# Evaluating Human Fecal Contamination Sources in Kranji Reservoir Catchment, Singapore

By

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**Abstract**

Singapore government through its Public Utilities Board is interested in opening Kranji Reservoir to recreational use. However, water courses within the Kranji Reservoir catchment contain human fecal indicator bacteria above recreational water quality criteria; their sources and distribution under dry and wet weather are also unknown. The goal of this study was to evaluate the distribution of *E. coli* under dry and wet weather, to determine the sources of the human fecal contamination, and to validate the use of human-specific 16S rRNA *Bacteroides* marker for human fecal source tracking in Singapore and tropical regions.

Environmental water and DNA water samples (332) collected in the Kranji catchment in January and July 2009, and January 2010 were analyzed for *E. coli* using Hach m-ColiBlue24® and IDEXX Colilert Quanti-Tray®/2000. Touchdown PCR and Nested-PCR HF183F assays were used to assess the absence or presence of the HF marker in Kranji catchment. Selected positive HF marker samples were sequenced and mapped using a phylogenetic tree to confirm their similarity in base order to the human factor identified in the temperate climate.

The indicator bacteria (*E. coli*) results showed consistently high *E. coli* concentrations (geometric mean 3240 CFU/100 ml) in dry and wet weather in residential, horticultural and animal farming areas. The DNA analysis results showed that 94% of the 34 environmental DNA water samples collected in residential, horticultural and animal farming areas were positive to the HF marker. Generally, 74% and 94% of DNA samples respectively collected in dry and wet weather in the Kranji catchment were positive. The sequence and phylogenetic tree analysis confirmed that the HF marker identified was similar to the HF marker identified in temperate climates.

Based on the results we conclude that human fecal contamination sources are widespread in the animal farming, horticultural and residential areas of Kranji catchment. The HF marker analysis validated its applicability as 16S rRNA gene of human-specific *Bacteroides* for human fecal source tracking in Singapore and elsewhere in tropical climates.

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# Chapter 1: Introduction

*Figure 1.1 Children in Dhaka Bangladesh Swimming in Polluted Water (photo by the Author)*

## 1.1 Project Scope

Point and nonpoint sources of human fecal contamination are a global threat to water quality. A variety of laboratory analysis techniques have been developed for the detection of fecal contaminants. Many of these methods rely on detection and quantification of indicator bacteria such as the total coliforms, *Enterococcus* and *Escherichia coli* (*E. coli*). These fecal indicator bacteria have proven to be good proxies for health risks associated with human sewage in many environments. When indicator bacteria levels exceed regulatory thresholds water recreational facilities, beaches and rivers are closed, thus protecting public health, but also reducing tourism, fishing, and boating income. In general, methods to detect indicator organisms do not link indicator organisms to their origins (i.e. human or animal) although human sewage is of particular concern because it presents the highest risk of transmitting human-infectious diseases (Anderson and Davidson 1997). Therefore, there is considerable interest in designing strategies to specifically monitor human sewage contamination to both maximize protection of public health and reduce economic losses due to unnecessary closures.

Recently, molecular microbiology techniques proposed a promising solution to the problem of ambiguity in bacterial source tracking. Methods targeting the 16S ribosomal RNA gene of bacteria only found in association with humans are used to detect nonpoint sources of human fecal bacteria pollution. This technique has been proved to be effective under the temperate climate of the United States of America where recent studies by Bernhard and Field (2000),

Forgarty and Voytek (2005), Santoro and Boehm (2007) and Shanks et al. (2006) demonstrated its applicability to monitor occurrence of the bacterial HF marker as a proxy for human fecal pollution in freshwaters. The research reported in this thesis uses this new laboratory technique, in conjunction with use of traditional fecal indicators (Coliforms and *E. coli*) and an analysis of land-use patterns, to identify the sources of human fecal pollution in the Kranji Reservoir catchment under dry and wet climate conditions.

A desire to increase public awareness of the importance of the water supply system has increased the Singapore government's interest in expanding water recreation facilities. Kranji Reservoir, a drinking water reservoir in the west of Singapore, has been included in the Western Catchment Masterplan (PUB 2007b), which includes the main upcoming Singapore water-recreation projects. However, human fecal bacteria pollution sources in the Kranji Reservoir catchment and their variation during wet and dry weather are unknown. The goal of this study was to determine the distribution of *E. coli* under dry and wet weather periods, to determine the sources of human fecal contamination, and to evaluate whether HF marker is a good indicator for human-associated wastes in Kranji catchment. We used Hach m-ColiBlue24® (Hach Company, 2008) and IDEXX Colilert Quanti-Tray®/2000 (IDEXX, 2009) methods to study the *E. coli* distribution, while Touchdown PCR and Nested-PCR to determine the distribution of the human-specific bacterial HF marker by the polymerase chain reaction (PCR) in Kranji catchment. In addition, selected positive HF marker samples were sequenced to confirm the similarity in base order to the HF marker identified in previous studies performed in the temperate climate.

The intent of this research is to help the Singapore Public Utilities Board in planning effective ways of managing and controlling nonpoint sources of human fecal contamination in the Kranji Reservoir. The results will also be used to evaluate the universality of using the HF marker found in human-associated *Bacteroides species* as an indicator of human fecal pollution.

This thesis is organized into seven chapters. Chapter One introduces the research project and gives the background of bacteriological pollution research in Kranji catchment and the water management system in Singapore. Chapter Two is a review of risks and regulatory guidelines associated with bacteriological pollution. Chapter Three is a review of challenges associated with fecal indicator bacteria and the methods for detection. Chapter Four is a presentation of the methodology used in this study. Finally, the Fifth, Sixth and Seventh Chapters present the study Results, Discussion, and Conclusions and Recommendations, respectively.

## 1.2 Water Pollution in the World

Water is essential to daily life. However, its quality is sometimes affected by water pollutants associated with human health risks. Water pollution originates from different sources such as municipal sewer systems, industries, farms and agriculture. The pollutants can be classified in

two major groups: chemical pollutants and bacterial pollutants. The bacteria pollutants include human fecal contamination, which has been a public health concern for centuries. During the 19th century, fecal contamination in water was recognized as related to a number of waterborne diseases and epidemic cases (Domingo and Ashbolt 2008). The palatability of water was a concern of humans for centuries and motivated water treatment to remove pollutants before water use. Filtration was the accepted treatment method used to improve the quality and the appearance of water. The people's awareness of the consequences of fecal pollution was firstly raised by the findings of John Snow in 1850s (Domingo and Ashbolt 2008). John Snow's research demonstrated the link between fecal contamination, drinking water supply, and a Cholera outbreak in London. Years later in the 1890s, chlorine was proved to be an effective water disinfectant. Retrospective epidemiological analysis has shown that in the United States of America the use of chlorine reduced the typhoid fever burden from 30 cases per 100,000 population before water chlorination in 1908 to 6 cases per 100,000 in 1990s (Figure 1.2) (Domingo and Ashbolt 2008).

Although fecal contamination was of interest due to its direct public health effects, industrial chemical pollutants were also becoming problematic in developed countries. In 1969 there was an incident in which the Cuyahoga River in Cleveland, Ohio actually caught fire due to industrial pollutants (GLIN 2010). This incident was one of many that prompted the policy makers to establish the Great Lakes Water Quality Act and Clean Water Act in 1970s to protect waterways in the United States of America (GLIN 2010). Generally, U.S. industry is estimated to cause more than half of the total USA water pollution (Bora 2010). The main chemical pollutants identified are acids, alkalis, toxic metals, oil, grease, dyes, pesticides, and even radioactive materials (Bora 2010). In addition, these chemicals have also killed many aquatic organisms, caused mutations, and included a number of chemicals that are considered carcinogenic. Moreover, the consequences of these pollutants are economically costly to manage.

On the other hand, developing countries present a different scenario. These countries are also concerned with the consequences of fecal pollution of surface waters. This situation is still manifested by the persistence of waterborne diseases and in some cases they have resulted in deadly epidemics. These diseases include cholera, typhoid, bacillary dysentery and diarrheal diseases (Cruz 2010). Nowadays, fecal contamination is still a huge concern of developed countries, although these countries have developed medicine, water treatment technologies, and policy development. There are some surface recreational facilities such as beaches in developed countries that have been closed due to fecal contamination. The United States has even established "the Total Coliform Rule," which emerged after the publication of water quality standards. The "Total Coliform Rule" was recently revised by the USEPA in 2007 (USEPA 2007).

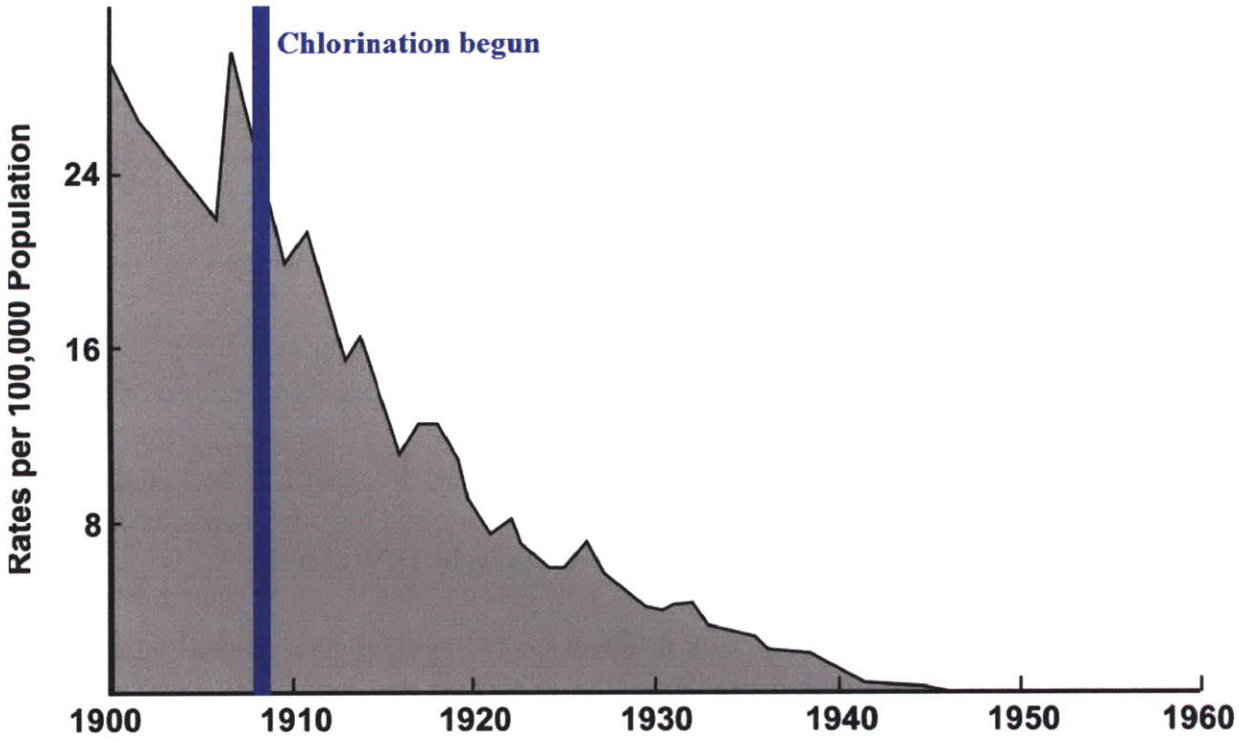


*Figure 1.2 Number of typhoid fever cases reported in the United States in the first half of the 20th century. The bar indicated the time chlorination was introduced as a disinfection treatment. (CDC, 1997)*

The United Nations International Drinking Water Supply and Sanitation decade (1981-1990) initiated many global activities that focused on the developing world and aimed at solving the water crisis. However, the major problems associated with high morbidity of waterborne diseases were not solved. The weaknesses identified were then discussed in the fourth Dublin Conference held in 1992. The resolutions of this conference were grouped under four strategies to accomplish the 1981-1990 decade agenda and introduce new water resources management approaches. The four principles are:

- Principle 1: Fresh water is a finite and vulnerable resource, essential to sustain life, development and the environment;
- Principle 2: water development and management should be based on a participatory approach, involving users, planners and policy makers at all the levels;
- Principle 3: 'Women play a central part in the provision, management and safeguarding of water';
- Principle 4: 'Water has an economic value in all its competing uses and should be recognized as an economic good' (UNESCO 2003).

These principles demonstrated the involvement of all the water stakeholders in protecting water quality. These resolutions were then revisited by the 2000 United Nations Summit, which established new protocols assembled under the "the Millennium Development Goals" with a 2015 achievement target. The millennium goals related to poverty and water are (UNESCO 2003):

1. To reduce the proportion of people living on less than 1 dollar per day;
2. To reduce the proportion of people suffering from hunger;
3. To reduce the proportion of people without access to safe drinking water;
4. To ensure that all children, boys and girls equally, can complete a course of primary education;
5. To reduce maternal mortality by 75 percent and under-five mortality by two thirds;
6. To halt and reverse the spread of HIV/AIDS, malaria and the other major diseases;
7. To provide special assistance to children orphaned by HIV/AIDS.

Despite the fact that there have been many different United Nations international programs to solve the water crisis and pollution problems, the impact at the community level in many developing nations is still hardly provable. While the United Nations Summit of 2000 was deciding about the next phase solutions, the reported data showed that fecal water contamination was still threatening lives in developing countries. The mortality rate related to fecal contamination and poor sanitation was estimated at 2,213,000 deaths annually (UNESCO 2003). In addition, the worldwide data showed that 2 billion people were contaminated with schistosomes and soil-transmitted helminthes (UNESCO 2003). Disease control strategies used in developed countries could be adopted and reshaped to fit the situation in developing countries.

The spread and distribution of waterborne diseases is related to the continuous loading of fecal contaminants into surface recreational waterways. This fecal loading is caused by a variety of sources such as birds, wild animals, leaking sewer and septic tanks, runoff, and wastewater discharge and it is observed in both developed and developing countries. The World Health Organization water-quality guidelines (WHO 2003) include guidelines for recreational waterways adoptable worldwide. These guidelines were introduced to help developing countries monitor surface recreational water contamination. However, the majority of these countries did not have enough resources to implement the program. This is primarily due to the high cost of equipment used for water quality analysis. On the other hand, developed countries have established fecal contamination monitoring programs for recreational waterways such as public beaches. In addition, progress in scientific research has reduced the cost of analysis making water analysis equipment accessible and easy to manipulate (WHO 2003).

However, water pollution is still problematic around the world. Developed countries and a few developing counties have managed to establish successful mechanisms to protect public health. Recreational surface water needs huge investments to ensure its safety. In 2010, the United

States through its Environmental Protection Agency (EPA) will spend nearly $100 million to ensure beach and coastal area safety (USEPA 2010). The USEPA program targets water pollution control and prevention strategies at these sites. Efforts are also remarkable in other developed and developing countries, which are seeking funding for implementing suitable water pollution control and prevention policies. The foundation of a joint action between developed and developing countries is encouraged to seek and reinforce "the world without water pollution" a strategy that I believe could help save the lives of millions of people who die every year from water pollution related illnesses.

## 1.3 Project Background

This section was written as part of a collaborative effort with Cameron Dixon, Kathleen B. Kerigan and Jessica M. Yeager.

### 1.3.1 Singapore Background

Singapore is an independent island city-state established in 1819 as a British trading colony in Southeast Asia (Figure 1.3) at the southern end of the Malaysian peninsula (Figure 1.4). It has a land area of 682.7 square kilometers and a water area of 10 square kilometers. It is 3.5 times the size of Washington, DC. Singapore became independent from Britain in 1963 after eighteen years of colonial rule. It was considered an important center for commerce and military exchange in Southeast Asia by the British Empire. During the independence period Singapore belonged to the Federation of Malaysia which included four areas: Malaya, Sabah, Singapore, and Sarawak. In 1965, after two successful years of developmental work, Singapore was recognized as an independent state by the Commonwealth of Nations, and was then detached from the Malaysian federation. From the time of independence, Singapore has emerged as a progressive and successful country with a large increase in the standard of living. Currently, the country's population is estimated at 4.7 million with a growth of 0.998% and a pyramid of age dominated by adults (15-64 years) totaling 76% (CIA 2010). Nevertheless, the gross domestic product (GDP) of Singapore dropped at 1.1% in 2008 due to the global economic crisis, and then increased at 2.6% in 2009. The GDP per capita is estimated at $50,300 and classified as the 8$^{th}$ worldwide before the United States of America, which is classified the 10$^{th}$ (CIA 2010).


*Figure 1.3 Map of Southeast Asia with Singapore Highlighted (NIE 2010)*


*Figure 1.4 Map of Singapore (CIA 2010)*

Environmentally, Singapore has a humid, rainy, and hot tropical climate with three different monsoon seasons: the northeastern monsoon that goes from December to March, the southwestern monsoon that goes from June to September, and the inter-monsoon period characterized by thunderstorms particularly in afternoons. The natural resources are mainly fish and deepwater ports. The freshwater withdrawals are divided between 45% for domestic, 51% for industrial, and 4% for agriculture uses (CIA 2010). This reflects the fact that the country's economy relies more on services than agriculture. In order to ensure a self-sufficient water supply system, Singapore allocated a part of its land to water reservoirs for storage. The majority of reservoirs are located in the western catchment which receives high quantities of rainwater, while the eastern catchment is drier and warmer.

The land altitude extends from 0 m up to 166 m, which is the highest point located in the Bukit Timah area. Although the country is known for its strict environmental protection rules, industrial pollution and waste disposal are of major concern due to constricted natural fresh water resources and land availability (CIA 2010).

Limited water resources have pushed Singapore to plan and implement an effective water resources management system that includes the Masterplan of different catchments areas in the country. The Public Utilities Board (PUB) has apportioned Singapore into three main catchment areas: the western catchment, the central catchment and the eastern catchment. The goal of PUB is not only to provide a suitable water management system that will capture freshwater and provide an effective management system, but also to provide people gratification through water recreational activities. The PUB, through its "Water for All: Conserve, Value and Enjoy" program, is also targeting an increase of the internal water supply and reduction of the national water demand. The program of increasing supply is composed of various steps, such as, to re-use more wastewater, increase the supply of desalinized water, and capture as much as possible of the considerable rainwater Singapore receives each year.

PUB has a goal of providing people with enjoyment through the recreation activities in Singapore's reservoirs. This program depends upon the status of water quality in this reservoir. PUB has established strategies to overcome the water quantity issue by increasing the water collected in Singapore. Water quality research that will provide the information needed for suitable water treatment, waster resources and pollution management strategies have been launched. The research reported in this thesis is among many that are currently ongoing, and the main focus is to evaluate the human fecal contaminations sources in the Kranji Reservoir Catchment located in Singapore Western Catchment.

### 1.3.2 Project location: Site Characteristics

This research was conducted in the catchment area of Kranji Reservoir in Singapore's Western Catchment (Figure 1.5). The Western Catchment encompasses the western third of the country and is home to about 1 million people or 27% of Singapore's total population (PUB 2007b). The catchment remained largely undeveloped until after Singapore achieved independence (PUB 2007b) and is currently an approximately equal mix of urban development, industrial development, and natural environment (PUB 2007a). Residential areas are concentrated on the southern edge of the catchment (PUB 2007b).

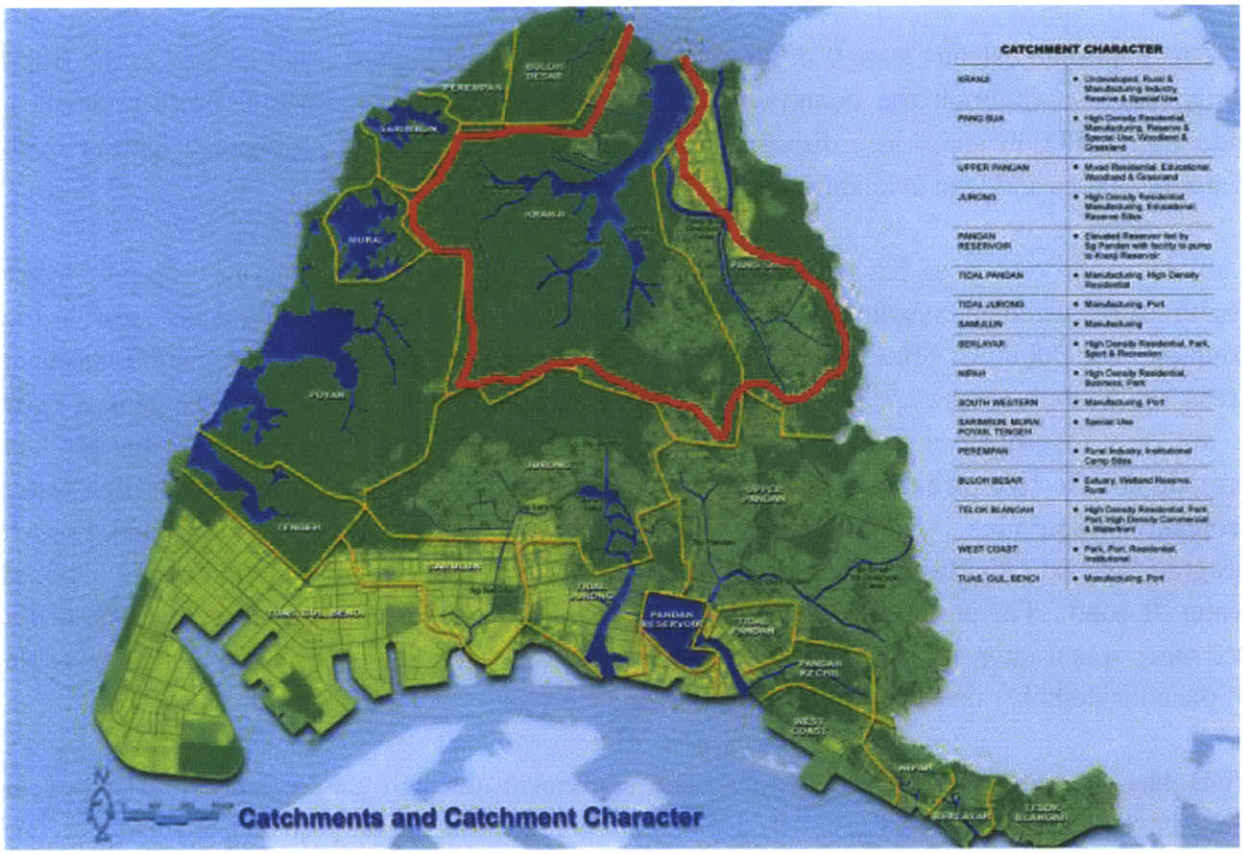


*Figure 1.5 Map of Singapore Western Catchment showing Kranji Reservoir Catchment (PUB 2007b)*

The Kranji Reservoir is located in the northwestern corner of the island (1°25'N, 103°43'E) (NTU 2008). The Kranji Reservoir was created in 1975 by the damming of an estuary which drained into the Johor Straits that separate the Malaysian mainland from Singapore. The reservoir is approximately 647 hectares in area and the catchment has four tributaries, Kangkar River, Tengah River, Pengsiang River in the south, and Pangsua River in the north (NTU 2008). The Kranji Catchment is approximately 6076 hectares in area (NTU 2008). The catchment has a

variety of land uses; including forests, reserved areas, agriculture, and residential areas (Figure 1.6 and Table 1.1). Table 1.1 shows a detailed land use

While the Kranji Reservoir is strong in many aspects (including beauty, ecological uniqueness and open spaces), the Western Catchment Master Plan identifies that the Kranji catchment currently has low visitor rates (PUB 2007b). This is due to a combination of factors. First, the site is relatively isolated since most of the catchment is undeveloped. Second, public transportation serving the area is limited. Third, there are only two entry points to the reservoir (one on either side of the dam) and poor connectivity within the site. Finally, public recreational activities are limited. Current recreational opportunities include cycling, park visits, and minor fishing areas.

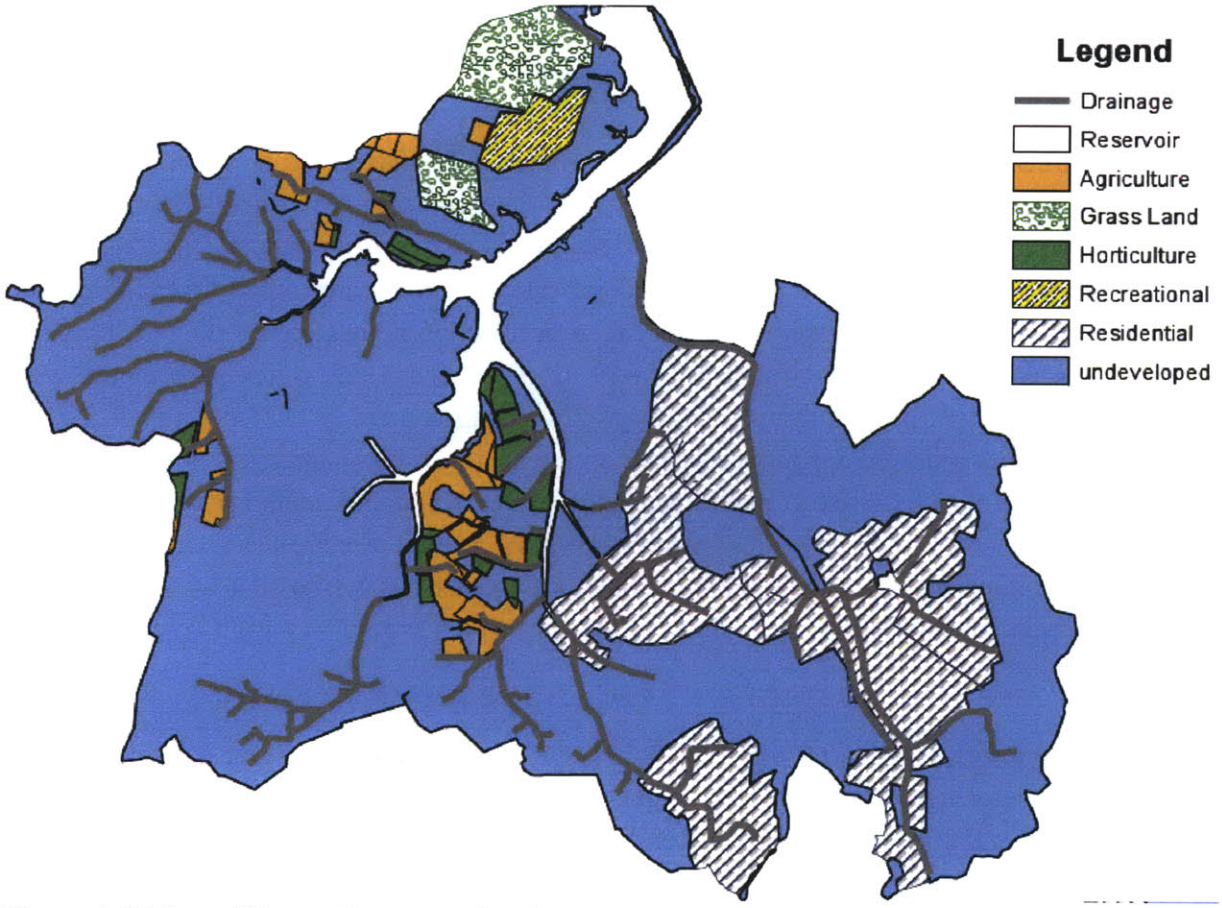


*Figure 1.6 Map of Kranji Reservoir Catchment land use.*

## 1.4 Project Motivation

### 1.4.1 Singapore Water Management Plan

When Sir Stamford Raffles arrived in Singapore in 1819, Singapore was self-sufficient in water supply with 150 residents (Lee 2005). Nearly 30 years later, the population had increased to 50,000 and water started to be scarce due to lack of a water supply system. In 1857, a donation was given by Tan Kim Seng to construct the first Singapore water distribution system (Lee 2005). Ten years after this work, the first municipal water supply system was completed at the same time as the first Singapore reservoir, MacRitchie Reservoir. The size of this dam was increased in response to the increase of water demand in 1890, 1894 and 1900 (Lee 2005). As Singapore development was progressing, the population number kept increasing until Singapore authorities realized that water resources available could not satisfy the demand. During this period, Singapore was still a territory of the Federation of Malaysia colonized by British. The neighboring province, Johore, in Malaysia was identified rich in water resources and selected then, by Singapore authorities, to be the source of the additional water quantity that the island was lacking to satisfy existing and future water demand.

The selection of Johore as source of complementary water to Singapore did not wait long to pass to action. In 1927 the first water agreement between the two areas was signed (Lee 2005 and Tortajada 2006). Five years later, Johore started supplying water to Singapore. The agreement stated the obligations of Singapore that included treatment of raw water piped from Johore and maintenance of the system and reservoir at the water connection point (Lee 2005). At this time, Singapore was withdrawing raw water for free. In 1961 and 1962 water agreements were reviewed and put under different terms than the first water agreement in 1927 (Tortajada 2006) . The new agreements required Singapore to pay 3 cents (US Dollars) for every 3.8 $m^3$ and the Johore Government was required to pay to Singapore 50 (US Dollars) cents for every 3.8 $m^3$ of treated water. The costs fixed by both agreements were supposed to be reviewed each 25 years, but this was not done because it was likely to increase tremendously the cost of treated water. Such a result was considered unfavorable to Malaysia, which would pay high cost for treated water (Lee 2005).

Currently, Singapore has rights to withdraw 1,271,898.4 $m^3$from Johore River according to the 1961 and 1962 water agreements. These water agreements will respectively be in force up to 2011 and 2061 (Segal 2004). Due to the latter deadlines, Singapore has been improving its water management systems in order to ensure that they are ready for self-sufficiency in meeting water needs at the end of both agreements. As the agreements are approaching deadlines, Singapore has already accomplished extensive work to guarantee Singapore water self-sufficiency. Various water programs have been completed such as reservoir enlargement, water catchments master plan, rainwater and runoff collection, NewWater production, desalination plant and community

involvement to increase the self-water supply system and reduce the purchasing water from Johore River.

MacRitchie Reservoir was the first to be built and enlarged in 1894 to meet the water demand that was increased with city development. Currently, Singapore has fifteen reservoirs after completing the construction of Marina reservoir (Lee 2005). Table 1.1 shows seven of the Singapore reservoirs, the year they were completed, and their storage capacity. Although water storage is the primary role of these reservoirs, they are also used to prevent and control flooding around the island (Lee 2005). Flood prevention and control is ensured through the "Reservoir Integration Scheme" completed in 2006 to share extra water among different reservoirs (Lee 2005). Singapore reservoirs are also being transformed into tourist and recreational areas allowing water games and tourism to bring additional wealth to the island.

Singapore reservoirs are not the only component included in the water self-sufficiency plan. NEWater is another part of the program that focuses on recycling wastewater for further reuse. The recycled water has characteristics of distilled water and is designated to be used for non-portable use. One part of the NEWater is purchased by companies that need highly treated water for their various industrial operations and another part is sent back to reservoirs where it is mixed with raw water in the drinking water reservoir (Lee 2005). Recycled water is projected to increase to 10 mgd and 55 mgd to drinking water reservoirs and non-potable use respectively by 2011 totaling 20% of Singapore water supply (Lee 2005).

*Table 1.1 Reservoirs of Singapore (Lee 2005)*

| Name of the Reservoir | Completion Period | Size (million $m^3$) |
|---|---|---|
| MacRitchie | 1867 (enlarged in 1894) | 4.2 |
| Lower Pierce | 1912 | 2.8 |
| Seletar | 1935 (enlarged in 1969) | 24.1 |
| Upper Pierce | 1974 | 27.8 |
| Kranji/Pandan | 1975 | 22.5 |
| Western Catchment | 1981 | 31.4 |
| Bedok/Sungei Seletar | 1986 | 23.2 |

Desalination is also another alternative that Singapore has been exploring as a strategy to achieve self-sufficiency in water supply. PUB started assessing this technology back in the 1970s, but the high cost of the system kept Singapore from implementation. In 1995, Singapore authorities decided the feasibility of the technology that was suggested for desalination (Lee 2005). The decision resulted from study tours done in different countries such as Saudi Arabia, the United Arab Emirates and Malta that use desalination as a key technology for water supply (Lee 2005). The desalination plant started to operate in 2005 producing 1.2 $10^5$ $m^3$, nearly 10% of the Singapore water supply system. The plant is projected to be producing approximately 3.4 $10^5$ $m^3$ by 2011 (Lee 2005).

The efforts of Singapore to be self-sufficient needed community involvement in order to reduce the water demand and increase the internal water supply coverage. PUB took action to involve the community by establishing the "Water for All: Conserve, Value and Enjoy" program. As the campaign to increase the water supply was about to be accomplished, the "Water for All" program was introduced to involve the community in conserving water and understanding its value and enjoyment. "Conserve" aims at involving Singaporeans in controlling the water demand and keeping it stable (PUB 2010). The "Water for All" program is implemented through the strategy known as the ABC campaign, which was launched to achieve national waters that are:

- Active – open for different recreational activities such as boating or fishing.
- Beautiful – aesthetically pleasing in a way that the nation's inhabitants can enjoy.
- Clean – of sufficient quality for domestic, industrial, and recreational uses.

The program engages different methods, which include using drinking water reservoirs for recreation. Improvement of quality, aesthetics and access to Singapore waterways is viewed by PUB as ways to promote sense of ownership and respect for water in Singaporean communities (PUB 2007a).

Singapore is closer to the self-sufficiency in water supply that was targeted after projecting the water crisis would follow dissolution of the water agreements with Malaysia. Extreme population growth is unlikely to occur in Singapore with a fertility rate of 0.98% and water demand per day per capita stable at 165 L for the last five years (CIA 2010 and PUB 2010). It is projected that Singapore will be water self-sufficient in 2011 relying on water supply from domestic reservoirs and catchments, desalination, and NEWater, which together will be providing 1.4 106 m3 (Lee 2005). However, total self-sufficiency requires more than what has been done since the estimations are subjected to many uncertainties, which could prompt Singapore to increase the water cost.

### 1.4.2 Singapore Recreational Water Initiative

Singapore's water management plan does not only aim at providing clean drinking water, but it also aims at providing enjoyment to the people through recreational activities. The section of the water management system directly related to recreation planning is the ABC Waters program. Two general recreational activities are pertinent to the ABC program: recreational activities in water reservoir and waterside activities along margins of reservoirs and waterways. Singapore has implemented and improved many of the margins along the drainage channels and reservoirs such as Marina Reservoir. Recreational activities that are currently well liked by Singaporeans include competitive sculling on Pandan Reservoir, kayaking on Jurong Lake and unauthorized fishing on different reservoirs (PUB 2007a). Swimming in reservoirs and other waterways is prohibited in Singapore. However, Singaporeans are allowed to swim at tourist beaches in coastal areas such as Sentosa Island and in swimming pools at sports clubs located at different apartments (PUB 2007a).

The water management plan encloses the recreation extension plans concerning nearly all the Singapore reservoirs. Selecting activities that match Singaporeans' recreational needs was also among the program priorities. The survey carried out by Keng et al. (2004) rated preferences of Singaporeans in regard to the recreational activities. The results showed that appreciated recreational activities are walking (42%), swimming (34%), jogging (27%), cycling (18%), beach activities (13%) and roller-blading (6%). Although the preferences seemed unpredictable, PUB believed that there could be other types of recreation that could be manifested once the reservoirs and waterways are made more attractive to the community. This PUB statement was based on the fact that Singaporeans have been practicing "water play" at the Singapore Science Center, Merchant Court, Clark Quay and the top of the Vivo City roof (PUB 2007a).

The Western Catchment and Eastern Catchment management strategies have been specified differently with the common goal of providing recreation spots. Water reservoirs and waterways in the Western Catchment have been classified depending on the type of recreational activities designated for each reservoir in the area. Pandan Reservoir has been selected as the sport center of the Western Catchment. Spots and recreational activities will be encouraged by increasing the sports variety in this reservoir. Pandan Reservoir will then have fishing, family boating, kayaking, and canoeing spots (PUB 2007a). As kayaking and canoeing have been selected to be the primary recreation activities in the Western Catchment, they will be encouraged at all the waterbodies in this catchment, which include Jurong Lake, Kranji Reservoir, and Sungei Ulu Pandan. These recreational activities will be placed in locations that will not interfere with wildlife. In addition, Kranji Reservoir will also have motorized boats and Eco-Tour cruises that will promote environment-friendly enjoyment (PUB 2007a).

Waterside recreational activities have also been planned for the Western Catchment. Fishing stations are operational on the side of the Jurong Lake and planned to be extended to the shores of Kranji Reservoir, Pandan Sua Diversion Canal, and Sungei Pang Sua. Additional recreational activities are to be promoted on the shores of reservoirs and other waterways, and they will include cycling, jogging, walking and strolling, Tai Chi, relaxing, bird watching, and quiet contemplation (PUB 2007a).

Kranji Reservoir in the Western Catchment has high recreational potential among other reservoirs of the Western Catchment. This is due to the availability of large undeveloped land in the Kranji Reservoir catchment (Figure 1.6). The recreational interest in Kranji Reservoir caused PUB to call for scientific research that would evaluate health risks related to the planned recreational activities. The identification of the human fecal pollution sources will help PUB to elaborate effective methods of managing and controlling this pollution, thus ensuring the safety of people using this reservoir for recreation. Previous studies by Dixon (2009), suggested various spots for recreational activities in Kranji Reservoir as demonstrated on Figure 1.7.

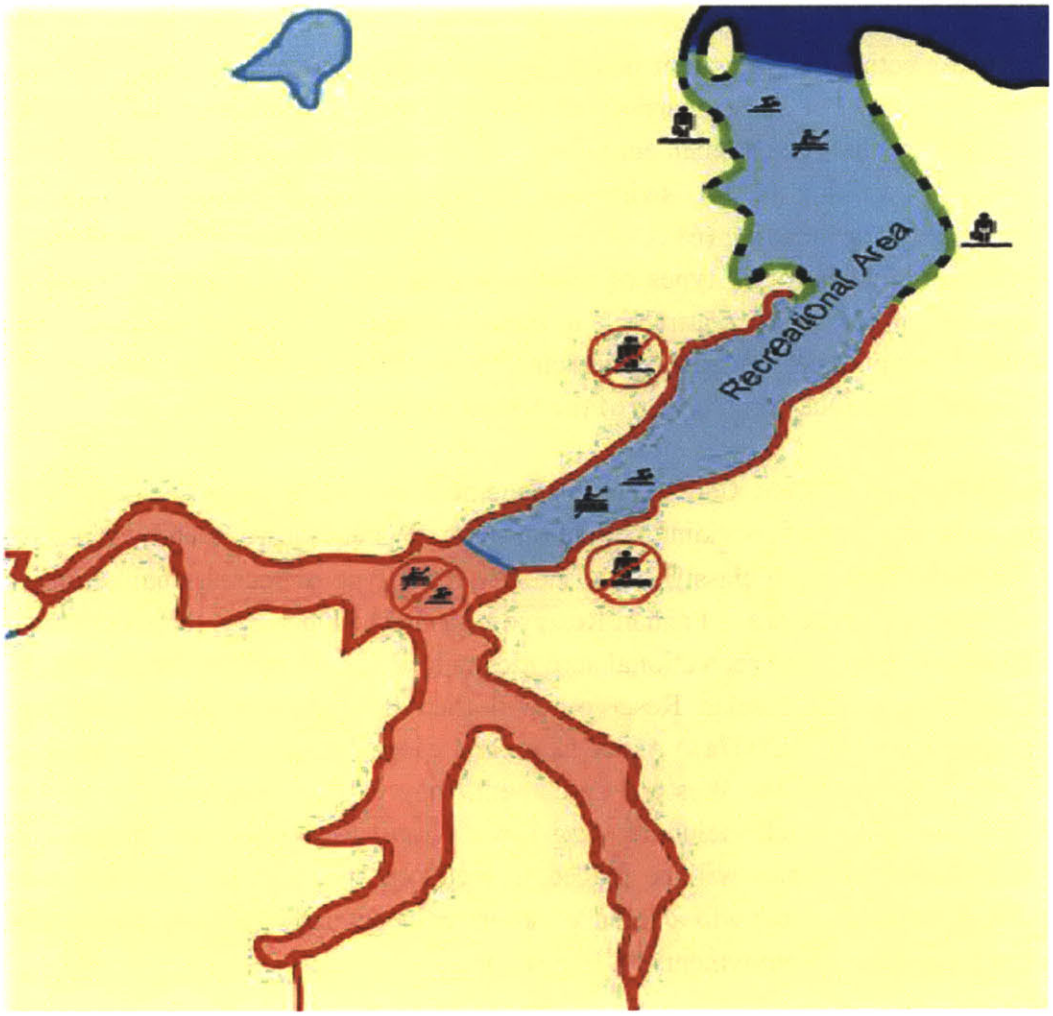


*Figure 1.7 Recommended and Prohibited Recreational Areas in Kranji Reservoir (Dixon 2009)*

### 1.4.3 NTU Bacterial Pollution Studies in Kranji Watershed

PUB sponsored a study by NTU to complete water pollution studies in Kranji watershed shortly after finalizing the Masterplan of the Western Water Catchment of Singapore. This work was designated to create a water quality model, which was planned to be accomplished prior to using Kranji Reservoir for recreational activities. The general study of water pollution was launched in May 2004 and extended until December 2007. This was the first bacteriological study in Kranji study by NTU (2008) and had the purpose of identifying baseline water quality and collecting information to design an integrated water quality model for the Kranji watershed. The results of this study (NTU 2008) led to recommendations that PUB carry out additional bacteriological studies to identify nonpoint sources and develop an appropriate model of bacterial attenuation in the tributaries of Kranji Reservoir. These recommended studies were carried out by MIT/NTU teams in January and July 2009 and January 2010 and will continue.

