm) was lower than GF (median=0.3 mS/cm), while NO_3^- in LV (median value 1.9 mg/L) was higher with respect to NP and SC (medians 0.02 and 0.003 mg/L). (Tab. 3.1)

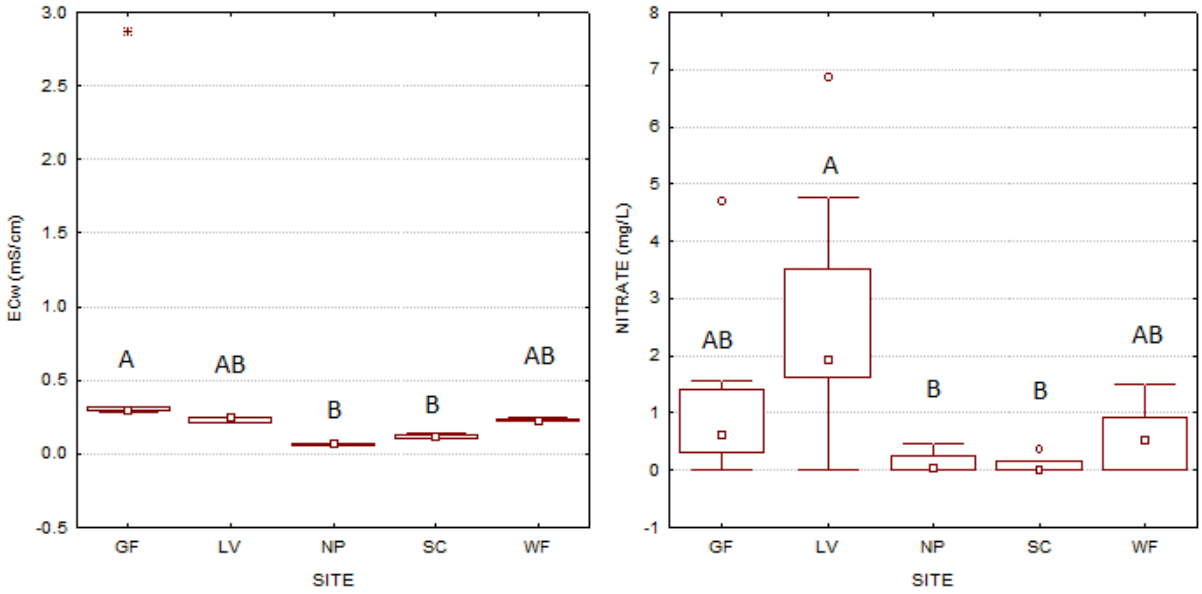

Figure 3.4 Box-plots of conductivity and nitrate concentration in the five ponds

In SC and WF significant higher values of Cl^- (medians 13.5 and 10.5 mg/L) were observed by comparison with other three ponds (almost one order of magnitude of difference). The median TSS in LV was 2.5 ppm significantly lower than the TSS in the other ponds (median of 10 ppm).