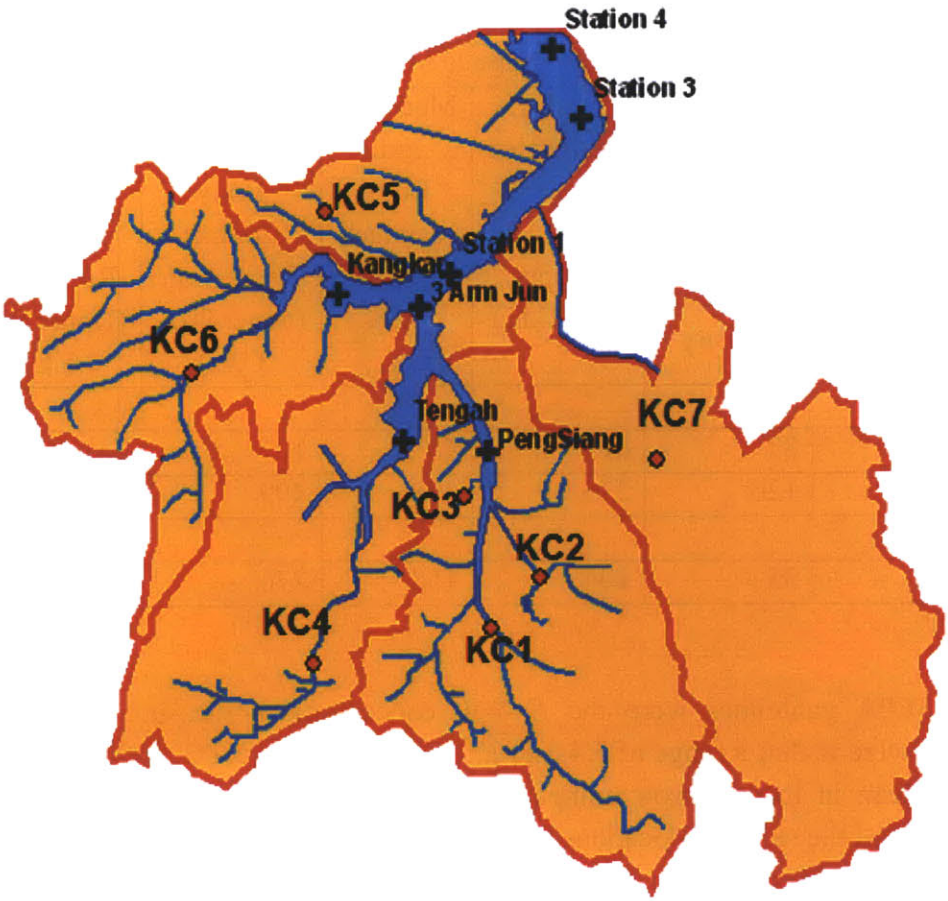


*Figure 1.8 NTU Catchment and Reservoir Sampling Locations (NTU 2008)*

As can be seen in Figure 1.8, samples were collected by NTU (2008) at seven sampling stations in the catchment and seven sampling stations inside the reservoir. The study tested water samples for *E. coli* and *Enterococci* as indicator bacteria for freshwater quality. The test results were then compared to the USEPA (1986) guidelines for *E. coli* and *Enterococci* as shown in Table 1.2. The geometric mean of *E. coli* concentration reported in the NTU (2008) study was above the recreational standard of 126 *E. coli* per 100 ml (USEPA 1986) at all sampled locations in Kranji catchment. The individual measurements ranged from 4,100 *E. coli*/100 ml to 24,000 *E. coli* per 100 ml (NTU 2008).

*Table 1.2 Indicator Bacteria Density Criteria for Freshwater and Marine Waters (USEPA 1986)*

| | | | Single-Sample Maximum Allowable Density | | | |
|---|---|---|---|---|---|---|
| | Acceptable Swimming Associated Gastroenteritis rate per 1000 swimmers | Steady State Geometric Mean Indicator Density | Designated Beach Area (Upper 75% C.L.) | Moderate Full Body Contact (upper 82% C.L.) | Lightly Used Full Body Contact Recreation (upper 90% C.L.) | Infrequently Used Full Body Contact Recreation (Upper 95% C.L.) |
| **Freshwater** | | | | | | |
| *Enterococci* | 8 | 33 | 61 | 78 | 107 | 151 |
| *E. coli* | 8 | 126 | 235 | 398 | 409 | 575 |
| **Marine Waters** | | | | | | |
| *Enterococci* | 19 | 35 | 104 | 158 | 276 | 501 |

C.L. Confidence Limit

Not violating the USEPA guidelines were the *E. coli* concentrations measured in Kranji Reservoir samples that were within a range of 3.4-100 *E. coli* per 100 ml (NTU. 2008). Despite the lower geometric mean in the reservoir compared to the catchment area results and the USEPA guidelines, some of the reservoir locations had spikes in the *E. coli* concentration results, which ranged from 130 to 2,400 *E. coli* per 100 ml (NTU 2008). In addition, the study showed an increase of *E. coli* indicator suggesting bacterial pollution after rainfall events, especially in residential areas. The overall results suggest the probable existence of nonpoint sources of fecal bacteria. On the other hand, the *Enterococci* readings showed geometric mean concentrations below the USEPA guidelines; however the maximum of samples taken in Kranji catchment area of Peng Siang was 2,000 MPN/100 ml. This peak of *Enterococci* in the Peng Siang water sample was higher than the USEPA guideline which is 33 MPN/100 ml (NTU 2008 and USEPA 1986).

Based on the results of the bacteriological analysis of the water pollution study of Kranji watershed, investigators from NTU (2008) recommended further studies to complete the first study's findings. The report of the first study was then used to identify and prioritize bacteriological studies that fulfilled the additional study needs. NTU collaborated with the Department of Civil and Environmental Engineering at MIT to carry out the bacteriological studies. The study by the MIT team started by analyzing samples collected from the locations previously used by NTU (2008) to study bacteria under dry-weather conditions during January 2009. In addition, the MIT team focused on different aspects of bacterial water pollution that include bacteria source tracking, bacteriological attenuation, and health risk assessment.

The study findings of the NTU team showed that the level of *E. coli* in dry weather exceeded the level determined by the USEPA (1986) at five locations in Kranji catchment (Chua et al. 2010). The locations with the highest concentrations among these five were identified to be in the most highly developed sub-catchments. Additionally, high wet-weather levels of *E. coli* and *Enterococci* were observed in the stormwater at KC1 and KC2 (Chua et al. 2010). The possible source of high *E. coli* concentrations in the KC2 sub-catchment was suggested to be located in 1.4 km along the drainage upstream of the KC2 sampling station. In general, the results at these different locations in the Kranji catchment suggest a positive correlation between *E. coli* concentration and land use development. The relationship between concentrations of *E. coli* and the degree of land development suggests that *E. coli* concentration increases as we go from undeveloped area to sewage treatment plants (STP) (Chua et al. 2010). These results suggest that the bacterial pollution control program that is being developed by PUB should include efforts to reduce nonpoint sources of bacterial pollution from residential areas and STPs.

The January 2009 bacterial pollution studies by the MIT team developed valuable information with regard to the locations of recreational sites along the reservoir and areas where pollution control should be implemented. As shown in Figure 1.7, the upper reaches of the reservoir have high concentrations of *E. coli* suggesting increased health risk. The areas selected for recreational activities had lower concentration. The residential zones in KC1 and KC2 sub-catchments were identified as areas with high predicted and measured concentrations of *E. coli*. Dixon et al. (2009) suggested additional monitoring of bacteria concentrations in order to establish a reasonable baseline for bacteria pollution control in the area.

The results of the studies by both MIT and NTU in 2009 suggested the need for further bacteriological studies to provide additional information regarding the sources of bacteria and other microorganisms that could affect human health. *E. coli* was detected in high concentrations, but its source was unknown. These concentrations could have been associated with human or animals. In addition, these sources could not be determined based on standard tests for *E.coli* and coliforms. Other studies showed that *E. coli* could growth in tropical freshwater environments (Hazen 1988). Therefore, there was a need of a monitoring technology that is specific for human wastes, which have been with the high risk of transmitting infectious diseases to humans. However, the "state of the art" monitoring technology for human waste (HF marker) has never been tested in tropics. Therefore the goal of this study was to evaluate whether HF marker is a good indicator for human-associated waste in Kranji catchment in Singapore. There recent development of DNA-based analysis for sources of tracking was then one of the motivations of this research. Therefore, we used the human host-specific 16S rRNA *Bacteroides* gene marker to study nonpoint sources of human fecal pollution in Kranji Reservoir.

## 1.5 Thesis Focus

This thesis reports the study carried out in Kranji catchment to identify the nonpoint sources of human fecal pollution. Waterways in Kranji catchment were identified to contain levels of human fecal indicator organisms above USEPA recreational water regulations (Table 1.2). Thus, there was a need to study the sources of human fecal contamination and their variation under dry and wet weather. The goal of the research reported in this thesis was to identify sources and origins of human fecal pollution in Kranji catchment. Recently developed molecular indicators that target a human-specific strain of *Bacteroides* were applied for source identification. Detailed approaches applied during this research are discussed in Chapter 4.

# Chapter 2: Fecal Bacteria Water Pollution

## 2.1 Introduction to Fecal Bacteria Water Pollution

Fecal bacteria water pollution has been a prominent problem that emerged with human development. Years ago, when the population could be supplied by existing clean water sources, natural springs and wells were the primary sources of drinking water supply. Water was fetched and used clean immediately from underground or flowing streams. Human wastes were dissipated in the environment where natural phenomena were basic to decomposition and use of waste products for vegetation regeneration. However, these natural processes were compromised by human development and discoveries. Urban development, the industrial revolution, mining, agriculture and livestock raising loaded pollutants into watercourses until the natural absorption capacity of rivers near populated areas was nearly exhausted. In addition, other parts of these pollutants infiltrated underground; they then turned groundwater-fed springs and wells unsafe for drinking water (Outwater 1996). A number of water supply systems and localized springs or wells established to provide clean water to urbanized regions were identified to be polluted, thus posing public health concerns. Ground-breaking research in epidemiology by John Snow (1854) discovered the link between cholera and human fecal pollution. Snow determined that the inhabitants of the Broad Street, London area were affected by a cholera outbreak caused by a shallow well that was contaminated by sewage (Steven 2006).

During the 19$^{th}$ century, the industrial revolution and urban development increased water bacterial pollution issues. Huge numbers of laborers were attracted by industrial jobs, creating cities and increasing water demand for both industries and households. Suitable living conditions implying standardized hygiene and wastewater management systems were established. Wastewater treatment plants discharged into waterways and waterbodies, thus exhausting the capacity of the local natural environment which could not handle the extra pollution.

At the beginning of the 20$^{th}$ century, filtration was introduced and applied as the way to remove contaminants from water. However, it had a weakness associated with particles that could pass through the filter pores. In addition, bacteria are of a small size and could not be seen in clear filtered water. A majority of bacteria was trapped in the filter, but some could go through it depending on their size. Concurrently, other researchers were looking into other ways of eliminating bacteria from water. Waterborne disease epidemics were still being recorded in some areas where the filtration technique was used. In 1910, chlorine was introduced as a chemical that could remove the bacteria left behind by the filtration method (Madigan and Martinko 2006). This discovery reduced remarkably the burden of infectious disease that was transmitted by unclean water (Figure 1.2).

Progress in water treatment prompted researchers to determine test methods that could prove the absence of pathogenic bacteria in treated water versus untreated water. Researchers defined indicator organisms, which were designated to indicate if any given water source was contaminated. The indicator that was mostly used, and is still currently used, is the coliform group. Coliforms are bacteria that live in humans' and animals' intestinal tracts. An assumption was made that their presence in water indicated that the water is unsafe for drinking, since coliforms may be associated with pathogens that are excreted by the same paths. The presence of coliforms in water may indicate that pathogens are present, but not necessarily in all cases. The pathogens require the presence of a host carrying an infectious disease transmitted through feces and have properties allowing them to use water as a development environment or transmission path (Madigan and Martinko 2006).

Water pollution research revealed that fecal bacteria pollution of water was from two major sources: identifiable single localized sources, which were grouped under "point sources" and diffuse sources, which were grouped under "nonpoint sources". Point sources of bacterial pollution were defined as identifiable sources such as municipal sewage treatment plants. Point sources are therefore subjected to regulations and standards before they can be discharged into waterbodies. On the other hand, nonpoint sources of pollution are diffuse sources such as leaking sewers and pet feces collected by stormwater. The differentiation of these two sources could be based on the characterization of their sources, one being identifiable single locations (point sources) and another being spread out and not easily narrowed to a single source (nonpoint sources). Beyond bacterial pollution, nonpoint sources of pollution include mining sites, urban runoff, construction site runoff, and pesticides from agricultural and livestock raising areas (USEPA 1999). Point sources include heavy metals and other toxic chemicals from industries (USEPA 1999 and 2000b, and Vigil 1996).

The 21$^{st}$ century started with new strategies of identifying and managing point and nonpoint sources of pollution. Progressive research achievements in chemistry and microbiology prompted design of chemical and biological pollution source-tracking techniques. Nonpoint sources, although scatted around and likely difficult to trace, were also tackled by a number of water pollution researchers. Bacterial water analysis techniques improved remarkably. Molecular microbiology-based methods were designed and used for identification of nonpoint sources of fecal bacteria pollution. One of the molecular microbiology methods used was based on the arrangement or sequence of nucleic acids within DNA of the environmental water sample and DNA of the source. A specific gene coded by the sequence of human or animal was targeted in the DNA of the environmental water sample to identify the source. Ram et al. (2007) used a sequence-based bacterial source-tracking method to demonstrate nonpoint sources of fecal bacteria pollution in two storm sewers located in a residential neighborhood in southeast Michigan. This study showed that the nonpoint sources of fecal bacteria pollution were generally pets, and cats' sequences were plentiful among sequences that were attributed to pets.

In addition, raccoons were also identified among the major sources in summer and fall. This seasonal effect was suggested to be due to density reduction of raccoons during the spring. Furthermore, other molecular microbiology-based studies were done around the world. Jenkins and others (2009) used host-specific bacteroidales assays to identify human and livestock sources of fecal contamination in Kenya, Africa. This study was carried out in the watershed of the River Njoro and was claimed to be the first of its kind on the African continent. The main sources of fecal bacteria pollution were identified to be humans and cows. Moreover, the study showed that the common occurrence of fecal contamination raised concerns for human and animal health in the area.

Although progress in laboratory methods is evolving towards the ability to trace sources of fecal bacteria pollution of any kind, point or nonpoint source, the techniques are still expensive and mostly restricted to developed countries. Developing countries with high rates of waterborne disease and lower income seem to be isolated from the benefits of these techniques. There is an emerging need to make these techniques accessible to the developing world where identifying nonpoint sources of fecal bacteria pollution could reduce death among children. Currently, it is estimated that 2 million children under age five die every year of diarrhea associated with unsafe drinking water and poor hygiene (Pontius 2008).

## 2.2 Point Sources and Nonpoint Sources of Fecal Bacteria Pollution

### 2.2.1 Point Sources of Fecal Bacteria Pollution

A point source of bacteria pollution is a known source that discharges bacteria into a watercourse or body of water. Point sources include municipal sewage treatment plants. Municipal treatment plants are presumed to contribute pathogens, organic matter and other nutrients that reduce oxygen in water. Nutrients discharged into water from these plants have been associated with fish and shellfish reduction, nuisance algae blooms and gradual decay of seagrasses and corals due to different diseases. This problem persists in bays, coastal areas, estuaries, and semi-closed waterbodies located downstream of a discharge point (NRC 1993). Nutrient loading is not the only concern associated with point sources. Fecal bacteria pollution associated with point-source discharges has also been a concern due to health risks. Public health risks were studied and prevented where point sources were identified as sources of pathogens, heavy metals and organic substances (NRC 1993). The recognition of the burden associated with point sources of water pollution was improved in 1970s when the Clean Water Act (CWA) (1972) was voted by the Congress of the United States (Vigil 1996). The CWA established baseline discharge guidelines and regulations that all point sources of water pollution should follow before discharging treated water into any watercourse or body of water.

Studies that examined pathogens associated with point sources of water pollution showed that the CWA of 1972 reduced disease outbreaks associated with these sources. Calderon (2010) showed that from 1971 to 2000 only 1% of the 24 major sources of fecal bacteria pollution in recreational water were associated with point sources of pollution. Nonpoint source of fecal bacteria pollutions were reported to have caused 83% of waterborne disease outbreak reported in recreational waters during the 30-year period. The link between sewer systems and recreational areas was generally due to leakage or overflow of sewage during storm events. The main contributors of fecal matter leading to disease outbreaks were human sources (Calderon 2010). The majority of the outbreaks recorded were caused by infectious bacteria or spores that included *Cryptosporidium, Giardia, Schistosomatidae, E. coli and Leptospira* (Calderon 2010).

At the present time, point sources of bacteria pollution have been subjected to effective control in developed countries. The CWA has served as a model to other many developed countries across the globe. The design of wastewater treatment plants has also been improved and the currently used technology gives clean water as the end product of the water treatment plant. Advanced water treatment has been pushed to the point that the content of bacteria or toxic chemicals in the effluent is expected to be zero concentration, thus allowing water to be reused as a source of drinking water. This technology is currently being applied in Singapore where sewage is treated to produce NEWater that is used in industries and drinking water systems (Luan 2010). Although this technology achieves management and control of point sources of fecal bacteria pollution, it is still used in few developed countries. In addition, the system requires huge investment and maintenance costs.

Despite the progress in developed countries in regards to the control and effective management of point sources of fecal bacteria pollution, the situation of developing counties is still alarming. The majority of the sewage treatment systems used in developing countries are lagoons, ponds, septic tanks, and pit latrines. These systems are compromised by malfunctions of different kinds. Some treatment systems leak into water springs, wells and boreholes located in the neighboring environment. Therefore, water sources change their quality and are turned into contaminated pools. The World Health Organization (2004) showed that poor water quality is still associated with diarrheal disease, with unsafe water supply, sanitation and hygiene causing 88% of the 1.8 million deaths per year due to diarrheal diseases. In addition, this burden affects mostly children in developing countries.

Point sources of fecal bacteria pollution were problematic in the $19^{th}$ century; however $20^{th}$-century treatment technology and water quality regulation left this problem almost solved around the globe. Some of the developing countries progressed as the developed countries in solving point source of fecal bacteria pollution problem, but the change is somewhat uneven. This slow pace in controlling point sources of pollution in these countries is principally due to three major reasons: high investment cost to acquire technology needed, lack of qualified technicians to

install and maintain the system, and a high percentage of population living in rural areas. There is a need to focus on creating affordable solutions for developing countries in order to reduce morbidity and mortality due to waterborne diseases.

### 2.2.2 Nonpoint Sources of Fecal Bacteria Pollution

Nonpoint sources of fecal bacteria pollution originate from diffuse sources such as wildlife, humans, seeping sewers, pets, and farms. The majority of nonpoint fecal bacteria pollutants are carried from their sources to waterways and waterbodies by runoff. In addition, agricultural areas where animal manure is produced and used as fertilizer are also major contributors of fecal bacteria to runoff. Furthermore, runoff from residential areas where pets are common could also be a major nonpoint contributor of fecal bacteria to the surrounding waterways (NOAA 2010).

Historically, the period after the World War II was characterized by huge demand of goods in Europe, while the United States of America was developing faster. Industrial and agricultural productions were increasing in response to the European markets and in other emerging countries around the world. The majority of agricultural fields and industries were established on the shores of waterbodies or closer to waterways to facilitate their production activities and wastewater discharge. Wastewater discharge was at the time not considered as an environmental issue in regards to discharge in freshwater. The scientific research was suggesting that "dilution is the solution to pollution" and discharging wastewaters from industries to a large lake or river would reduce their toxicity. This practice did not last long. Deterioration of the environment started to be evident in the industrialized areas of the United States and the Congress started establishing regulations governing pollution controls during the 1950s – 1970s. However, the control of nonpoint sources of fecal bacteria pollution was still challenging.

Interest in nonpoint sources of fecal bacteria pollution was increased by related human health risks. Pathogens found in water were believed to be associated with human or animal feces, but scientists were still uncertain in identifying the source. It was imperative to identify the source in order to control and prevent bacteria from the water accessed by the public. The indicator bacteria were to be used to know if these pathogens were present, but did not determine their sources (Anderson and Davison, 1997). Studies carried out for different land-uses such as agriculture, livestock-raising farms, residences and industries showed different concentrations of fecal coliforms. Residential, livestock raising and agricultural areas were reported to contain high fecal coliform counts. These studies suggested that the presence of pathogenic microorganisms could probably be correlated to the land-use with high counts in residential and livestock raising farm areas. Studies in the 1980s investigated the sources of pathogenic microorganisms in water. However, it was still not common to identify specific pathogenic microorganisms (Anderson and Davison, 1997).

The 2000's decade started with promising microbiology discoveries resulting from extensive development of DNA-based laboratory analysis. Apart from the membrane filtration technique used to identify the fecal bacteria indicators, molecular microbiology analysis became also cheaper and applicable. Nowadays, pathogenic microorganisms are studied in detail compared to the situation of 1980s and 1990s. Bernhard and Field (2000) were the first to prove the use of 16s host-specific-based analysis in identifying nonpoint sources of fecal bacteria pollution. Five years later, Forgarty and Voytek (2005) used terminal restriction fragment length polymorphism (T-RFLP) of 16s rRNA genetic markers to identify fecal sources from environmental water samples. The study showed that the anaerobic bacterium from the guts of animals, *Bacteroides Prevotella*, responds with a different T-RFLP peak depending on the type of fecal bacteria host present in water. The peaks produced were then associated with different sources of contamination such as chickens, cows, deer, dogs, geese, horses, humans, pigs, and seagulls.

Despite tangible scientific progress in tracking nonpoint sources of fecal bacteria pollution, there is need of additional research to validate the efficacy and sensitivity of emerging laboratory techniques. DNA-based analysis should be validated in various areas of the world where infectious diseases are still an issue to the public health.

## 2.3 Bacterial Water Pollution Guidelines

Bacteria water pollution guidelines are determined to protect humans from exposure to illness associated with water fecal contamination. The microorganisms of concern may reach water from different fecal contamination sources. The guidelines are established to determine whether water contains levels of pathogenic bacteria sufficient to cause disease in humans. The pathogenic bacteria targeted are those from humans or warm-blooded animals that have water as a transmission phase. Since specific pathogen species cannot rapidly be studied during emergence of a waterborne disease outbreak, indicator bacteria were selected as the bacteria pollution bottom-line. Therefore guidelines were determined depending on indicator bacteria concentrations in waterways, waterbodies and drinking water supply systems.

As water is a natural resource and its composition may vary depending on the geology and land use, fecal bacteria pollution may also vary from place to place depending on wildlife diversity. Hence the determination of guidelines depends on different factors such as nature, local endemic illness, population behavior, exposure patterns, and sociocultural, economy, environment, and technical aspects, as well as competing health risks from other diseases that are not related to water (WHO 2003). The variability of factors associated with guidelines prompted different countries to develop their own local guidelines. In addition, guidelines were also extended from drinking water, then recreational water and other water criteria determined relevant in accordance with recent study findings. These studies showed also discrepancies in time-duration of human exposure. Drinking water consumption represents therefore higher exposure than

recreational water, which is in contact with human occasionally while drinking water represents a life-time exposure (WHO 2003).

### 2.3.1 Bacterial Recreational Water Guidelines

Recreational waters include coastal and freshwater reserved for the use of human enjoyment. These recreational areas receive many people per day for different activities such as kayaking, sailing, swimming and fishing. Through these different activities, humans get in contact with water by oral or direct skin contact. During contact events, if water is contaminated by pathogenic microorganisms, recreating people could be exposed to waterborne diseases. Guidelines for fecal bacteria pollution in recreational water were established to protect human health, specifically the protection of the public from pathogenic bacteria discharged to recreational water and free-living pathogens found in recreational water (WHO 2003). Therefore, guidelines are not established to discourage water recreational activities, but they assist managers to ensure safe recreation. In addition, guidelines should be considered as the level of management that ensures safety of important groups of users, thus minimizing exposure (WHO 2003). When guidelines fail, they should trigger additional investigations to identify the cause of failure and project future effects. Thereby, water regulation authorities should be involved in upgrading the guidelines to respond to the existing situation.

Guidelines for bacteria pollution of recreational water were first established in the United States of America. Descriptive water quality documents published in 1967 and 1976 demonstrated bacteria guidelines based on concentrations of fecal coliforms in recreational water. They suggested that the maximum densities of fecal coliforms in recreational water should not exceed a geometric mean of 200 organisms per 100ml (USEPA 1986). Apart from the 1967 and 1976 documents, the U.S. National Technical Advisory Committee (NTAC) of the Department of the Interior (1968) also suggested a guideline value of 200 organisms per 100ml in recreational water. This NTAC proposal (1968) was developed based on research carried out in 1940s and 1950s by the U.S. Public Health Service (NTAC 1968). These studies were conducted at different beaches in the United States such as Lake Michigan at Chicago, Illinois; on the Ohio River at Dayton, Kentucky; and on Long Island Sound at Mamaroneck and New Rochelle, New York (USEPA 2003). The results of these studies confirmed the range of microorganism concentration above 200 as a probable cause of contamination and gastrointestinal illnesses (USEPA 2003). However, this criterion was criticized by different scientists who were concerned by the research methodology used in the aforementioned study. The calendar methodology that was used consisted of distributing calendars on which residents filled in the days they visited the beach. This method was criticized since being on beach did not mean swimming or being in contact with water. A number of people could go to the beach and avoid total body contact with water (USEPA 1986). As a result, the USEPA (1986) improved the criteria based on improved methodology, which included all variables related to being in contact

with water when visiting the beach. The findings from the new study updated also guideline values that were used as recreational water guidelines in 1976. *E. coli* was recommended as a fresh recreational water indicator regulated at a level of 126/100ml, and *Enterococci* was recommended for fresh and marine recreational waters regulated at levels of 33/100ml and 35/100ml respectively (Table 2.2) (USEPA 1986).

Recreational water bacteria standards were therefore determined based on two bacteria indicators *Enterococci* and *E. coli*. The progress of research by USEPA (2004) showed that *E. coli* was not as good an indicator as *Enterococci* because it was suspected to also develop in soil and could bias the criteria for recreational water. *Enterococci* concentration was then correlated to illnesses identified among people who used the beaches. In addition, the USEPA (1986) showed that pollution at a level of 200 microorganisms per 100ml could cause 8 illnesses per 1,000 swimmers at freshwater beaches and 19 illnesses per 1,000 swimmers at a marine beach. Since 1986, these illness rates have been used to determine levels of exposure among swimmers on beaches. Nevertheless, the exposure estimation improves with scientific research and should be updated together with recreational water regulation reviews. The criteria put forth by USEPA (1986) are summarized in Table 1.2 (fresh and marine waters).

The bacteriological recreational water guidelines developed by the USEPA were used as a model to develop local bacteriological recreational water standards around the world. The WHO (2003) published basic guidelines that developing countries could use as a reference for different cases. The WHO document suggests that developing countries require 5 years data collection of at least 100 samples per year per recreational area (WHO 2003). The data would then be used to classify recreational areas as very good, good, fair, poor or very poor depending on results. If any of the locations sampled had high concentrations of gastrointestinal *Enterococci,* continuous follow-up was recommended. In addition, it was suggested that these investigations be coupled with frequent sanitary inspections in order to ensure source control. The basic purpose of this work is to help managers to identify the sources of fecal pollution and prevent further contamination, thus ensuring the safe health of swimmers and others who could be exposed to different health risks (WHO 2003).

Although guidelines for recreational water have been established and are used to protect human health, they are limited to a few microorganisms. WHO (2003) demonstrated that free-living organisms were neglected in previous guideline determinations. Free-living organisms are microorganisms from humans or animals that have been excreted into water and adapted to prevalent environmental conditions in the water. These microorganisms have the capacity to stay in surface water for long periods. Free-living organisms include *amoeba*, *leptospires*, *Aeromonas, Acanthamoeba*, *Naegleria fowleri* and *Balamuthia mandrillaris.* The latter three are known to infect and cause fatal conditions in humans.

In addition, beach sand should also be included in areas to be regulated because some protozoa and fungi were identified at some beaches (WHO 2003). People contaminated by fungi and protozoa at the beach are at high risk of contaminating others through person-to-person contact. Furthermore, it was also demonstrated that some pets brought to the beach may contaminate people by cross-transmission diseases or contaminate the beach sand. The WHO (2003) report recommended that in such cases restrictions should be placed on dogs and other pets that can put the health of recreating people in danger.

Bacteriological guidelines for recreational water developed in the USA and by WHO should be used as models to design localized recreational water guidelines in developing countries where waterborne diseases are still a major challenge. Water quality testing is also still expensive for these countries, but currently cheaper laboratory methods are being developed and could reduce total costs, thus promoting health.

### 2.3.2 Bacterial Drinking Water Guidelines

Drinking water guidelines were developed based on the same philosophy of protecting public health. The Safe Drinking Water Act of 1974 (SDWA) in the U.S. was partly prompted by the identification of organic contamination and other pollutants in public drinking water and the need for national standards. Currently the SDWA has been amended twice: 1986 and 1996. The 1986 SDWA amendment intended to increase the impact to water systems for communities. The new additions included well head protection, prohibition on the use of lead in solder and plumbing, increase in the use of disinfection for groundwater systems, increase of the use of filtration for surface water systems, and addition of new substances to be monitored. The progress in development of laboratory techniques, identification of new microorganisms in drinking water and the increase of community water supply systems were among the motivations of the SDWA amendment of 1996. The amendment of the SDWA (1996) included also the involvement of the community that was structured through public information, consultation events, and consumer confidence reports. This new strategies were aiming at involving the community in maintaining drinking water quality. The SDWA (1996) increased also the list of microorganisms tested in water. *Cryptosporidium* was added to the new list of standards (USEPA 1996).

The SDWA determined also Maximum Contaminant Level Goals (MCLGs) and Maximum Contaminant Levels (MCLs). The MCLG represents the quantity of a pollutant in drinking water below which there is no known or expected risk to health. The MCLGs were established to allow margins of safety. The MCL represent the highest quantity of a pollutant that is allowed in drinking water. The MCLs are fixed as close to MCLGs as achievable using the best treatment technology and considering costs. The MCLs are regulated standards (USEPA 2009).

The MCL and MCLGs determined by the USEPA (2009) for microorganisms are shown in Table 2.3.

Although the SDWA is considered as a model for other countries to improve their drinking water quality, recent reports showed it is commonly violated in the U.S. The recent report showed that 20% of the U.S. water treatment systems violated the SDWA for the past five years (Duhigg 2009). Most of these violations were recorded at water treatment systems supplying fewer than 20,000 residents. Currently, the USEPA is establishing new policies that will upgrade these systems and ensure safe drinking water supply at all treatment plants.

The bacterial guidelines for drinking water are critical because they regulate water that directly affects human health and represents life-time exposure. The violation of guidelines has probable direct impact on human health. It is thus imperative to ensure safe and bacteria-free drinking water. However, drinking water criteria are not universal; they can also vary between countries. This variation could be due to available budgets to implement an effective water treatment technology and other reasonable strategies to ensure safe drinking water (WHO 2006).

*Table 2.1 Standards of Microorganisms in the U.S. (USEPA 2009)*

| **Pollutants** | **MCLG (mg/l)** | **MCL or TT** | **Potential Health Exposure** | **Sources** |
|---|---|---|---|---|
| ***Cryptosporidium*** | Zero | unfiltered systems are required to include Cryptosporidium in their existing watershed control provisions | Gastrointestinal illness (e.g., diarrhea, vomiting, cramps) | Human and animal fecal waste |
| ***Giardia lamblia*** | Zero | 99.9% removal/inactivation | Gastrointestinal illness (e.g., diarrhea, vomiting, cramps) | Human and animal fecal waste |
| ***Legionella*** | Zero | Removed/inactivated as Giardia and viruses according to the treatment techniques | legionnaire's Disease, a type of pneumonia | Natural lives in water and multiplies in heating systems |
| **Total coliforms (including fecal coliform and *E. coli*)** | Zero | No more than 5.0% samples total coliform-positive in a month | Used as indicator bacteria | Coliforms are naturally present in the environment; fecal coliforms and E. coli come from human and animal fecal waste |
| **Viruses (enteric)** | Zero | 99.99% removal/inactivation | Gastrointestinal illness (e.g., diarrhea, vomiting, cramps) | Human and animal fecal waste |

As drinking water is a vulnerable resource, regular risk assessments and risk management of supply systems could increase confidence in providing clean drinking water. This regular work includes systematic assessment from the drinking water source and its catchment to the consumers. Furthermore, assessment could be coupled with strategies before consumers could access the water (WHO 2006).

All consumers are concerned with the safety of their drinking water. Human and wildlife feces are considered as a source of pathogenic bacteria, viruses, protozoa and helminthes (WHO 2006). In addition, long-term data collection has shown that fecal contamination can change quickly and may be characterized by peaks of fecal contamination, which could cause outbreaks of waterborne diseases such as cholera (WHO 2006).

Bacteriological guidelines for drinking water are critical for the safety of public health. Monitoring systems are one of the strategies to reinforce prevention and control. Prüss et al. (2008) demonstrated that 3.575 million people die each year from water-related disease and 98% of these deaths are recorded in developing countries. In addition, they demonstrated that 43% of water-related deaths are due to diarrhea and 84% of water-related deaths are among children of zero to fourteen years (Prüss et al. 2008). Collaborative programs between countries should be encouraged in order to share experience and successful strategies. Cheap clean water treatment technologies should be designed and distributed to markets in developing countries. Financial support is also still needed to increase daily water quality monitoring and other water research initiatives aiming at increasing local safe drinking water accessibility.

## 2.4 Fecal Bacteria Water Pollution in Urban Watersheds

Urban areas are generally characterized by high population density, intensified residential development and development work, construction sites, transportation ways, water supply pipelines, sewers and stormwater ditches. A number of these urban characteristics are associated with pollution of different type, and could be hazardous or toxic to the public health or wildlife. Water supply systems are among the vulnerable and protected elements of urban areas due to the connection with the public health. In some areas, water supply systems have been damaged by roots of plants, thus exposing them to fecal or chemical pollution. As urban areas are highly exposed to nonpoint sources of fecal contamination, they are likely to be carried by runoff and become the first pollutants to reach the damaged point on the pipe system. Runoff collects many different objects such as debris, and wildlife, pet and livestock fecal excretions (Table 2.3) (CWP 1999). In addition, runoff could also flood sewer systems and septic tanks, thus spreading contaminants around the urban area. All this different fecal matter carried with stormwater is grouped under nonpoint sources of fecal bacteria pollution (GLSAB 2000).

The Center for Watershed Protection (CWP, 1999) demonstrated to water management planners important facts to consider when organizing bacteria pollution management in an urban area. The ubiquity of fecal contamination in urban runoff made it a priority to planners. Apart from fecal bacteria, urban stormwater may also carry pathogens such as S*higella spp.* that can cause dysentery, S*almonella spp.* that can cause gastrointestinal illness and *Pseudomonas auerognosa* that can cause swimmer's itch (CWP 1999). In addition, protozoa such as *Giardia* and *Cryptosporidium* characterized by their hard casings called "cysts" are also present in urban stormwater (CWP 1999). These organisms tend to last longer in water due to their casing protection and could trigger a disease anytime swallowed by humans. Table 2.5 shows sources of fecal coliform bacteria in urban watersheds and Table 2.6 shows different microorganisms found in urban stormwater

Water pollution studies carried out in the United States in the 1980s reported high fecal coliform levels in urban stormwater. The Nationwide Urban Runoff Program (NURP) (1983) reported that fecal coliform counts in urban runoff ranged from 10,000 to 100,000 per 100 ml under warm weather conditions (USEPA 1983). The median of colony counts of samples from all sites was reported to range around 21,000 per 100 ml. The data collected in cold weather showed that the median fecal densities from all sites were in the range of thousands of colonies per 100 ml. Although a number of NURP projects reported fecal coliform densities that violated water standards, the runoff was considered unlikely to be a danger to the public health. Most of discharge areas of the urban runoff were located at relatively long distances from swimming areas and shellfish beds. Therefore, the degree of dilution and dispersion was considered reasonably high to reduce the concentration of transported pathogenic microorganisms (USEPA 1983).

In addition, Varner (1995) carried out a comparison of fecal coliforms between dry and wet weather in Bellevue City, Washington, U.S. The results were compiled for 11 sampling stations. The results showed a large difference between wet- and dry-weather periods. The mean value of samples collected during rain events was 4,500 MPN/100 ml, while the mean for dry-weather or base-flow samples was 600 MPN/100ml.

These research reports show that fecal bacteria pollution from nonpoint sources is still an issue in urban areas. Integrated fecal bacteria pollution management in urban areas that includes all potential sources of contamination should be encouraged. This integrated management should establish collaboration among authorities, sewer and pipeline management contractors, community, researchers, and other stakeholders. In addition, the integrated solution will be to establish public education program for all involved parties that will help fix major causes of uncontrolled nonpoint sources of fecal loading (e.g. leaking septic tanks).

*Table 2.2 Sources of Fecal Coliform Bacteria in Urban Watersheds (CWP 1999).*

| Major Sources | Categories | Specific Sources |
|---|---|---|
| **Human Sources** | Watershed with a sewer system | • Combined sewer overflows<br>• Sanitary sewer overflows<br>• Illegal sanitary connections to storm drains<br>• Illegal disposal to storm drains. |
| | Watershed without a sewer system | • Failing septic systems<br>• Poorly operated package plant<br>• Landfills<br>• Marinas and pumpout facilities |
| **Non-human Sources** | Domestic animals and urban wildlife | • Dogs, cats<br>• Rats, raccoons<br>• Pigeons, gulls, ducks, geese |
| | Livestock and rural wildlife | • Cattle, horse, poultry<br>• Beaver, muskrats, deer, waterfowl<br>• Hobby farms |

*Table 2.3 Microorganisms Found in Stormwater (CWP 1999).*

| Microbial Indicator | Present in Urban Runoff | Fecal origin | Non-human sources | Information use |
|---|---|---|---|---|
| **Total coliforms (counts/100ml)** | All samples | Most | Animals, plants, soil | Historical, seldom used |
| **Fecal coliforms (counts/100ml)** | All samples | Most | Animals, plants, soil | Water contact, shellfish, drinking water |
| **Fecal streptococci (counts/100ml)** | All samples | Yes | Warm-blooded animals | Sometimes used to determine waste source |
| ***Escherichia coli* (counts/100ml)** | Nearly all | Yes | Mammals, soils | Water contact, shellfish, drinking water |
| ***Salmonella spp* (counts/100ml)** | About half | Yes | Mammals (dogs) | Food safety |
| ***Pseudomonas aeruginosa* (counts/100ml)** | All samples | Yes | Mammals | Drinking water |
| ***Cryptosporidium spp (Oocysts/1L)*** | Less than half | Yes | Mammals (livestock) | Drinking water |
| ***Giardia spp. (Cysts /1L)*** | Less than half | Yes | Mammals (dogs and wildlife) | Drinking water |

Control of both volumes of runoff and pollution sources should be included in this strategy. Local urban institutions and water pollution regulation boards should also be involved by organizing joint meetings and involving the community in this process (GLSAB 2000). Urban nonpoint sources of fecal bacteria pollution could be controlled in all seasons, thus minimizing health risks and waterborne disease burdens.

## 2.5 Fecal Bacteria Water Pollution and Seasonal Variation

Evidence of the variation in the concentration of fecal contamination across seasons has been demonstrated by different researchers from 1950s. Geldreich et al. (1968) showed variation of fecal bacteria densities associated with seasonal variation. During rainy periods, peaks were observed in fecal bacteria density in runoff water samples. The peaks commonly observed were those of total coliforms, fecal coliforms and fecal streptococci in stormwater samples taken in autumn. These results were generated from 294 samples taken in drainages of the business district of Cincinnati in 1962-1966. Figure 2.9 shows the seasonal variation of fecal bacteria counts observed by Geldreich et al. (1968) in Cincinnati. As can be seen, the autumn had peaks in all three fecal bacteria (total coliforms, fecal coliforms, and fecal streptococcus). The plotted counts are the median values from the data compiled during the four years of study. On the other hand, the spring season was characterized by the lowest median values among other seasons due to the climate characteristics of the period (Geldreich et al. 1968).

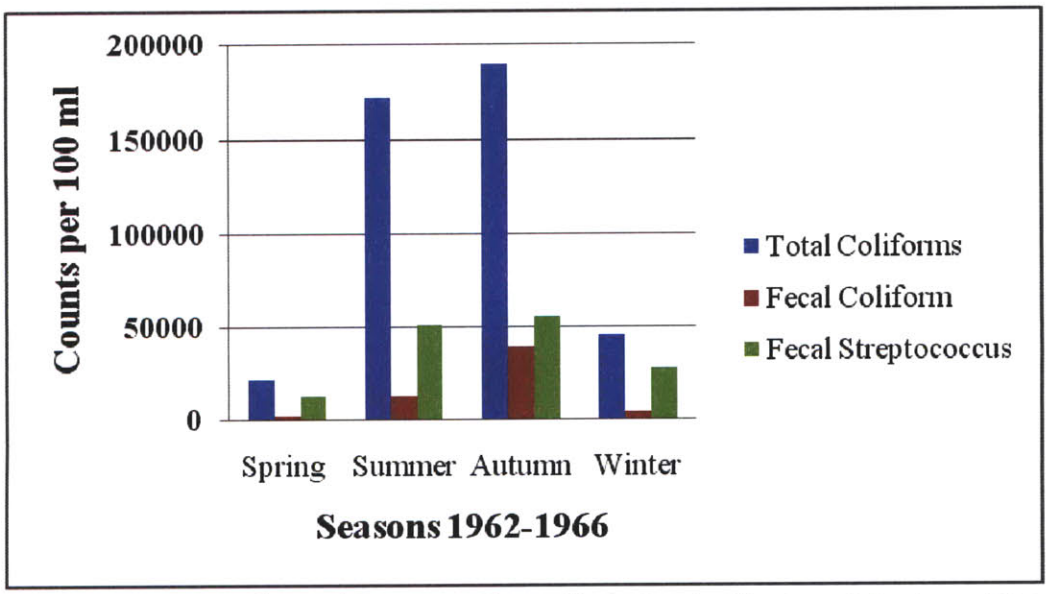


*Figure 2.1 Seasonal Fecal Bacteria Count Variation in Cincinnati Business District (1962-1966) (Geldreich et al., 1968)*

The seasonal variation of the fecal bacteria water pollution in the tropics has different characteristics from the aforementioned temperate climate study. The seasonality of the tropical

region is characterized by dry and wet weather, in contrast to the four seasons of temperate climates. Wright (1986) reported seasonal variation of fecal contamination under the tropical climate of Sierra Leone. During his study, water samples were taken in rivers and streams during different seasons, and they were analyzed for fecal coliform and fecal streptococci (Wright 1986). The results of the analysis showed higher counts in the dry season than in the wet season. High peaks were observed from April to June, which is the transitional period between dry and wet season. This phenomenon was attributed to climate factors that increase stream dilution during the wet season, and leave behind dry stagnant water that will increasingly be contaminated by fecal bacteria from water fetchers (Wright 1986), a phenomenon known as "concentration effects". After heavy rainfall, runoff will run in the streams and dilute the fecal bacteria. Therefore, the dry season stagnant water will have high counts of fecal coliforms and fecal streptococci, while the counts of diluted streams are very low. The dilution of streams happens gradually as the rainfall increases with the rain season.

The seasonal variation of fecal bacteria pollution is a problem that has been observed under different climate zones of the globe. Temperate and tropical climates are both characterized by the seasonal variation of fecal bacteria pollution, but the period in which this phenomenon takes place is different. The epidemiologic studies in developing countries such as Sierra Leone showed a high frequency of waterborne diseases correlated with high concentration during the April-June period (Wright 1986). Continuous fecal bacteria monitoring with emphasis on seasons with high risk is advised to ensure the safety of urban citizens.

# Chapter 3: Challenges Associated with Fecal Indicator Bacteria and Methods for Detection

## 3.1 Introduction to Indicator Bacteria

Indicator bacteria are particular bacteria species used to indicate probable presence of pathogenic microorganisms in water or food. The bacteria species used as indicator bacteria are total coliforms, fecal coliforms, *Escherichia coli* and *Enterococci* (Anderson and Davidson 1997).

The progress of water bacteriological research revealed uncertainties associated with the use of coliform indicator bacteria. Cabelli (1977) suggested four major criteria that should be fulfilled when determining an indicator bacterium. These four criteria included:

- Indicator bacteria are consistently and strongly related to the source of pathogens,
- Indicator bacteria density should be high and correlated to a high density of pathogens to confirm an unacceptable risk of sickness,
- Indicator bacteria resistance to environmental stress and disinfectant should match the resistance of the important pathogen at the source,
- Indicator bacteria should be measurable with cheap, accurate, precise and specific laboratory analysis methods.

Based on these criteria, Cabelli (1977) recommended total coliforms (TC), fecal coliforms (FC), *E. coli*, fecal *streptococci* (FS), and fecal *enterococci* (FE) as indicator bacteria.

Although Geldreich (1978) showed a correlation between total coliforms or fecal coliforms with the presence of pathogens, Dufour (1977) demonstrated that TC and FC could also be found in soil, on plants and in fish ponds. Dufour's (1977) research revealed therefore uncertainties related to the use of TC and FC as indicator bacteria. The presence of TC and FC in water would not necessarily be associated with the presence of pathogenic microorganisms in water. Therefore, TC and FC violated the first Cabelli (1977) criterion of suitable indicator bacteria.

The uncertainties concerning indicator bacteria raised in the 1970s were reduced by water treatment technology and improvements in water quality monitoring in the 1980s. Water treatment technology reduced the uncertainties of the 1970s due to fact that it facilitated the establishment of a controlled volume of safe water quality. The presence of indicator bacteria in this controlled water volume was considered as sign of fecal contamination, thus presenting health risk to users. The progress of this water treatment technology started in 1900s by the combination of disinfectants and filtration for water purification. Drinking water and swimming pool water were therefore protected and indicator bacteria were used to provide information in regards with fecal contamination. The amendment of the Clean Water Act of 1986 presented

new sets of guidelines to protect recreational waters and concluded on concentrations of indicator used as guideline. Further studies of 1990s showed that most of the indicator bacteria were not correlated with fecal epidemics such gastroenteritis. The concentration of streptococci was the only linearly correlated with the presence of gastroenteritis diseases (Kay et al. 1994). On the other hand, the drinking water quality criteria recommended zero as concentration of indicator bacteria (coliform group) in pipelines from the treatment plant to consumers. However, scientists realized some parasites such as *giardia spp.* and *cryptosporidium* were detected in water at zero coliform concentration. USEPA (2000) reported that giardia levels were detected at 10 cysts per liter of drinking water in Northern America. Giardia was also reported to range from 10,000 to 100,000 cysts per liter in raw sewage and 10 to 100 cysts per liter in treated sewage (USEPA 2000a). In addition, cysts were detected in cisterns and wells that were contaminated by surface water or leaking sewer systems. Furthermore, USEPA (2000) showed different probable animal sources of giardia spp. such as beaver, muskrats, wading birds, voles, mice, shrews, gerbils, rats, deer, native marsupials, Australian brush-tail possums, ringed seals, and llamas. The *giardia* species that were identified by USEPA (2000) to be associated with waterborne diseases outbreak were *G. lamblia, G. duodenalis, or G. intestinalis.* In addition, USEPA recorded 130 *giardia* related outbreaks in the U.S. from 1971 to 2000 (USEPA 2000a). Furthermore, the health risk assessment predicted that 250 infections per 10,000 people will to be associated with *giardia* each year in the USA. These different findings helped the USEPA to determine specific guidelines, laboratory analysis methods and regulation of *giardia* and other microorganisms such as cryptosporidium. These regulations were amended by the SDWA of 1996 and were recommended to different US states, which were required to make them effective by 2002 (USEPA 1998). These solutions reestablished the full trust of different water treatment technologies in providing clean and safe water.

Concurrent studies of indicator bacteria have improved laboratory analysis methods to accurately detect them in environmental water samples. The membrane filter and growth media method, and most probable number (MPN) method, concepts were improved from the fermentation concept to the enzymatic approach (Baker 1995). New water analysis methods aiming at simultaneously detecting fecal coliforms and E. coli were also developed. IDEXX Colilert-18TM/Quanti-TrayTM was developed based on a defined substrate technology in which a microorganism with a particular enzyme is detected (Hanko 2000). As the substrate technology was a 1990s invention, another new detection technique was developed based on nucleic acids of the cell of bacteria. DNA-based method was developed by Bernhard and Field (2000). The basic concept of this method differs from the growth medium and substrate technologies. The culture medium and the substrate technologies are respectively based on fermentation and enzymatic metabolism concepts. The culture medium has relative ability to identify the sources of fecal bacteria pollution.

The seasonal variation in the concentration of the indicator bacteria was also another aspect that was investigated for decades. Ruediger (1911) in his earlier research demonstrated that indicator bacteria survival varied with seasonally. Kittrell and Furfari (1963), and Cohen and others (1973) confirmed Ruediger's assertion. They demonstrated that indicator bacteria survived in water longer in winter than in summer. In addition, Johnstone et al. (1974) demonstrated that at $0^{o}C$, indicator bacteria survival time varies from total coliforms with the highest, then fecal coliforms and fecal streptococci with lowest time.

Indicator bacteria investigations would be valuable due to the changes the global warming that may influence indicators' growth conditions in the aquatic environment. The survival of indicator bacteria from which we determine the validity of indicators in time should also be updated in order to include the majority of prevalent pathogens associated with fecal pollutions. . Not only survival rate studies are recommended, but laboratory analysis technique improvement is also encouraged.

## 3.2 Indicator Bacteria Classification

The coliform bacteria group includes many individual organisms of the intestinal flora of human and warm blooded-animal. These bacteria share general characteristics such as being gram-negative, not having spore form, being aerobic and facultative anaerobic, being able to ferment glucose and lactose, growing at $37^{0}C$ with formation of gas residue, being saprophyte and being potential pathogens. (Malcolm 1938). It is estimated that each person excretes from 100 to 400 billion coliform bacteria per day (Metcalf and Eddy 2003). Pathogens of fecal origin include protozoa (e.g. *giardia*), viruses (e.g. hepatitis A), bacteria (e.g. *salmonella*) and other parasites (e.g. *schistosomiasis)* (USEPA 2000a). Although coliform bacteria are abundant in feces, they can also be found in aquatic environments, soil and vegetation (EA 2002). .

Scientifically, coliforms are classified in the kingdom of bacteria; phylum of proteobacteria; class of gamma proteobacteria; order of enterobacteriales and in the family of *Enterobacteriaceae* (Leclerc et al. 2001). Furthermore, the coliform group includes also bacteria from different related genera such as *Enterobacter, Klebsiella, Aeromonas and E. coli.* For the purpose of use the coliform group is classified as shown in Figure 3.1.

Another indicator bacterium is the fecal streptococci group. Oppenheim (1920) confirmed that fecal streptococci were associated with diarrheal diseases and gastro-intestinal illnesses among adults and infants. The fecal streptococci bacteria group (streptococci and enterococci) are therefore included among indicator bacteria of water pollution. Further studies revealed that fecal streptococci were even better indicators of some pathogenic virus than coliforms and fecal coliforms. Cohen and Shuval (1973) demonstrated that streptococci had higher survival rates compared to coliforms and fecal coliforms. In addition, they showed that the presence of fecal

streptococci in water could also be associated with the presence of pathogenic microorganisms such as viruses.

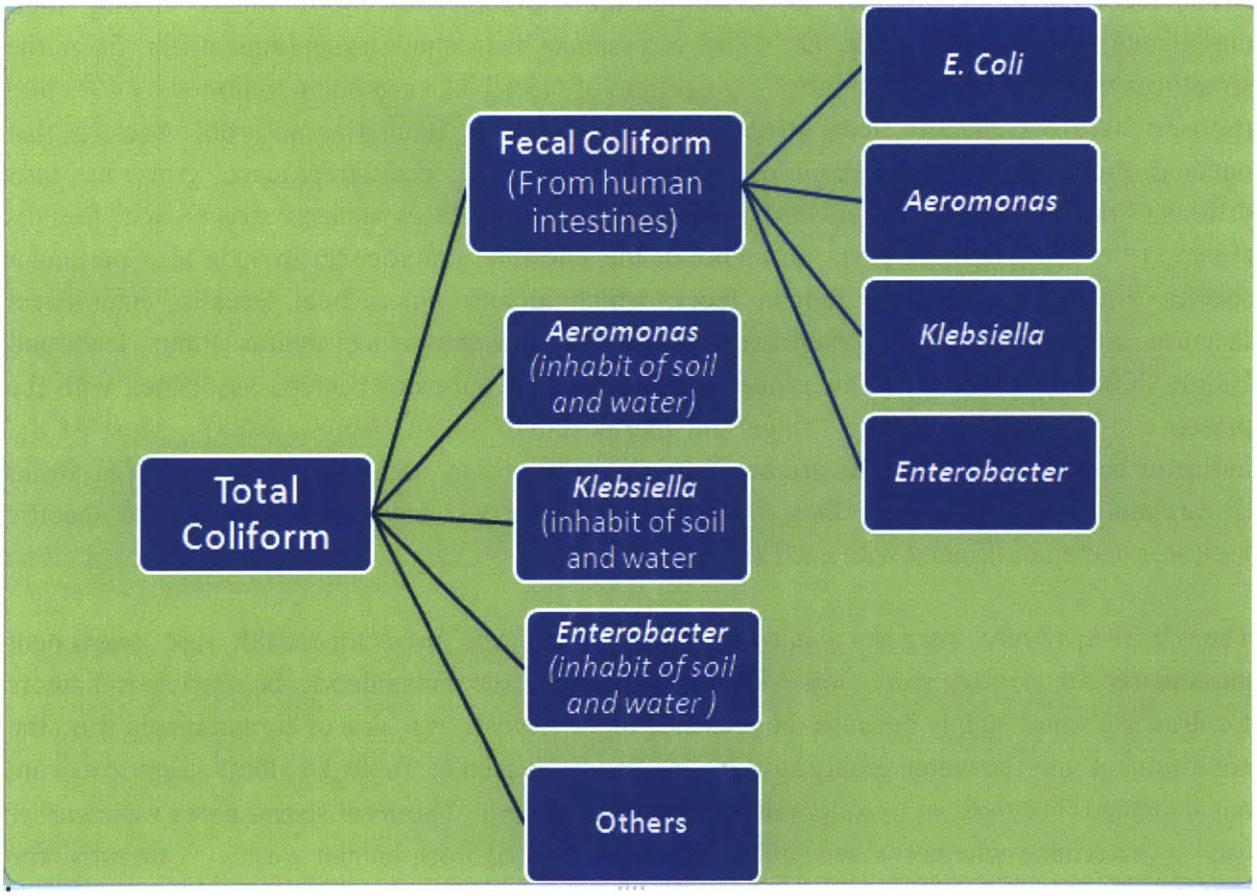


*Figure 3.1 Classification of coliform bacteria (Leclerc et al. 2001, and Anderson and Davidson 1997)*

Fecal streptococci bacteria are classified as species of the genus streptococcus, which are gram-negative, somewhat tolerant to sodium chloride and alkaline pH levels. The scientific classification of this species refers to various criteria such as (Sobsey 2006):

Lancefield antigens (A, B, C, D, and E-T),

Colony morphology and hemolysis,

Biochemical reactions,

Resistance to physical and chemical agents,

Antigenic composition and serological reactions,

Ecology, and

Molecular properties.

Based on Lancefield (date) the classification stipulates that fecal streptococci and *enterococci* are characterized by the serogroup D. This grouping was based on the carbohydrates of the cell wall of these bacteria. The majority of the group D streptococci are non-hemolytic. Since the fecal streptococci have enterococci as a sub-group, discrepancies were found among their classification under the group D. The enterococci that show exceptions differ from the streptococci in their inability to grow in a medium of 6.5% NaCl and being inhibited by 40% bile (Sobsey 2006). Generally, fecal streptococci are known as facultative anaerobic bacteria that occur in the environment as single or as short chains. The fecal streptococci group has also different species which are frequently found in the human feces such as streptococci faecalis (Pinto, 1999 and Bitton, 2005). In addition, the intestinal enterococci include also particular species found exclusively in human feces, which include enterococci faecalis, enterococci faecium, enterococci durans, enterococci gallinarum, and enterococci avium (Pinto, 1999 and Bitton, 2005). Furthermore, this group has been used as indicator bacteria associated with the presence of viruses in sewage sludge and marine environment (Bitton, 2005). Most of the indicator bacteria classifications are based on their physiology and taxonomy. However, water researchers classify them according to water use. Table 3.1 shows the water use and specific indicator bacteria affiliated with each use.

The USEPA (2006) suggests *enterococci* and *E. coli* be used for health risk assessment particularly for primary recreation. Total coliforms are recommended to be used as indicators for drinking water supply because the presence of TC reveals intrusion of contaminants from the environment into the water supply system. As can been seen in Table 3.1, fecal streptococci are not indicated, but their sub-group, *enterococci*, is included. The fecal streptococci were earlier used to determine whether water contamination originated from human wastes. Currently, the USEPA (2006) no longer recommends coliform/streptococci ratio method which was proven unreliable. On the other hand, the *enterococci* have been identified as more related to human intestines than other members of the fecal streptococci group. Therefore, *enterococci* have been recommended by USEPA (2006) as suitable indicator bacteria for recreational salt water and health risk assessments.

### 3.2.1 Type and Use of Indicator Bacteria

Indicator bacteria commonly used are Total Coliform, Fecal Coliform, *E .coli*, and *Enterococci.* As shown in Table 3.1, these indicators are used differently according to the water use.

**Total coliform bacteria:** Total coliforms are common bacteria in environment. Different bacteria species that constitute this group can be found in human feces, animal manure, soil, submerged wood, and at various external locations of the human body (USEPA 2006). The ubiquity of total coliform has limited its use to drinking water systems, while they were previously used for recreational water as well (USEPA 2006). The presence of total coliforms in

drinking water supply systems proves the entrance of environmental contaminants into a water supply system.

*Table 3.1 Indicator Bacteria Classification Based on Water Use (Metcalf and Eddy 2003 and USEPA 2006)*

| **Water Use** | **Indicator Bacteria** |
|---|---|
| Drinking Water | Total coliform |
| Freshwater used for recreation | Fecal coliform, *E. coli*, and *enterococci* |
| Saltwater used for recreation | Fecal coliform, total coliform, *enterococci* |
| Shellfish growing zones | Total coliform and fecal coliform |
| Irrigation | Total coliform |
| Wastewater effluent | Total coliform |
| Disinfected water | Fecal coliform |

**Fecal coliform bacteria:** Fecal coliform bacteria are a sub-group of total coliform and they are exclusively from feces of humans or animals where they are found in intestines. Fecal coliforms are known to produce gas at high temperature of 44.5±0.2 degrees Celsius during 24±2hr (Metcalf and Eddy 2003). Fecal coliform contain also *Klebsiella* in large number from feces and they are also found in the environment. *Klebsiella* have been identified to be related to textile, pulp, and paper mill wastes (USEPA 2006). These findings have raised concerns of false positive associated with monitoring human fecal coliform in waterways. Fecal coliforms are used as an indicator for recreational waters, although recent updates within USEPA (2006) state *E. coli* and *Enterococci* to be better indicators for this purpose.

***Escherichia coli:*** *Escherichia coli (E. coli)* are a species of bacteria that is a subgroup of total coliforms and fecal coliform. *Escherichia coli* are generally found in human and warm blooded-animal intestinal trucks. They are referred to as reliable indicator bacteria for health risk studies in recreational water (USEPA 2006). Although *Escherichia coli* are used as an indicator, they have subgroups related to pathogenic strains such as enteropathogenic, enteroinvasive, enterotoxigenic, and enterohemorrhagic. The general symptoms of disease caused by these *E. coli* subgroups strains are diarrhea and bloody diarrhea (Anderson and Davidson 1997). The presence of *Escherichia coli* is not always associated with pathogenic microorganisms. In addition, Tallon and others (2005) showed that *E. coli* is the best indicator in regards with fecal contamination. .This recommendation was based on three major factors:

- *E. coli* are rarely thermotolerant, thus reducing their abundance in temperate environment compared to fecal coliforms,
- *E. coli* are predominant in human and animal feces and,
- Cheap, rapid, sensitive, specific and easier analysis methods are available.

### 3.2.2 Weaknesses and Advantages of Indicator Bacteria

#### *3.2.2.1 Advantages of Indicator Bacteria*

Since the introduction of indicator bacteria, the lives of many have been saved and many waterborne disease outbreaks have been controlled and prevented. The most important advantage of indicator bacteria is associated with their non-pathogenicity and their correlation to the presence of pathogenic microorganisms. This factor has helped scientists to carry out many studies and has improved water quality around the world. It has also helped to improve water analysis technology in the laboratory and the field. Additional indicator bacteria advantages include the following: indicator bacteria are easier to detect than pathogens, pathogens are diverse and it is impossible to use direct detection to monitor for all pathogens, and high concentrations of fecal indicator bacteria suggest a pathway exists for pathogens in feces to come into contact with humans via drinking or recreational contact.

Tallon and others (2005) discussed current perspectives on indicator bacteria and acknowledged their contribution in fighting disease outbreaks. In addition, they demonstrated the usefulness of indicator bacteria in facilitating the establishment of regulations in developed and developing countries. Guidelines for drinking and recreational water have been established and chemical and bacteriological pollutants are monitored daily in some areas of the world. Bitton (2005) supported that indicator bacteria were needed to control pathogenic microorganisms, thus controlling water-contamination-related outbreaks. However, Bitton (2005) showed how the list of indicator bacteria should be increased based on progress in water research. He argued that bacteria such as *clostridium perfringens* should be added to the list of indicator bacteria since it has the ability to form spores, which are resistant to climate conditions and may last much longer in water than bacteria. Bitton (2005) also showed how C*lostridium perfringens* is currently used in Europe where it is set at 0/100 ml as a drinking water standard.

Indicator bacteria are of great importance in protecting lives. The addition of any additional species to the list of indicators should comply with the recently updated criteria determined by Bitton (2005). This will avoid including any bacteria that may compromise the main goal and advantages of indicator bacteria as aforementioned.
The Bitton (2005) criteria are a new version of Cabelli's (1977) criteria and they are the following:

- An indicator bacterium has to be a bacteria present in intestinal flora of human and warm - blooded animals,
- An indicator bacterium has to be present when pathogens are present in water and absent when they are absent,
- An indicator bacterium has to be present in larger number than pathogens,

- An indicator bacterium has to be as resistant to environmental factors and disinfection of water and treated wastewater as pathogens,
- An indicator bacterium should not reproduce in the environment,
- An indicator bacterium has to be easily, rapidly and cheaply detectable by laboratory procedures,
- An indicator bacterium has to be non-pathogenic.

#### *3.2.2.2 Weaknesses of Indicator Bacteria*

Fecal indicator concentrations are only correlated to diseases transmitted by the fecal-oral and are not indicative of all waterborne diseases (e.g. they do not correlate with *Legionella* or endemic pathogens). Deeper understanding of fecal indicator bacteria showed that some of the selected bacteria species could also grow and multiply in the environment. This point manifested the first strong weakness of indicator bacteria. They were no longer uniquely associated with their sources as expected. The second important weakness lies in the failure of some indicators to fulfill the criteria as updated by Bitton (2005) (e.g. ability of *E. coli* and other total coliforms to grow in environment (soil and water)).

Field and Samadpour (2007) discussed how *E. coli* and *Enterococci* could reproduce, survive and form population in natural environments such as freshwater (e.g. lakes and streams). They also added that these bacteria could be found in plant cavities. Furthermore, the genetic research proved also that the strain of this bacterium found in plant cavities was not associated with human fecal strains, but were identified as originating from a single environmental strain (Power et al. 2005). Moreover, indicator bacteria are also supposed to have relatively equal environmental survival periods. However, Lemarchand and Lebaron (2003) demonstrated that *E. coli* and *Enterococci* could not be correlated with the presence of *Salmonella spp.*, *Campylobacter* spp., *Cryptosporidium*, *Giardia*, and human enteroviruses, implying different survival periods for these different species.

The weakness of indicator bacteria related to their existence in the environment has also been observed for other indicators such as total coliforms and fecal coliforms. Gavini and others (1985) demonstrated that 61% of 1,000 coliform strains from various water samples had an origin different than fecal coliforms. Their findings included results from 23 different laboratories around the world. In addition, Camper et al. (1991) confirmed the capability of total coliforms to grow in natural environments and drinking water distribution systems.

Recognition of the weaknesses of indicator bacteria has helped water regulators to design strategies to evaluate water quality. Tallon et al. (2005) demonstrated how UK regulators determined to use total coliforms in the case of drinking water distribution. Despite the

weaknesses described above, the presence of coliform bacteria in water distribution systems reflects high probability of the system being contaminated. However, further research is encouraged to respond to these weaknesses that are affecting the use of indicator bacteria.

#### *3.2.2.3 Indicator Bacteria Survival in Tropical Climate*

The ideal indicator bacterium would not grow nor persist in environmental water, and their presence would indicate recent water fecal contamination. However, research has shown that some indicator bacteria have high survival rates and can reproduce under environmental conditions in tropical climates. These studies are further discussed in this section.

The severity of waterborne disease outbreaks in different areas located in the tropics motivated indicator bacteria studies focused on the tropics. These studies emerged in the 1980s when the international community started recognizing health problems of the area. Barbara (1986) reported that 250 million new cases of waterborne disease and 10 million deaths were registered worldwide each year and that 75% of waterborne disease occurred in tropical areas. Earlier in the same decade Snyder and Merson (1982) reported that 4.6 million diarrheal deaths were occurring in the tropics among children under the age of five. The response to this situation was water research and supply since it was assumed that this high morbidity and mortality were associated with the use of fecal contaminated water. As the prior decade of the 1970s was marked by a big improvement of water regulation in the USA, researchers felt the need to determine affordable indicator bacteria for tropical regions. Although a number of countries under temperate climate had determined indicator bacteria, the tropical areas needed additional specific studies due to climate differences.

Indicator bacteria studies under the tropical climate were thus intensified. Evison and James (1973) examined the literature of studies carried out in different tropical countries including Egypt, India and Singapore. They showed that all the studies suggested high counts of *E. coli* that were not associated with known sources of *E. coli*. In addition, other studies of the ratio FC/FS showed that the source was more likely to be livestock than human (Feachem 1974). Furthermore, no correlation was observed between *E. coli* and water pathogens such as *Salmonella spp.* Thomson (1981) showed the absence of correlation between *Salmonella spp.* and tested indicator bacteria (coliforms, fecal coliforms and *E. coli*) in water samples taken from drinking water wells in Botswana.

The environmental conditions in the tropics favor the growth of *E. coli* in the environment due to the presence of favorable growth temperature and high nutrients. Therefore, *E. coli* is not considered a suitable bacteriological indicator for this climate. Apart from growth, *E. coli* does not comply with the criteria suggested by Bitton (2005) for suitable indicators for tropical area.

Rivera et al. (1988) isolated *E. coli* from pristine areas of tropical forest in Puerto Rico. Their results showed that *E. coli* strains isolated from an epiphyte in 15-m-tall trees were similar to clinical *E. coli* strains collected from humans. This was confirmed through characterizing procedures, which included plasmid profiles, antibiotic sensitivities, coliphage vulnerability, and physiological and biochemical descriptions. The DNA isolated from both a clinical *E. coli* strain and an *E. coli* strain from the epiphyte of the tree showed 85% of DNA homology by antibiotic susceptible test. Therefore, it was concluded that tropical water sources do not only have high densities of *E. coli* in the absence of fecal sources, but *E. coli* could probably occur naturally under a tropical climate. In addition, this suggestion was supported by high densities of *E. coli* found in waterways or waterbodies of different tropical countries (Hazen 1988). The reliability of *E. coli* as indicator bacteria in the tropics was therefore questioned.

Different environmental factors drive the change in survival rate under tropical climate, but the temperature has been identified as the principal factor. *E. coli* and coliforms have been identified to survive long periods in tropical streams and high temperature. Table 3.2 shows *in situ* survival time of *E. coli* under tropical and temperate climates. As can be seen in the table, *E. coli* survival time is higher in the tropics and lower in temperate climates. In addition, Hazen (1988) suggested that these variations depend on the fact that the microbial diversity of tropical waters is considerably greater than that of temperate waters.

*Table 3.2 Comparison of Tropical and Temperate In Situ Survival Rate of E. coli (Hazen 1988)*

| Climate | Initial Density | Survival time (hours) | Reference |
|---|---|---|---|
| **Temperate Climate** | $10^9$ | 50 | Gordon and Fliermans (1978) |
| | $10^5$ | 30.6 | McFeters et al. (1974) |
| | $10^8$ | 24 | Sjogren and Gilbson (1985) |
| **Tropical** | $10^7$ | 294 | Lopez-Torres et al. (1987) |
| | $10^6$ | 206 | Valdes-Collazo et al. (1987) |

**Survival time**: time to reach 90% (1 log or $T_{90}$) reduction of initial cell density

Nowadays, it is recognized that fecal indicator bacteria (fecal coliforms, *E. coli*, *Enterococci*) can grow in soil, sediment, and water in tropical and subtropical environments of Hawaii, Puerto Rico, south Florida and elsewhere (USEPA 2001). These findings call into question the applicability of USEPA guidelines in these regions. Current suggestions stipulate additional guidelines for tropical and subtropical regions that will use alternative indicators such as *Clostridium perfringens* and coliphages (USEPA 2001).

## 3.3 Indicator Bacteria Analysis Methods

Research for improving water quality analysis method is an active field. The developing world still relies on membrane filtration and most probable number methods of detecting indicator organisms as these are the methods on which water quality criteria and standards are based. However, further water microbiology research is encouraged to provide low-cost methods and increase the specificity of existing methods for contamination with human origin.

The most recent water analysis methods are based on nucleic acids. These methods are known by their capacity to identify sources of contamination such as humans and wildlife. The PCR-based method is one of the methods recently developed based on this concept. PCR (Polymerase Chain Reaction) implies the amplification of a targeted DNA by cycling replication (copying by cycle the targeted DNA gene; all the copies are supposed to be identical.) (Tallon et al. 2005).

### 3.3.1 Traditional Indicator Bacteria Analysis Methods

#### *3.3.1.1 Membrane Filtration Method (MF) and Most Probable Number Tubes (MPN)*

The membrane filtration (MF) and most probable number (MPN) methods have improved throughout years. Clark and Kabler (1951) first demonstrated the procedures of membrane filtration to test coliforms in environmental water samples. The method was composed of two steps and two media. After the filtration of the environmental water sample, the membrane was enriched with an albimi medium for 2 hours before being transferred to a Petri dish, which contained an absorbent pad completely soaked with Endo broth. The sample was thereafter incubated for 12 to 15 hours at a designated temperature depending to the type of indicator bacteria analyzed. According to Clark and Kabler (1951), the Endo broth medium was composed of the following chemicals: lactose (20 mg), neopeptone (20 mg), potassium dihydrogen phosphate (7 mg), distilled water (1,000 mg) and a solution of potassium hydroxide to maintain the pH of the medium at 7.5. In addition, the medium was prepared daily due to lack of appropriate storage conditions.

Currently, the culture media that are mostly used for culturing indicator organism on membrane filters include m-Endo-type media largely used in North America and Tergitol-TTC medium largely used in Europe (Rompre et al. 2002). The coliform bacteria analyzed using the Endo-type medium will form colonies with red color and a metallic sheen after an incubation period. On the other hand, coliforms analyzed using Tergitol-TTC media will form colonies with yellow-orange color after an incubation period (Rompre et al. 2002). In addition, Mac Conkey agar and teepol media were respectively used in South Africa and Britain. A comparison of the yield of these media showed that m-Endo agar had higher counts than Mac Conkey or teepol agar (Grabow and du Preez 1979).

Despite the worldwide use of the membrane filtration technique, it is still associated with weaknesses that have not yet been addressed. Rompre and others (2002) showed that a major concern results from the inability of the media used to grow stressed coliforms cells. These stressed indicator bacteria cells may be dormant after exposure to chemicals used during water treatment. In addition, Rompre et al. (2002) pointed out that chlorination could cause inhibition of metabolism and enzymatic activities. Since these bacteria are not dead pathogens may have also survived the treatment process and pose a risk. Thus, undetectable dormant cells may bias the water quality results suggesting water as safe when it is actually not.

Furthermore, Szewzyk et al. (2000) demonstrated that the lactose fermentation and gas production methods could misidentify coliform strains leading to false positive results. This method was also criticized based on the fact that certain conditions could lead to lack of lactose fermentation (Szewzyk et al. 2000). These concerns were again discussed by Bitton (2005), who pointed out various factors that can influence the performance of the membrane filtration technique. These factors include the type of growth medium, solution used for dilution, type of membrane filter, presence of non-coliforms bacteria, occurrence of injured coliforms, and water turbidity. The current membrane filtration methods are globally used because they are cheap, easy to use and they have increased sensitivity compared to the methods used decades ago.

Apart from the membrane filtration method that is used almost everywhere, the MPN method is also sometimes used under specific conditions (Hanko 2000). The MPN method consists of a number of tubes in which aliquots of sample are inoculated into selective culture medium. This method is used for high turbidity samples where membrane filters are likely to be clogged with suspended material before a representative sample can be completely filtered (Hanko 2000).

### 3.3.2 Emerging Indicator Bacteria Analysis Methods

Emerging analysis methods for indicator bacteria (Table 3.3) are based on substrate (enzyme) and nucleic acid concepts. The enzymatic method was introduced to increase the sensitivity and speed of water quality analysis. The specificity was based on designing a method that would increase the detection of indicator bacteria and isolate environmental bacteria of similar characteristics (Bitton 2005). Various chromogenic enzyme substrates were applied to detect indicator bacteria. Table 3.4 summarizes enzyme substrates that are used and specific bacteria targeted. Currently, enzyme-substrate-based technology is the most used and several methods have been developed around it. These methods are improved membrane filtration and most probable number techniques. The results obtained from these methods are of various types depending on the goal of the monitoring or investigation. The types of results are presence/absence and colony counts in CFU (colony-forming units) per 100 ml. The new version of MPN uses multiple-well trays in the place of multiple tubes. The dilution that used to be done in multiple tubes is then performed on a tray where a100-ml sample of water is mixed

with the culture medium and distributed evenly on the tray. In addition, the enzyme substrate method has permitted microbiologists to design test methods capable of detecting simultaneously coliforms and *E. coli* (Bitton 2005). The observations are done with a naked eye for coliform reading and under UV light for *E. coli* counting.

IDEXX Colilert (IDEXX 2009) and m-ColiBlue24 are two of the commercial test methods that have been developed based on the enzyme substrate concept. The IDEXX Colilert test was designed to facilitate the concurrent enumeration of total coliforms and *E. coli* in environmental water samples (Edberg et al. 1990). In addition, Edberg et al. (1990) confirmed the validity of the IDEXX Colilert test results (IDEXX 2009). Their investigation consisted of enumeration of coliforms and *E. coli* in surface water samples. Samples were analyzed using both IDEXX Colilert and standardized multiple-tube fermentation method (MTF). Comparison of the results by statistical methods proved IDEXX Colilert to be a reliable water analysis method (Edberg et al. 1990). Furthermore, this investigation confirmed that the method was sensitive, specific, required little labor, provided results rapidly and without any interference of non-coliform heterotrophy. Nowadays, the most used Colilert method is IDEXX Colilert-18 or 20/ Quanti-Tray, which has also been used in this study (IDEXX 2009).

*Table 3.3 Indicator Bacteria and their respective enzymatic reactions (Bitton 2005)*

| **Enzyme** | **Bacteria** | **Reaction** |
|---|---|---|
| **β-D-glucuronidase** | 94-96% of *E. coli* | Product (4-methylumbelliferone) of 4-methylumbelliferyl-β-D-glucoronide (MUG) hydrolysis, fluoresces under UV light. |
| **β-D-galactosidase** | 100% of coliforms in the *Enterobacteriacea* family (defined as lactose fermenting). | Ortho-nitrophenyl-β-Dgalactopyranoside (ONPG) hydrolysis releases the yellow chromogen, ortho-nitrophenol. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) breakdown produces blue colonies. |

On the other hand, the m-ColiBlue24 method is also an enzyme-substrate-based method that has been approved by the Federation Registration (FR) (1999) to be used for simultaneous enumeration of *E. coli* and total coliforms. The *E. coli* colonies are identified by a blue color that results from a reaction of the enzyme β-D-glucuronidase and 5-bromo-4-chloro-3-indoyl-β-D glucuronidase (Vail et al. 2003). Apart from the medium, other laboratory procedures follow the membrane filtration approach. The environmental water sample is then incubated for 24 hours at 35±0.5 Celsius degrees (Vail et al. 2003). Although the m-ColiBlue24 method was approved as an analytical method suitable for enumeration of total coliforms and *E. coli* in the U. S., Jensen and others (2001) criticized its application under tropical climates.

*Table 3.4 Methods for Water Microbiological Source Tracking (Scott et al. 2002)*

| Method | Bacteria Tested | Targeted Sources | Current Status |
|---|---|---|---|
| **Ratio of Fecal Coliform to Streptococci** | *Streptococci* and fecal coliforms | Human and Animal | No longer in use |
| **Conventional Membrane filtration** | *Bifidobacterium spp.*, *Bacteroides fragilis* bacteriophage, | Human fecal pollution | Approved by EPA |
| **Hybridization approach (nucleic acid)** | F-specific RNA coliphage : F+ RNA coliphages (Leviviridae) | Human fecal pollution | Not yet validated |
| **Bacteria cultivation followed by Reverse Transcription-PCR** | Human enteric viruses | Human fecal pollution | Not largely applied |
| **Phenotypic method**: **(1)** MAR analysis (use common antibiotics for human and animal), and | *E. coli* and fecal streptococci | Human and animal fecal pollution | Not validated, additional research need |
| **Phenotypic method: (2)** immunology(targets antigens) | *E. coli* and fecal streptococci | Human and animal fecal pollution | Not validated, additional research need |
| **Genotypic method: (1)** Pulsed-field gel electrophoresis (PFGE) (DNA fingerprint) | All indicator bacteria | Human and specific animal(s) | Not validated, additional research need |
| **Genotypic method: (2)** Repetitive element PCR (Rep-PCR) (genomic fingerprint) | All indicator bacteria | Human and specific animal(s) | Not validated, additional research need |
| **Genotypic method: (3)** ribotyping (DNA fingerprint) | All indicator bacteria | Human and specific animal | Not validated, additional research need |
| **Genotypic method: (4)** Host-specific molecular markers | *Bacteroides spp.* | Human and specific animal(s) | Recommended, but not largely applied |

Jensen et al.'s (2001) investigation results proved that the specificity of m-ColiBlue was lower in Pakistan (65%) than in the U.S. (95%). It was also concluded that the m-ColiBlue24 method applied to surface water under tropical climates is associated with a large number of false positive errors. Jensen and others (2001) recommended including samples from tropical climates when designing alternative growth medium to m-ColiBlue24 method.

The decade of the 1990s was marked by the launch of another category of water analysis methods based on analysis of nucleic acids, which helped researchers to resolve uncertainties associated with indicator bacteria detection. The PCR-based method (Polymerase Chain Reaction) uses specific primers to detect and identify bacteria. Primers are designed based on specific genes that reliably code different specific enzymes used in previously described enzyme based method.

Coliform bacteria were identified and detected using a primer called "*LacZ*" that was designed based on the genes that code $\beta$-D-galactosidase (Bej et al. 1990). However, Fricker and coworkers (1994) showed the inability of this assay to distinguish between coliforms and the non-coliform bacteria *Hafnia alvei* and *Serratia aborifera* strains proving that the primer could give false positive results. On the other hand, the detection of *E. coli* targeting the gene for the $\beta$-glucuronidase enzyme (*uidA*) was promising. The primers designed based on genes that code for *uidA* detected the *E. coli* with high sensitivity compared to the previous enzyme substrate-based method (Fricker 1994).

These PCR-based method trials have been followed by other method development based on the same concepts in order to increase the detection, specificity and identification of specific pathogens. Bej et al. (1991) used a Multiplex PCR method to identify *lacZ* (coliform) and *uidA* (*E. coli*) genes, thus monitoring the presence of coliform and *E. coli*. The Multiplex PCR is a method that uses concurrent amplification of different gene sequences. The findings of Bej et al. (1991) showed an increased sensitivity and detection of both *lacZ* and *uidA*, but recommended additional research using various environmental water samples before validating the efficacy of the method. In addition, other trials focused on improving the capacity of the method to detect lower concentrations of *E. coli* within short time. Juck and others (1996) used the "Nested PCR" method as a rapid method to detect *E. coli* in drinking water. The Nested PCR method consists at using two successive rounds of PCR amplification. The second round is added to raise PCR products to detectable levels (Juck et al. 1996). The results of Juck et al. (1996) confirmed that the method could detect a lower concentration of *E. coli* ranging in the interval of 1-10 bacterial cells per 50 ml after6-8 hours. Another method designed with similar goals as the method by Juck et al. (1996) is the "*In situ* PCR method." This method consists of fixing the cellular wall and permeabilizing it, thus enabling diffusion of relevant reagents into the cell. After "*in vivo*" amplification, the images are observed to enumerate the targeted *E. coli* cells. These cells are easily observed since they are labeled by fluorescent PCR products (Tallon et al. 2005).

Nowadays, investigators are focusing on genotypic methods designed based on the nucleic acid concept to identify the source of fecal contamination. The goal of this new approach is to match bacteria identified to the contaminated site, and animals or humans as the origin of fecal contamination (Shanks 2005). Various methods such as pulsed-field gel electrophoresis (PFGE), repetitive element PCR, ribotyping, and host-specific molecular makers have been documented (Scott et al. 2002).

The PFGE is a DNA fingerprint method, which implies the purification of genomic bacterial DNA with rare-cutting restriction endonuclease (enzyme that recognizes a DNA sequence that occurs infrequently in the genome) before generating a DNA fingerprint. This method has been used to study bacteria connectedness and to carry out epidemiology studies (Scott et al. 2002). Simmons et al. (2000) used the latter method to study nonpoint sources of fecal pollution in the Four Mile Run watershed in the Northern Virginia. Their investigation used the *E. coli* DNA from environmental samples taken from the stream based on seasonal variation and sediment in the ultra-urban Four Mile Run watershed. Simmons and others (2000) concluded that human sources were dominant followed by the waterfowl estimated at 37%. Other sources identified included nonhuman mammals such as raccoons, dogs, deer, and Norway rats.

The repetitive element PCR method is based on the use of primers analogous to interspersed repetitive DNA (allowing the evolution of new genes during meiosis by uncoupling similar DNA sequences from gene convention) elements present in various locations within the procokaryotic genome (total number of genes within a reproductive cell) to produce highly specific genomic fingerprints. This method uses three methods of repetitive sequence analysis where each repetitive sequence targets a specific family of repetitive element. These three methods are: (1) repetitive extragenic palindromic sequence PCR (REP-PCR), (2) enterobacterial repetitive intergenic consensus sequence PCR, and (3) PCR with extragenic repeating elements (Box-PCR) (Scott et al. 2002). The Box-PCR primer has been used to differentiate bacteria with close strains. Dombek et al. (2000) used repetitive DNA sequences and the Box-PCR primer to differentiate *E. coli* strains from humans and animals. They showed that the Box-PCR primers were more efficient for putting *E. coli* strains into groups than the rep-PCR primers. Furthermore, Box-PCR primers were used to classify suitably 100% of 154 strains of *E. coli* from cow and chicken, and 70%-90% of strains of human, goose, duck, pig, and sheep strains to their specific sources. Moreover, the genetic data created when the Box-PCR method is used can also be used to identify other DNA fingerprints from unknown strains identified in environmental water samples.

Ribotyping is a method of DNA fingerprinting used to identify conserved rRNA genes using oligonucleotide probes. These probes are used after purifying genomic DNA with restriction endonuclease. This method is known to require labor-intensive procedures including culture and

identification of bacteria, DNA extraction, gel electrophoresis, southern blotting and discriminant analysis of obtained DNA fingerprints. This method has been used in epidemiological studies since it was found useful in investigations that involve different bacteria species (e.g. *E. coli*, *S. enteric*, *Vibrio cholera*, and *Vibrio vulnificus*) (Scott et al. 2002).

Methods targeting host-specific molecular markers are current strategies for tracking species genotypes of bacteria found only in certain animal hosts. This method consists of detecting a host-specific molecular marker in environmental water samples. It does not require pre-culturing the studied organisms. In addition, this method is promoted as the most rapid in identifying the source of bacterial pollution (Scott et al. 2002). The source identification is done through discrimination of human fecal contamination and other animal fecal pollution. The discrimination-based methods that are currently used include length heterogeneity PCR (LH-PCR) and terminal restriction fragment length polymorphism analysis (TRFLP-PCR) (Scott 2002). Bernhard and Field (2000) designed and used these methods to discriminate human and ruminant feces based on host differences in *Bacteroides Prevotella* genes coding 16S rRNA. This method is described further in Section 3.3.2.1 below.

#### *3.3.2.1 Bacteroides Prevotella 16S rRNA Gene-Based Method*

*Bacteroides* spp. are non-spore-forming obligate anaerobic bacteria that live in the intestinal tract of warm-blooded animals including humans. *Bacteroides* are estimated to have a concentration of $10^{10}$ cells per gram of human feces in which their quantity is estimated at one-third of human fecal bacteria (Holdeman et al. 1976). They have limited survival in the environment and host-specific variation in animals (Shanks 2005). Therefore, the *Bacteroides Prevotella* group was selected to be used in research to find methods to link fecal contamination to human or animal sources.

Some advantages and limitations of this host-specific PCR method have been identified. The advantages of this genotypic approach include no need to pre-culture the studied strain, rapidity, increased sensitivity, specificity of target, isolation of target strain from a complex environment, and applicability of automated technology (Shanks 2005). On the other hand, the common limitations include inhibition of PCR, few experiences with the method, and targeting small sequence database of the targeted strain (Shanks 2005). However, this method is most promising due to its rapidity in identifying targeted organisms (Scott et al. 2002).

Many PCR-based tests for identification of particular bacterial strains target the 16S ribosomal RNA gene. This gene encodes for a subunit of the bacterial ribosome. The ribosome is known for its important role in mass production of protein in living cells. The 16S rRNA gene is applied in phylogenetic studies that use the genetic approach to study evolution (Weisberg et al. 1991). The 16S rRNA gene sequences have hypervariable regions that can be use as species-

specific signature sequences. These signature sequences have turned the 16S rRNA gene into a powerful tool used for rapid identification studies in medical microbiology and phenotypic studies of bacteria (Weisberg et al. 1991).