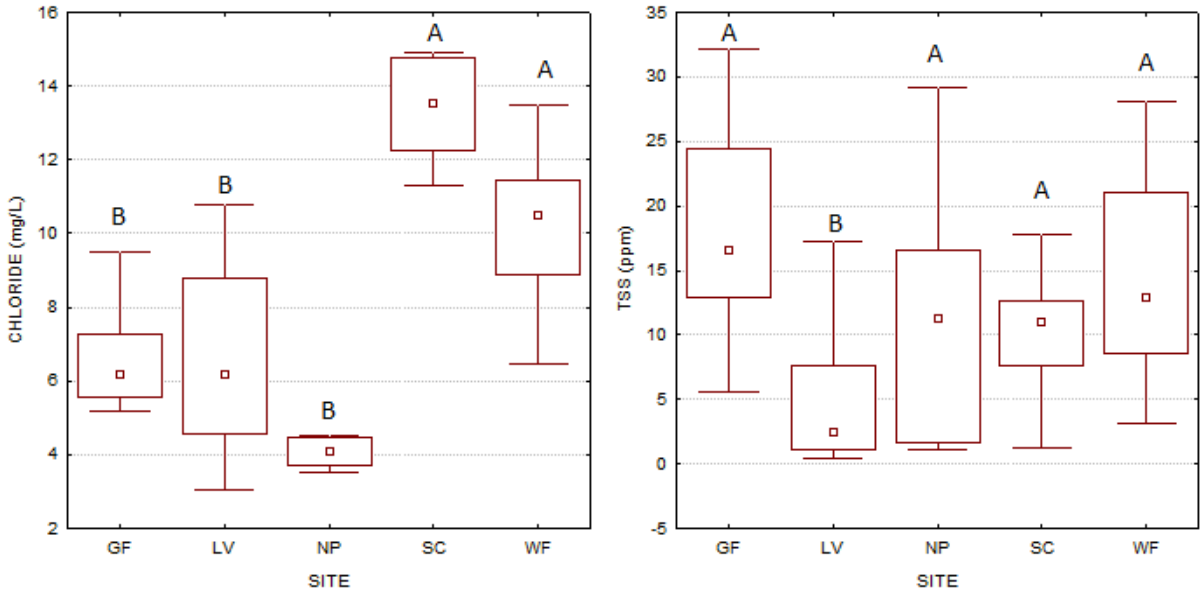

Figure 3.5 Box-plots of Chloride and Total Suspended Solids concentration in the five ponds

The fecal indicator bacteria concentration varied during the season according to the rainfall trend. Indeed the higher values were observed in coincidence with the highest 3 rainfall events of August 25th, October 27th and December 10th 2008 (Fig. 3.6).