Because the 16S rRNA gene has proven to be useful for bacterial identification, researchers have developed its application in bacteria water research. The main goal is to design a rapid analysis method that can provide information on the source of fecal contamination within a short time. Bernhard and Field (2000) used the PCR assay to discriminate human and ruminant feces based on variation in *Bacteroides Prevotella* 16S rRNA genes where *Bacteroides* strains only found in either human or ruminant feces could be identified by their unique DNA sequences. This research used environmental water samples collected from different frequently fecal contaminated locations in Tillamook Bay in Oregon. The purified DNA from these samples was then amplified with *Bacteroides Prevotella* specific primers, Bac32F and Bac708R. The results showed that it was possible to detect different sources of fecal contamination such as human and wildlife based on the type of *Bacteroides Prevotella* group detected in the sample. The study developed primers that are specific for strains of *Bacteroides-Prevotella* found exclusively in humans (HF183F) or in both humans and animals (Bac32).

Nowadays, the human host-specific molecular primer (HF183F) determined by Bernhard and Field (2000) is a promising globally reliable marker for detection of human fecal contamination. Recent fecal bacteria pollution studies carried out in the USA by Shanks et al. (2006) and Santoro and Boehm (2007) confirmed the HF183F applicability in identifying humans as sources of nonpoint fecal bacteria pollution. Other studies carried out in Europe, Africa and Australia confirmed also the applicability of this method. Jenkins et al. (2009) identified human and livestock sources of fecal contamination in Kenya and confirmed HF183F primer suitability in the area. Pickering et al. (2010) confirmed also the applicability of HF183F primer in tropical regions when examining fecal contamination in Tanzanian communities with improved and non-networked water supplies. Gawler et al. (2007) validated HF183F primer as a tool to identify the origin of human fecal pollution in Atlantic Rim countries of the European Union. Finally, Ahamed and others (2008) evaluated *Bacteroides* markers for the detection of human fecal pollution in Southeast Queensland, Australia. They confirmed that HF183F is a reliable marker to detect human fecal pollution.

#### *3.3.2.2 Clone Library Formation and Phylogenetic Analysis*

Clone library formation and phylogenetic analysis are important to confirm the accuracy of the genotypic method used in microbial source tracking. Such analyses allow us to determine whether a target sequence detected by a PCR-based method (e.g. the HF183F primer) matches the original sequence for which the test was designed. This provides a quality-control check to confirm the specificity of the DNA-based test. The main steps of the PCR-based method from

collection of water environmental samples to phylogenetic tree analysis are summarized in Figure 3.2. As can been seen in Figure 3.2, the procedure starts with the collection of environmental water samples, which will be taken through the entire procedure until a phylogenetic tree is generated and analyzed. In this case, we are considering that the host-specific primer is being used to target human fecal bacteria that may be among the water bacteria pollutants of a given surface water sample. After the amplification of the extracted DNA samples using the HF183F host-specific and Bac708R primers, the results are electrophoresed on 1% agarose gel. Amplicons that are positive to HF183F primer are selected (depending on the type of environmental samples collected), then purified using a gel extraction kit of choice (e.g. Qiaquick gel extraction kit (QIAGEN). The purified amplicons are then cloned into *E. coli* cells that will be grown with this new DNA, thus facilitating the sequencing processes. The major cloning steps are ligation, transformation, colony screening, and liquid culture (Hewson 2002).

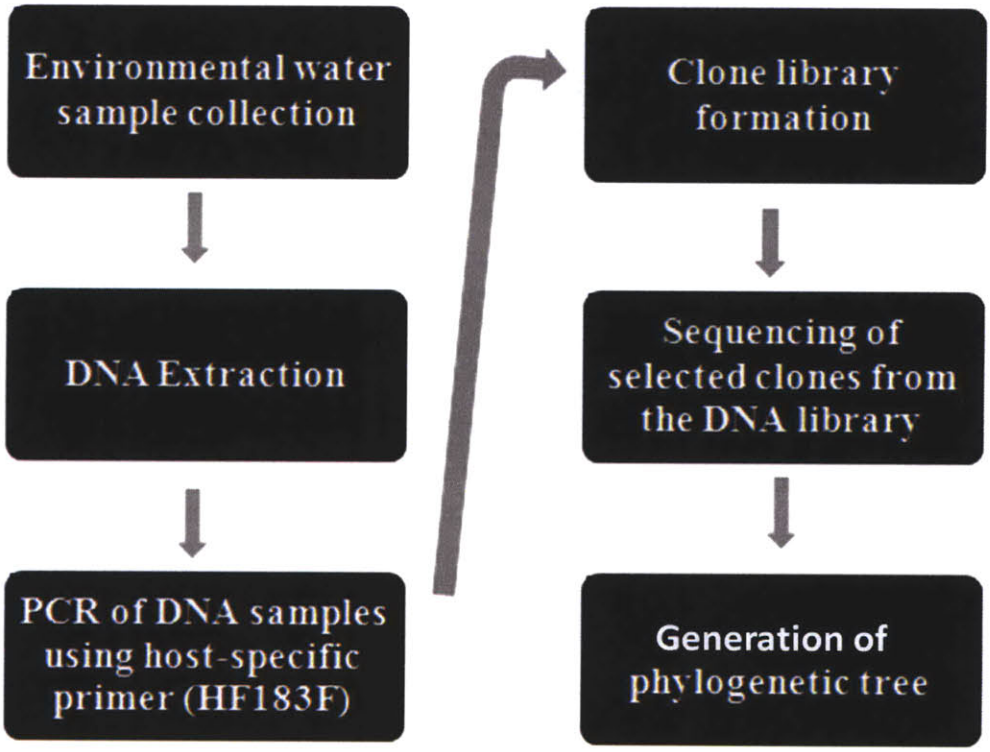


*Figure 3.2 Main Steps of PCR-based Method*

A number of *E. coli* colonies resulting from cloning are selected for sequencing. During colony screening, the *E. coli* colonies with white color are selected. The white color proves that the insert has been taken as new DNA in the *E. coli* cells used during the insertion. The *E. coli* colonies characterized with the blue color did not grow with the inserted DNA and cannot be used for further steps (Hewson 2002). The white-color cells are sequenced in order to check the order of the nucleic acid bases (Adenine, Guanine, Cytosine and Thymine) of the inserted DNA.

Recall from above that the DNA that was inserted resulted from the PCR of 16S rRNA amplified with the HF183F primer targeting human-specific bacteroides. Therefore, the DNA sequences obtained from the environment are expected to match the 16S rRNA from the human-specific 16S rRNA *Bacteroides* (Figure 3.2). The obtained sequences are used to generate a phylogenetic tree, which represents evolutionary relationships among sequences obtained from the environmental water sample being analyzed.

The phylogenetic tree is a genetic tool used to demonstrate evolutionary relationships among groups of organisms or a family of related nucleic acid or protein sequences. Continuing from the above discussion, the phylogenetic tree is then generated from sequences generated from sequenced clones that have been randomly selected from the clone library. Before generating the tree, a multiple sequence alignment is created. The sequence alignment consist of arranging the sequences of DNA, RNA or protein to determine regions of resemblance that could be results of functional, structural or evolutionary relationships between sequences (Mount 2004). Sequences of nucleotides or amino acids that have been aligned have an image of a matrix. Currently, the alignment is done using software such as ClustalX, MUSCLE or ARB. In addition, the quality of the sequence alignment is generally verified by manual inspection of the alignment. The alignment is then followed by the sequence distance matrix calculation. This distance is estimated by comparing sequences to one another, then taking the number of exact matches dived by the sequence length. The results of the distance matrix are used to generate the tree that shows in which order the sequences are aligned. The alignment based on sequence distances is done by aligning the most closely related sequences first, then adding the distant ones which are aligned to the first alignment. Finally the phylogenetic tree is completed and interpreted. Since this work is done using specialized software, the tree is tested, using a bootstrap test, for robustness and branching patterns. Briefly, the phylogenic tree analysis is used to confirm if the DNA extracted from the environmental water sample and confirmed positive to HF183F host-specific primer is evolutionally related to human bacteroides. Further, the phylogenetic tree results are compared to results from other studies to confirm the validity and specificity of the method.

The discussion of the phylogenetic analysis provided above is supported by an example discussed below. This example was generated based on the online material provided by the University of Auckland (2008). During the description of our example we will refer to the phylogenetic tree in Figure 3.4 that illustrates the evolution of different animals.

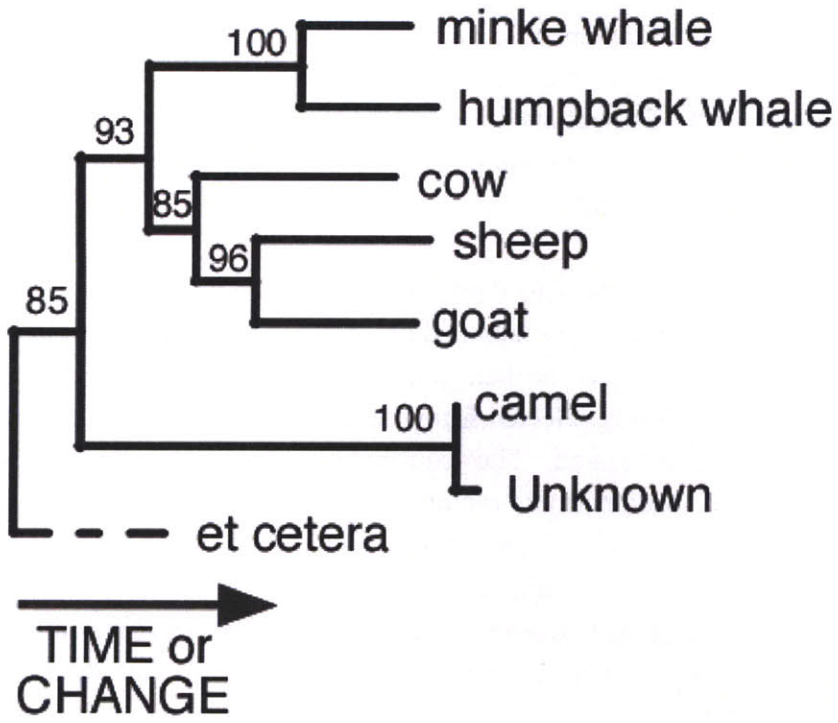


*Figure 3.3 Phylogenetic Tree Generated for the Illustration of the Phylogenetic Analysis (UA, 2008)*

In this context, we will consider the tree on Figure 3.4 as the family tree of species. The horizontal illustration of the tree makes the related species to set at the end of the branches (horizontal lines on Figure 3.4). These horizontal lines have an origin from the node formed between vertical and horizontal lines of the tree. The length of the branch is associated with the time of evolution or changes between the first node and the next. In addition, the node is considered as the representative of a common ancestor to all the organisms to its right-side. The relation of two species is determined by the leftward length needed to reach the common ancestor. Based on the latter, Sheep and Goat on Figure 3.4 have an equal and closer relationship, than neither of them is with the cow. This analysis shows that the phylogenetic tree can also be used for identification of unknown organisms by identifying their closer match. This can be done by using sequences from known animals (as reference) and sequence of unknown animals. The phylogenetic three produced from these sequences will show the known specimen closely related to the unknown. As can be seen on Figure 3.4, the unknown specimen is extremely related to the Camel. According to their branching, the unknown specimen is probably from the camel. Nevertheless, this approach is limited by different scenarios where some unknown specimen may not be classified with the known specimen since their sequences have not yet been identified (UA, 2008).

Confidence in the predicted evolutionary relationships can be examined based on the data used to generate it. The analysis used to test the robustness of branching patterns inferred from sequence of data is known as "bootstrapping". The bootstrapping method can be demonstrated as follow: first we will take a sample of 100 beads from a container, then count reds in the sample and thereafter turn the beads back into the container. Mix well the beads in the container and randomly select another sample of 100 beads, then do the same procedures many times and each time count the number of reds. After this session, check how frequent you got at least 50 reds, 60 reds, 70 reds, 80 reds, 90 reds and 100 reds out 100 beads picked at each time. Finally, this will give you a sampling distribution of reds (AU, 2008).

A similar approach is also used with gene sequences. Individual nucleotide positions are randomly selected from the sequence alignment until a sample of the same length as the original sequence is obtained. Then this information is used to generate a phylogenetic tree. The same procedures are repeated many times and at the end we examine the frequency of individual relationship identified in the first phylogenetic tree generated. The numbers on Figure 3.4 illustrated the final results of the bootstrap. As can be seen on Figure 3.4, the two species of whale have a bootstrap number of 100%, meaning that they have appeared together all the times that similar procedure as described above were repeated. Since all the numbers are above 70% we are confident of the evolutionary relationship of these animals used as example. During the phylogenetic analysis of sequences from environmental samples the reasonable bootstrap percentage is above or equal to 50% (AU, 2008).

# Chapter 4: Methods and Site Characterization

## 4.1 Site Characterization

### 4.1.1 Land Use

Sampling was carried out across the five sub-catchments of Kranji Reservoir: Kangkar, Neo Tiew, Tengah, Peng Siang and Pang Sua (Figure 4.1) (NTU 2008). The sub-catchments are generally characterized by undeveloped (76%), residential (19%) and agricultural (5%) area (Table 4.1). Kangakar sub-catchment is the most undeveloped and Pang Sua sub-catchment is the highly populated among the rest of the sub-catchments (Table 4.1).

The geography of the Kranji catchment areas is complex. It is characterized by a topography dominated by lower elevations with an estimation of altitude averaged in the interval of zero meters at sea level to 152m at Bukit Gombak (PUB 2007a). The hydrology of the area is dominated by man-made concrete drainage channels. There are a small number of natural streams in the catchment. In addition, the geology of the area comprises various sedimentary rocks, which have been metamorphosed. The high lands of Bukit Gombak are generally granite and norite. The geological characteristics of the area create limitations for holding groundwater (PUB 2007a).

### 4.1.2 Rainfall Data

The rainfall of the Western Catchment is influenced by the seasonality of the area. Generally, the northeast monsoon (December-March) has an average rainfall of 623 mm and the southwest monsoon (June-September) has 647 mm. The rainfall in between these two monsoons is averaged at 432 mm (March-May) and 574 mm (October-December). Total annual rainfall in the Western Catchment varies between 2300 mm and 2400 mm (PUB 2007a).

Rainfall was measured (NTU 2009) June-July 2009 from stations located in Pang Siang (2 stations), Tengah (1 station), Kangarkar (1 station) and Pang Sua (1 station) in Kranji Reservoir catchment and showed the beginning of the southwest monsoon. The total rainfall in the period of June and July was 124 mm. Figure 5.2 shows the rainfall distribution by sub-catchment in the Kranji catchment. The rainfall during the period of January 2009 was 23 mm, which is five times smaller than the rainfall of the period of June-July 2009. Therefore, the field work was classified under to major rounds: January 2009 was considered a dry period, and June–July 2009

a wet period. Figures 4.3 and 4.4 show the variation of rainfall in Kranji Reservoir catchment during June and July respectively.

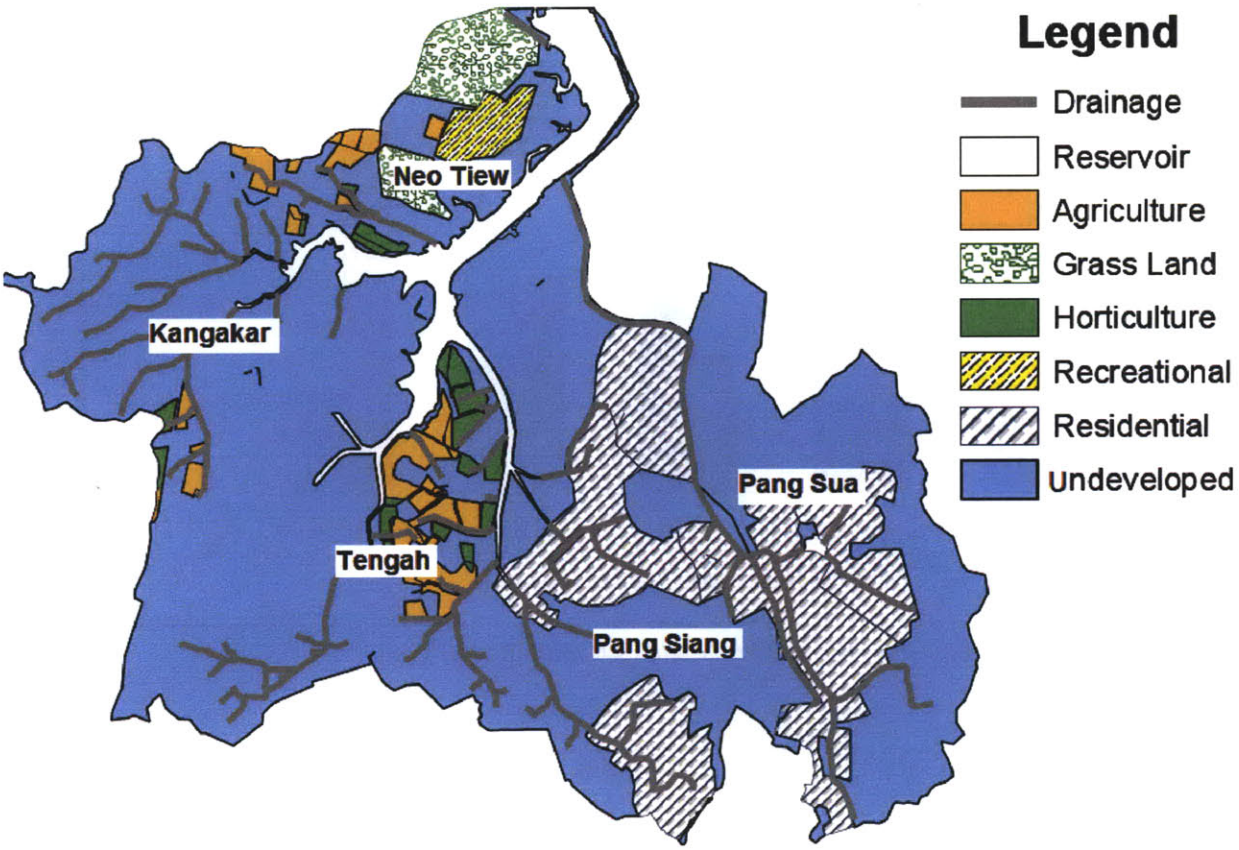


*Figure 4.1 Map of Kranji Reservoir Catchment land use.*

*Table 4.1 Sub-catchment Information (NTU 2008)*

| Sub-catchment | Area, ha | Residential, % | Agricultural, % | Undeveloped,% |
|---|---|---|---|---|
| **Kangakar** | 872 | 0 | 4 | 96 |
| **Tengah** | 993 | 0 | 9 | 91 |
| **Peng Siang** | 1334 | 32 | 6 | 62 |
| **Pang Sua** | 1570 | 40 | 0 | 60 |
| **Neo Tiew** | 660 | 0 | 10 | 90 |
| **Total** | 5429 | 19 | 5 | 76 |

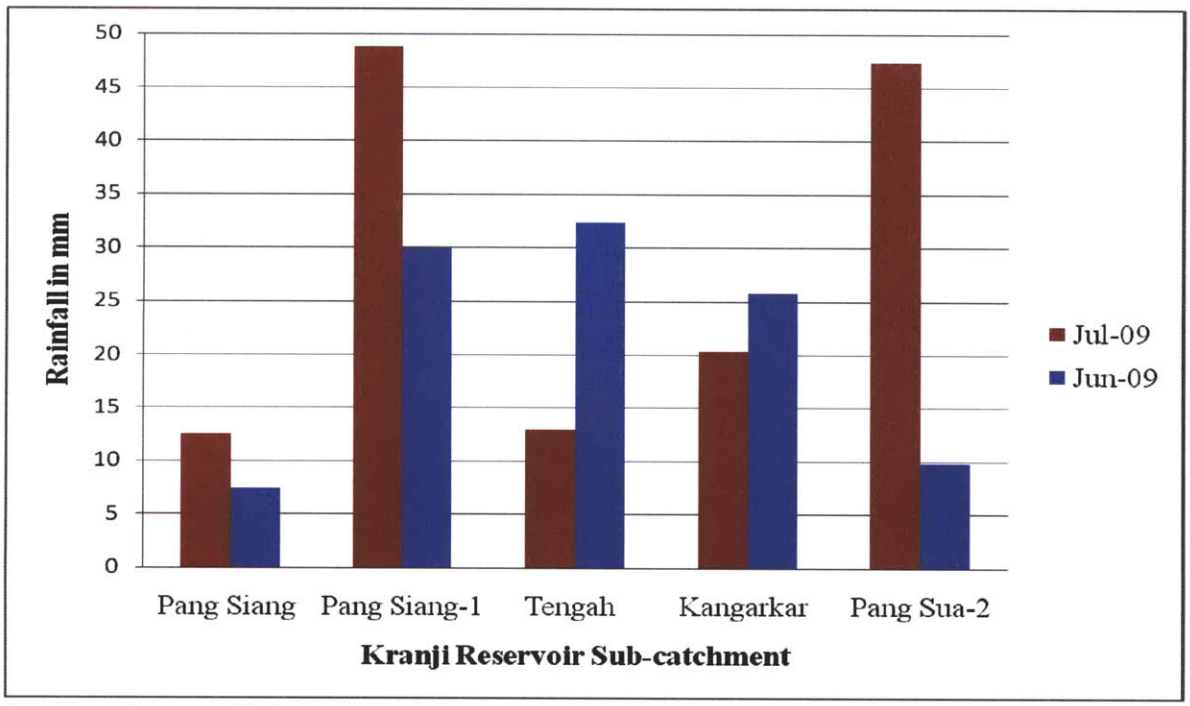


*Figure 4.2 Rainfall data, June-July 2009, Kranji Catchment (NTU 2009)*

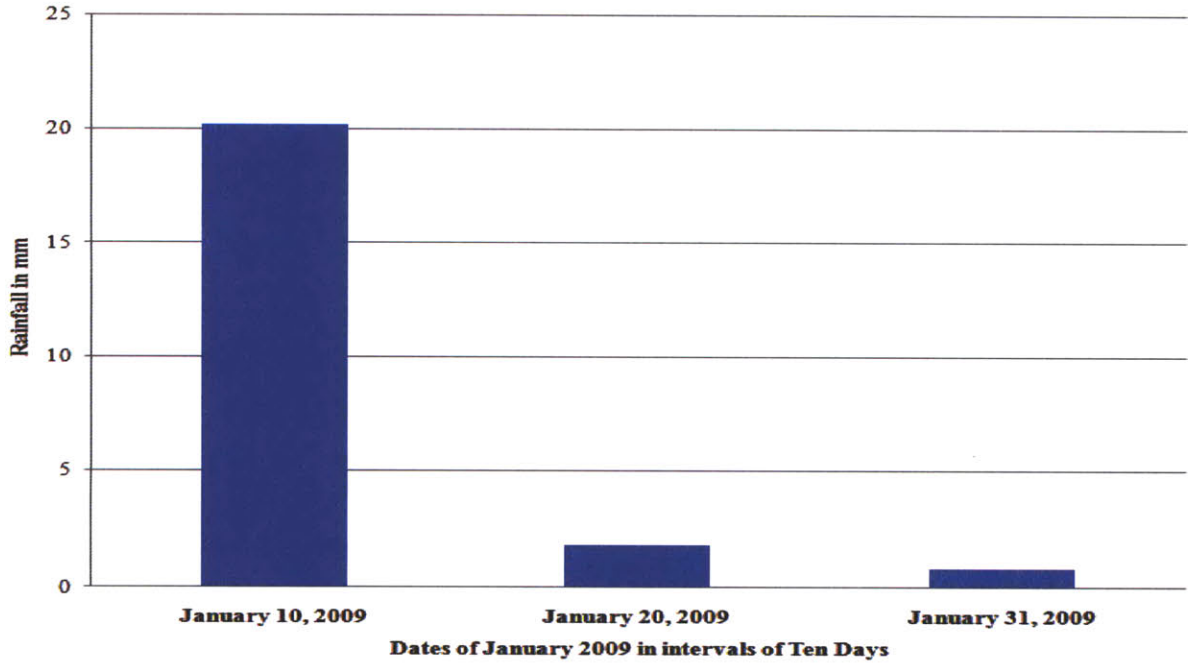


*Figure 4.3 Rainfall Data, January 2009, Kranji Catchment (NTU 2009)*

## 4.2 Field Methods

The field work was carried out to collect samples for the bacteriological analysis of water quality in Kranji Reservoir. Samples were collected during two different seasons: a dry period (January 2009) and a wet period (July 2009). This classification depended on the rainfall data collected during the two periods. During the preparation of the field work we were targeting the wet weather since NTU (2008) research covered the dry weather. However, during the field work of January 2009 the weather was unusually dry (Figure 4.3) and Kranji catchment received low rainfall. Therefore, there was a need to carry out additional investigation during the wet weather. July 2009 was selected as wet period (Figure 4.2) based on the Singapore climate information and additional environmental water samples were collected during this period. Thus, the field method discusses the work done during these two periods (January and July 2009). *E. coli* samples collected in January 2010 were also used in this study to understand the distribution of indicator bacteria in dry and wet weather. January 2010 was a wet period.

### 4.2.1 Geographical International System (GIS)

A Global Positioning System (GPS) device was used in the field to record the geographical location of the sampling sites. ArcGIS Version 9.3 software (ESRI®, 2008) was used to present Singapore GIS data provided by PUB and the sampling locations on the Kranji watershed map. The maps produced included maps of sampling sites (DNA environmental water sampling locations) and results (*E. coli* and HF marker) for January and July 2009. The results illustrated the distribution of HF and *E. coli* in the Kranji Reservoir and the Kranji catchment.

### 4.2.2 January 2009 Field Sampling Location

The aforementioned characteristics of the Kranji Reservoir catchment and previous studies were considered in determining the sampling locations of this study. The main goal of the field work was to obtain samples from the Kranji catchment that are representative of its diverse nature and inhabitants in order to determine the sources of human fecal pollution.

During January 2009, a total of 139 environmental water samples were taken in Kranji Reservoir catchment. These samples were analyzed for total coliforms and *E. coli*. Twenty seven were environmental DNA water samples which were analyzed using a PCR-based technique. Seven blank samples (Appendix A) were also taken as a check on potential contamination during transportation of the environmental water samples from the field to the laboratory and also during the laboratory processing.

These environmental water samples were collected at different locations within Kranji Reservoir catchment and in Kranji Reservoir (Figure 4.4). The approach was to start sampling based on the stations established in the previous study by NTU (2008). Based on these stations we

determined new upstream sampling locations for environmental DNA water samples. The NTU stations considered are seven: KC1, KC2, KC3, KC4, KC5, KC6, and KC7 (Table 4.2). These sampling stations were distributed mainly in residential and farming areas, with two in the military area (KC4 and KC6). The distribution of sampling stations was planned in order to confirm the previous study by NTU (2008) and from these new results determine the areas to focus on for additional upstream sampling.

*Table 4.2 NTU (2008) Sampling Locations and New Name Codes According to Kranji Reservoir Sub-catchments*

| **Kranji Reservoir Sub-catchments** | **NTU (2008) Sites** | **New Name Code (2009)** |
|---|---|---|
| **Neo Tiew** | KC5 | NT and FM |
| **Kangkar** | KC6 | KK |
| **Tengah** | KC4 and KC3 | TH, TA and FM |
| **Peng Siang** | KC1 and KC2 | PS and PB |
| **Pang Sua** | KC7 | PU and PUt |

**TA**: Tengah Airbase, **PB**: Peng Siang Brickland Highway, **PU**: Peng Sua. **PUt**: Pang Sua-Bukit Timah Road, **FM**: Farming (fish and chicken farms) and Horticulture (flowers and vegetables)

DNA samples were taken based on various criteria. A high concentration of *E. coli* and total coliform at a given location was among the selection criteria for DNA sampling locations. In addition, we also included other sampling locations that did not have high colony counts in order to have a suitable representation of the range of environmental DNA water samples within Kranji Reservoir catchment. Table 4.3 and Figure 4.4 describe and present the location of the 27 environmental DNA water samples collected.

Table 4.3 shows details related to the locations in Kranji Reservoir catchment where the DNA samples were taken. As can be seen in Table 4.3, the field notes column explains the field location of each sampling site. The names of samples were generated from the names of the Kranji Reservoir sub-catchments as shown in Table 4.2. Water samples and DNA samples collected in the farming and horticulture areas were represented by FM. The farming areas include chicken and fish farms, whereas, horticulture include areas used for growing flowers and vegetables within the area of Tengah. The areas are mostly located in Neo Tiew and Tengah sub-catchments.

*Table 4.3 Environmental DNA Water Sampling Locations in January and July 2009*

| Names | Sub-Catchments | Field notes |
|---|---|---|
| FM07 | Tengah | Outlet of the Aqua Tropical Fish Farm, Sungei Tengah Rd |
| FM08 | Tengah | Drainage from Farmart fish pond and restaurants, Sungei Tengah Rd. |
| FM05 | Tengah | Drainage from the left end of the chicken farm at the end of Sungei Tengah Rd. |
| FM03 | Tengah | Outlet of the fish farm on the left hand of the Jalan Lekar Rd. |
| FM01 | Tengah | Outlet in the front side of the chicken farm at Neo Tiew Lane 1 |
| FM06 | Tengah | Ditch in front of the aqua tropical fish farm, Sungei Tengah Rd. |
| PB02 | Pang Siang | Upstream KC1, left side, bridge on Choa Chu Kang Ave 3 |
| PB03 | Pang Siang | Upstream KC1, right side, bridge on Brick land Rd |
| PS02 | Pang Siang | Upstream KC2, natural drainage, left side of Choa Chu Kang Loop across block 342 - 341 |
| PS05 | Pang Siang | Upstream KC2 at the right side entrance of the 296 block at Choa Chu Kang Ave 2 |
| PS04 | Pang Siang | Upstream KC2, drainage in front of block 123 left side of Keat Hong SMRT station |
| PS03 | Pang Siang | Upstream KC2, natural drainage, bridge on Choa Chu Kang Ave 1 across Keat Hong BP3 SMRT station |
| TH01 | Tengah | Drainage bridge on Sungei Tengah Road, behind Ton Orchid |
| TA01 | Tengah | Upstream KC4 left side, 400m from the KC4 auto-sampler |
| TA02 | Tengah | Upstream KC4 right side, 400m from the KC4 auto-sampler |
| NT02 | Neo Tiew | Bridge on Neo Tiew Rd, between Bollywood farm and Gan Aquarium fish farm |
| NT01 | Neo Tiew | Bridge on Neo Tiew Road, right end of the Bollywood farm |
| KK02 | Kangakar | Upstream KC6 right side, Lim Chu Kang Rd, 300m away |
| KK03 | Kangakar | Upstream KC6 left side, left side of Lim Chu Kang Rd |
| KK04 | Kangakar | Upstream KC6, left side drainage of Lim Chu Kang Rd, Sunnyville home corner |
| KK05 | Kangakar | Bridge in undeveloped (military) area on Lim Chu Kang Rd, first after KC6 auto-sampler |
| KK06 | Kangakar | Military area, Lim Chu Kang Rd, closer to the camp entrance, Lamppost 191/11 |
| PU02 | Pang Sua | Upstream KC7 right side, block 158 Jalan Teck Whye Ave. |
| PU03 | Pang Sua | Upstream KC7 north-south drainage, left side of the train rail to Singapore from Malaysia |
| PU04 | Pang Sua | Upstream KC7 left side, drainage from the residential area, from Bukit Panjang area |
| ResA | Reservoir | Point located near the Kranji reservoir dam |
| ResB | Reservoir | Point located near the golf course on the Northwest of Kranji Reservoir |
| ResC | Reservoir | Point on the convergence of tributaries from Pang Siang and Tengah in the reservoir |
| ResD | Reservoir | Point inside the Kranji Reservoir on the main drainage from Kangakar |

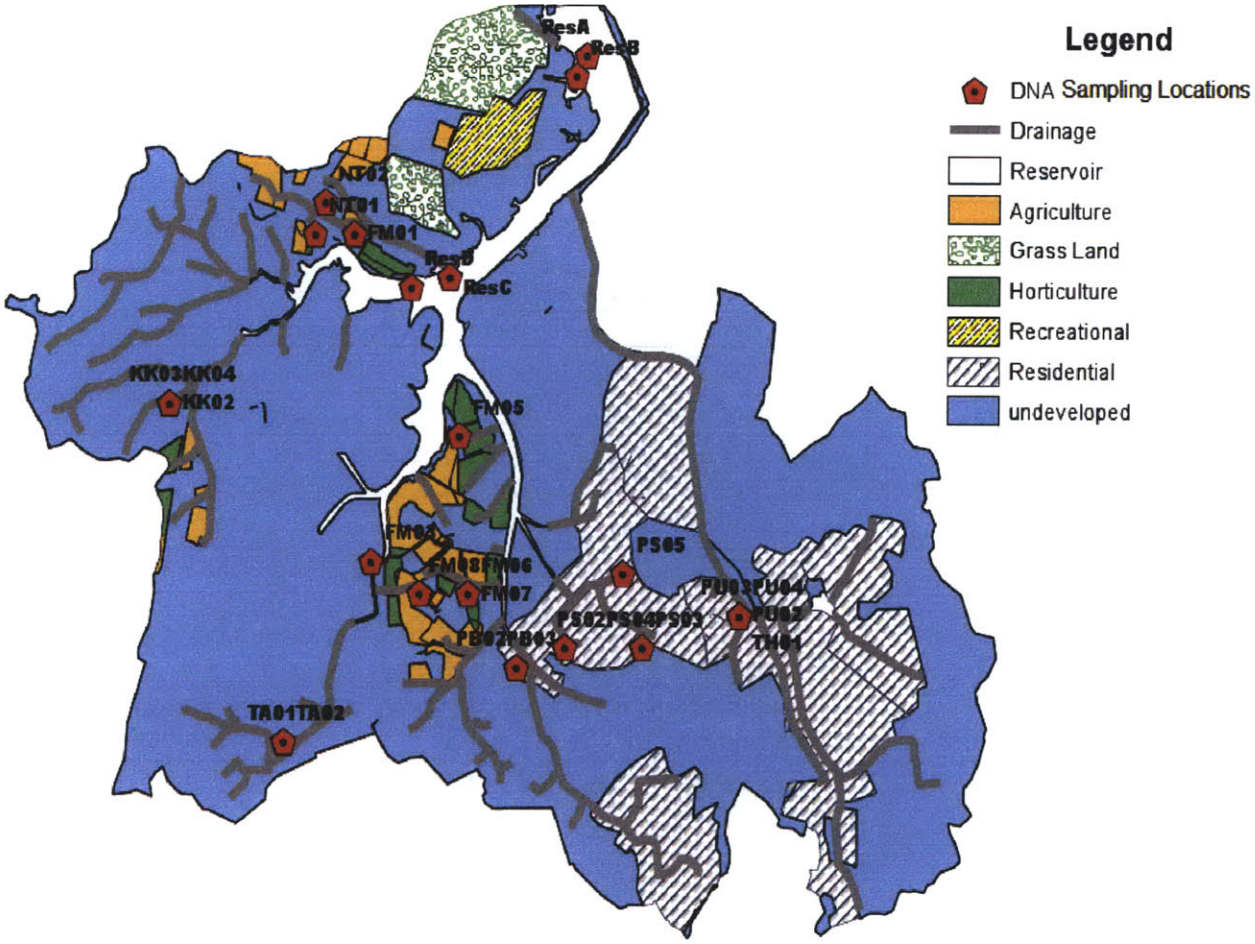


*Figure 4.4 Water and DNA Environmental Sampling Sites, January 2009*

### 4.2.3 July 2009 Field Sampling Locations

The field work of July 2009 was organized to collect samples under weather conditions different than the dry weather of January 2009. July 2009 was a wet period. It was critical to know how the indicator bacteria concentrations (total coliforms and *E. coli*) vary with during wet and dry periods of Kranji catchment. During the field work we collected samples based on the sites that were used in January 2009 and based on the preliminary results of January 2009. Additional upstream sampling locations were added in order to understand obscure results from January 2009.

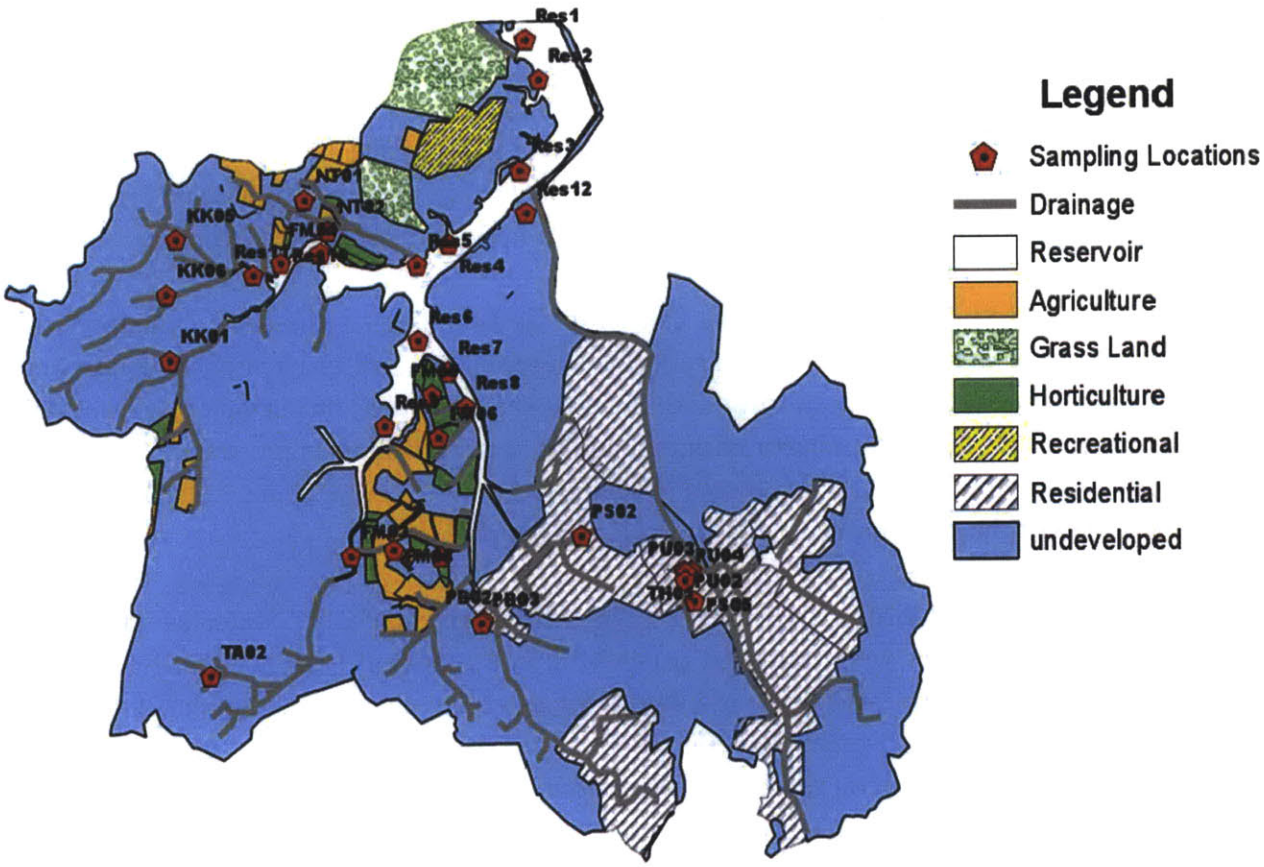


*Figure 4.5 Environmental DNA Water Sampling Stations, July 2009*

During the period of three weeks in July 2009, a total of 168 environmental water samples were taken for total coliform and *E. coli* analysis. The total number of samples includes 34 blanks, 34 duplicates, 94 analyzed for *E. coli* and 58 environmental DNA water samples. Figure 4.5 shows DNA sampling sites of July 2009 and Table 4.3 shows the DNA sampling locations. The 58 DNA samples include also 12 environmental DNA water samples (not represented in Table 4.3) collected in Kranji Reservoir for a better understanding of the distribution of human fecal pollution and to identify potential sources of human fecal contamination that are not associated with the main tributaries of the reservoir. In addition, environmental DNA water samples were taken from a fish pond and sewage treatment plants (STPs). These samples were taken in January 2010 after recognizing a need to use them during the DNA sequence analysis.

## 4.3 Laboratory Methods

### 4.3.1 Environmental Water Sampling Techniques: Water and DNA Sampling Techniques

During the field work, environmental water samples collected were identified by a labeling system that used two stages: labeling the bottle of the environmental water samples and

recording the information of each water sample. Each label of the water sample bottle had the following information: a sample name, type of the sample (DNA), date, hour, minute and GPS coordinates to record the geographical information. Similar information was also recorded on the field sheet that sometimes included a small sketch to record the field situation during the sampling. The samples taken in the reservoir were collected in the depth of at least 1m. Water samples were then transported in a cooler filled with ice from the field to the laboratory.

Blank samples were taken in the field and carried to the laboratory in the same container with other samples to control for cross contamination among samples. All blank sample results were zero colonies for both total coliforms and *E. coli* showing that the samples were safely transported from the field to the laboratory. In addition, a duplicate sample was also taken in accordance with Eaton et al.'s (2005) recommended procedures. The sampling conditions of the duplicate followed the same procedures as the original sample.

Apart from the total coliform and *E. coli* analysis, environmental DNA water samples were taken. The DNA samples were collected using Millipore Sterivex™-GS 0.22μm Filter Units (Figure 4.6). The Sterivex™-GS 0.22μm Filter Units used to collect DNA samples in the field accumulate sediments and bacteria on the membrane incorporated into their interior section. These sediments and the bacteria represent the environmental sample from which a DNA sample is extracted. Theoretically, one liter of water sample should be pumped through the filter in order to have a representative DNA sample. The turbidity of the water in the drainages of the Kranji Reservoir Catchment and the reservoir did not always allow a full liter to pass through before the filter became clogged. The range in the amount of water pumped through the filter was seventy milliliters to one liter. Once collected, the Sterivex™-GS 0.22μm Filter Units containing the DNA samples were kept frozen at negative 80°C.

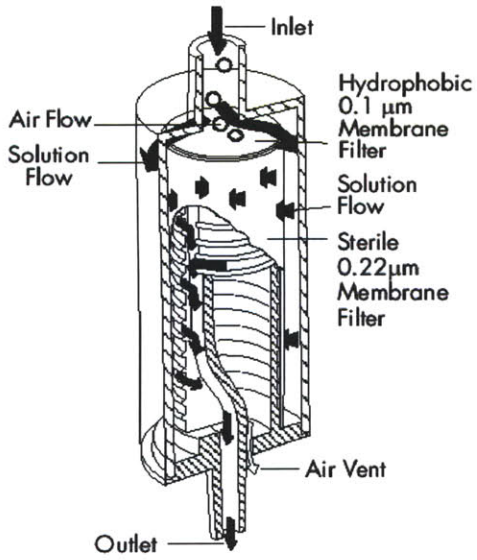


*Figure 4.6 Millipore Sterivex™-GS 0.22µm Filter Unit (Millipore, 2003)*

Environmental DNA water samples were taken from the field and filtered in the laboratory. Environmental water samples were pumped thought the filter using a syringe. The samples were held from 1 hour to 6 hours before they were pumped through the filter. In filtering, a syringe full of water sample is connected onto the filter unit inlet. The quantity of the water filtered is then collected at the outlet and its volume is measured to know the volume passed through the water filter.