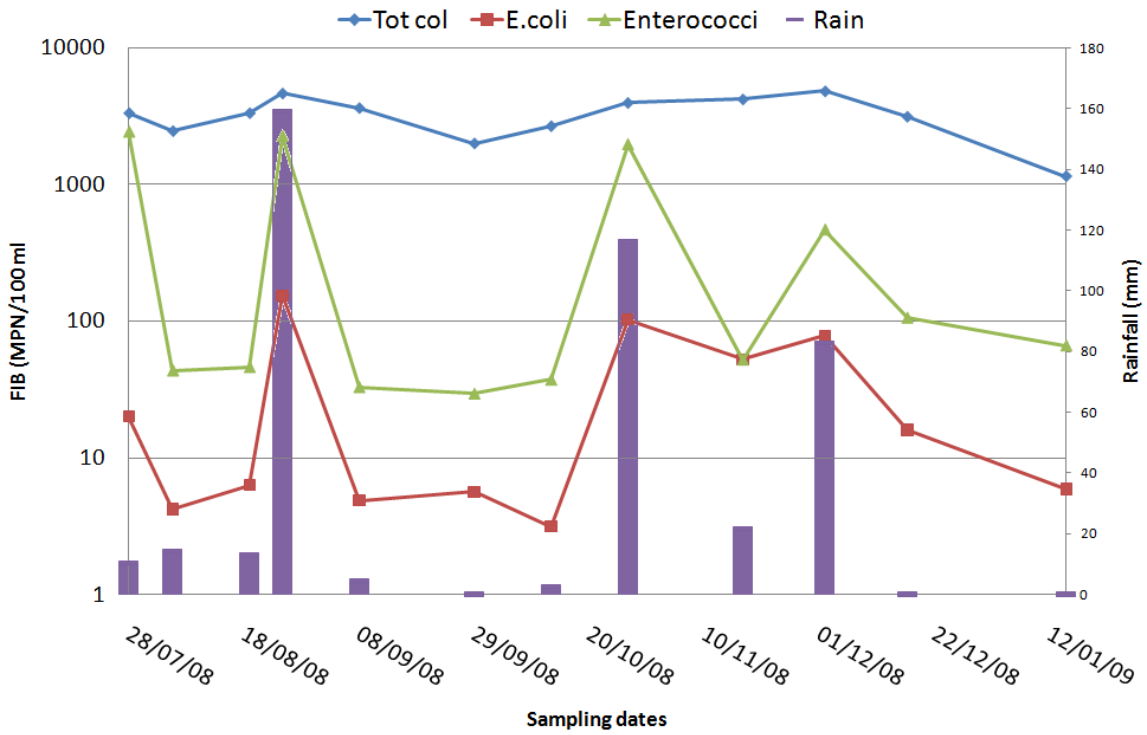

Figure 3.6 Fecal indicator bacteria concentration and rainfalls during the experimental period

The median values of TC, E. coli, and Enterococci were 4405.4, 8.0 and 49.9 MPN/100 ml respectively with a maximum value of 4838.4, 551.0 and 4838.4 and a minimum of 204.6, 0.0 and 1.0 MPN/100 ml (Tab. 3-1).

Significant correlations were observed between rainfall and TC (R=0.558), E. coli (R=0.571) and Enterococci (R=0.420). Significant correlation were observed also between TSS and FIB, with a higher value for enterococci (R=0.636), (Tab. 3-2).

The fecal indicator bacteria concentration showed a large variability among the five pounds. Median concentration of TC and E. coli was apparently higher in GF and WF (Fig. 3.7). However, only Enterococci significantly differed between the ponds with lower values in LV (median 13.2 MPN/100 ml) than GF and WF. Intermediate values (median 66.6 and 15.4 MPN/100 ml) were measured in NP and SC.