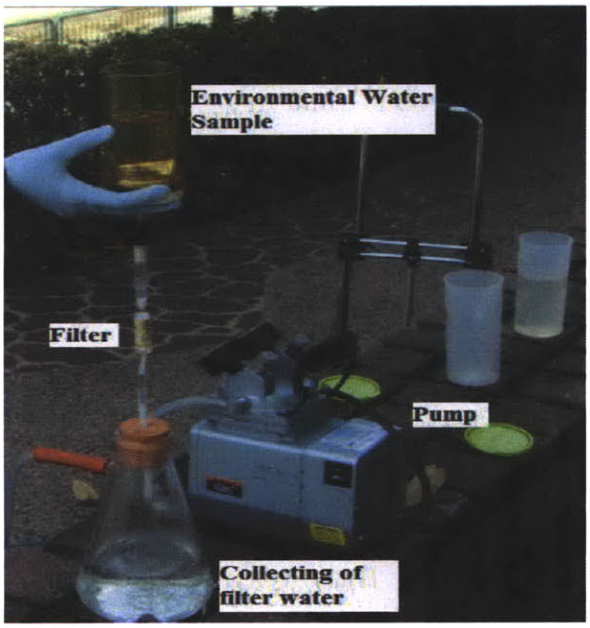


*Figure 4.7 Field Collection of Environmental DNA Water Sample – July 2009*

During July 2009, the method of filtering the environmental water sample was improved allowing the DNA samples to be taken in the field (Figure 4.7). Figure 4.7 demonstrates the method used when collecting DNA samples from the environmental water samples. As mentioned, the standard volume that has to be filtered through Sterivex filter is one liter. The experience of the January 2009 sampling during which only small volumes were filtered through the filter prompted the design of this mechanism that increases the vacuum using a pressure pump powered by a generator. The method helped to increase the volume filtered through, although the turbidity, another major factor, did sometime lead us to using more than one filter in order to filter the appropriate water volume. The environmental DNA water samples were collected in Kranji Reservoir using an AquaStore Model 1010 Niskin water sampler following the manufacturer's protocols (Figure 4.8). This apparatus was used to collect water samples at a depth of 1m within the reservoir.

*Figure 4.8 Niskin Water Sampler (AquaStore, 2005)*

### 4.3.2 Water Analysis

#### *4.3.2.1 Total Coliform and E. coli Analysis Techniques (January and July 2009)*

During January 2009, the water samples were analyzed for total coliforms and *E. coli* using the Hach m-ColiBlue24® method. The Hach m-ColiBlue24® method uses an enzyme-based substrate culture medium and membrane filtration technique. After an incubation period of 24

hours, the total coliform colonies appeared red and *E. coli* appeared blue, and they were then counted (Hach Company 1999). The results of the colony counts were used to find the final concentrations of total coliform and *E. coli* since environmental samples were diluted during the analysis. The reservoir samples did not give reasonable results of *E. coli* because the high turbidity of the reservoir interfered with the m-ColiBlue24 method.

Dilution was performed on the samples analyzed using Hach m-ColiBlue24® (Hach Company 1999) test method. The latter method results are considered valid when the *E. coli* counts range between 12 and 200. However, some of the *E. coli* densities from the first field *E. coli* analysis were out of the range and most of them fall into the range of the too numerous to count. The purpose of the dilution was therefore increasing the range of *E. coli* densities in order to include the high range counts. The dilution sequences used were 1:1, 1:10, 1:100, 1:1,000, 1:10,000, 1:,000,000.
The accuracy of the Hach m-ColiBlue24® method is questionable due to culture medium problems reported by the Hach Company (2008). The company reported a lack of sensitivity in random tests of the product. The communication from the company specified that the problem is associated with the test method giving lower colony counts of *E. coli* than expected (Hach Customer Service 2009). This problem was identified after our use of this product; therefore the *E. coli* concentrations that we obtained may be biased low.

During July 2009 we switched the analysis method from Hach m-ColiBlue24® (Hach Company, 2008) to Colilert Quanti-Tray®/2000 (IDEXX, 2009). The IDEXX Colilert Quanti-Tray®/2000 (IDEXX, 2009) was used to estimate the MPN of total coliform and *E. coli*. Environmental water samples were diluted to in order to get the MPN counts densities in the admissible range of MPN useful for statistical analysis. As with Hach m-ColiBlue24®, the Colilert Quanti-Tray®/2000 uses also an enzyme substrate medium and includes a fluorescent product that is used to identify the *E. coli* under UV light. The total coliforms are identified by a yellow color that appears after the incubation period. The *E. coli* colonies are identified by a blue color that appears under UV light. Figure 4.9 shows the illustration of the Colilert Quanti-Tray®/2000 results from the analysis carried out in July 2009. The total coliform (yellow color) and *E. coli* (blue) Quanti-Tray are labeled. As can be seen, all the wells of the Quanti-Trays are either yellow or blue. This means that the water sample tested had the MPN numbers of > 2,419.6 per 100 ml. This number (>2,416.9) is the upper detection limit of the MPN countable on the tray of Colilert Quanti-Tray®/2000. This value indicates that the dilution is needed or the analyzed water sample has undetermined results.

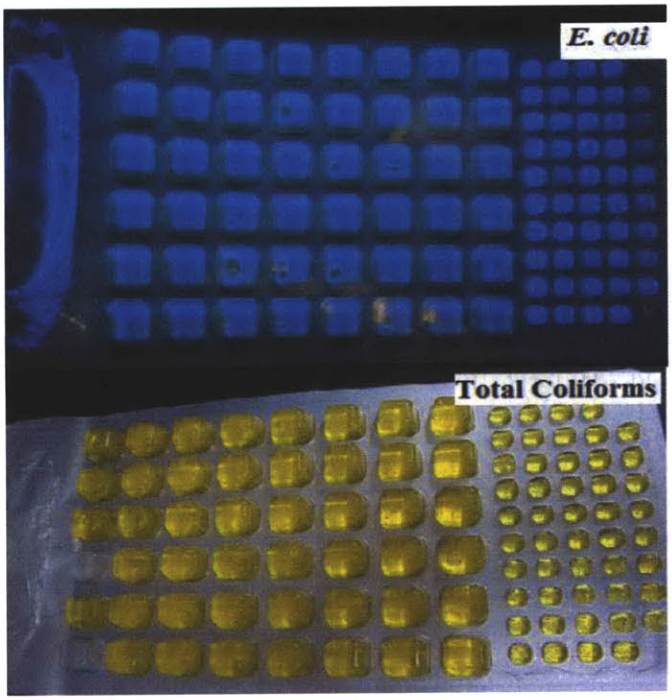


*Figure 4.9 Quanti-Tray/2000 of E. coli (blue color) and Total Coliform (Yellow color)*

#### *4.3.2.2 Environmental DNA Water Sample Analysis: January and July 2009*

##### *4.3.2.2.1 DNA Extraction*

The DNA analysis of samples taken in January and July 2009 was done at the Parsons Laboratory at the Massachusetts Institute of Technology. Samples were shipped from Singapore to MIT on dry ice via overnight delivery service. The laboratory analysis started with extracting the environmental DNA water samples taken in Singapore. This extraction procedure had the purpose of separating the cells from the sediments that were accumulated on the Sterivex filter unit used to collect the DNA sample. In addition, the extraction removed other substances contained in the sediments that could lead to inhibitions in the subsequent analysis. The Ultraclean™ Soil DNA isolation kit protocol (Mo Bio Laboratories, Inc. 2009) was followed according to the manufacturer's instructions and the DNA samples were then stored at negative 20°C before the following analysis (Mo Bio Laboratories, Inc. 2009). The quality of the DNA samples resulting from the extraction was evaluated when these samples were electrophoresed on 1% agarose, stained with ethidium bromide dye, and then observed under UV-light.

##### *4.3.2.2.2 PCR Assay Techniques*

In this study, DNA methods were used to identify the presence of a targeted gene: the 16S ribosomal RNA gene of members of the *Bacteroides Prevotella* group that are found only in humans (Bernhard and Field, 2000). HF183F is generally used to track human fecal pollution

and is therefore called the "human factor." Two different PCR (polymerase chain reaction) assays were used: Touchdown PCR and Nested-PCR. Touchdown PCR was applied to the 27 DNA water samples collected in January 2009, while Nested-PCR was applied to samples collected in January and July 2009. The environmental DNA water samples were subjected to polymerase chain reaction (PCR) to test for the presence of human-specific Bacteroides molecular marker (HF marker). The methods used for the touchdown and Nested-PCR assays were adopted from Bernhard and Field (2000), Shanks et al. (2006), and Santoro and Boehm (2007). The PCR assay reagents used were: (1) buffer solution which provides an appropriate chemical environmental for DNA polymerase consistency and efficient activity, (2) Deoxynucleoside triphosphates (dNTPs) which are the building blocks for new DNA strand production by the DNA polymerase, (3) primer mixture, which contain short DNA sequences that hybridize to the HF marker and direct the polymerase to replicate this specific sequence, (4) *Taq* polymerase which is the enzyme that catalyzes the DNA replication due to its ability to add new nucleotides when copying the existing DNA strand, (5) the DNA sample that carries the target DNA gene to be amplified, and (6) distilled water. The PCR reagents and concentrations used in this study are summarized in Table 4.4.

*Table 4.4 PCR Reagents and Quantities*

| **Reagents** | **Touchdown PCR(µl)** | **Nested-PCR – Bac32F (µl) Step 1** | **Nested-PCR – HF183F (µl) Step 2** |
|---|---|---|---|
| 10xBuffer | 5 | 2.5 | 2.5 |
| dNTP (10 µlM) | 1 | 0.5 | 0.5 |
| Primer mixture (25µM) | 1 | 0.5 | 0.5 |
| Taq Polymerase | 0.5 | 0.125 | 0.125 |
| DNA | 5 | 3 | 1 |
| Distilled water | 37.5 | 18.375 | 20.375 |
| **Total** | **50** | **25** | **25** |

Generally, PCR amplification happens under repeated cycles of three steps that depend on temperature. (1) denaturation consists of denaturing of the DNA at 94°C for two minutes, before PCR, (2) annealing temperatures falls in 50°C and 60°C, this temperature favors the amplification by heating and cooling mechanisms, and (3) extension time, which happens at 72°C for a predetermined time depending on the number of base pairs of genes being amplified. The sequence of these tree different steps of alternative temperature is usually known as the thermocycle reaction. These thermo-cycles are essential in PCR amplification to favor the chemical reactions that support the amplification. The typical cycling process of the Touchdown PCR is illustrated on Figure 4.11: The thermal cycle of the Touchdown PCR included 50 cycles, whereby the 10 first cycles were characterized by one degree Celsius decrease in annealing

temperature per cycle, then stabilized at a constant annealing temperature until the end of the 40 cycles. At the end of the PCR cycle, we electrophoresed 10µl of each DNA sample on the 1% agarose gel to verify the quality of amplicons. Table 4.5 shows primers (HF283F and 708R) used during the Touchdown PCR amplification.

The Nested-PCR had a quite similar thermocycle, which differed from the Touchdown PCR by the absence of temperature variations during the amplification processes and fewer cycles in two separate stages. The thermocycle of the nested PCR used 35 cycles that were organized as illustrated on Figure 4.12. As this amplification is carried out in two stages, Figure 4.12 differentiates the first thermocycle, where the Bac32F primer is used, from the second thermocycle, where HF183F primer (Table 4.5) is used. Notice that the Nested-PCR used 35 cycles for each stage for a combined and a total of 70 cycles, while the Touchdown PCR used 50 cycles in a single stage. Thus the amplification potential in the nested procedure is greater (with higher sensitivity). At the end of the first thermocycle, the positive amplification results and negative control reactions are purified using QIAquick® PCR purification (QIAGEN® 2006). The purified amplicons are then used in the second thermocycle as templates (DNA sample). The results of both thermocycles, Nested and Touchdown PCR, are electrophoresed and compared to the ladder as described in the case of the Touchdown PCR. Table 4.5 shows the primers that have been used during this analysis (Bac32F, HF183F, and 708R).

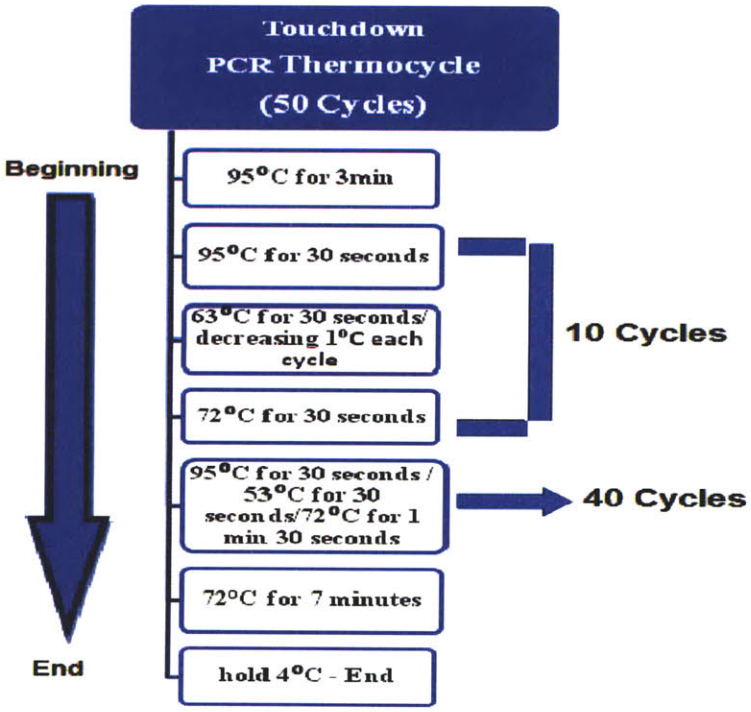


*Figure 4.10 Thermocycle of Touchdown PCR (Applied to January 2009 Samples)*

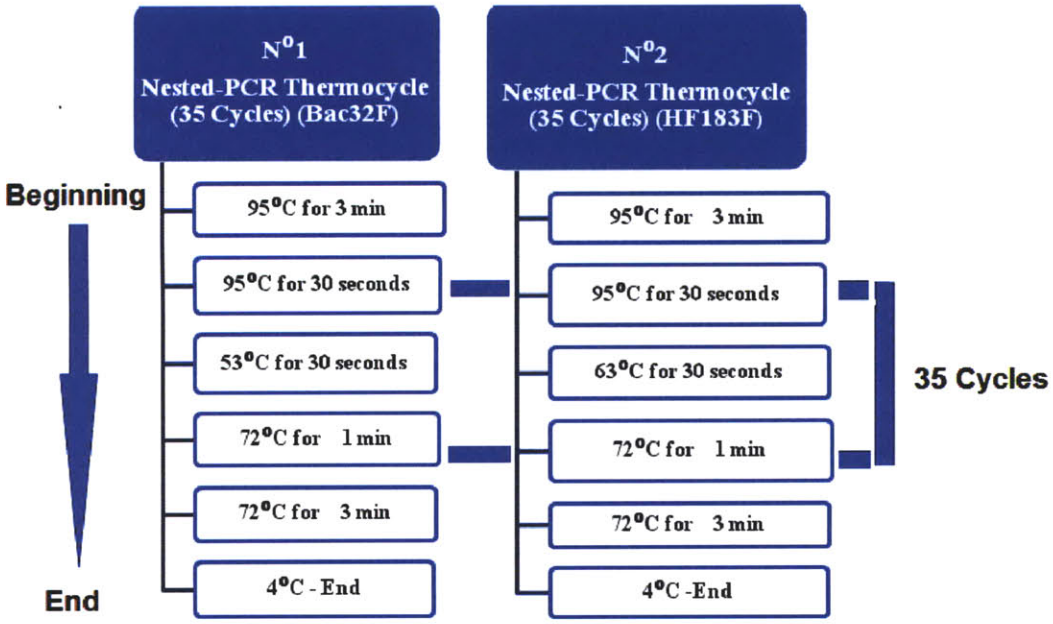


*Figure 4.11 Thermocycle of Nested-PCR (Applied to July 2009 Samples)*

*Table 4.5 PCR Amplification Primers and their Mixture (Fogarty and Voytek 2005)*

| Primer | Target | Sequences (5'-3') |
|---|---|---|
| Bac32F | *Bacteroides-Prevotella* group | AACGCTAGCTACAGGCTT |
| Bac708R | *Bacteroides-Prevotella* group | CAATCGGAGTTCTTCGTG |
| HF183F | Human marker | ATCATGAGTTCACATGTCCG |

The Nested PCR method described above was also used by Shanks and others (2006) to identify nonpoint sources of fecal contamination. The Touchdown PCR method used in this study worked successfully with the January 2009 samples. When this method was used on the DNA samples collected in July 2009 we experienced a streaking (Figure 4.12) problem that prevented to get usable results. A streaking problem is experienced when after the electrophoresis of the amplicons; a straight smear is observed from the well where the sample was loaded. The smear takes place of a consistent band that was predicted to be observed. The Nested-PCR approach was then applied and proved able to overcome the streaking problem. The Nested-PCR was therefore adopted and used for both January and July 2009 environmental DNA water samples analysis.

*4.3.2.2.3 Clone Library Formation*

The DNA environmental water samples that were identified to contain HF183F marker were taken through cloning in order to create a clone library used later for sequencing and phylogenetic analysis. DNA samples with strong bands and from locations of the Kranji

catchment with different characteristics were selected for cloning. We cloned one sample from the residential area, one sample from the outlet of the fish pond, one sample from the STPs and one sample from the reservoir. The Zero Blunt® TOPO® cloning protocol (Invitrogen™, 2006) was used for cloning. $PO_4$ was added at the 5' end in order to favor further reactions. The main stages of this process were PCR assay using the Taq polymerase enzyme, gel purification based on QIAquick® gel extraction kit protocol (QIAGEN 2006), ligation, transformation and PCR using M13 to confirm the clone results. After these steps the end product was used to generate sequences, which were analyzed using the phylogenetic tree. The sequences were analyzed using the Sequencher® 4.10.1 software Version 2009 (Gene Codes Corporation, 2009).

*4.3.2.2.4 Bacteroides Phylogenetic Tree Analysis*

The phylogenetic analysis was done using the Mobyle@pasteur and iTOL (Interactive Tree Of Life) online software for the display and manipulation of phylogenetic trees (http://mobyle.pasteur.fr/cgi-bin/portal.py and http://itol.embl.de/). The sequences generated from the clone library described in Section 4.2.2.2.3 were used to generate the tree. The NCBI blast software (http://blast.ncbi.nlm.nih.gov/) was used to identify the closest metagenomic match, which identifies a specific bacterium with similar characteristics to the studied one. The phylogenetic tree is created through the following main steps: alignment, which uses the MUSCLE (MUltiple Sequence Comparison by Log-Expectation) program (Mobyle@pasteur, 2010) that creates multiple alignments of nucleic acids or protein sequence, and PhyML, which is a simple and fast algorithm used to estimate the phylogenies of big distances (give citation). The product of PhyML was copied onto the itol.embl.de uploading window to have a visual phylogenetic tree.

*4.3.2.2.5 Sensitivity and Specificity Analysis*

The sensitivity determined based on the standard curve established using the positive control and the PCR assay designated for this study as mentioned above. This sensitivity was determined by amplification of known quantities of positive-control DNA containing the human factor (HF) marker.

The specificity of the HF183F assay for identifying the human-specific *Bacteroides* targeted was tested by using negative control DNA containing a homologous non-HF factor *Bacteroides* sequence (clones D01 and 7G03), provided by Jia Y. Har and a positive control (an environmental clone of the PCR 183F-708R DNA cloned into plasmid (PCR2.1)) provided by Professor Alexandra Boehm, of Stanford University. The negative clones were purified using the plasmid DNA purification protocol of the QIAprep Spin Miniprep Kit (QIAGEN). After the extraction these two strains were tested for purity and quality to determine their reliability in providing suitable information. Their concentrations were 285.1ng/µl of D01 and 347.05ng/µl of 7G03.

Known quantities of the positive control of HF marker were used to determine the detection limit of the PCR assay for each environmental sample collected as part of this study. The detection limit was estimated by determining the lowest number of positive control HF marker copies that could be detected by the PCR when spiked into a PCR assay containing the environmental sample.

*4.3.2.2.6 Positive and Negative Control*

During the PCR reaction processes issues related to contamination and inhibition could affect the PCR amplification results. These concerns are addressed by using two experimental controls: the positive and negative controls. The negative control verifies that the PCR mixture was not contaminated during laboratory manipulations and the positive control verifies that the DNA samples do not have any inhibiting substances that affect the amplification. The positive control was a recombinant plasmid (PCR2.1) containing the HF marker (16S ribosomal RNA gene positions 183-708 from an uncultured *Bacteroides*). The plasmid containing the HF marker was prepared as part of the study by Santoro and Boehm (2007) and was generously provided by Prof. A. Boehm, Stanford University. The negative control was distilled water subjected to the HF marker PCR assay. The PCR results of the negative control are supposed to not show a band under UV-light. If the latter shows a band, the PCR mixture is considered contaminated and the PCR results are invalidated. The experiment is then taken afresh.

### 4.3.3 Data Analysis Methods

#### *4.3.3.1 Touchdown PCR Analysis (January 2009)*

The positive control is spiked in the PCR DNA sample tubes in order to check occurrences of inhibition in the PCR assay. To perform sample analysis with estimation of sample detection limits each sample PCR assay is split into three PCR tubes, which are organized as follows:

- The 1st PCR tube contains the PCR mixture and the DNA sample.
- The 2nd contains the PCR mixture, the DNA sample and 10 copies of the positive control. This minimum is also considered as the optimal sensitivity of the assay.
- The 3rd PCR tube contains the PCR mixture, the DNA sample and the positive control corresponding to the upper detection limit, which is estimated at 1000. The PCR results from these three tubes are interpreted as shown in Table 4.22.

Table 4.6 shows the method of reading the DNA sample results as they were observed under UV-light after electrophoreses on agarose 1% gel. The 2nd and 3rd PCR tubes were expected to always show a band because the positive control was spiked into these tubes and was from a sample known to have a high concentration of the human factor. As can be seen in the first row

of Table 4.6, the human factor was recorded as being present in the sample when all the three tubes showed a band (positive sign in Table 4.6). The human factor was considered absent by our analysis when the 1st PCR tube of the DNA sample did not show the band (negative sign in Table 4.6), while other two (2nd and 3rd) containing the positive control showed the bands. When all three tubes did not show the bands, we did not have any conclusion about them because there were inhibitors in the DNA samples.

#### *4.3.3.2 Nested PCR Results Interpretation*

Nested PCR results were interpreted based on the general goals of the method. The main goal was to determine the presence or absence of the HF marker using HF183F assays. The presence of HF marker was confirmed if the DNA sample was positive to both the Bac32F-708R and HF183F-708R primers. The absence of HF marker was confirmed when the sample was positive to Bac32F and negative to HF marker. When both Bac32F and HF183F assays were negative, the DNA sample was considered inhibited

*Table 4.6 PCR DNA Sample Tube Results Interpretation*

| | **DNA Sample** | **Lower Detection Limit ($1 \times 10^1$)** | **Upper Detection Limit ($1 \times 10^3$)** |
|---|---|---|---|
| | **1st PCR Tube** | **2nd PCR Tube** | **3rd PCR Tube** |
| Presence of HF in environmental sample | + | + | + |
| HF absent or below lower detection limit | – | + | + |
| HF absent or below upper detection limit – PCR inhibitors are reducing assay sensitivity | – | – | + |
| No conclusion due to presence of PCR inhibitors in the DNA sample | – | – | – |

Notes: +: Presence of band, –: Absence of band (observed under UV-light) and
HF: Human Factor

# Chapter 5: Results

## 5.1 GIS Mapping

GIS data provided by PUB was used to generate various Kranji watershed maps that illustrate land use (Figure 4.1), sampling locations (Figure 4.4 and Figure 4.5) and the distribution of *E. coli* and HF marker results in the watershed (maps of results in Chapter 5). These maps were also used to identify different trends in the distribution of HF and *E. coli* concentration results with different land use of the Kranji catchment. Maps were generated using ArcGIS Version 9.3 software (ESRI, 2008).

## 5.2 *E. coli* Analysis Results

### 5.2.1 *E. coli* Analysis Results: January 2009

A total of 139 water samples were collected and analyzed for *E. coli* colony counts in three weeks during January 2009 using the Hach m-ColiBlue24® method. Water samples from this area were taken from the outlet drainages that discharge wastewaters from chicken and fish farms and horticulture areas into the public drainage. The maximum *E. coli* count from all 139 samples was 2,900,000 CFU/100 ml from a water sample collected in Tengah drainage (Appendix E). The minimum was zero CFU/100 ml recorded in water samples taken in Kranji Reservoir and some other water samples collected in the residential area and in the Tengah (TA01 and TA02) area.

As can be seen in Figure 5.1, the water samples with concentrations higher than the guidelines were 78% of 131 water samples analyzed for *E. coli* concentration. The map of the *E. coli* concentration results (Figure 5.2) shows also that most of the water samples with high *E. coli* counts were collected in the residential, horticultural and chicken and fish farming areas. Farming and horticulture areas are located in the Tengah and Neo Tiew sub-catchments. The residential area had also one of the six too numerous to count (TNTC) peaks (sample collected in Pang Sua sub-catchment), but generally, residential areas were identified to have *E. coli* concentrations ranging from 1,000 CFU/100 ml to 10,000 CFU/100 ml (Figure 5.1).

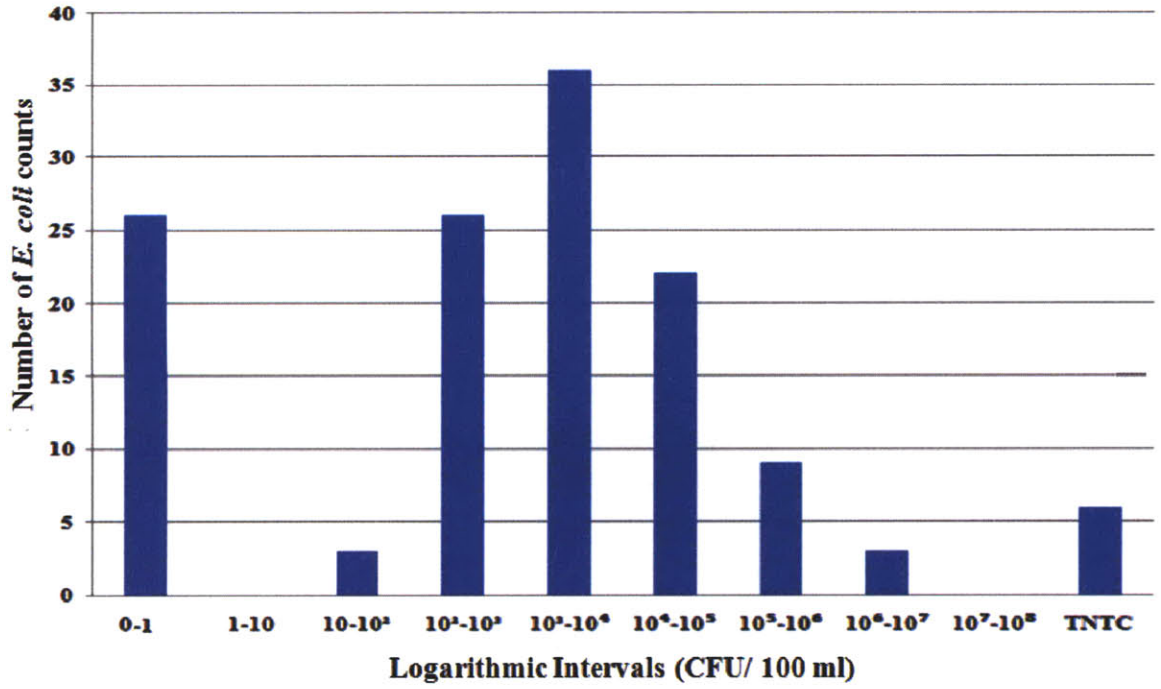


*Figure 5.1 Histogram of E. coli Concentration in January 2009*

**Note TNTC:** Too Numerous To Count.

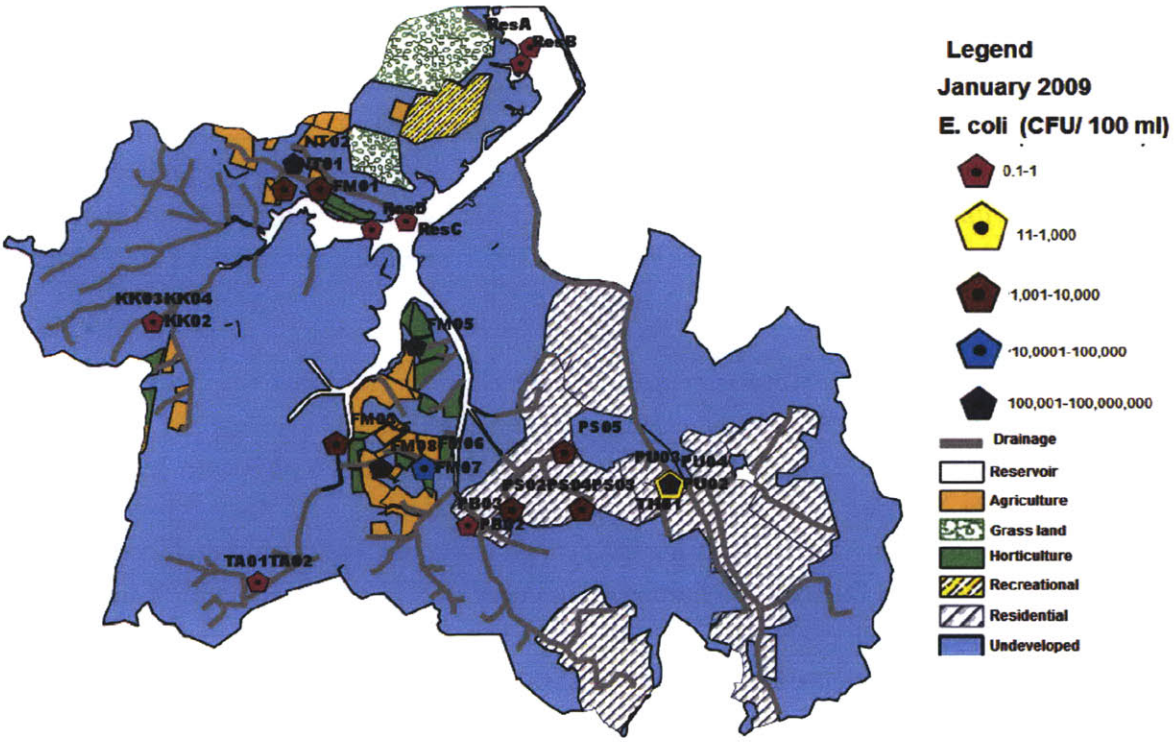


*Figure 5.2 E. coli Concentrations at the DNA Sampling Locations of January 2009*

### 5.2.2 *E. coli* Analysis Results: July 2009 and January 2010

The second part of the field work of this study was carried out in July 2009 and January 2010. One hundred and sixty-eight environmental water samples were collected for *E. coli* and total coliform analysis in July 2009 and an additional twenty-five samples were collected in January 2010. The IDEXX Colilert Quanti-Tray®/2000 (IDEXX, 2010) method was used for the analysis to generate MPN/100ml concentrations of *E. coli* and total coliforms. The total coliforms were not considered for this analysis since they are not a good indicator of human contamination in freshwater as *E. coli.*

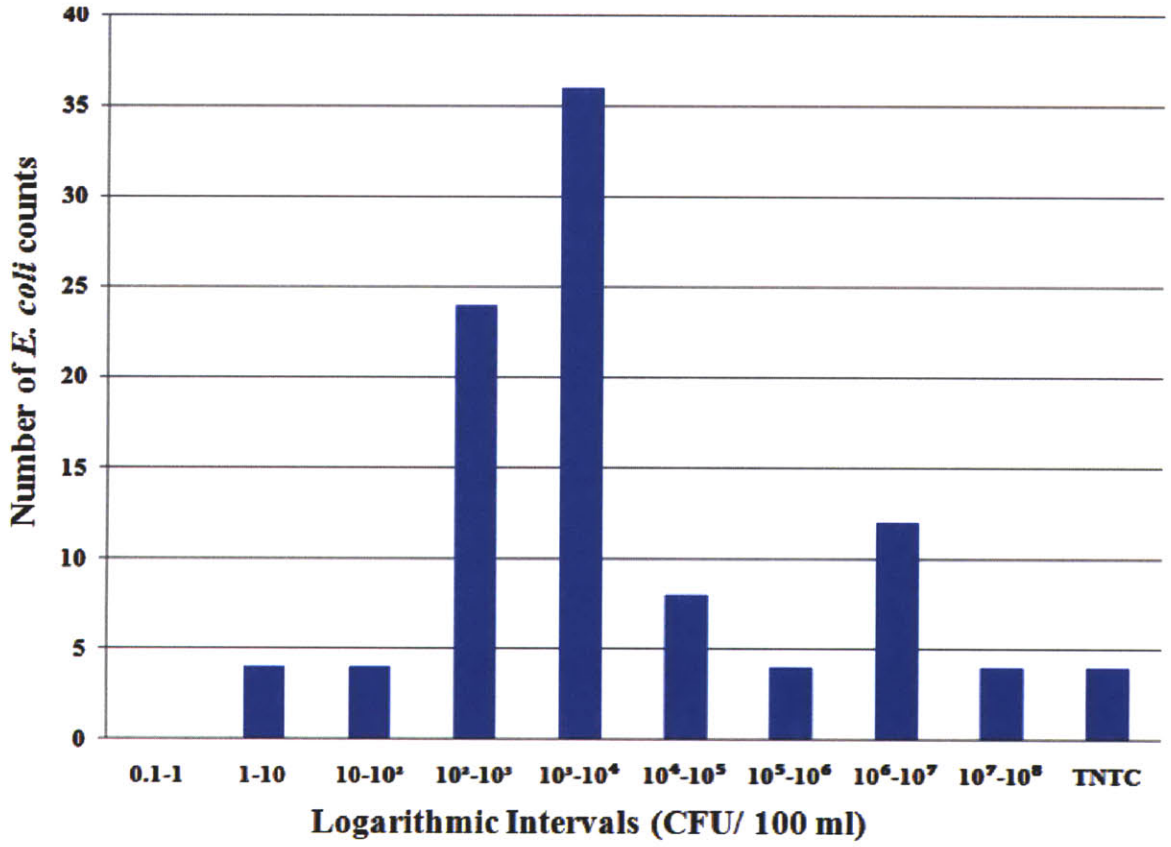


*Figure 5.3 Histogram of E. coli Concentration in January 2010*

The majority of the environmental water samples of July 2009 and January 2010 were collected after or during rainfall events. The overall (July 2009 and January 2010) results of the samples tested demonstrate that 64 (53.7%) of the stations had *E. coli* counts higher than the recreational freshwater limit (235 CFU/100 ml). All categories of watershed land use were identified to be contributors. Residential, horticultural, and chicken and fish farming areas were among the locations that had *E. coli* values above >2419.6 (26%) (Figure 5.3 and Figure 5.4: Noted as TNTC) (Section 4.3.2.1 Chapter 4). The maximum *E. coli* count (after sample dilution) was 241,960 CFU/100 ml and was detected at FM08, which is the outlet of the Farmart fish farm and

commercial area complex located in Tengah sub-catchment on Sungeih Tengah Road. Other high counts were also detected from samples FM02, FM03 and FM04 collected in the same sub-catchment. These water samples had *E. coli* counts of 198,630 CFU/100 ml, 155,310 CFU/100ml and 155,310 CFU/100 ml respectively. The effluent of sanitary facilities collected during January 2010 had the peak *E. coli* concentrations. These samples are BJ820 (2,247,000 CFU/100 ml), BJ856 (2,100,000 CFU/100 ml), BJ800 (17,100,000 CFU/100 ml) and FM08 (2,187,000 CFU/100 ml) (in the range of $10^6$-$10^7$ and $10^7$-$10^8$ on Figure 5.3).

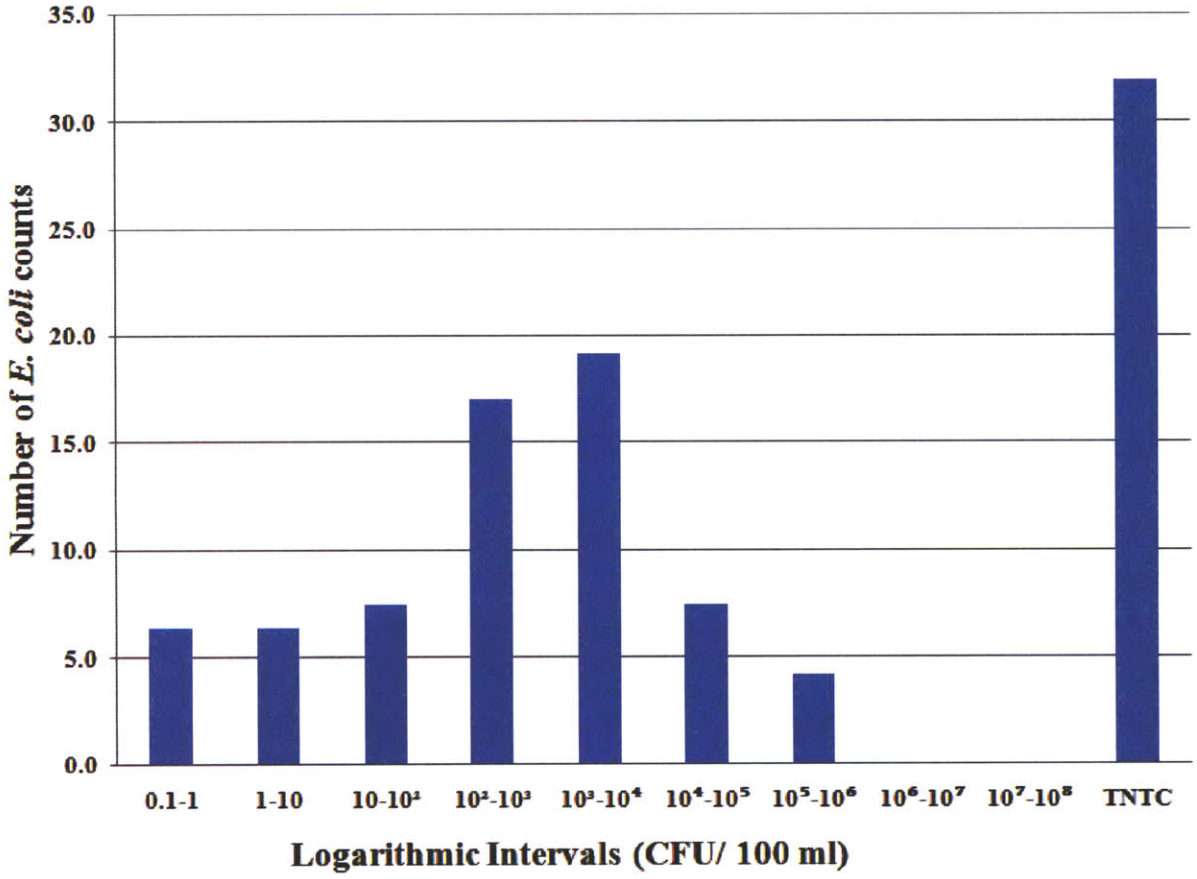


*Figure 5.4 Histogram of E. coli Concentration in July 2009*

During the field work in July 2009, we also saw inappropriate sanitation practices in the Tengah sub-catchment. Figure 5.5 shows a toilet suspended over the drainage system at FM06. The water sample collected at this location had 6,440 CFU/100 ml and was identified positive for the HF marker. This drainage is located behind the Cheng Farm, 98 Sungeih Tengah Road. The owner mentioned that the toilet was used by the farm workers.

All twelve environmental water samples collected in Kranji Reservoir in July 2009 had *E. coli* counts below the recreational freshwater. The majority of the reservoir water samples had zero CFU/100 ml and the maximum was 34.5 CFU/100 ml from the sample collected at Res9 (Table

5.3). The lower colony counts in Kranji Reservoir could be associated with the fact that our samples were taken at a depth of one meter at which solar radiation could contribute to the reduction of *E. coli* concentration. Kleinheinz et al. (2006) for example demonstrated changes in *E. coli* concentration in samples that were taken at different depths of five northern beaches of Lake Michigan (30 cm, 60 cm and 120 cm). The majority of the samples that were taken by Kleinheinz at 120 cm had *E. coli* MPN/100 ml ranged between 0 and 100 MPN/100 ml.

*Figure 5.5 Toilet used by Farm Workers in Tengah Sub-catchment (author and Shawkat Q. looking at the toilet) (Picture by Syed Alwi B.S.H.A)*

Figure 5.6 illustrated the distribution of measured *E. coli* concentrations during the month of July 2009. As can be seen, the peak readings (100,000-100,000,000 MPN/100 ml) were recorded in water samples collected from the horticultural and farming area (Tengah sub-catchment). The minimum concentrations (0-1 MPN/100 ml) were observed in water samples collected in Kranji Reservoir. The majority of the residential areas water samples collected had concentrations ranging between 1,000-10,000 MPN/100 ml. The water samples collected in Neo Tiew sub-catchment were also high and ranged between 10,000 and 100,000 MPN/100 ml.

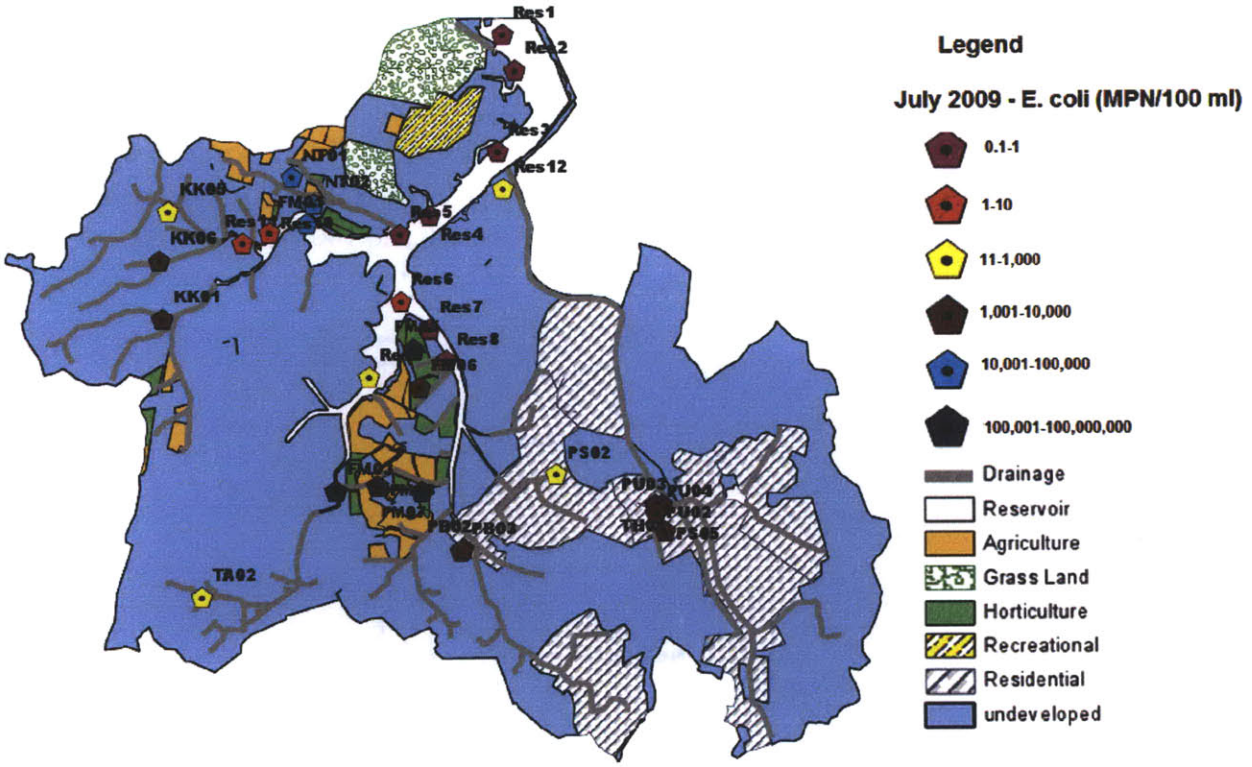


*Figure 5.6 E. coli Concentrations in Water Samples Collected in July 2009*

## 5.3 Environmental DNA Water Analysis Results (January and July 2009)

### 5.3.1 DNA Extraction Results

All the environmental DNA water samples collected were of good quality after extraction. The DNA extraction results are represented by the example shown in Figure 5.7. Figure 5.7 shows pictures of the UV-light observation of selected DNA samples. An extraction of good quality is shown by the band of the samples, which is above and to the right of the left-hand side band of alternative light and dark color usually called the "ladder." The ladder represents the possible range in the size of molecular fragments of DNA, and is of known size and quality. When an extracted DNA sample has a band lower than the maximum band of the ladder then there is concern that the DNA sample may have been deteriorated.

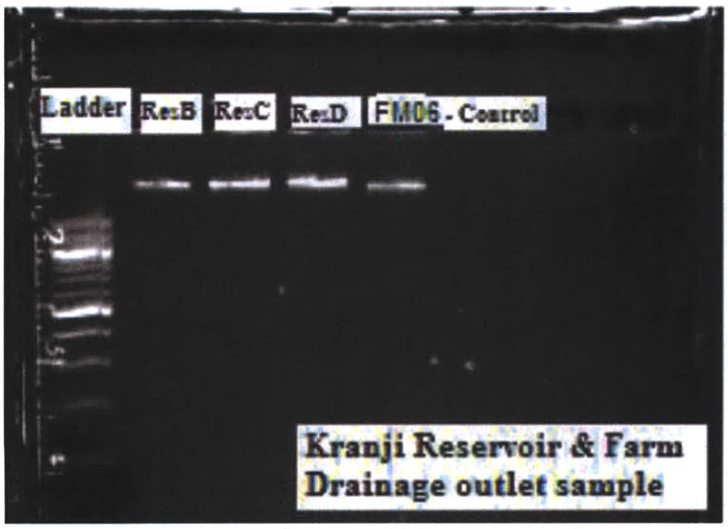


*Figure 5.7 Picture of Some of the Extracted DNA Samples Electrophoresed on 1% Agarose Gel and Observed under UV-Light (January 2009)*

### 5.3.2 HF Marker Assay Verification

#### *5.3.2.1 Sensitivity*

The quantity of human factor target genes that could be optimally detected per PCR assay was determined to be 10 copies per microliter (10/µl) (Figure 5.8) using Nested-PCR. This sensitivity was determined by amplification of known quantities of positive-control DNA containing the human factor (HF) marker. This assay sensitivity was higher than the published HF marker assay sensitivities that range from 100 copies per PCR assay (Shanks et al. 2006) to 100,000 copies per PCR assay (Bernhard and Field 2000). Touchdown-PCR was able to detect only 1000 copies per microliter.

#### *5.3.2.2 Specificity*

All PCR assays of the negative control (D01 and 7G03) run to test against the host-specific primer HF183F showed negative response. All PCR assays of the positive control (a PCR 183F-708R cloned into plasmid PCR2.1) run against the human-specific marker showed positive results. The specificity of the HF183F in identifying the human *Bacteroides Prevotella* gene was thus confirmed (Section 4.3.2.2.5 of Chapter 4).

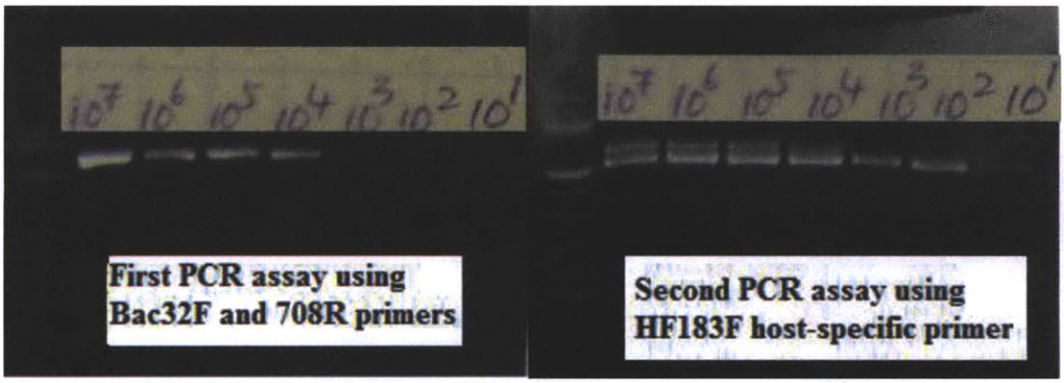


*Figure 5.8 Nested-PCR – Electrophoresis Gel Picture of the Standard Curve of Detection Limit. Left image: Initial PCR using Primers Bac32F and 708R and indicated concentration of positive control template. Right image: second "nested" PCR using Primers HF183F-708R and an aliquot of the first PCR as template. Both the no-template control (0 copies/µl) and the second "nested" PCR did not manifest a PCR band, indicating the PCR was free of contamination (data not shown).*

#### *5.3.2.3 Phylogenetic Tree*

The major goal of the phylogenetic analysis was to confirm the specificity of the HF183F assays used in identifying the HF group of *Bacteroides* and to also confirm its applicability under a tropical climate. The phylogenetic analysis was therefore performed on two (PU04 and FM05) DNA environmental water samples collected in January 2009 in Kranji catchment. The first was PU04 collected in the residential area of Bukit Panjang in the sub-catchment of Pang Sua. The second was FM05 collected in the outlet drainage of the chicken farm at the end of Sungei Tengah Rd in the Tengah sub-catchment. These samples were selected in order to include different land uses and because both were positive for the human factor marker amplified by the HF183F primer. After the Touchdown PCR assay amplification, human-specific *Bacteroides* molecular marker clone libraries were generated and 36 clones from each sample library were randomly selected to be sequenced. Out of a total of 72 sequences, 68 sequences were of good quality and were therefore used for phylogenetic tree generation.

Before generating the phylogenetic tree (Figure 5.9), these 68 sequences were processed and analyzed using Sequencher® to check the diversity in the human factor marker. The Sequencher software at 99% threshold following the methodology of Santoro and Boehm (2007) was used to classify sequences in different groups according to similar operational taxonomic units (OTUs). The analysis classified these sequences into six clusters where some sequences were grouped under three groups with the same operational taxonomic units ("Contig" on the phylogenetic tree in Figure 5.5) and three others classified under individual OTUs.

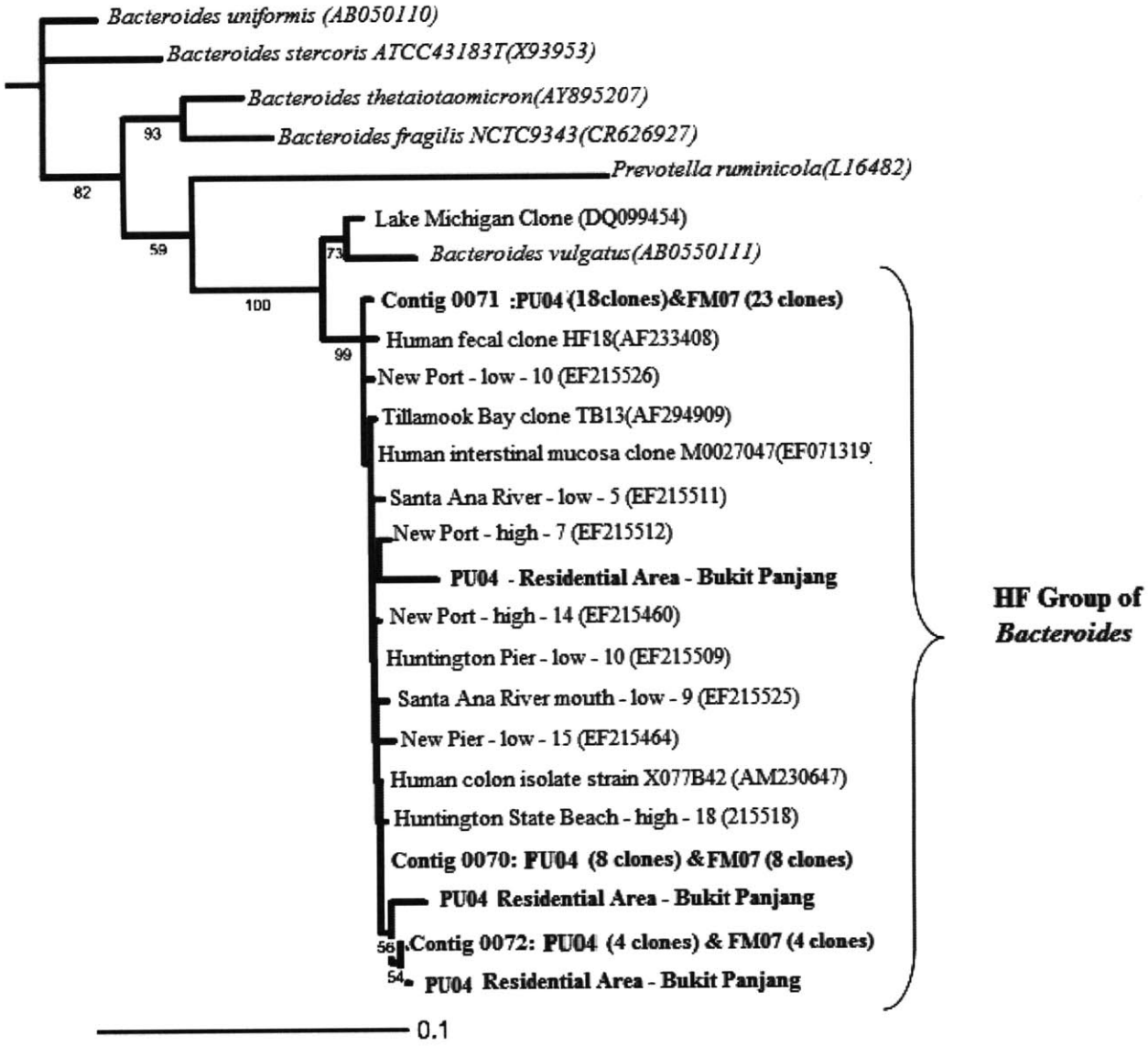


*Figure 5.9 Phylogenetic Tree of 16S rRNA Gene Segment for Fecal Bacteroides*

**Note:** Names in bold type are the clones generated from two Kranji Catchment samples: PU04 from the residential area and FM05 from the farming area. Remaining names indicate referencing clones from Santoro and Boehm (2007). The numbers on the tree branches indicate the bootstrap values. The bootstraps below 50% were not represented on the tree.

The 99% threshold was used in order to have similar conditions as the reference sequences of Santoro and Boehm (2007). Under the 99% threshold, the Sequencher analyses led to six OTUs. These six clusters are in bold in Figure 5.9 and labeled as follows: three contig (contig 0070, contig 0071 and contig 0072) and three individuals (branch cluster of PU04). Contig clusters contain both PU04 and FM05. Finally, the 68 sequences were analyzed using NCBI Blast

software (NCBI, 2010) to identify the closest matches to each sequence. The peak of the reads was 99% similar to the human microbiome, GQ493974, which appeared in the metagenomic study of Turnbaugh et al. (2009).

The phylogenetic tree generated (Figure 5.9) shows that the sequences of Kranji catchment environmental samples are very closely related to other samples with a human fecal origin and other environmental clones obtained using HF183F primer. This demonstrates the HF183F test was specific for the targeted organisms. Although the Santoro and Boehm's (2007) sequences are among the closest database matches, the detailed analysis through all clusters shows that the HF sequences from Kranji catchment have a different genetic diversity compared to Santoro and Boehm's sequences. However, the difference in the genetic diversity is expected given that the sequences were obtained from different environments. The difference in genetic diversity is shown by the fact that four of the six groups are classified in branch clusters on the bottom of the phylogenetic three. The contig 0071 group and the individual PU04 sequence group are classified in similar branch clusters with the Santoro and Boehm (2007) sequences. This suggests that the HF groups of *Bacteroides* found in Singapore, a tropical climate, are closely related to HF found under temperate climate by Santoro and Boehm (2007).

### 5.3.3 Touchdown PCR Assay Results: DNA Samples Collected January 2009

During January 2009, 27 DNA environmental water samples were collected and analyzed using Touchdown and Nested PCR. Table 5.1 shows results for these 27 DNA samples and Figure 5.10 presents them on the Kranji catchment map. As can be seen in Table 5.1, nine of the 27 samples (33%) were identified positive to the HF marker.

These positive samples are distributed throughout the different sampling areas of Kranji Reservoir catchment, but most of them were found in the farming area (Neo Tiew and Tengah), where five of the six samples were positive. This could be due to the combined drainage system (combination of sanitary wastewater, farm wastewater, and storm water into the same discharge drain) inside the farms (FM01, FM05, and FM07) and the direct discharge of septic tank wastewater into the main drainage (FM06 and FM08). The four remaining positive samples are from residential areas, the reservoir, and the drainage from the restricted military area (NT02) where we were not able to know the activities inside in order to understand the probable sources of this human factor maker.

*Table 5.1 DNA Analysis Results January 2009 (PCR)*

| Sampling Locations | *E. coli* (CFU/ 100ml) | DNA Results | | | Interpretation |
|---|---|---|---|---|---|
| | | HF Marker | Detection Limit ($10^3$) | Detection Limit ($10^5$) | |
| FM01 | 3700 | + | – | + | Presence of HF |
| FM03 | 1500 | – | + | + | Absence of HF |
| FM05 | TNTC | + | + | + | Presence of HF |
| FM06 | 100,000 | + | + | + | Presence of HF |
| FM07 | 400,000 | + | + | + | Presence of HF |
| FM08 | TNTC | + | + | + | Presence of HF |
| PB02 | 0 | – | – | + | Absence of HF at upper detection limit |
| PB03 | 0 | – | – | + | Absence of HF at upper detection limit |
| PS02 | 2100 | – | + | + | Absence of HF at assay sensitivity |
| PS03 | 1100 | + | – | + | Presence of HF |
| PS04 | 7900 | – | – | + | Absence of HF at upper detection limit |
| PS05 | 4900 | – | – | + | Absence of HF at upper detection limit |
| TH01 | 200 | – | – | + | Absence of HF at upper detection limit |
| TA01 | 0 | – | – | – | Presence of inhibitors |
| TA02 | 0 | – | – | – | Presence of inhibitors |
| NT01 | 500 | – | – | + | Absence of HF at upper detection limit |
| NT02 | 290,000 | + | + | + | Presence of HF |
| KK02 | 0 | – | – | + | Absence of HF at upper detection limit |
| KK03 | 0 | – | – | + | Absence of HF at upper detection limit |
| KK04 | 0 | – | – | + | Absence of HF at upper detection limit |
| PU02 | 400,000 | – | – | + | Absence of HF at upper detection limit |
| PU03 | 500 | – | – | – | Presence of inhibitors |
| PU04 | 1000 | + | + | + | Presence of HF |
| ResA | 0 | – | + | + | Absence of HF at assay sensitivity |
| ResB | 0 | + | + | + | Presence of HF |
| ResC | 0 | – | – | + | Absence of HF at upper detection limit |
| ResD | 0 | – | – | + | Absence of HF at upper detection limit |

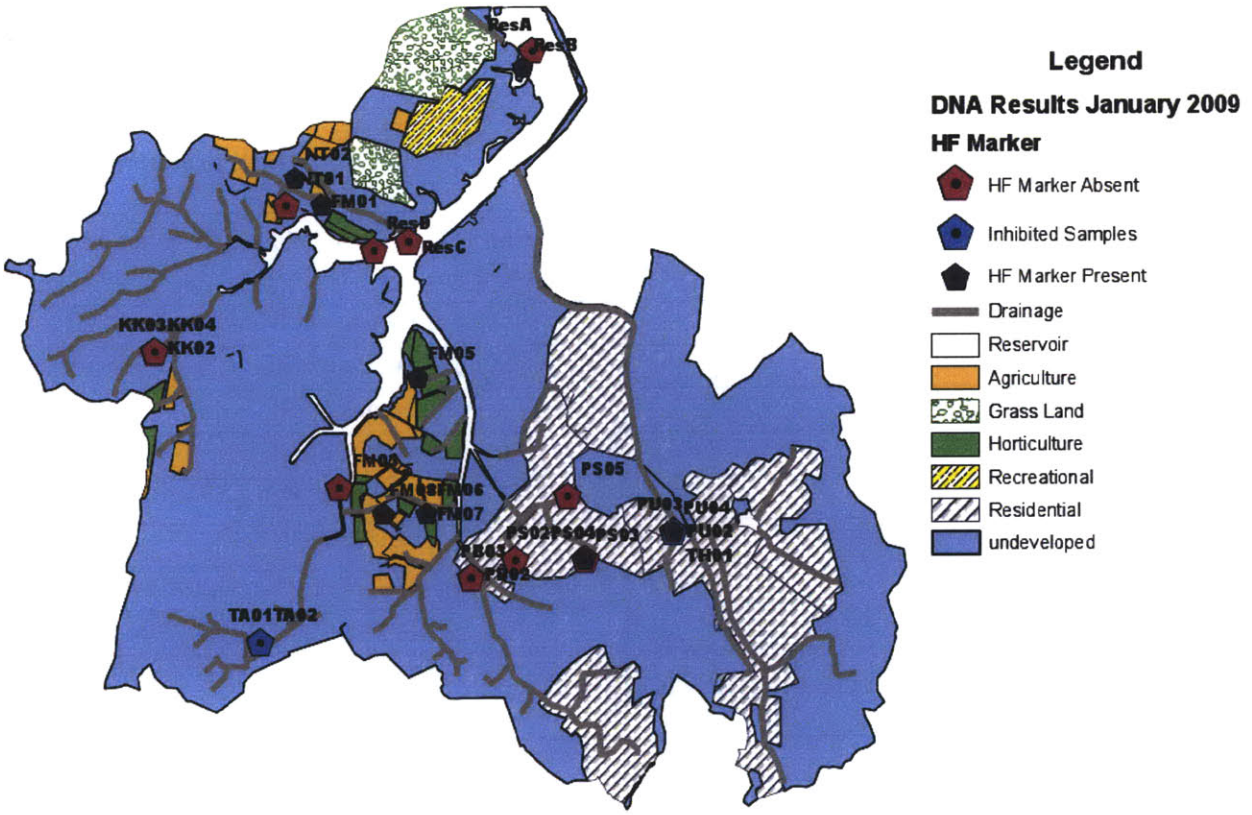


*Figure 5.10 Results of DNA Environmental Samples Collected January 2009 in Kranji Catchment (Results of Touchdown RCR Assays)*

The two positive residential sampling locations (PS03 and PU04) were identified as having sewage odor during field sampling, but these odors do not indicate the exact sources of the human factor marker. It is possible that the positive samples in the residential area could be due to leaking sanitary sewers. The reservoir sample that was identified as positive is located near the Kranji Dam (ResB). According to Dr. Maszenan bin Abdul Majid of NTU, camping activities and intense movement of workers with poor sanitary behavior in this area may be among the causes behind this presence of the human factor marker (Maszenan 2009).

Although nine of the DNA samples showed positive results by the Touchdown PCR assay, three (TA01, TA02 and PU03) of the 27 showed questionable results. The PCR assays of these three samples showed complete PCR inhibition (Table 5.1). PCR inhibition can be caused by contaminating molecules that are not removed sufficiently during the DNA extraction procedure. Furthermore, natural inhibitory factors may include high levels of humic acids or plant- or algae-derived organic matter. The data suggest that a substance existing in the KC4 sub-catchment (TA: Tengah Airbase station) water sample was capable of inhibiting the PCR amplification, but we did not carry out any chemical analysis of the water samples to identify the substance.

The Touchdown PCR HF183F assay was successfully applied to the DNA water samples collected in January 2009, but presented smear streaking patterns with the July 2009 DNA samples. These streaking patterns resulted in rejecting the method and using the Nested PCR for the July 2009 DNA environmental water samples.

### 5.3.4 Nested PCR Assay Analysis Results: DNA Samples Collected January 2009

The nested PCR assay detection limit (10 copies per microliter) was intended to be used only for the environmental DNA water samples collected in July 2009, but its high sensitivity in detecting the HF183F marker motivated a reanalysis of the January 2009 samples. Table 5.2 and Figure 5.11 show the results of the nested PCR assay applied to the January 2009 DNA water samples. As can been seen in Table 5.2, 20 of the 27 samples (74%) were identified positive for the HF marker. The positive samples were distributed in all different sampled tributaries of Kranji catchment. Four (FM01, FM03, PS02 and ResA) (Figure 5.11) of the 27 samples were confirmed negative for HF, while three were identified inhibited (TA01, TA02 and PU03). The inhibited samples were the same as defined by the first method (Touchdown PCR assay). FM03 was the only sample that was negative for Bac32F and HF183F. It was also confirmed negative for the HF marker by the Touchdown PCR assay.

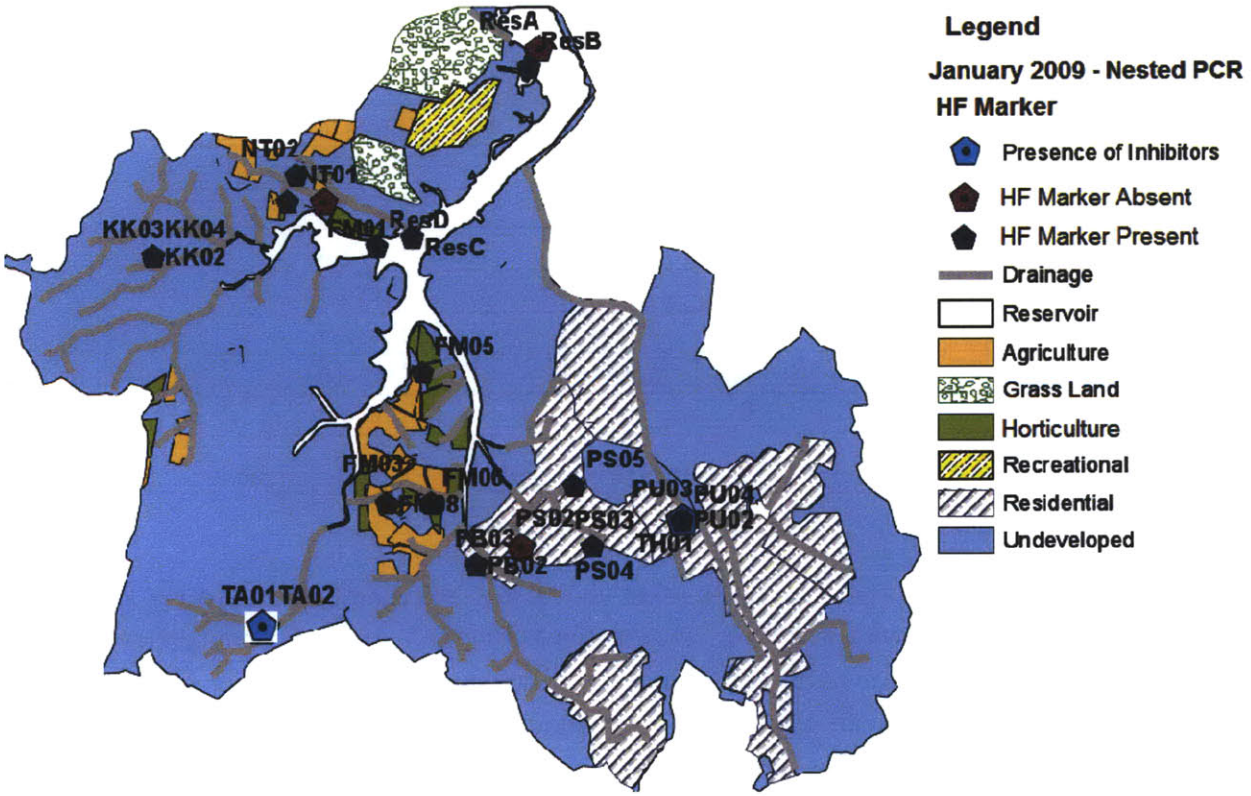


*Figure 5.11 DNA Results of Environmental Samples Analyzed with Nested PCR – January 2009*

*Table 5.2 DNA Analysis Results January 2009 (Nested PCR)*

| Sampling Location Name | Alternate Location Name | *E. coli* (CFU/100ml) | Bac32F Primer | HF183F Primer | Interpretation |
|---|---|---|---|---|---|
| FM01 | F5 | 3700 | + | – | Absent |
| FM03 | F4 | 1500 | – | – | Absent |
| FM05 | F3 | TNTC | + | + | Present |
| FM06 | F4A | 100,000 | + | + | Present |
| FM07 | F1 | 400,000 | + | + | Present |
| FM08 | F2 | TNTC | + | + | Present |
| PB02 | KC1.1 | 0 | + | + | Present |
| PB03 | KC1.2 | 0 | + | + | Present |
| PS02 | KC2.3 | 2100 | + | – | Negative |
| PS03 | KC2.6 | 1100 | + | + | Present |
| PS04 | KC2.5 | 7900 | + | + | Present |
| PS05 | KC2.4 | 4900 | + | + | Present |
| TH01 | KC3.1 | 200 | + | + | Present |
| TA01 | KC4.2 | 0 | – | – | Inhibited |
| TA02 | KC4.3 | 0 | – | – | Inhibited |
| NT01 | KC5.5 | 500 | + | + | Present |
| NT02 | KC5.4 | 290,000 | + | + | Present |
| KK02 | KC6.6 | 0 | + | + | Present |
| KK03 | KC6.1 | 0 | + | + | Present |
| KK04 | KC6.2 | 0 | + | + | Present |
| PU02 | KC7.3 | 400,000 | + | + | Present |
| PU03 | KC7.4 | 500 | – | – | Inhibited |
| PU04 | KC7.5 | 1000 | + | + | Present |
| ResA | R1/ResA | 0 | + | - | Present |
| ResB | R6/ResB | 0 | + | + | Negative |
| ResC | R2/ResC | 0 | + | + | Present |
| ResD | R3/ResD | 0 | + | + | Present |

The increased prevalence of positive HF marker should not be regarded as alarming since it is not necessarily correlated with risk as a relationship between *Bacteroides* concentrations and elevated human health risk has not been established. The increase in the number of positive HF marker sites is associated with the use of a method with higher sensitivity. This higher sensitivity is likely the reason that samples previously identified as negative by the Touchdown PCR were viewed as positive by the Nested PCR assay. The results of four DNA samples taken in the reservoir showed that three of them were positive by Nested PCR (ResB, ResC to ResD)

(Figure 5.11) and one negative (ResA). The negative sample was closer to the Dam. These results are consistent with Yeager and Kerigan's (2009) attenuation results. They predicted that the concentration of bacteria (*E. coli*) reduces from the confluence of the main tributaries of Kranji Reservoir to the dam at the reservoir's downstream end.

### 5.3.5 Nested PCR Assay Analysis Results: DNA Samples Collected July 2009

Table 5.3 and Figures 5.12 and 5.13 present the results of the Nested PCR assay used to analyze the DNA environmental water samples collected in July 2009. A total of 59 DNA samples were taken around Kranji catchment during July 2009. Thirty-two DNA environmental water samples were analyzed in the laboratory. We prioritized the samples from the sampling locations used in January 2009 and added five sites to increase the diversity of the area sampled. As can be seen in Table 5.3, 30 samples (93%) were identified positive for the HF marker. Two of the 32 analyzed DNA samples were negative. No sample was identified inhibited.

The majority of the DNA water samples were collected during or slightly after (1-2 hours) rainfall events. This factor could have increased the chance of collecting environmental water samples with high concentrations of fecal content carried by runoff. As can been seen in Table 5.3 and on Figure 5.13 the samples positive for the human factor are distributed throughout all the sub-catchments of the watershed.

The environmental DNA water samples collected in the undeveloped areas were also positive for the human factor (KK01, KK05, and KK06). Although the sub-catchment of Kangakar is characterized as undeveloped, it is covered with a tropical forest used for military training. Since the area is not allowed to civilians we did not have a chance to explore the possible causes of fecal contamination. All samples from Tengah sub-catchment were also identified positive for the human factor.

Figure 5.12 is a photograph of the electrophoresis gel 1% of the second Nested PCR of HF183F assays of July 2009 DNA samples. As can be seen in Figure 5.12, the strong white bands represent samples that are positive to the HF marker. The figure shows also Sample TA02, which does not have any strong white band. This illustrates that HF marker is absent in the environmental DNA water sample collected at T02. In addition, this sample is one of the two samples that were tested negative to HF marker, the other being Res10. The ladder is used as a reference to generate the size (number of base pairs) of the band. During the DNA analysis were interested with band with size ranging between 500–600 bp. The white strong bands recognized on the picture are therefore of size ranging between 500–600 bp, which is consistent with the size of HF marker that we intended to observe from a successfully amplified positive DNA sample.

*Table 5.3 DNA Analysis Results July 2009 (Nested PCR)*

| Sampling Locations | *E. coli* (MPN/100 ml) | Bac32F Primer | HF183F Primer | Interpretation |
|---|---|---|---|---|
| FM01 | 11,060 | + | + | Present |
| FM04 | 155,310 | + | + | Present |
| FM05 | >2419.6 | + | + | Present |
| FM06 | 6440 | + | + | Present |
| FM07 | >2419.6 | + | + | Present |
| FM08 | 241,960 | + | + | Present |
| PB02 | 649 | + | + | Present |
| PB03 | 92,080 | + | + | Present |
| PS02 | 125.9 | + | + | Present |
| PS05 | >2419.6 | + | + | Present |
| TH01 | 27,500 | + | + | Present |
| TA02 | 200 | + | - | Absent |
| NT01 | 16,000 | + | + | Present |
| NT02 | 88,200 | + | + | Present |
| KK01 | 7500 | + | + | Present |
| KK05 | 40 | + | + | Present |
| KK06 | 30 | + | + | Present |
| PU02 | 1119.9 | + | + | Present |
| PU03 | 285 | + | + | Present |
| PU04 | 720 | + | + | Present |
| Res1 | 0 | + | + | Present |
| Res2 | 0 | + | + | Present |
| Res3 | 0 | + | + | Present |
| Res4 | 3 | + | + | Present |
| Res5 | 0 | + | + | Present |
| Res6 | 2 | + | + | Present |
| Res7 | 0 | + | + | Present |
| Res8 | 0 | + | + | Present |
| Res9 | 34.5 | + | + | Present |
| Res10 | 5.2 | - | - | Absent |
| Res11 | 4.1 | + | + | Present |
| Res12 | 11 | + | + | Present |

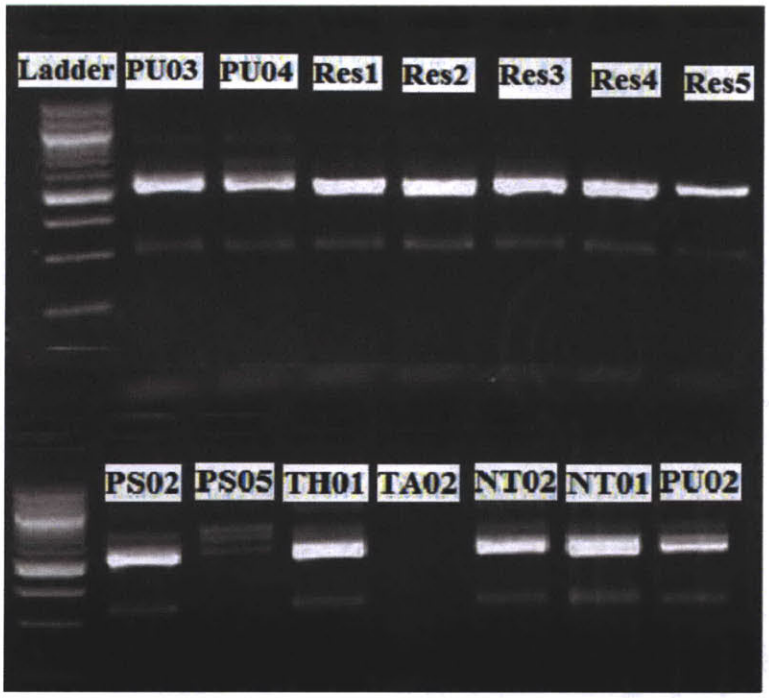


*Figure 5.12 Electrophoresis Gel Picture of Some of the Nested PCR Results of July 2009 DNA Water Samples*

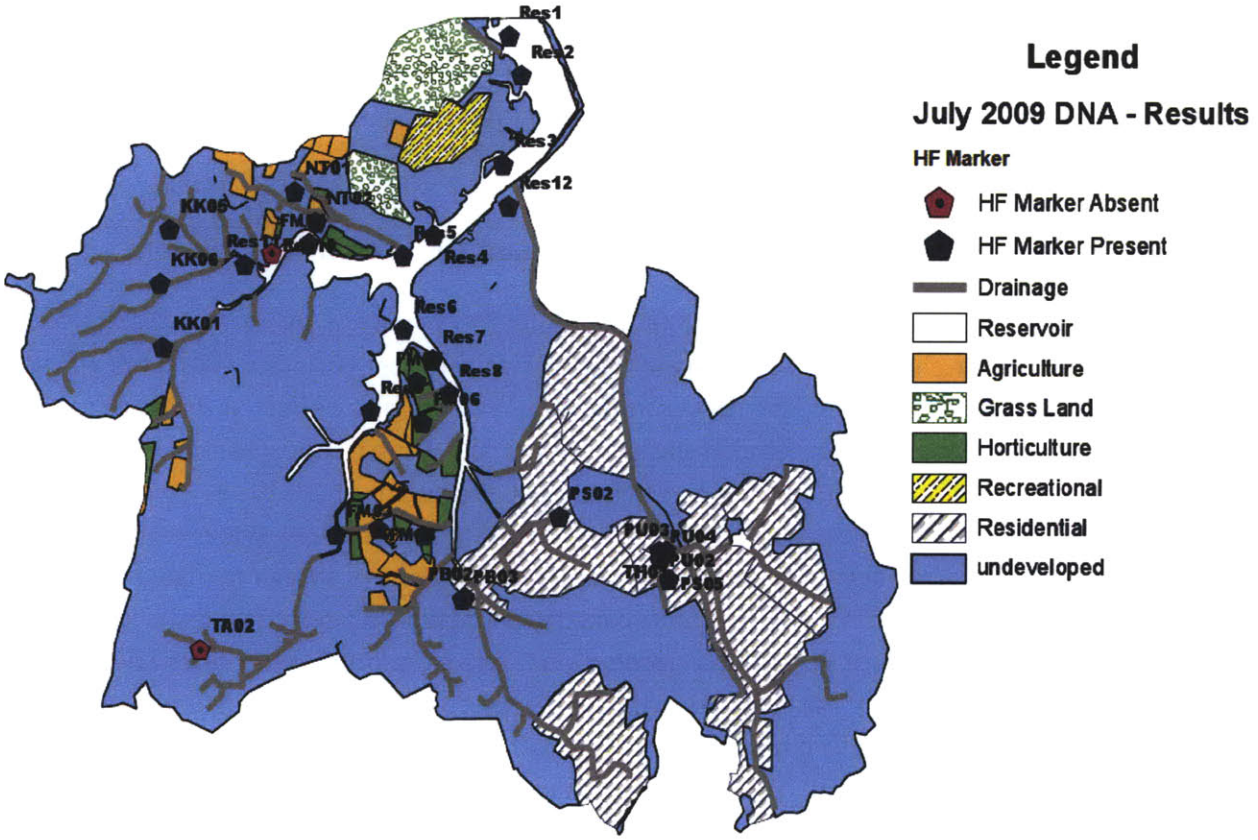


*Figure 5.13 Results of DNA Analysis July 2009 (Nested PCR)*

The majority of the drainages sampled in the Tengah sub-catchment receive discharges from a combined drainage system in which rainfall runoff is mixed with domestic sanitary wastewater. We suspected that the positive results were caused by sanitary wastewaters. This was also the case of the DNA environmental water samples collected in January 2009 in the same locations. Both Nested PCR and Touchdown PCR assay results proved that FM07, FM08, FM01, and FM06 were positive for the human factor marker. The results of the July 2009 DNA samples at TA01 and Res10 were negative. Samples from TA01 were identified inhibited during the analysis of January 2009 samples. Other samples that were identified inhibited (TA02 and PU03) did not have inhibition in the July 2009 analysis. In addition, samples that were identified negative (PS02) by both PCR assays applied to the January 2009 samples were positive when re-sampled in July 2009. The reason behind these changes could be attributed to seasonal variations between the two periods. As shown by the rainfall data, January 2009 was almost completely dry, while July 2009 had intense rainfall events that started in June 2009 (Section 4.1.2).

The percent of Nested PCR positive sites increased from 74% to 93% from the drier month of January (20 positive sites /27 total) to the wetter month of July (30 positive sites / 32 total) which is consistent with a model of increased fecal contamination from rainfall runoff, however a higher sampling frequency would be needed to determine whether a correlation existed. The 33% frequency of HF-positive sites detected in January 2009 by Touchdown PCR could not be compared to the July 2009 results by Nested PCR since the two methods have different sensitivities (1000 copies/µl and 10 copies/µl respectively). Positive results at sites using the less sensitive Touchdown PCR assay may suggest sites with higher concentrations of the HF marker for human fecal pollution.

## 5.4 Correlation of Land Use, *E. coli* Concentration and HF Marker Results

The locations that were sampled January 2009 and July 2009 were of interest to this analysis since they could reflect changes in water quality within two different periods (wet and dry). We also compared the presence of the HF marker in the 27 DNA samples collected in January 2009 with the *E. coli* concentrations measured at the same sites. We wanted to assess if the high the *E. coli* concentration could mean the presence of HF marker, bearing in mind that *E. coli* may grow in the environment under tropical conditions. The 27 environmental DNA water samples were analyzed for both *E. coli* concentration and HF marker presence. As shown in Table 5.1 some sampling locations had zero CFU/100ml *E. coli* concentration, but they tested positive for the HF marker. Figure 5.14 shows *E. coli* concentrations results plotted with the HF marker presence/absence results from the Touchdown PCR. As can be seen in Figure 5.14, water samples with the highest *E. coli* counts tended also to be positive for the HF marker. Closer analysis of Figure 5.14 shows that 100% of the samples with *E. coli* above $10^5$ CFU/100 ml and 64% of samples with *E. coli* count above $10^3$ CFU/ 100 ml were positive to HF marker. The

elimination of inconclusive samples among water samples analyzed to have *E. coli* concentration below $10^3$ CFU/100 showed that only 10% were positive to HF marker. Interestingly, some water samples that were identified to have small low counts of *E. coli*, (i.e. in the range of water recreational guidelines <235CFU/100 ml), were identified to be positive for the HF marker.

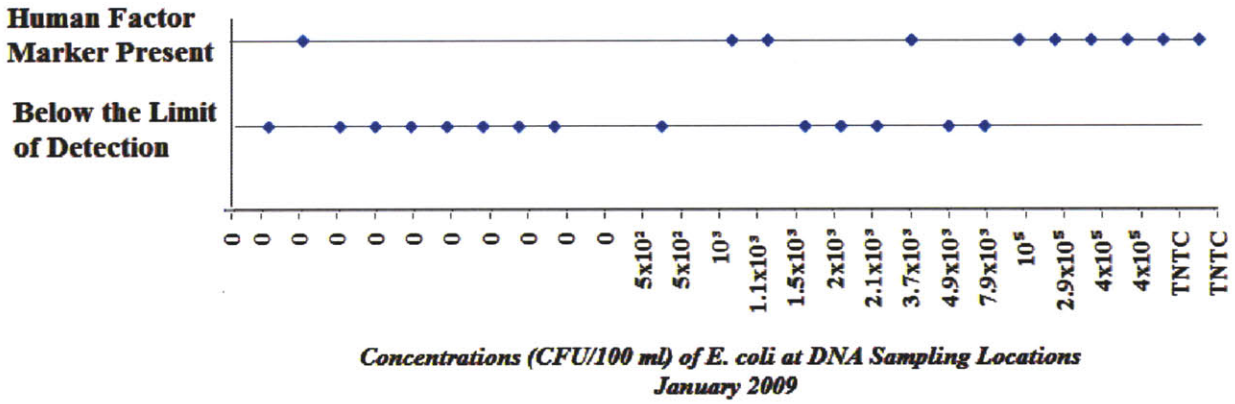


*Figure 5.14 Comparison of E. coli concentration and Presence of HF Marker for January 2009*

The results obtained using the Nested PCR for the January 2009 DNA water samples were also compared to the *E. coli* concentrations in January 2009. Table 5.2 shows that FM03 (1,500 CFU/100 ml), FM01 (2,100 CFU/100 ml), PS02 (2,100 CFU/100 ml), PS04 (7,900 CFU/100 ml), PS05 (4,900 CFU/100 ml), and PU02 (400,000 CFU/100 ml) had elevated *E. coli* densities, but the DNA analysis of the same water samples was identified negative to the HF marker based on the Touchdown PCR analysis results. Another observation is that water samples with zero colony counts were also identified as positive for HF marker as was the case for the Touchdown PCR results.

Table 5.4 shows *E. coli* concentration results and Nested PCR results from sampling locations used in both January 2009 and July 2009. As can be seen in Table 5.4, these results are distributed over different sub-catchments of the Kranji Reservoir catchment including samples from the reservoir itself. The sub-catchments were therefore classified under four main groups of land use: undeveloped, animal farming, horticultural and residential areas. Residential area is located in two sub-catchments: Pang Siang and Pang Sua: animal farming and horticultural areas are located in two sub-catchments: Neo Tiew and Tengah: and undeveloped area is located in Kangarkar sub-catchment. The distribution of *E. coli* concentrations in these areas suggest that the peak *E. coli* concentrations shifted from residential areas during dry weather to farming areas in wet weather. The peak *E. coli* concentration was 2,900,000 CFU/100 ml (PU03) detected in January 2009 (dry period) in the drainage from the residential area of Pang Siang sub-catchment. The peak *E. coli* concentration in July 2009 (wet weather) was 241,960 CFU/100 ml detected from the farming area (FM08) of Tengah sub-catchment.