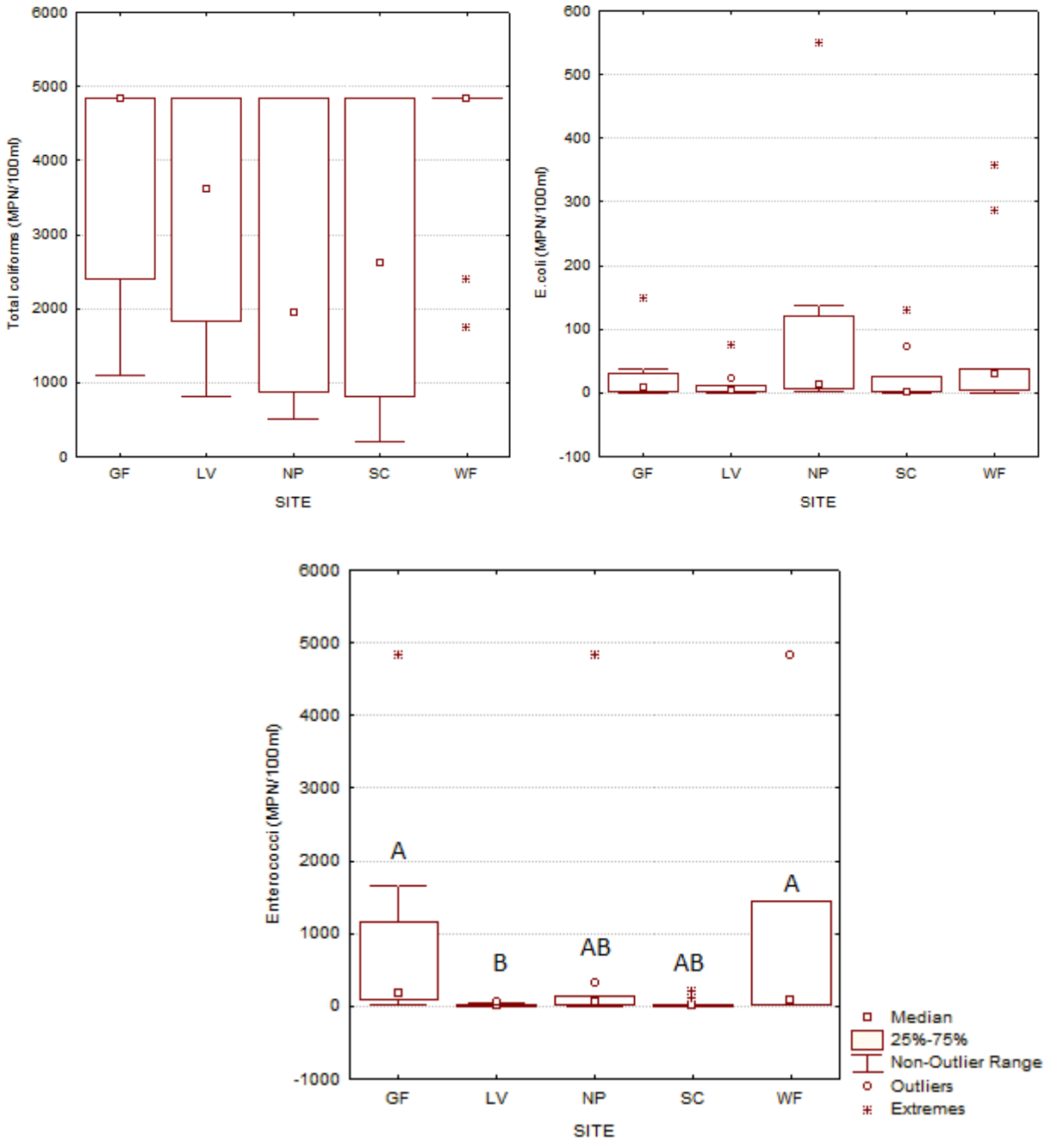

Figure 3.7 Box-plots of TC and E. coli and Enterococci concentration in the five ponds

Table 3-2 Spearman correlation matrix

O157:H7	Salmonella	EC	E. coli	TC	TSS	Tot N	Tot P	$NH4^+$	PO_4^{3-}	Cl^-	$N\text{-}NO_3^-$	ECw	Rain	T min	T max	
-0.481	0.014	-0.187	-0.289	-0.078	-0.083	-0.199	0.128	-0.087	-0.337	-0.118	-0.417	-0.064	-0.096	0.913	1.000	**T max**
-0.437	0.026	-0.016	-0.089	0.072	0.010	-0.017	0.137	0.014	-0.192	-0.169	-0.349	-0.016	0.162	1.000	0.913	**T min**
0.221	0.137	0.420	0.571	0.558	0.333	0.313	0.077	0.168	0.004	-0.173	0.157	0.161	1.000	0.162	-0.096	**Rain**
-0.308	-0.054	0.097	-0.350	0.077	0.059	0.061	0.206	-0.504	-0.289	0.082	0.479	1.000	0.161	-0.016	-0.064	**ECw**
0.060	-0.020	0.150	0.175	0.274	0.007	0.701	-0.002	0.142	0.087	0.002	1.000	0.479	0.157	-0.349	-0.417	**$N\text{-}NO_3^-$**
0.101	0.086	-0.167	-0.251	0.049	0.003	0.015	0.092	-0.072	-0.090	1.000	0.002	0.082	-0.173	-0.169	-0.118	**Cl^-**
0.209	-0.210	0.414	0.363	0.092	0.175	0.093	0.132	0.328	1.000	-0.090	0.087	-0.289	0.004	-0.192	-0.337	**PO_4^{3-}**
0.155	-0.118	0.345	0.409	0.037	0.255	0.228	0.073	1.000	0.328	-0.072	0.142	-0.504	0.168	0.014	-0.087	**NH_4^+**
-0.288	-0.014	0.151	-0.081	0.140	0.171	0.031	1.000	0.073	0.132	0.092	-0.002	0.206	0.077	0.137	0.128	**TP**
0.090	0.050	0.196	0.255	0.210	0.091	1.000	0.031	0.228	0.093	0.015	0.701	0.061	0.313	-0.017	-0.199	**TN**
-0.050	0.167	0.636	0.389	0.360	1.000	0.091	0.171	0.255	0.175	0.003	0.007	0.059	0.333	0.010	-0.083	**TTS**
0.235	0.406	0.521	0.560	1.000	0.360	0.210	0.140	0.037	0.092	0.049	0.274	0.077	0.558	0.072	-0.078	**TC**
0.455	0.231	0.734	1.000	0.560	0.389	0.255	-0.081	0.409	0.363	-0.251	0.175	-0.350	0.571	-0.089	-0.289	**E. coli**
0.215	0.138	1.000	0.734	0.521	0.636	0.196	0.151	0.345	0.414	-0.167	0.150	0.097	0.420	-0.016	-0.187	**EC**
0.176	1.000	0.138	0.231	0.406	0.167	0.050	-0.014	-0.118	-0.210	0.086	-0.020	-0.054	0.137	0.026	0.014	**Salmonella**
1.000	0.176	0.215	0.455	0.235	-0.050	0.090	-0.288	0.155	0.209	0.101	0.060	-0.308	0.221	-0.437	-0.481	**O157:H7**