*Table 5. 4 E. coli Concentrations and DNA Analysis Results January and July 2009 (Similar Sampling Locations)*

| Names | January 2009 (CFU/100 ml) | July 2009 (CFU/100 ml)* | | HF Marker (January 2009) | HF Marker (July 2009) | Sub-Catchments (number of samples) |
|---|---|---|---|---|---|---|
| PB01 | 87 | 1732.9 | ↑ | | | Pang Siang (9) |
| PB02 | 0 | 284 | ↑ | + | + | |
| PB03 | 0 | 1413.6 | ↑ | + | + | |
| PS01 | 2900 | 30,900 | ↑ | | | |
| PS01 | 4300 | 1119.9 | ↓ | | | |
| PS02 | 2100 | 125.9 | ↓ | - | + | |
| PS03 | 1100 | 2419.6 | ↑ | + | | |
| PS04 | 7900 | 1732.9 | ↓ | + | | |
| PS05 | 4900 | >2419.6 | | + | + | |
| KK01 | 10,700 | 7500 | ↓ | | + | Kangarkar (4) |
| KK02 | 0 | 159.7 | ↑ | + | | |
| KK03 | 0 | 613.1 | ↑ | + | | |
| KK04 | 0 | 307.6 | ↑ | + | | |
| TA01 | 0 | 435.2 | ↑ | - | | Tengah (10) |
| TA02 | 0 | 200 | ↑ | - | - | |
| TH01 | 500 | 27,500 | ↑ | + | + | |
| TH02 | 290,000 | >2419.6 | | | | |
| TH03 | 400,000 | >2419.6 | | | | |
| FM03 | 1500 | 155,310 | ↑ | - | | |
| FM05 | 100,000 | >2419.6 | | + | + | |
| FM06 | 400,000 | 6440 | ↓ | + | + | |
| FM07 | TNTC | 833.5 | | + | + | |
| FM08 | TNTC | 241,960 | | + | + | |
| PU02 | 1500 | 1119.9 | ↓ | + | + | Pang Sua (3) |
| PU03 | 2,100,000 | 285 | ↓ | - | + | |
| PU04 | 650 | 720 | ↑ | + | + | |
| NT01 | 500 | 16,000 | ↑ | + | + | Neo Tiew (3) |
| NT02 | 1000 | 88,200 | ↑ | + | + | |
| FM01 | 3700 | 11,060 | ↑ | - | + | |
| Res01 | 0 | 0 | | _ | + | Reservoir (4) |
| Res02 | 0 | 0 | | + | + | |
| Res05 | 0 | 0 | | + | + | |
| Res06 | 0 | 2 | ↑ | + | + | |

* Arrow indicates direction of change in concentration from January to July

The shift in the peak concentration may be attributable to the dilution mechanism that changes with the variation in rainfall. The residential areas with large impervious areas are likely to favor collection of rainfall, which increases the degree of water dilution in drainages. However, this dilution is also limited by the high concentration of *E. coli* in runoff. The undeveloped area of Kangarkar had samples with lower *E. coli* concentration in dry weather (January 2009), while the concentration increases slightly in wet weather (July 2009). In general, the residential and farming areas had high *E. coli* counts for both periods, dry and wet, while the undeveloped areas had lower *E. coli* densities in dry weather, then slightly high densities in wet weather (Table 5.4).

HF marker results (Nested-PCR) showed that all samples collected in residential in July 2009 were positive for the HF marker. The January 2009 results showed that two of the samples collected in the same areas were negative (PS02 and PU03). PU03 was the samples with high *E. coli* concentration in the same month. The touchdown PCR results confirmed that this sample was inhibited. PS02 had *E. coli* concentration of 2100 CFU/100 ml. The results from the sub-catchment with animal farming and horticultural activities were positive in wet weather (July 2009) to the HF marker except TA02 (200 CFU/100 ml). The samples collected from the undeveloped area (Kangarkar) under dry weather (January 2009) had a lower density of *E. coli* (0 CFU/100 ml), but they were positive to the HF marker (KK02, KK03 and KK04). This inconsistence of *E. coli* density and HF marker confirmed the absence of correlation between both indicators. Generally, the results suggested that HF marker and *E. coli* cannot be correlated to each other, and moreover, that these two indicators are distributed in all of the land use categories. The high densities of *E. coli* could not necessarily mean the presence of HF marker since at PU03location HF marker was absent while *E. coli* concentration was 2,100,000 CFU/100 ml. However, their distribution is persistent in dry and wet weathers in residential, animal farming and horticultural area.

The correlation of *E. coli* concentrations in January 2009 (dry season) and July 2009 (wet season) was examined. 28 sampling locations were included in the analysis as 5 locations supported *E. coli* counts that were above the countable range (either To Numerous To Count (TNTC) CFU/100 ml (January 2009) or >2419.6 MPN/ 100 ml (July 2009)). For samples that were negative for *E. coli* (0/100ml) a detection limit of 1/100ml was used in the analysis. The Pearson coefficient R (0.64) suggested that the *E. coli* counts of the dry weather (January 2009) were correlated to the *E.coli* counts of the wet period (July 2009). As can been seen, on Figure 5.15 the Kranji Reservoir samples were negative for *E. coli* during both dry and wet weather with the exception of one sample that supported low levels (2 MPN/100ml) during the wet season. The undeveloped area had *E. coli* counts below the detection limit (<1 CFU/100 ml) for the period of January 2009, while the counts increased during wet weather. The *E. coli* counts residential, horticultural and animal farming areas had *E. coli* counts higher compared to the Kranji Reservoir and the undeveloped areas *E. coli* counts. This analysis was based on two different analysis methods were used to generate *E. coli* counts (membrane filtration in January

and the MPN method in July). The use of a single method may increase the correlation at these sites.

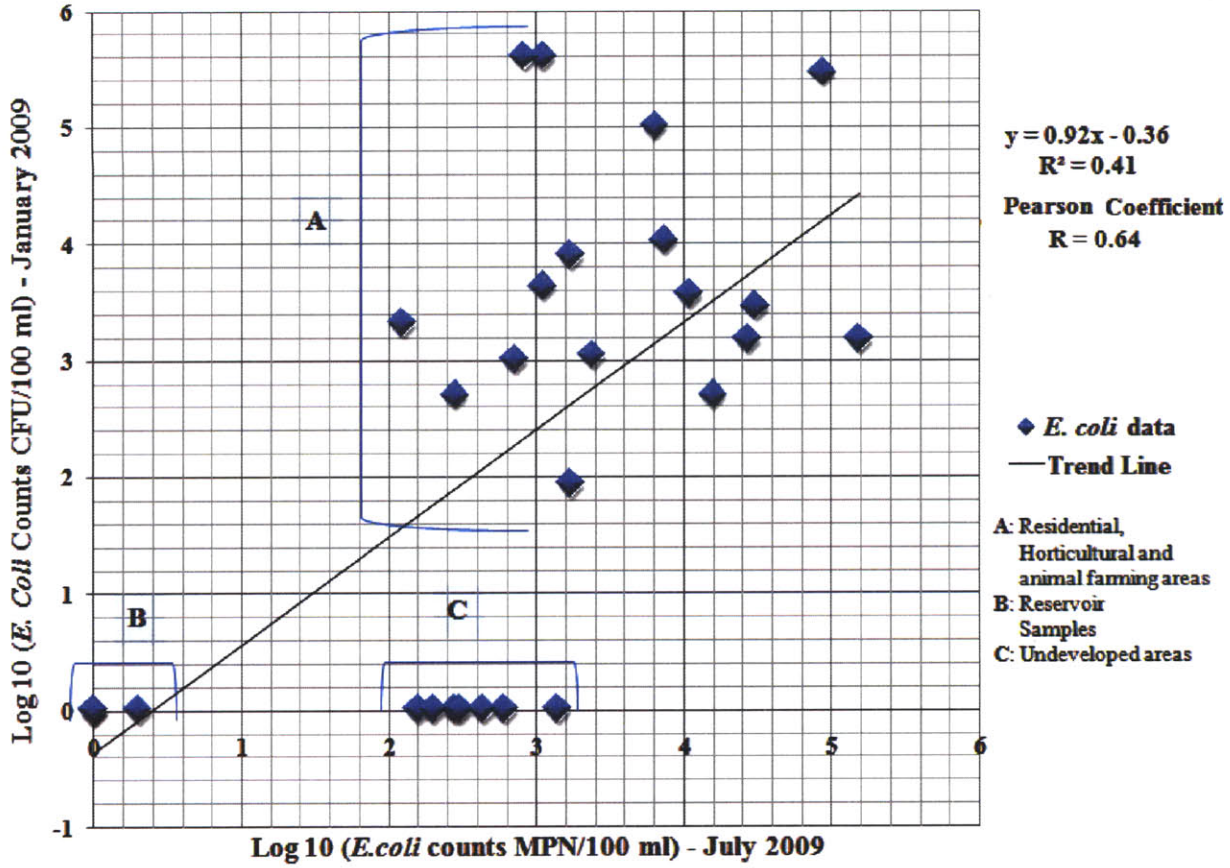


*Figure 5.15 Correlation of E. coli concentrations in Dry (January 2009) and Wet (July 2009) Weather.*

**Notes**: (1) For samples with zero E. coli count we adapted their lower limit of detection, which is <1 *E. coli*/100ml. (2) 0 on Figure 5.15 represents the detection limit

# Chapter 6: Discussion

## 6.1 Introduction

The ubiquity of *Bacteroides* species in humans and other warm blooded animals has been monitored and confirmed by different researchers who applied different detection techniques to prove their existence. Holdeman and others (1976) used culturing methods, Wang and others (1996) used PCR-based techniques and Lay and others (2005) used fluorescent in-situ hybridization to study *Bacteroides* species. The predominance of these *Bacteroides* in animals and their particular host-dependent character motivated the development of the different markers to relate them to their fecal origins, thus determining fecal pollution sources (Bernhard and Field 2000). The primer HF183F in conjunction with Bac708F primer was developed and used to amplify a specific segment of the 16S rRNA gene of *Bacteroides* strains found only in humans. Therefore, this primer (HF183F) has been recently widely used to assess human fecal contamination sources in USA, Europe, Africa (Kenya) and Austria (Bernhard and Field 2000, Gawler et al. 2007, Santoro and Boehm 2007, Jenkins et al. 2009 and Ahmed et al. 2008). The aim of this research reported in this thesis was to identify potential nonpoint sources of human fecal contamination, to validate the use of HF183F assays under tropical climates, and to evaluate the implications of seasonal variations in rainfall in Kranji watershed of Singapore..

## 6.2 Human as Sources of Fecal Pollution in Kranji Catchment

The host-specific markers developed by Bernhard and Field (2000) provided a means of identifying the source of fecal pollution. Since the publication of this method, the detection of human-specific *Bacteroides* by targeting its 16S rRNA gene by PCR has been applied in different studies of human fecal source monitoring in the USA and around the world. This technique has helped to collect and analyze environmental DNA water samples in order link the bacteria to their sources. The emphasis on human fecal sources of contamination relies on the fact that health threats from human fecal contamination are known and documented (Field and Samadpour 2007). Therefore, the identification of human fecal sources is associated with suitable and effective human fecal pollution control strategies.

Human health risks associated with human fecal pollution have been considered more dangerous than health risks that are associated with domesticated and agricultural animals. As the current study focused on Kranji catchment which includes various type of land development (residences, animal farming and horticulture), it was imperative to understand the problem of the human fecal pollution in order to elaborate an effective source-control strategy.

Detection of the 16S rRNA gene of human-specific *Bacteroides* was therefore applied to identify the source of human fecal pollution in Kranji catchment. The DNA analysis of 65 environmental DNA water samples collected in Kranji Reservoir catchment under dry and wet weather showed that 85% were positive for the human-specific *Bacteroides* molecular marker (HF marker). These findings show that human fecal sources are widespread in the Kranji catchment. The phylogenetic analysis confirmed that the human-specific *Bacteroides* molecular marker identified in Kranji catchment was of human origin. In addition phylogenetic analysis of results obtained by Touchdown PCR showed that the identified HF markers were clustered within the same evolutionary group as other HF markers identified in human fecal source tracking studies completed in the United States (Fogarty and Voytek 2005, Santoro and Boehm 2007, Bernhard and Field 2000, and Shanks et al. 2007).

## 6.3 Magnitude of Human Fecal Pollution Sources in Residential, Horticultural and Animal Farming Areas

The coexistence of urban land use, animal farming and wildlife in a watershed creates challenges for source tracking of nonpoint sources of fecal pollution in the watershed. Urban stormwater, animal farming activities, and wildlife are usual considered as major sources of fecal pollution of surface waters (James and Joyce 2004). The approach used to study human fecal pollution sources in Kranji catchment included *E. coli* and HF marker analysis. The overall results of *E. coli* and HF marker analysis revealed an interesting scenario.

Of the 34 environmental DNA water samples collected in residential, horticultural and animal farming areas, 94% were positive to the HF marker. This finding confirmed that these areas are potential sources of human fecal pollution in Kranji catchment. The consistency of high *E. coli* average counts under dry and wet weather in residential, horticultural and animal farming areas (geometric mean 3240 CFU/100 ml) suggests the presence of continuous fecal sources. The detection of HF marker DNA and the phylogenetic similarity to other human fecal bacteria supported the presence of human fecal pollution in these areas.

The persistence of high *E. coli* counts in both dry and wet weather is associated with two different scenarios where each scenario is particular to the weather considered. During the wet weather, the drainage channels and paved spaces in residential areas increase the transport of fecal pollution by runoff, thus maintaining high *E. coli* concentrations. Effluents from sanitary systems serving farming and horticultural areas are diluted by rainfall runoff during wet weather, but the dilution is not enough to reduce the fecal bacteria load to insignificant levels. This is because the concentration of bacteria in the runoff, while less than that in sanitary effluents, is still high. During dry weather, the bacteria loads (particularly of sanitary effluents) is high and the intensity of rainfall is small, thus reducing dilution by runoff and maintaining *E. coli* concentrations slightly higher than in the wet season. As Kranji Reservoir is intended for

recreational activities, residential, horticultural and animal farming areas in the catchment area should be monitored during every time of the year in order to ensure the safety of users of recreational areas of Kranji Reservoir.

## 6.4 Human Fecal Pollution Sources: Comparison Dry and Wet Weather

We discussed above factors that maintain the high *E. coli* densities in some of the areas of the Kranji catchment. Based on the *E. coli* densities from wet (July 2009 and January 2009) and dry (January 2009) weather we selected 33 locations where samples were collected during both wet and dry periods and we compared the frequency distributions of *E. coli* densities among these locations. Figure 6.1 shows the comparison of dry and wet weather percentage frequencies of the 33 sampling locations.

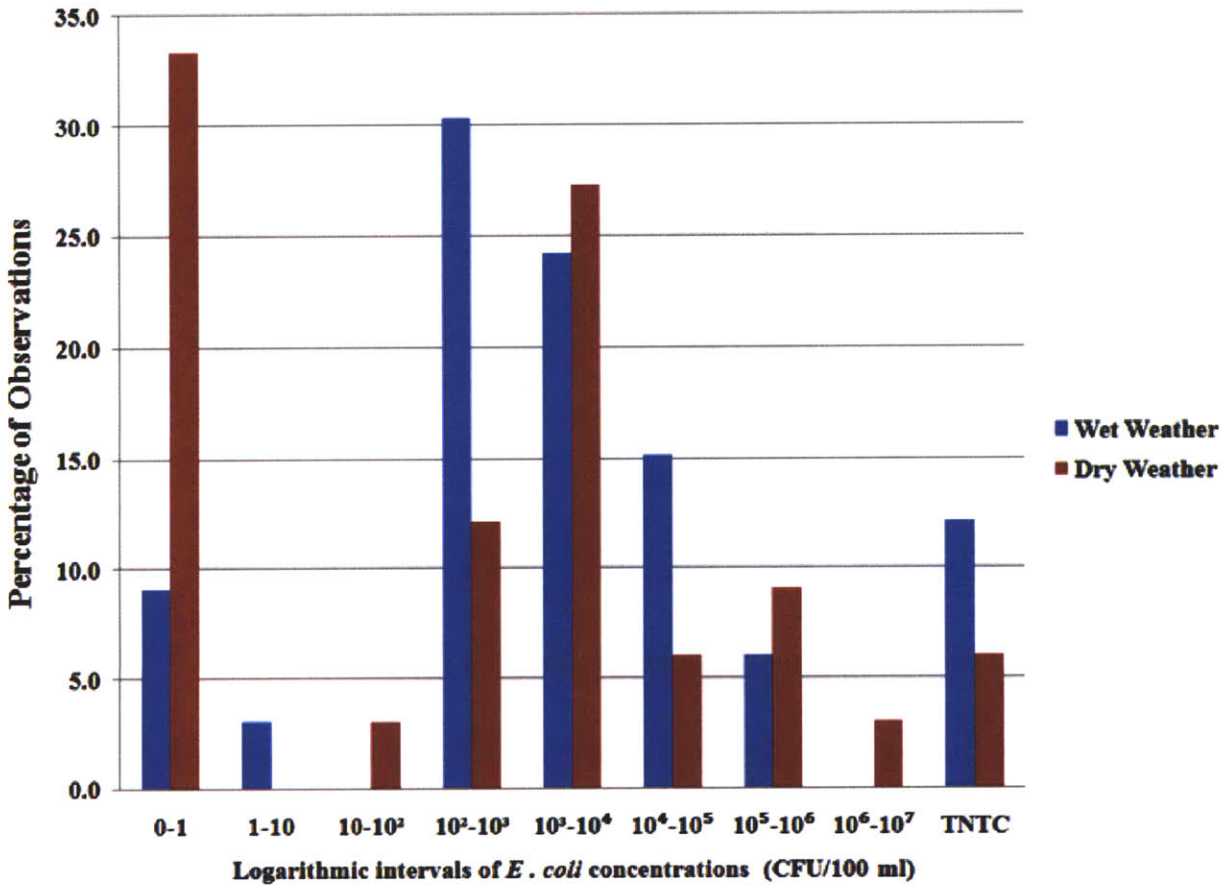


*Figure 6.1 Comparison of Frequency Percentages of 33 Similar Sampling Locations during Dry and Wet Weather*

As can be seen in Figure 6.1, there is a small but significant difference between the frequency distributions of wet and dry weather. The dry weather has a high percentage (33%) of frequency of *E. coli* counts ranging between 0 to 1 CFU/100 ml, while the wet weather frequency

percentage at the same interval is much lower at 9%. The high percentage observed in dry weather is presumably associated with the present of less nonpoint sources fecal pollution in runoff; while the high *E. coli* counts in the top intervals in dry weather (e.g. $10^6$- to-$10^7$ CFU/100 ml) is presumably associated with little dilution associated with less rainfall. In general, the frequency of the *E. coli* concentrations will tend to be high in the dry weather due to little dilution of the points sources of fecal pollutions in Kranji Reservoir catchment (e.g. sanitary systems in horticultural and animal farming areas of Tengah and Neo Tiew).

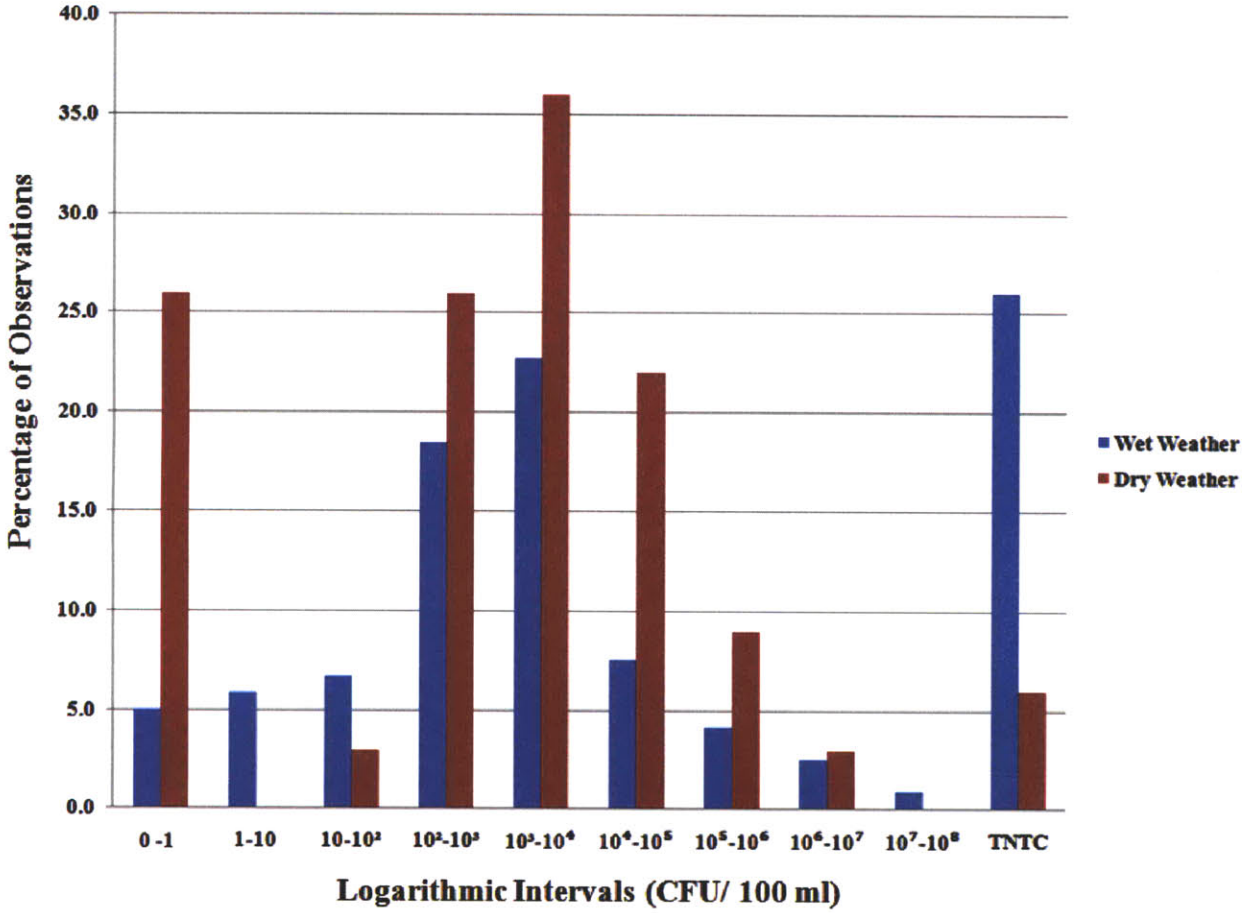


*Figure 6.2 Comparison of Frequency Percentages of All E. coli Data Recorded under Dry (January 2009) and Wet Weather (July 2009 and January 2010)*

Figure 6.2 compares the frequency percentage of all *E. coli* data collected in dry and wet weather during this study period. The small but significant *E. coli* concentration difference in the interval of 0 to 1 *E. coli* counts is also observed in this distribution. As can be seen in Figure 6.2, the distribution under dry weather is similar to that of Figure 6.1. A difference between the distributions in Figures 6.1 and 6.2 is that the overall data in Figure 6.2 show a small number of detections (0.8%) under wet weather in the interval of $10^7$-$10^8$ CFU/100 ml, which are the

highest concentrations detected. The frequency distribution for wet weather shows concentrations at all levels (from 0.1-1 CFU/100 ml to $10^7$-$10^8$ CFU/100 ml).

There is a small but significant difference between the two different weather conditions. The consistency of high *E. coli* counts in wet and dry weather is associated with the fact that the majority of water samples collected for *E. coli* analysis was located in residential, horticultural and animal farming areas. The reasons for high *E. coli* in these areas are discussed in detail above in Section 6.3. These findings are slightly different from the findings of Wright (1986) who showed that dry weather conditions were characterized by high *E. coli* counts. In the particular situation of Kranji Reservoir catchment (mixed activities including urban residential, horticultural and animal farming areas), it is obvious to have somewhat different findings from other studies comparing seasonal patterns under very different land uses. This shows also the role of human development and activities in influencing the distribution of fecal bacteria pollution under different weather conditions. This study confirms the variation of *E. coli* concentration for both wet and dry weather that was previously suggested by Chua and others (2010) for dry weather. The concentration of *E. coli* increases according to the sequence "Undeveloped → Farm → Residential → Sewage Treatment Plant (STP)" (Chua et al. 2010).

The small but significant difference in fecal contamination patterns between dry and wet weather in Kranji catchment was also supported by the findings of the DNA-based analysis. The Nested-PCR was used to analyze DNA samples collected under dry and wet weather. The frequency of HF marker was found to be high (92% of 38 DNA samples analyzed) in DNA environmental water samples collected and analyzed under wet weather. On the other hand, the DNA environmental samples collected and analyzed had an HF frequency of only 74% among the 27 samples collected under dry weather.

## 6.5 Human-Specific Marker for Human Fecal Pollution Tracking under the Tropical Climate

The human-specific *Bacteroides* 16S rRNA gene identified in studies carried out in the USA (Santoro and Boehm 2007) was also detected in the environmental DNA water samples collected in the Kranji Reservoir catchment of Singapore. The 68 gene sequences generated during this study were phylogenetically analyzed together with genes detected by Santoro and Boehm (2007). Figure 5.5 shows how all of these sequences have been aligned in clusters under the human *Bacteroides* group. Similar alignment clusters of sequences of clones of temperate climate samples and Kranji Reservoir samples proved that the HF183F assays used under temperate climates are also effective in tropical climates. In addition, the HF marker identified in Singapore was clustered with other HF markers found in water samples collected fromKenya (Jenkins et al. 2009), Europe (Gawler et al. 2007) and Australia (Ahmed et al. 2008) as shown by the findings of DNA phylogenetic analysis.

Jenkins et al. (2009) recently demonstrated the use of HF183F assays in detecting human fecal pollution and separating them from cow sources in the Njoro watershed of Kenya. As this area is also under a tropical climate it was interesting to see that the results of HF183F PCR assays of the DNA water samples collected in Njoro watershed were correlated to the locations where human fecal pollution were suspected to be originating. Similarly, the sanitary systems in the horticultural and animal farming areas of Kranji catchment were suspected to contain human fecal pollution based on the drainage and piping systems. The HF183F assays of environmental water samples collected in these areas identified them as containing human fecal pollution (positive to HF marker).

Apart from the phylogenetic analysis, sequences generated from the clone library created using Kranji catchment DNA samples positive to HF marker were analyzed using Blast online software (NCBI, 2010) to check the 99% similarities to other studies. The top hit was of the human microbiome guts (GQ493974) (Turnbaugh et al. 2009). This confirmed also that the HF marker identified in DNA samples collected in Kranji catchment is similar to HF markers identified in gut flora of other humans living in other regions. These results and the phylogenetic analysis and its comparison to similar HF marker identified in other studies under the temperate climate (Bernarhard and Field 2000, Shanks et al. 2006, and Santoro and Boehm 2007) and tropical climate (Jenkins et al. 2009 and Pickering et al. 2010) confirm the applicability of the human-specific *Bacteroides* marker in fecal source tracking under a tropical climate.

# Chapter 7: Conclusions and Recommendations

## 7.1 Conclusions

### 7.1.1 Human Fecal Contamination Sources in Kranji Reservoir

The evaluation of human fecal contamination sources in Kranji Reservoir catchment in Singapore was carried out during dry (January 2009) and wet (July 2009) periods. This study was motivated by the program of the Public Utilities Board to open Kranji Reservoir for recreational use. In additional we wanted to confirm the applicability of the DNA-based method (host-specific 16S rRNA *Bacteroides* gene human marker) under the tropical climate of Singapore. This DNA-based method was recently developed and applied in human fecal source tracking under the temperate climates of the USA, Europe, and Australia, and tropical climate of Kenya and Tanzania. The identification of the major sources of human fecal pollution within the Kranji catchment area was considered critical in order to help ensure the safety of users of recreational areas. The data gathered from the field and the laboratory analysis for *E. coli* and the presence/absence of human-specific 16S rRNA of *Bacteroides* gene lead to the following conclusions:

- The results from Touchdown PCR and Nested PCR analysis with the HF183F assays show that it is broadly distributed suggesting that human fecal contamination in dry and wet periods within Kranji catchment may originate in residential (Pang Sua and Pang Siang) and horticultural and animal farming (Tengah and Neo Tiew Farms) areas.

- The effluent samples from animal farming areas and their associated sanitary systems were confirmed to contain human fecal contamination and other bacteria. In addition, during the analysis, all the samples were tested positive to the Bac32F assays, confirming them to contain other *Bacteroides*, such as from warm-blooded animals other than human. Bac32F assays are capable of identifying other different *Bacteroides* groups that are indicative of other warm-blooded animal sources such as dogs, cows, and chickens.

- The overall results of the DNA analysis suggested human fecal pollution to be a major source of fecal bacteria pollution during both dry and wet weather in Kranji catchment; with a slight increase during wet weather. This is supported by the Nested PCR analysis, which showed that 74% of 27 environmental DNA water samples collected under dry weather (January 2009) contained HF marker, while 92% of 38 environmental DNA water samples collected under wet weather (July 2009 and January 2010) contained HF marker

The frequency distributions of *E. coli* concentrations under wet (July 2009 and January 2010) and dry (January 2009) weather suggested variation patterns of *E. coli* concentration between land uses of residential, horticultural and animal farming areas. The major reasons associated with this slight change are discussed in sections 6.3 and 6.4 of Chapter 6.

- The distribution *E. coli* concentration in the logarithmic intervals showed that there is a significant difference of *E. coli* concentrations in wet and dry weather in the distribution interval of 0 to 1 CFU/100 ml as discussed in section 6.4 of chapter 6.

- The results environmental DNA samples collected in Kranji Reservoir catchment during the dry period of January 2009, and the wet period of July 2009 and January 2010 suggested that human fecal contamination is reaching the Kranji Reservoir from the upstream areas.

- Although Kangakar area seems to be undeveloped, it is also confirmed to be a source of human fecal contamination during both dry and wet periods. The major drainages contributing to the sampling location in this area are from a military training area with restricted access. Due to these conditions we have not been able to check the probable upstream causes of these findings.

### 7.1.2 Use of the Human Host-specific 16S rRNA Genetic Marker under Tropical Climate

- The phylogenetic analysis of the environmental DNA samples from locations FM05 and PU04, which were found positive to human-specific gene, revealed that the human host-specific 16S rRNA *Bacteroides* gene found in Kranji catchment samples were phylogenetically related to genes identified recently under temperate climate conditions by Santoro and Boehm (2007). This confirms that the HF marker technique based on Touchdown PCR is effective under the tropical climate of Singapore. In addition, the phylogenetic tree confirmed also that the HF marker identified in DNA water samples collected in Kranji catchment have been identified under other tropical climate regions such as Njoro, Kenya. Future work will be needed to perform phylogenetic analysis on Nested PCR samples to confirm that these results have the same high level of specificity for *Bacteroides* from human origins.

### 7.1.3 Human Host-specific 16S rRNA genetic Marker and Freshwater Indicator Bacteria

- The *E. coli* results of water samples collected in January and July 2009 and January 2010 showed that the majority of the water samples collected in Tengah, Pang Sua and Pang Siang violated the guidelines for freshwater recreation (235 CFU/100 ml) (Table 5.4). The same areas were also identified by the HF maker to be potential contributors of

human fecal contamination. However, these regions are not intended for recreational activities, thus guidelines relevant to their potential role as sources of bacterial pollution to the Kranji Reservoir would need to be established.

- The *E. coli* concentrations in water samples collected in Kranji Reservoir in January and July 2009 did not violate the freshwater recreational guidelines (235 CFU/100 ml) (Table 5.4, Figure 5.2 and Figure 5.6) . However, since the water samples were collected at only one meter depth and other studies demonstrated the variation of *E.coli* concentrations with the increase of the depth (20 cm, 30 cm and 120 cm), the results could not be considered conclusive. Recent studies have shown decreases in *E. coli* concentration with depth in reservoirs and lakes. In addition, the majority of all the water samples collected in the reservoir were also tested positive to the HF marker (87.5% of 16 environmental DNA water samples collected in Kranji Reservoir were positive).

### 7.1.4 Results and Kranji Reservoir Recreational Activities

- The different information provided by these studies confirms that major sources of human fecal contamination are consistent in both dry and wet periods. However, these results do not provide information regarding the risk associated with these sources. The majority of DNA samples analyzed were confirmed positive for the HF marker, but the HF concentrations have not yet been translated to human risk.

- The Kranji Reservoir *E. coli* results for both dry and wet periods did not violate the single-sample recreational guidelines for freshwater (235 CFU/100 ml). However, these results are less conclusive since coliform concentrations could have been reduced by exposure to solar radiation the shallow depth at which samples were collected.

## 7.2 Recommendations

### 7.2.1 Recommendations to Singapore Public Utilities Board

The recommendations provided to PUB are in accordance with the findings of our study. They are intended to provide recommendation to the PUB on remaining research needs and the way forward for the suitable management of human fecal contaminations within Kranji catchment within dry and wet periods.

- The horticultural and animal farming areas of Tengah and Neo Tiew pose a particular threat to the water quality of Kranji Reservoir due to their close proximity. The majorities of the effluent samples in these areas during both dry and wet periods had high levels of *E. coli* and were positive to HF marker. More vigorous monitoring and tighter wastewater permit restrictions should be evaluated for these areas.

- The residential areas of Pang Sua and Pang Siang were associated with high *E. coli* concentration and the HF marker in dry and wet weather. As the drainages channels from these areas are also tributaries of Kranji Reservoir, they should be examined for permanent (during dry and wet weather) active monitoring of fecal contamination.

- The horticultural and animal farming facilities located within Tengah were identified by this study to be among the origins of human fecal pollution (HF marker results). Apart from an active monitoring of HF marker and indicator bacteria (e.g. *E. coli* and *Enterococcus*), there should be an evaluation of improving the sanitary treatment system to levels of discharge that could reduce the human fecal loadings in Kranji Reservoir and its tributaries.

- Human fecal contamination monitoring sectors should be established within the reservoir during wet periods. This study showed that the frequency of human fecal pollution was higher during wet-weather periods. Therefore the safety of users within the reservoir can be effectively protected by increased monitoring during this period. In addition, all tributaries of the Kranji Reservoir carry runoff, which is one of the major contributors of human fecal contamination during wet periods.

- The assay for detection of the HF maker in this study recovered sequences similar to other studies targeting human fecal pollution, thereby supporting the use of the HF marker in the tropical climate of Singapore. In addition, HF marker DNA-based analysis provides results rapidly (within 4 hours for presence/absence test) compared to traditional culture based methods (24 hours of incubation period).

### 7.2.2 Recommendations for Future Research

- This study has supported the use of the HF marker as a tracer for human fecal contamination in Singapore, however additional work is necessary in order to evaluate whether this is a robust indicator for risks associated with human sewage. There is therefore a need to study quantitatively the relationship between *Bacteroides* 16S rRNA genetic markers (from human and other animals) and the presence of pathogens within Kranji Reservoir and catchment. This study should be structured to quantitatively show the correlation between pathogens and the presence of HF marker.

- There is a need to develop a quantitative assay for the HF marker using QPCR since the results of this study indicated that increased sensitivity resulted in a higher number of positive samples. After developing a quantitative assay, we could then relate HF marker levels to levels of pathogens and other sewage indicators during different seasons and at different locations. This correlation could indicate how well the abundance of the HF

marker correlates to other indicators that do not grow under tropical climate (e.g. *Enterococcus* and caffeine). Ultimately a quantitative epidemiological study relating HF marker levels to public health outcomes would be necessary to determine whether it serves as a proxy for human health risk. Ideally, such a study would be combined with evaluation of other sewage indicators and would be performed in both tropical and temperate environments, as the majority of published epidemiological studies on the use of fecal indicators have been done in temperate climates.

- The concentration of *E. coli* in the majority of the water samples collected within Kranji Reservoir was low (less than 1 MPN/100 ml), however *E. coli* concentrations throughout the catchment were significantly higher, suggesting potential sources of fecal contamination that could be targeted to reduce risk of contaminating the reservoir waters. However, *E. coli* may not be an ideal indicator for Singapore because some studies suggest it can grow in tropical environments (Hazen 1988), thus Singapore has recently adopted *Enterococcus* as an indicator organism and is evaluating use of alternative indicators. Additional work is needed to validate use of such alternative markers (such as the HF marker) as described below. In addition, recent studies have shown that the *E. coli* concentration decreases with the depth (20 cm, 30 cm and 120 cm) of the sample collection. Thus a study of the depth profile of fecal indicator concentrations could help improve sampling strategies to monitor fecal indicators in the Kranji Reservoir.