The MPN method for *E. coli* 157:H7 and *Salmonella* Spp confirmed the presence of low numbers of these zoonotic pathogens (Figure 3.8).

PCR analysis detected that *E. coli* O157:H7 was present in 17.1% of the samples. The MPN determinations ranged from below the detection limit (<0.1 mpn/10 L) to the maximum of 0.98 MPN/L (it was observed in NP November 17th 2008). With regard to Salmonella, PCR was positive for 31.8% of the total samples and the MPN determinations ranged from below detection limit to the maximum of 2.07 mpn/L (measured in WF pond September 8th 2008) for *Salmonella* (Tab. 3-1).

On one sampling day, October 27th, an additional 10-L water sample was gathered 50 cm below the surface in each pond. Also a sample of sediments was collected from the bottom of the ponds. The results of analysis for FIB and pathogens was in line with the results obtained from surface water samples, therefore reputedly there is no difference between surface water, 50-cm deep water, and bacteria presence within the bottom sediments. Nevertheless, one single data is not enough to do a significant statistic consideration; hence further samplings will be necessary in order to confirm this hypothesis.

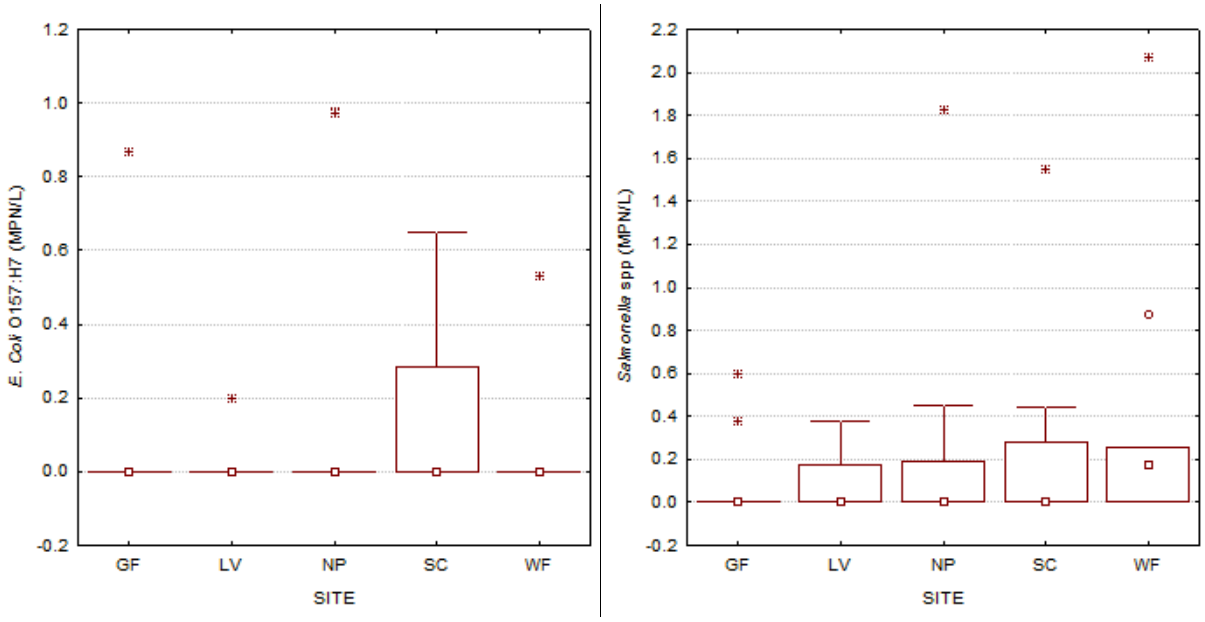

Figure 3.8 Box-plots of *E. coli* O157:H7 and *Salmonella* spp. concentration in the five ponds

In the majority of the samples, concentrations of both pathogens were below the detection limit, however SC pond there exhibited the highest detection rate of *E. coli* O157:H7 (33% of the samples) while for Salmonella, the maximum detection rate (50% of the samples) occurred in WF (Table 3-3).

	Salmonella spp. (%)	*E. coli* O157:H7 (%)
GF	22.2	11.1
LV	33.3	11.1
NP	20.0	20.0
SC	33.3	33.3
WF	50.0	10.0
Total	31.8	17.1

Table 3-3 Percentages of Salmonella and E. coli positive PCR samples for each pond

There were no significant differences in concentration for both E. coli and Salmonella between the ponds even if Salmonella apparently had a higher median in WF pond (0.17 MPN/L). Both E. coli and Salmonella showed a median of zero MPN/L (Tab. 3.1).

A significant correlation (Tab. 3-2) was found between *E. coli* O157:H7 and FC (R=0.455) and between *Salmonella* spp. and TC (R=0.287).

Choosing those variables presenting a significant correlation (Tab. 3-2) the Principal Component Analysis was run in order to have a better statistical interpretation of the system and to reduce the quantity of the variables. Using this analysis, three principal components were created: the first PC was linked to water runoff system and groups positively precipitation, TSS, and FIB. The second PC put together pathogen *E. coli* and the maximum and minimum temperatures in negative way. The last PC linked nutrients and pathogen Salmonella (Tab. 3-4).

Table 3-4 PCA loadings spreadsheet and percentage of single and total variance explained by each component

	PC 1	PC 2	PC 3	
T max	0.419709	0.832390	0.193055	
T min	0.263132	0.894771	0.115841	
Rain	-0.736356	0.267906	-0.320933	
PO_4^{3-}	-0.288461	-0.035924	-0.383914	
TN	-0.063048	0.343118	-0.615988	
TSS	-0.606336	0.232020	0.484672	
E. coli	-0.838813	0.239204	0.150988	
Enterococci	-0.716780	0.480112	-0.091070	
Salmonella	-0.234092	-0.148365	0.634254	
***E. coli* O157:H7**	-0.452771	-0.606208	-0.013939	**Total**
% variance explained	27.2	24.2	13.5	**64.8**

4 DISCUSSION

Baseline sensitivity for this MPN method was based on the MPN for one positive tube at the lowest dilution of a five-tube dilution series, and was considered to be the most likely low value of the 95% CL (Garthright and Blodgett 2003): 0.01 MPN L^{-1}. Variables controlling the method's detection limits included:

- volume of the sample passed through the FALP filter (V_f)
- recovery of organisms (R)
- baseline sensitivity (S)
- fraction of volume of filter concentrate used to inoculate the first replicate tubes of LTB (always 1.0 ml)
- Inhibition factor (I) that could prevent the selection and confirmation of *E. coli* O157:H7 and *Salmonella* spp. by the TaqMan assay.

Inhibition (I) was sample specific and represents the dilution necessary to produce a positive-PCR result. (I) is expressed as the inverse of the highest dilution with a positive tube (Loge et al. 2002).

The equation for determining the overall detection limit of the PCR-based method can be expressed as

$$\text{Detection limit} = \frac{I \cdot S}{R \cdot V_f} \qquad \text{(Equation 3)}$$

Thus, the overall detection limit was highly dependent on the potential inhibition factor (I) because it ranged from 10 to 1000; whereas the recovery factor (R) and volume of sample (V_f) had smaller ranges and had relatively little effect on the range of overall detection limit (i.e. 0.01–1 MPN L^{-1}). Biochemical confirmation is suggested to be done in conjunction with the method to further validity results. Given the range of extraction efficiency, variables affecting detection limits, and the probability of *E. coli* O157:H7 and *Salmonella* cells being components of biofilms or particulate aggregates, our MPN determinations were likely underestimations.

Given all variables at our disposal associated with the ponds and those that better fit by matrix correlation, a system of three uncorrelated principal factors took place. Run off factor explained about 27% of total variability: it's very likely that water runoff coming from surrounding agricultural field and riparian zones may transport soil particles, organic matter and bacteria. Agricultural soils in the area I operated, are very rich in sand and this facilitates

particle erosion from the soil matrix, particularly in conjunction with heavy rainfall events such as rainstorms and hurricanes so likely in this part of the country.

The second factor involved the presence of *E. coli* O157:H7 that is negatively correlated with the temperatures system: pathogenic E. coli concentration increased at decreasing temperatures. This PC was able to explain 24.2% of the total variability. It's well known, in fact, that temperature has an inverse relationship with the survival of microorganisms. *Escherichia coli* O157:H7 is more sensitive to competition by the native microbial community than *Salmonella* spp (Semenov et al. 2007). Apparently, at intermediate and high temperatures, microorganisms antagonistic to enteropathogens are more competitive than at low temperatures, possibly because of faster growth or increased production of antimicrobials at higher temperatures. As the total bacterial populations were independent of incubation temperature, there could have been shifts in microbial composition with the changes in temperature (Panswad et al. 2003).

The third factor didn't show clear relationships between Salmonella presence and nutrient concentrations in the water. In general, nutrients should promote bacteria life but only under controlled conditions: in an open ecosystem more factors weigh upon it which could obscure this correlation. With regard to this, a thorough literature review did not provide any insight thus further studies and analysis should be done.

USEPA guidelines for microbial water quality criteria vary depending on water destination use (drinking, recreational, irrigation waters) and water sources (surface and waste waters). Guidelines for the microbial quality of surface water tend to be more lenient than those for wastewater because of the belief that human pathogens are less likely to be present or less numerous (Steele and Odumeru 2004). Criteria are defined as a quantifiable relationship between the density of indicators in the water and the potential human health risks involved in the water use (Cabelli 1983).

For drinking water USEPA requires a maximum contaminant level (MCL) goal of zero for TC and FC per 100 mL of drinking water (USEPA 1989). USEPA requires not more than 61 enterococci and 235 E. coli per 100 ml in primary contact recreational water, whereas 89 enterococci and 298 E. coli per 100 ml in secondary contact recreational water (USEPA 1986).

USEPA irrigation water quality criteria recommend an absence of detectable FC (E. coli) in wastewater used to irrigate crops likely to be eaten uncooked and less than 200 FC per 100 ml in that used to irrigate processed or fodder crops (USEPA 1992). For surface water EPA

guideline recommends fewer than 1000 FC per 100 ml (USEPA 1973). Although these standards are widely accepted in USA some states also publish their own irrigation water quality standards.

Working on surface waters, the threshold of 1000 FC per 100 ml was never exceeded in any of the samples from any of the ponds (Figure 3.9), so these waters are suitable to be used as irrigation water.

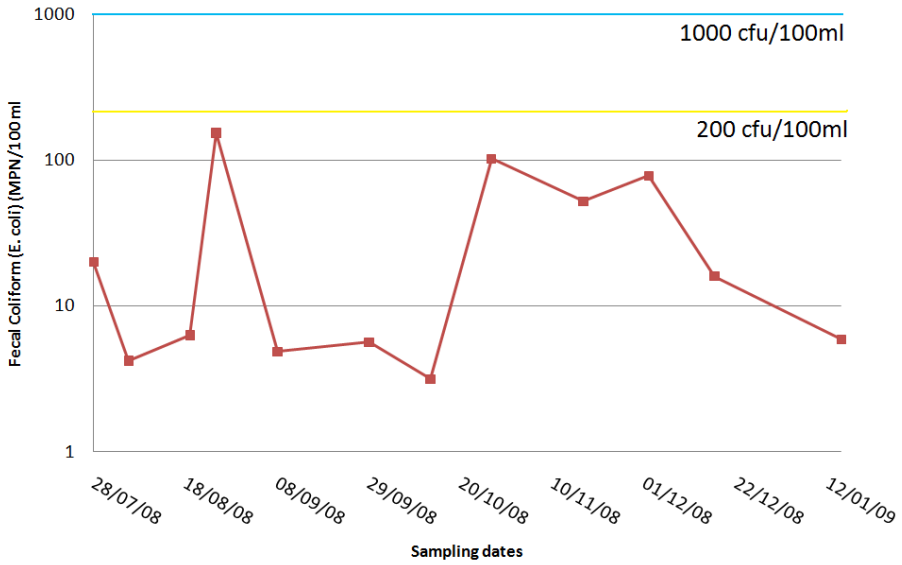

Figure 4.1 Distribution of Fecal Coliform by sampling date and reference guideline criteria

In addition, the limit of 200 FC per 100 ml is not exceeded, so you might use these water reservoirs to irrigate crops to be eaten raw as well.

Considering pathogen concentrations, even in the worst case scenario, it doesn't awaken concerns for human health because they have a maximum concentration of 2.02 MPN/L for Salmonella and 0.98 MPN/L for *E. coli* O157:H7, which are very below the infective dose for both bacteria.

5 GENERAL CONCLUSION AND FUTURE PROSPECTS

The culture-based MPN method described and tested in this study has quantified dilute densities of *E. coli* O157:H7 and *Salmonella* spp in environmental surface waters of a watershed containing animal agriculture and wildlife. It provided a quantitative tool for improving our understanding of the pathogens in association with indicator bacteria, nutrients and others environmental parameters we measured.

In relation to the goals of the thesis, this method was able to identify the presence of target pathogenic bacteria within the irrigation reservoirs and also a correlation between these pathogens and indicator bacteria (in particular for TC and Enterococci) was found indicating that the detection method described is a valid tool for further similar analysis.

With variables at disposal, we were able to explain about 65% of what occurs in this system showing that there might be other variables governing the occurrence such as field management, animal agricultural and wildlife habits, soil composition, and not least, sampling frequency. Because treatment options are limited, it's better to prevent contamination of surface water. When access to good-quality irrigation water is limited, one alternative is to treat crops that are not consumed raw with lower quality irrigation water and use higher quality water for crops which are eaten with little or no processing. An alternative might be to irrigate with lower quality water early in the growing season while irrigating with better quality water closer to harvest.

In systems such as those described here, a deeper study and more frequent sampling plan can be developed and correlated with the best management practices such as run off control, fertilization and irrigation techniques, soil cover and residues management, in order to lessen the agricultural impact on surface waters.

Significant improvements are still needed at all levels of sample preparation, including filtration, template recovery, and sample purification. Once these improvements are made, monitoring protocols can be used in quantitative analyses or as a standard method to assess ambient water quality.

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