## Chapter 8: References


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# APPENDIX

## Appendix A: Blank Samples

### Table A.1: Field Blank (B) and Laboratory Sterilization Blank (BS) Samples

| | | | Total Coliform | | | E. coli | | |
|---|---|---|---|---|---|---|---|---|
| **Names** | **Explanation** | **Dates** | **# Large** | **# Small** | **MPN** | **# Large** | **# Small** | **MPN** |
| **B01** | Blank | 7/7/2009 | 0 | 0 | 0 | 0 | 0 | 0 |
| **B02** | Blank | 7/7/2009 | 0 | 0 | 0 | 0 | 0 | 0 |
| **B03** | Blank | 7/7/2009 | 0 | 0 | 0 | 0 | 0 | 0 |
| **B04** | Blank | 7/7/2009 | 0 | 0 | 0 | 0 | 0 | 0 |
| **B05** | Blank | 7/8/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **B06** | Blank | 7/8/2009 | 4 | 0 | 4.1 | 1 | 0 | 1 |
| **B07** | Blank | 7/8/2009 | 4 | 0 | 4.1 | 1 | 0 | 1 |
| **B08** | Blank | 7/9/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **B09** | Blank | 7/9/2009 | 0 | 0 | 0 | 0 | 0 | 0 |
| **B10** | Blank | 7/9/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **B11** | Blank | 7/9/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **B12** | Blank | 7/13/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **B13** | Blank | 7/13/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **B14** | Blank | 7/14/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **B15** | Blank | 7/14/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **B16** | Blank | 7/14/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **B17** | Blank | 7/16/2009 | 0 | 0 | <1 | 0 | 0 | <0 |
| **B18** | Blank | 7/17/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS01** | Blank/Sterilization | 7/7/2009 | 0 | 0 | 0 | 0 | 0 | 0 |
| **BS02** | Blank/Sterilization | 7/7/2009 | 0 | 0 | 0 | 0 | 0 | 0 |
| **BS03** | Blank/Sterilization | 7/8/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS04** | Blank/Sterilization | 7/8/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS05** | Blank/Sterilization | 7/9/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS06** | Blank/Sterilization | 7/9/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS07** | Blank/Sterilization | 7/13/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS08** | Blank/Sterilization | 7/13/2009 | 0 | 0 | <1 | 0 | 0 | <1 |

| | | | Total Coliform | | | E. coli | | |
|---|---|---|---|---|---|---|---|---|
| **Names** | **Explanation** | **Dates** | **# Large** | **# Small** | **MPN** | **# Large** | **# Small** | **MPN** |
| **BS09** | Blank/Sterilization | 7/14/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS10** | Blank/Sterilization | 7/14/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS11** | Blank/Sterilization | 7/15/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS12** | Blank/Sterilization | 7/15/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS13** | Blank/Sterilization | 7/16/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS14** | Blank/Sterilization | 7/16/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS15** | Blank/Sterilization | 7/17/2009 | 0 | 0 | <1 | 0 | 0 | <1 |
| **BS16** | Blank/Sterilization | 7/20/2009 | 0 | 0 | <1 | 0 | 0 | <1 |

## Appendix B: Comparison of *E. coli* Concentrations Wet and Dry Periods

### Table B.1: *E. coli* Counts from Similar Sampling Locations: January 2009 and July 2009

| July 2009 | | January 2009 | |
|---|---|---|---|
| Station Name | *E. coli* (MPN/100 ml) | Station Name | *E. coli* (CFU/100 ml) |
| PB01 | 1732.9 | PB01 | 87 |
| PB02 | 284 | PB02 | 0 |
| PB03 | 1413.6 | PB03 | 0 |
| PS01 | 30,900 | PS01 | 2900 |
| PS01 | 1119.9 | PS01 | 4300 |
| PS02 | 125.9 | PS02 | 2100 |
| PS03 | 2419.6 | PS03 | 1100 |
| PS04 | 1732.9 | PS04 | 7900 |
| PS05 | >2419.6 | PS05 | 4900 |
| KK01 | 7500 | KK01 | 10,700 |
| KK02 | 159.7 | KK02 | 0 |
| KK03 | 613.1 | KK03 | 0 |
| KK04 | 307.6 | KK04 | 0 |
| TA01 | 435.2 | TA01 | 0 |
| TA02 | 200 | TA02 | 0 |
| TH01 | 27,500 | TH01 | 1500 |
| TH02 | >2419.6 | TH02 | 2,100,000 |
| TH03 | >2419.6 | TH03 | 650 |
| NT01 | 16,000 | NT01 | 500 |
| NT02 | 88,200 | NT02 | 290,000 |
| PU02 | 1119.9 | PU02 | 400,000 |
| PU03 | 285 | PU03 | 500 |
| PU04 | 720 | PU04 | 1000 |
| FM01 | 11,060 | FM01 | 3700 |
| FM03 | 155,310 | FM03 | 1500 |
| FM05 | >2419.6 | FM05 | TNTC |
| FM06 | 6440 | FM06 | 100,000 |
| FM07 | 833.5 | FM07 | 400,000 |
| FM08 | 241,960 | FM08 | TNTC |
| Res01 | 0 | ResA | 0 |
| Res02 | 0 | ResB | 0 |
| Res05 | 0 | ResC | 0 |
| Res06 | 2 | ResD | 0 |

## Appendix C: Table of the Data Used to Generate the GIS Map

### Table C.1 : Sampling Locations, *E. coli* and Nested PCR Results: January 2009

| FID | Station Name | *E. coli* (CFU/100 ml) | HF Marker (HF183F Primer) | Latitude | Longitude |
|---|---|---|---|---|---|
| 504 | FM01 | 3700 | 0 | 1.417 | 103.719 |
| 503 | FM03 | 1500 | -1 | 1.386 | 103.721 |
| 502 | FM05 | TNTC | 1 | 1.398 | 103.73 |
| 505 | FM06 | 50,000 | 1 | 1.383 | 103.731 |
| 500 | FM07 | 400,000 | 1 | 1.383 | 103.731 |
| 501 | FM08 | TNTC | 1 | 1.383 | 103.726 |
| 506 | PB02 | 0 | 1 | 1.376 | 103.736 |
| 507 | PB03 | 0 | 1 | 1.376 | 103.736 |
| 508 | PS02 | 2100 | 0 | 1.378 | 103.741 |
| 509 | PS05 | 4900 | 1 | 1.385 | 103.747 |
| 510 | PS04 | 7900 | 1 | 1.378 | 103.749 |
| 511 | PS03 | 1100 | 1 | 1.378 | 103.749 |
| 512 | TH01 | 0 | 1 | 1.381 | 103.759 |
| 513 | TA01 | 0 | -1 | 1.369 | 103.712 |
| 514 | TA02 | 0 | -1 | 1.369 | 103.712 |
| 515 | NT02 | 290,000 | 1 | 1.42 | 103.716 |
| 516 | NT01 | 3500 | 1 | 1.417 | 103.715 |
| 517 | KK02 | 0 | 1 | 1.411 | 103.7 |
| 518 | KK03 | 0 | 1 | 1.411 | 103.7 |
| 519 | KK04 | 0 | 1 | 1.411 | 103.7 |
| 520 | PU02 | 2100 | 1 | 1.381 | 103.759 |
| 521 | PU03 | 500 | -1 | 1.381 | 103.759 |
| 522 | PU04 | 337,000 | 1 | 1.381 | 103.759 |
| 523 | ResA | 0 | 0 | 1.433 | 103.742 |
| 524 | ResB | 0 | 1 | 1.435 | 103.705 |
| 525 | ResC | 0 | 1 | 1.412 | 103.729 |
| 526 | ResD | 0 | 1 | 1.413 | 103.726 |

Notes: HF results: 1: Present, -1: Inhibited, 0: Absent

**Table C.2: Sampling Locations, *E. coli* Concentration and Nested-PCR DNA Results: July 2009**

| FID | Station Name | *E. coli* (MPN/100 ml) | HF Marker (HF183F Primer) | Latitude | Longitude |
|---|---|---|---|---|---|
| 104 | FM01 | 11,060 | 1 | 1.41446 | 103.718 |
| 103 | FM03 | 155,310 | 1 | 1.38274 | 103.7212 |
| 102 | FM05 | >2419.6 | 1 | 1.39955 | 103.7304 |
| 105 | FM06 | 6440 | 1 | 1.39502 | 103.7311 |
| 100 | FM07 | 833.5 | 1 | 1.38268 | 103.7312 |
| 101 | FM08 | 241,960 | 1 | 1.38338 | 103.7261 |
| 106 | PB02 | 2841 | 1 | 1.37587 | 103.736 |
| 107 | PB03 | 1414 | 1 | 1.37559 | 103.7357 |
| 108 | PS02 | 126 | 1 | 1.38468 | 103.7468 |
| 109 | PS05 | >2416.9 | 1 | 1.37783 | 103.7594 |
| 110 | TH01 | 27,500 | 1 | 1.381 | 103.759 |
| 111 | TA02 | 200 | 0 | 1.37022 | 103.7057 |
| 112 | NT02 | 88,200 | 1 | 1.41682 | 103.7188 |
| 113 | NT01 | 16,000 | 1 | 1.42005 | 103.7163 |
| 114 | KK01 | 7500 | 1 | 1.40331 | 103.7013 |
| 115 | PU02 | 1119.9 | 1 | 1.38094 | 103.7582 |
| 116 | PU03 | 285 | 1 | 1.38078 | 103.7585 |
| 117 | PU04 | 720 | 1 | 1.38 | 103.7583 |
| 118 | Res1 | 0 | 1 | 1.43685 | 103.7408 |
| 119 | Res2 | 0 | 1 | 1.43264 | 103.7422 |
| 120 | Res3 | 0 | 1 | 1.42296 | 103.7402 |
| 121 | Res4 | 3 | 1 | 1.41539 | 103.7323 |
| 122 | Res5 | 0 | 1 | 1.41315 | 103.7288 |
| 123 | Res6 | 2 | 1 | 1.40524 | 103.7289 |
| 124 | Res7 | 0 | 1 | 1.40196 | 103.7322 |
| 125 | Res8 | 0 | 1 | 1.3982 | 103.7341 |
| 126 | Res9 | 34.5 | 1 | 1.39628 | 103.7251 |
| 127 | Res10 | 5.2 | 0 | 1.41342 | 103.7137 |
| 128 | Res11 | 4.1 | 1 | 1.41217 | 103.7106 |
| 129 | Res12 | 11 | 1 | 1.41857 | 103.7408 |
| 130 | KK06 | 30 | 1 | 1.41019 | 103.7009 |
| 131 | KK05 | 40 | 1 | 1.41603 | 103.702 |

Notes: HF Marker Results: 1: HF marker Present and 0: HF marker absent

**Table C.3: DNA Results of Touchdown PCR Analysis**

| FID | Name | *E. coli* (/100 ml) | HF Marker Results | Latitude | Longitude |
|---|---|---|---|---|---|
| 126 | FM01 | 3700 | 1 | 1.417 | 103.719 |
| 127 | FM03 | 1500 | 0 | 1.386 | 103.721 |
| 128 | FM05 | TNTC | 1 | 1.398 | 103.73 |
| 129 | FM06 | 50,000 | 1 | 1.383 | 103.731 |
| 130 | FM07 | 400,000 | 1 | 1.383 | 103.731 |
| 131 | FM08 | TNTC | 1 | 1.383 | 103.726 |
| 132 | PB02 | 0 | 0 | 1.376 | 103.736 |
| 133 | PB03 | 0 | 0 | 1.376 | 103.736 |
| 134 | PS02 | 2100 | 0 | 1.378 | 103.741 |
| 135 | PS05 | 4900 | 0 | 1.385 | 103.747 |
| 136 | PS04 | 7900 | 0 | 1.378 | 103.749 |
| 137 | PS03 | 1100 | 1 | 1.378 | 103.749 |
| 138 | TH01 | 0 | 0 | 1.381 | 103.759 |
| 139 | TA01 | 0 | -1 | 1.369 | 103.712 |
| 140 | TA02 | 0 | -1 | 1.369 | 103.712 |
| 141 | NT02 | 290,000 | 1 | 1.42 | 103.716 |
| 142 | NT01 | 3500 | 0 | 1.417 | 103.715 |
| 143 | KK02 | 0 | 0 | 1.401 | 103.7 |
| 144 | KK03 | 0 | 0 | 1.401 | 103.7 |
| 145 | KK04 | 0 | 0 | 1.401 | 103.7 |
| 146 | PU02 | 2100 | 0 | 1.381 | 103.759 |
| 147 | PU03 | 500 | -1 | 1.381 | 103.759 |
| 148 | PU04 | 337,000 | 1 | 1.381 | 103.759 |
| 149 | ResA | 0 | 0 | 1.434 | 103.743 |
| 150 | ResB | 0 | 1 | 1.432 | 103.742 |
| 151 | ResC | 0 | 0 | 1.413 | 103.729 |
| 152 | ResD | 0 | 0 | 1.412 | 103.725 |

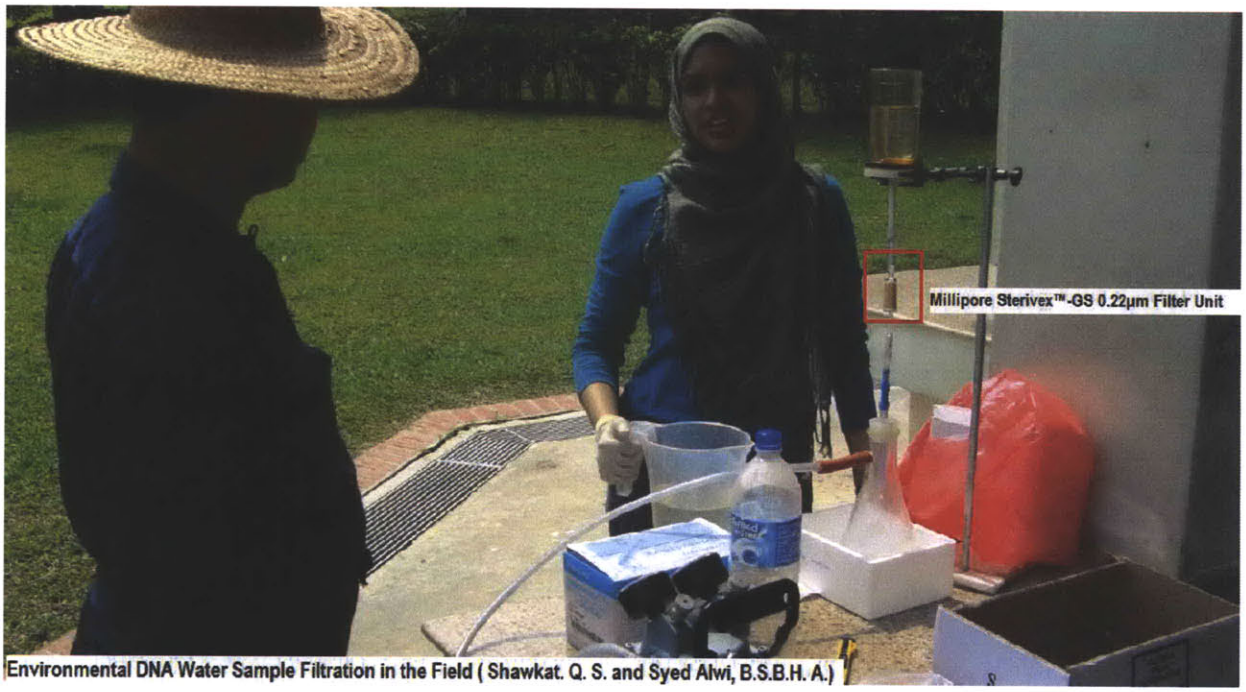
Notes of HF Marker: 1: HF Marker Present, -1: HF Marker Inhibited and 0: Absent Samples

## Appendix D: Pictures of Sampling

Animal Farming Activities on shores of Kranji Reservoir, Tengah Sub-catchment

Industrial Zone on Shores of Kranji Reservoir, Pang Sua Sub-catchment

PS01, Pang Siang : Runoff Water Sampling in Residential Area




Environmental DNA Water Sample Filtration in the Field ( Shawkat. Q. S. and Syed Alwi, B.S.B.H. A.)

TA02: Tengah, Airbase Area: Water Sampling in Undeveloped Area (Syed Alwi, B.S.B.H.A. Collecting the Water

Sanitary System in a Fish Farm on Jalan Lekar Rd, Tengah Sub-catchment


FM08, Sampling Location in Tengah Sub-catchment (Farmart Fish Farm)

FM07 : Drainage of the Outlet of the Aqua Tropical Fish Farm, Sungei Tengah Rd

## Appendix E: MPN of Total Coliforms July 2009, January 2010 and January 2009

### Table E. 1: MPN of Total Coliforms and E. coli July 2009

| | | | Total Coliform | E. coli | TC | E. coli | TC | E. coli | TC | E. coli | Average E coli | Location | | DNA Samples | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Names** | **Dates** | **Volume** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **Latitude** | **Longitude** | **Samples** | **Results** |
| **Dilutions** | | | **1** | **1** | **100** | **100** | **1,000** | **1,000** | **10,000** | **10,000** | | | | | |
| | | | | | | | | | | | | | | | |
| **PB02** | 7/7/2009 | - | 437.5 | 284.1 | - | - | - | - | - | - | 2841.1 | 1.37587 | 103.73596 | | |
| **PB03** | 7/7/2009 | - | >2419.6 | 1413.6 | - | - | - | - | - | - | 1413.6 | 1.37559 | 103.73567 | | |
| **PB01** | 7/7/2009 | - | >2419.6 | 1732.9 | - | - | - | - | - | - | 1732.9 | 1.37661 | 103.7354 | | |
| **PS01** | 7/7/2009 | - | >2419.6 | 1119.9 | - | - | - | - | - | - | 1119.9 | 1.38283 | 103.73948 | | |
| **PS02** | 7/7/2009 | - | >2419.6 | 125.9 | - | - | - | - | - | - | 125.9 | 1.38468 | 103.74676 | | |
| **PS03** | 7/7/2009 | - | >2419.6 | 2419.6 | - | - | - | - | - | - | 2419.6 | 1.37822 | 103.7486 | | |
| **PS04** | 7/7/2009 | - | >2419.6 | 1732.9 | - | - | - | - | - | - | 1732.9 | 1.37938 | 103.74899 | | |
| **PS05** | 7/7/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.37783 | 103.75935 | | |
| **PU02** | 7/7/2009 | | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.38094 | 103.75824 | | |
| **PU03** | 7/7/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.38078 | 103.75853 | | |
| **PU04** | 7/7/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.38 | 103.75829 | | |
| **PU05** | 7/7/2009 | - | >2419.8 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.38 | 103.7583 | | |
| **NT01** | 7/8/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.42005 | 103.71631 | | |
| **NT02** | 7/8/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | | | | |
| **KK01** | 7/8/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.40331 | 103.70127 | | |
| **KK02** | 7/8/2009 | - | >2419.6 | 159.7 | - | - | - | - | - | - | 159.7 | 1.40107 | 103.70049 | | |
| **KK03** | 7/8/2009 | | >2419.6 | 613.1 | - | - | - | - | - | - | 613.1 | 1.40141 | 103.70056 | | |
| **KK04** | 7/8/2009 | - | >2419.6 | 307.6 | - | - | - | - | - | - | 307.6 | 1.40116 | 103.7009 | | |
| **TA01** | 7/8/2009 | - | >2419.6 | 435.2 | - | - | - | - | - | - | 435.2 | 1.369 | 103.712 | | |

| | | | Total coliform | E. coli | Total Coliform | E. coli | Total Coliform | E. coli | Total Coliform | E. coli | Average E coli | Location | | DNA Samples | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Names** | **Dates** | **Volume** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **Latitude** | **Longitude** | **Samples** | **Results** |
| **Dilutions** | | | **1** | **1** | **100** | **100** | **1,000** | **1,000** | **10,000** | **10,000** | | | | | |
| **TH01** | 7/8/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | | | | |
| **TH02** | 7/8/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | 2419.6 | 1.381 | 103.759 | | |
| **TH03** | 7/8/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.383 | 103.731 | | |
| **PB01** | 7/9/2009 | - | 533.5 | 490.7 | - | - | - | - | - | - | 409.7 | 1.37713 | 103.73456 | 1 | |
| **PB04** | 7/9/2009 | - | >2419.6 | 30.0 | - | - | - | - | - | - | 30.0 | 1.37218 | 103.74472 | | |
| **PB05** | 7/9/2009 | | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.36089 | 103.7457 | | |
| **PB06** | 7/9/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.36081 | 103.74577 | | |
| **PB07** | 7/9/2009 | - | >2419.6 | 1299.7 | - | - | - | - | - | - | 1299.7 | 1.36112 | 103.74567 | | |
| **PB08** | 7/9/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.36634 | 103.75121 | | |
| **PB09** | 7/9/2009 | - | >2419.6 | 2419.6 | - | - | - | - | - | - | 2419.6 | 1.36634 | 103.75121 | | |
| **PB10** | 7/9/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.3668 | 103.7536 | | |
| **PB11** | 7/9/2009 | - | >2419.6 | 14.5 | - | - | - | - | - | - | 14.5 | 1.35644 | 103.75301 | | |
| **PU06** | 7/9/2009 | - | >2419.6 | 2419.6 | - | - | - | - | - | - | 2419.6 | 1.38008 | 103.76166 | | |
| **PU07** | 7/9/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.38051 | 103.76244 | | |
| **PU08** | 7/9/2009 | - | >2419.6 | 2419.6 | - | - | - | - | - | - | 2419.6 | 1.38125 | 103.7666 | | |
| **PU09** | 7/9/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.38086 | 103.76683 | | |
| **PU10** | 7/9/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.38244 | 103.76696 | | |
| **PU11** | 7/9/2009 | - | >2419.6 | >2419.6 | - | - | - | - | - | - | >2419.6 | 1.3825 | 103.76681 | | |
| **PB02** | 7/10/2009 | 1 liter | - | - | - | - | - | - | - | - | | 1.37587 | 103.73596 | 1 | |
| **PB03** | 7/10/2009 | 1 liter | - | - | - | - | - | - | - | - | | 1.37559 | 103.73567 | 1 | |
| **PS02** | 7/10/2009 | 1 liter | - | - | - | - | - | - | - | - | | 1.38468 | 103.74676 | 1 | |
| **PS05** | 7/10/2009 | 1 liter | - | - | - | - | - | - | - | - | | 1.37783 | 103.75935 | 1 | |
| **PS05** | 7/10/2009 | 1 liter | - | - | - | - | - | - | - | - | | 1.37783 | 103.75935 | 1 | |
| **Res01** | 7/13/2009 | 200ml | 1413.6 | <1 | - | - | <1 | <1 | <1 | <1 | 0.0 | 1.43685 | 103.7408 | 1 | P |
| **Res02** | 7/13/2009 | 200ml | 721.5 | <1 | - | - | 0 | <1 | 0 | 0 | 0.0 | 1.43264 | 103.7422 | 1 | P |

| | | | Total Coliform | E. coli | Total Coliform | E. coli | Total Coliform | E. coli | Total Coliform | E. coli | Average E coli | Location | | DNA Samples | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Names** | **Dates** | **Volume** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **Latitude** | **Longitude** | **Samples** | **Results** |
| **Dilutions** | | | 1 | 1 | 100 | 100 | 1,000 | 1,000 | 10,000 | 10,000 | | | | | |
| **Res03** | 7/13/2009 | 200ml | 148.3 | <1 | - | - | <1 | <1 | <1 | <1 | 0.0 | 1.42296 | 103.74017 | 1 | P |
| **Res04** | 7/13/2009 | 180ml | 1732.9 | 3.0 | - | - | 0 | 0 | 0 | 0 | 3.0 | 1.41539 | 103.73225 | 1 | P |
| **Res07** | 7/14/2009 | 160ml | 218.7 | <1 | - | - | <1 | <1 | <1 | <1 | 0.0 | 1.40196 | 103.73219 | 1 | P |
| **Res08** | 7/13/2009 | 150ml | 46.4 | <1 | - | - | <1 | <1 | <1 | <1 | 0.0 | 1.3982 | 103.73405 | 1 | P |
| **Res09** | 7/13/2009 | 220ml | 686.7 | 21.6 | - | - | <1 | <1 | <1 | <1 | 21.6 | 1.39628 | 103.72505 | 1 | P |
| **Res10** | 7/13/2009 | 210ml | >2419.6 | 5.2 | - | - | <1 | <1 | <1 | <1 | 5.2 | 1.41342 | 103.71373 | 1 | A |
| **Res11** | 7/13/2009 | 250ml | >2419.6 | 3.0 | - | - | <1 | <1 | <1 | <1 | 3.0 | 1.41217 | 103.71055 | 1 | P |
| **Res12** | 7/13/2009 | 400ml | >2419.6 | 11.0 | - | - | <1 | <1 | <1 | <1 | 11.0 | 1.41857 | 103.74084 | 1 | P |
| **PU06** | 7/14/2009 | 1 Liter | >2419.6 | 816.4 | - | - | <1 | <1 | - | - | 816.4 | 1.37736 | 103.76264 | 1 | |
| **PUt01** | 7/14/2009 | 1 Liter | >2419.6 | >2419.6 | - | - | <1 | <1 | - | - | >2419.6 | 1.3775 | 103.76274 | 1 | |
| **PUt02** | 7/14/2009 | 1 Liter | >2419.6 | >2419.6 | - | - | <1 | <1 | - | - | >2419.6 | 1.37413 | 103.7633 | 1 | |
| **PUt03** | 7/14/2009 | 1 Liter | >2419.6 | >2419.6 | - | - | <1 | <1 | - | - | >2419.6 | 1.37005 | 103.76434 | 1 | |
| **PUt04** | 7/14/2009 | 1 liter | >2419.6 | >2419.6 | - | - | <1 | <1 | - | - | >2419.6 | 1.36383 | 103.76788 | 1 | |
| **PUt05** | 7/14/2009 | 1 Liter | >2419.6 | >2419.6 | - | - | <1 | <1 | - | - | >2419.6 | 1.3638 | 103.76788 | 1 | |
| **PUt06** | 7/14/2009 | 750 ml | >2419.6 | >2419.6 | - | - | <1 | <1 | - | - | >2419.6 | 1.36624 | 103.76933 | 1 | |
| **PUt07** | 7/14/2009 | 300 ml | >2419.6 | >2419.6 | - | - | 2 | <1 | - | - | >2419.6 | 1.36614 | 103.76933 | 1 | |
| **PUt08** | 7/14/2009 | 300 ml | 1119.9 | 25.6 | - | - | < | <1 | - | - | 25.6 | 1.37097 | 103.76263 | 1 | |
| **PUt09** | 7/14/2009 | 1 Liter | >2419.6 | >2419.6 | - | - | <1 | <1 | - | - | >2419.6 | 1.39604 | 103.76366 | 1 | |
| **PU02** | 7/14/2009 | 1 Liter | >2419.6 | 1119.9 | - | - | <1 | <1 | - | - | 1119.9 | 1.38094 | 103.75824 | 1 | P |
| **PU03** | 7/14/2009 | 1 Liter | >2419.6 | 285.1 | - | - | 27.9 | <1 | - | - | 285.1 | 1.38078 | 103.75853 | 1 | P |
| **PB01** | 7/15/2009 | 500 ml | >2419.6 | >2419.6 | - | - | <1 | <1 | - | - | 0.0 | 1.37652 | 103.73577 | 1 | |
| **PS01** | 7/15/2009 | 450 ml | >2419.6 | >2419.6 | - | - | 365.4 | 30.9 | - | - | 30900.0 | 1.38243 | 103.73985 | 1 | |

| | | | Total Coliform | E. coli | Total Coliform | E. coli | Total Coliform | E. coli | Total Coliform | E. coli | Average E coli | Location | | DNA Samples | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Names** | **Dates** | **Volume** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **Latitude** | **Longitude** | **Samples** | **Results** |
| **Dilutions** | | | **1** | **1** | **100** | **100** | **1,000** | **1,000** | **10,000** | **10,000** | | | | | |
| **TH01** | 7/15/2009 | 350 ml | >2419.6 | >2419.6 | - | - | 365.4 | 27.5 | - | - | 27500.0 | 1.39061 | 103.73154 | 1 | P |
| **KK01** | 7/15/2009 | 300 ml | >2419.6 | >2419.6 | - | - | 44.4 | 7.5 | - | - | 7500.0 | 1.4033 | 103.70126 | 1 | P |
| **NT01** | 7/15/2009 | 600 ml | >2419.6 | >2419.6 | | | 235.9 | 16.0 | | - | 1,600 | 1.42018 | 103.71627 | 1 | P |
| **NT02** | 7/15/2009 | 600 ml | >2419.6 | >2419.6 | | | 1299.7 | 88.2 | | - | 88,200 | 1.41799 | 103.71532 | 1 | P |
| **PB10** | 7/16/2009 | 650 ml | >2419.6 | 307.6 | 156.5 | 3.1 | - | - | - | - | 310.0 | 1.36679 | 103.75368 | 1 | |
| **PB06** | 7/16/2009 | 1 Liter | >2419.6 | 1119.9 | 461.6 | 13.4 | - | - | - | - | 1340.0 | 1.36083 | 103.74568 | 1 | |
| **PB07** | 7/16/2009 | 600 ml | >2419.6 | 133.3 | 325.5 | 7.4 | - | - | - | - | 740.0 | 1.36107 | 103.74552 | 1 | |
| **PB12** | 7/16/2009 | - | >2419.6 | >2419.6 | >2419.6 | >2419.6 | - | - | - | - | >2419.6 | 1.36094 | 103.74579 | | |
| **PB03** | 7/16/2009 | 1 Liter | >2419.6 | 1046.2 | >2419.6 | 920.8 | - | - | - | - | 92080.0 | 1.37236 | 103.74374 | 1 | P |
| **PB02** | 7/16/2009 | 600 ml | >2419.6 | 648.8 | 2 | <1 | - | - | - | - | 0.0 | 1.3724 | 103.74356 | 1 | P |
| **PU07** | 7/17/2009 | 500 ml | >2419.6 | >2419.6 | 248.9 | 42.6 | - | - | - | - | 4260 | 1.38118 | 103.76353 | 1 | |
| **PU12** | 7/17/2009 | 500 ml | 87.8 | 1.0 | 50.4 | <1 | - | - | - | - | 0 | 1.38119 | 103.76316 | 1 | |
| **PU09** | 7/17/2009 | 500 ml | >2419.6 | >2419.6 | 435.2 | 165.8 | - | - | - | - | 16580 | 1.38238 | 103.76684 | 1 | |
| **PU13** | 7/17/2009 | 350 ml | >2419.6 | >2419.6 | 547.5 | 68.9 | - | - | - | - | 6890 | 1.37995 | 103.76784 | 1 | |
| **PU14** | 7/17/2009 | 280 ml | 13.5 | 1.0 | <1 | <1 | - | - | - | - | 0 | 1.38005 | 103.76796 | 1 | |
| **PU04** | 7/17/2009 | 600 ml | >2419.6 | >2419.6 | 307.6 | 7.2 | - | - | - | - | 720 | 1.38 | 103.75829 | 1 | P |
| **FM01** | 7/20/2009 | 1 Liter | >2419.6 | >2419.6 | 517.2 | 110.6 | - | - | - | - | 11060 | 1.41446 | 103.71801 | 1 | P |
| **FM02** | 7/20/2009 | 300 ml | >2419.6 | >2419.6 | >2419.6 | 1553.1 | - | - | - | - | 155310 | 1.378 | 103.72829 | 1 | |
| **FM03** | 7/20/2009 | - | >2419.6 | >2419.6 | >2419.6 | 1986.3 | - | - | - | - | 198630 | 1.38274 | 103.72119 | | |
| **FM04** | 7/20/2009 | 1 Liter | 2419.6 | 960.6 | >2419.6 | 1553.1 | - | - | - | - | 155310 | 1.38647 | 103.72121 | 1 | P |
| **FM05** | 7/20/2009 | 250 ml | >2419.6 | >2419.6 | >2419.6 | >2419.6 | - | - | - | - | >2419.6 | 1.39955 | 103.73038 | 1 | P |
| **FM06** | 7/20/2009 | 150 ml | >2419.6 | >2419.6 | 1299.1 | 64.4 | - | - | - | - | 6440 | 1.39502 | 103.73109 | 1 | P |

| | | | Total Coliform | E. coli | Total Coliform | E. coli | Total Coliform | E. coli | Total Coliform | E. coli | Average E coli | Location | | DNA Samples | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Names** | **Dates** | **Volume** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **MPN** | **Latitude** | **Longitude** | **Samples** | **Results** |
| **Dilutions** | | | **1** | **1** | **100** | **100** | **1,000** | **1,000** | **10,000** | **10,000** | | | | | |
| **FM07** | 7/20/2009 | 150 ml | >2419.6 | >2419.6 | >2419.6 | >2419.6 | - | - | - | - | >2419.6 | 1.38268 | 103.73119 | 1 | P |
| **FM08** | 7/20/2009 | 140 ml | >2419.6 | >2419.6 | >2419.6 | >2419.6 | - | - | - | - | >2419.6 | 1.38338 | 103.72613 | 1 | P |
| **KK05** | 7/22/2009 | 160 ml | >2419.6 | 30.9 | 30.5 | <1 | - | - | - | - | 30.9 | 1.41019 | 103.70086 | 1 | P |
| **KK06** | 7/22/2009 | 185 ml | >2419.6 | 22.8 | 35.5 | 1.0 | - | - | - | - | 100 | 1.41603 | 103.70198 | 1 | P |
| **TA02** | 7/22/2009 | 1 Liter | >2419.6 | 127.4 | 160.7 | 2.0 | - | - | - | - | 200 | 1.37022 | 103.70567 | 1 | A |

## Table E. 2: *E. coli* and DNA (Touchdown PCR) Results: January 2010

| Names | Dates | Total Coliform | E. coli | Total Coliform | E. coli | Total Coliform | E. coli | Total Coliform | E coli | Total Coliform | E. coli | Averaged MPN | DNA Samples | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MPN | MPN | MPN | MPN | MPN | MPN | MPN | MPN | MPN | MPN | | Samples | Results |
| **Dilutions** | | **1:1** | **1:1** | **1:10** | **1:10** | **$1:10^2$** | **$1:10^2$** | **$1:10^4$** | **$1:10^4$** | **$1:10^6$** | **$1:10^6$** | | | |
| NT02 | 7-Jan | >2419.6 | >2419.6 | - | - | >2419.6 | 344.8 | 133.3 | 4.1 | - | - | 41,000 | 0 | |
| NT01 | 7-Jan | >2419.6 | >2419.6 | - | - | 2419.6 | 45.9 | 18.3 | 0 | - | - | 4,590 | 0 | |
| PU02 | 13-Jan | >2419.6 | >2419.6 | - | - | >2419.6 | >2419.6 | 209.8 | 88.4 | - | - | 884,000 | 0 | |
| PU03 | 13-Jan | >2419.6 | >2419.6 | - | - | 2419.6 | 39.9 | 37.9 | 0 | - | - | 3,990 | 0 | |
| PU04 | 13-Jan | >2419.6 | >2419.6 | - | - | >2419.6 | 95.9 | 18.3 | 1 | - | - | 9,590 | 0 | |
| PU05 | 13-Jan | >2419.6 | >2419.6 | - | - | >2419.6 | 54.6 | 37.3 | 1 | - | - | 5,460 | 0 | |
| PU07 | 13-Jan | >2419.6 | >2419.6 | - | - | 387.3 | 49.9 | 5.2 | 1 | - | - | 4,990 | 0 | |
| PU08 | 13-Jan | >2419.6 | >2419.6 | - | - | 1553.1 | 1 | 13.2 | 0 | - | - | >2419.6 | 0 | |
| PU09 | 13-Jan | 2419.6 | 98.7 | - | - | 34.5 | 0 | 0 | 0 | - | - | 98.7 | 0 | |
| BJ820 | 28-Jan | >2419.6 | >2419.6 | - | - | >2419.6 | >2419.6 | 224.7 | 224.7 | 3.1 | 2.1 | 2,247,000 | 1 | P |
| BJ856 | 28-Jan | >2419.6 | >2419.6 | - | - | >2419.6 | >2419.6 | 118.7 | 18.7 | 4.1 | 2.1 | 2,100,000 | 1 | P |
| BJ030 | 28-Jan | >2419.6 | 153.9 | - | - | >2419.6 | 31.8 | 93.3 | 0 | 0 | 0 | 3,180 | 1 | A |
| BJ800 | 28-Jan | >2419.6 | >2419.6 | - | - | >2419.6 | >2419.6 | >2419.6 | 1986.3 | 101.4 | 17.1 | 17,100,000 | 1 | P |
| FPF | 28-Jan | 1297.7 | 1 | - | - | 186 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | P |
| FM08 | 28-Jan | >2419.6 | >2419.6 | | | >2419.6 | >2419.6 | >2419.6 | 218.7 | 36.4 | 2 | 2,187,000 | 1 | P |
| PUt01 | 25-Jan | - | - | >2419.6 | 307.6 | >2419.6 | 52 | 547.5 | 0 | - | - | 5,200 | 0 | |
| PB08 | 25-Jan | - | - | >2419.6 | 77.6 | 685.7 | 7.5 | 8.4 | 0 | - | - | 776 | 0 | |
| PB05 | 25-Jan | - | - | >2419.6 | 70 | 0 | 0 | 0 | 0 | - | - | 700 | 0 | |
| PB12 | 25-Jan | - | - | >2419.6 | 488.4 | >2419.6 | 35.5 | 290.9 | 0 | - | - | 4,884 | 0 | |
| PUt02 | 25-Jan | - | - | 1046.2 | 16.1 | 172.2 | 4.1 | 0 | 0 | - | - | 410 | 0 | |
| PB06 | 25-Jan | - | - | >2419.6 | 25.3 | 461.1 | 3.1 | 4.5 | 0 | - | - | 310 | 0 | |
| PUt03 | 25-Jan | - | - | >2419.6 | 272.3 | >2419.6 | 46.4 | 36.4 | 0 | - | - | 4,640 | 0 | |
| PB10 | 25-Jan | - | - | >2419.6 | 53.8 | 1732.9 | 6.3 | 13.5 | 0 | - | - | 630 | 0 | |
| PUt04 | 25-Jan | - | - | >2419.6 | 76.7 | 387.3 | 6.3 | 5.2 | 0 | - | - | 767 | 0 | |
| PUt04 | 25-Jan | - | - | >2419.6 | 1553.1 | >2419.6 | 128.1 | 88.4 | 5.1 | - | - | 51,000 | 0 | |

## Table E. 3: E. coli and DNA Results January 2009

| Date | Names | Sub-catchment | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | Averaged *E. coli* | DNA | Latitude | Longitude | DNA Results | DNA ID |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dilutions** | | | **1** | **10** | **100** | **1000** | **10000** | **100000** | | | | | | |
| 1/14/2009 | Blank | Pang Siang | 0 | | | | | | 0 | | | | | |
| 1/15/2009 | Blank | Pang Siang | 0 | | | | | | 0 | | | | | |
| 1/12/2009 | Blank | Pang Siang | 0 | | | | | | 0 | | | | | |
| 1/14/2009 | Blank | | 0 | | | | | | 0 | | | | | |
| 1/13/2009 | Blank | Pang Siang | 0 | | | | | | 0 | | | | | |
| 1/12/2009 | Blank | Pang Siang | 0 | | | | | | 0 | | | | | |
| 1/16/2009 | Blank | | 0 | | | | | | 0 | | | | | |
| 1/14/2009 | 25.4-43.2 | Neo Tiew | | 39 | 10 | 0 | 0 | | 390 | 0 | 1.424 | 103.721 | | |
| 1/19/2009 | 25.2-42.9 | Neo Tiew | | | TNTC | | 29 | 0 | 290,000 | 1 | 1.420 | 103.716 | P | KC5.4 |
| 1/16/2009 | 25.2-42.9 | Neo Tiew | | | 35 | | 1 | 0 | 3500 | 0 | 1.420 | 103.716 | | |
| 1/7/2009 | 25.2-42.9 | Neo Tiew | TNTC | TNTC | 31 | | | | 3100 | 0 | 1.420 | 103.716 | | |
| 1/14/2009 | 25.2-42.9 | Neo Tiew | | 86 | 9 | 1 | 0 | | 860 | 0 | 1.420 | 103.716 | | |
| 1/20/2009 | 25.0-43.1 | Neo Tiew | | | 37 | | 4 | 0 | 3700 | 1 | 1.417 | 103.719 | P | F5 |
| 1/14/2009 | 25.0-42.9 | Neo Tiew | | | 125 | 7 | 2 | | 12,500 | 0 | 1.417 | 103.715 | | |
| 1/19/2009 | 25.0-42.9 | Neo Tiew | | | 5 | | 0 | 0 | 500 | 1 | 1.417 | 103.715 | A | KC5.5 |
| 1/7/2009 | 24.1-42.0 | Kangarkar | TNTC | TNTC | 107 | | | | 10,700 | 0 | 1.403 | 103.701 | | |
| 1/15/2009 | 24.1-42.0 | Kangarkar | | | 10 | 0 | 1 | | 5500 | 0 | 1.403 | 103.701 | | |
| 1/15/2009 | 24.0-42.0-E | Kangarkar | | | 0 | | 0 | 0 | 0 | 0 | 1.401 | 103.700 | | |
| 1/15/2009 | 24.0-42.0-D | Kangarkar | | | 6 | | 4 | 0 | 40,000 | 0 | 1.401 | 103.701 | | |
| 1/15/2009 | 24.0-42.0-D | Kangarkar | | | 0 | | 0 | 0 | 0 | 0 | 1.401 | 103.701 | | |
| 1/19/2009 | 24.0-42.0-C | Kangarkar | | | 0 | | 0 | 0 | 0 | 1 | 1.411 | 103.700 | A | KC6.2 |
| 1/15/2009 | 24.0-42.0-C | Kangarkar | | | 0 | | 0 | 0 | 0 | 0 | 1.401 | 103.701 | | |
| 1/15/2009 | 24.0-42.0-B | Kangarkar | | | 1 | | 0 | 0 | 100 | 0 | 1.402 | 103.701 | | |

| Date | Names | Sub-catchment | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | Averaged *E. coli* | DNA | Latitude | Longitude | DNA Results | DNA ID |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dilutions** | | | **1** | **10** | **100** | **1000** | **10000** | **100000** | | | | | | |
| 1/19/2009 | 24.0-42.0-B | Kangarkar | | | 0 | | 0 | 0 | 0 | 1 | 1.411 | 103.700 | A | KC6.1 |
| 1/15/2009 | 24.0-42.0-A | Kangarkar | | | 0 | | 0 | 0 | 0 | 0 | 1.402 | 103.701 | | |
| 1/19/2009 | 24.0-42.0-A | Kangarkar | | | 0 | | 0 | 0 | 0 | 1 | 1.411 | 103.700 | A | KC6.6 |
| 1/16/2009 | 23.9-43.8 | Kangarkar | | | TNTC | | TNTC | TNTC | TNTC | 1 | 1.398 | 103.730 | P | F3 |
| 1/14/2009 | 23.9-43.8 | Kangarkar | | | TNTC | TNTC | TNTC | | TNTC | 0 | 1.399 | 103.730 | | |
| 1/15/2009 | 23.9-42.0-B | Kangarkar | | | 0 | | 0 | 1 | 0 | 0 | 1.399 | 103.701 | | |
| 1/15/2009 | 23.9-42.0-A | Kangarkar | | | 2 | | 0 | 0 | 200 | 0 | 1.399 | 103.701 | | |
| 1/20/2009 | 23.8-43.4-B | Neo Tiew | | | 0 | | 0 | 0 | 0 | 0 | 1.414 | 103.724 | | |
| 1/20/2009 | 23.8-43.4-A | Neo Tiew | | | TNTC | | 83 | 0 | 830,000 | 0 | 1.414 | 103.724 | | |
| 1/7/2009 | 23.6-45.1 | Pang Sua | TNTC | 70 | 5 | | | | 700 | 0 | 1.395 | 103.753 | | |
| 1/7/2009 | 23.4-43.8-C | Tengah | TNTC | 96 | error | | | | 960 | 0 | 1.391 | 103.731 | | |
| 1/7/2009 | 23.4-43.8-B | Tengah | TNTC | 38 | 7 | | | | 380 | 0 | 1.391 | 103.731 | | |
| 1/7/2009 | 23.4-43.8-A | Tengah | TNTC | 65 | 2 | | | | 650 | 0 | 1.391 | 103.731 | | |
| 1/16/2009 | 23.4-43.8 | Tengah | | | 7 | | 2 | 0 | 700 | 0 | 1.391 | 103.731 | | |
| 1/21/2009 | 23.4-43.8 | Tengah | | | 2 | | 0 | 0 | 200 | 1 | 1.381 | 103.759 | A | KC3.1 |
| 1/20/2009 | 23.2-43.3 | Tengah | | | 15 | | 1 | 0 | 1500 | 1 | 1.386 | 103.721 | A | F4 |
| 1/12/2009 | 23.0-44.7-C | Pang Siang | | | 6 | 7 | 0 | | 600 | 0 | 1.385 | 103.747 | | |
| 1/9/2009 | 23.0-44.7-B | Pang Siang | | | 4 | 0 | 0 | | 400 | 0 | 1.385 | 103.746 | | |
| 1/14/2009 | 23.0-44.7-A | Pang Siang | | | 49 | 0 | 0 | | 4900 | 1 | 1.385 | 103.747 | A | KC2.4 |
| 1/12/2009 | 23.0-44.7-A | Pang Siang | | | 3 | 1 | 0 | | 300 | 0 | 1.385 | 103.747 | | |
| 1/9/2009 | 23.0-44.7-A | Pang Siang | | | 0 | 0 | 0 | | 0 | 0 | 1.385 | 103.747 | | |
| 1/9/2009 | 23.0-44.6 | Pang Siang | | | 3 | 0 | 0 | | 300 | 0 | 1.384 | 103.744 | | |
| 1/9/2009 | 23.0-44.5 | Pang Siang | | | error | error | error | | 0 | 0 | 1.384 | 103.743 | | |
| 1/14/2009 | 23.0-44.5 | Pang Siang | | | 5 | 1 | 0 | | 500 | 0 | 1.384 | 103.743 | | |

| Date | Names | Sub-catchment | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | Averaged *E. coli* | DNA | Latitude | Longitude | DNA Results | DNA ID |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dilutions** | | | **1** | **10** | **100** | **1000** | **10000** | **100000** | | | | | | |
| 1/16/2009 | 23.0-43.8-B | Pang Siang | | | TNTC | | TNTC | 29 | 2,900,000 | 0 | 1.383 | 103.731 | | |
| 1/16/2009 | 23.0-43.8-B | Pang Siang | | | TNTC | | TNTC | 4 | 400,000 | 1 | 1.383 | 103.731 | P | F1 |
| 1/14/2009 | 23.0-43.8-B | Pang Siang | | | TNTC | TNTC | TNTC | | TNTC | 0 | 1.383 | 103.731 | | |
| 1/14/2009 | 23.0-43.8-B | Pang Siang | | | TNTC | TNTC | TNTC | | TNTC | 0 | 1.383 | 103.731 | | |
| 1/16/2009 | 23.0-43.8-A | Pang Siang | | | TNTC | | 10 | 0 | 100,000 | 1 | 1.383 | 103.731 | P | F4A |
| 1/14/2009 | 23.0-43.8-A | Pang Siang | | | TNTC | 49 | 2 | | 49,000 | 0 | 1.383 | 103.731 | | |
| 1/20/2009 | 23.0-43.6 | Pang Siang | | | TNTC | | TNTC | TNTC | TNTC | 1 | 1.383 | 103.726 | P | F2 |
| 1/9/2009 | 22.9-44.6 | Pang Siang | | | 0 | 0 | 1 | | 0 | 0 | 1.382 | 103.743 | | |
| 1/9/2009 | 22.9-44.5 | Pang Siang | | | 0 | 0 | 0 | | 0 | 0 | 1.383 | 103.743 | | |
| 1/13/2009 | 22.9-44.4-C | Pang Siang | | | 137 | 49 | 6 | | 60,000 | 0 | 1.383 | 103.741 | | |
| 1/13/2009 | 22.9-44.4-B | Pang Siang | | | 74 | 12 | 2 | | 20,000 | 0 | 1.383 | 103.741 | | |
| 1/7/2009 | 22.9-44.3-E | Pang Siang | TNTC | 72 | 13 | | | | 1300 | 0 | 1.383 | 103.739 | | |
| 1/9/2009 | 22.9-44.3-E | Pang Siang | | | 2 | 0 | 0 | | 200 | 0 | 1.383 | 103.739 | | |
| 1/7/2009 | 22.9-44.3-D | Pang Siang | TNTC | TNTC | TNTC | | | | TNTC | 0 | 1.383 | 103.739 | | |
| 1/9/2009 | 22.9-44.3-D | Pang Siang | | | 34 | 21 | 1 | | 21,000 | 0 | 1.383 | 103.739 | | |
| 1/9/2009 | 22.9-44.3-D | Pang Siang | | | 47 | 0 | 1 | | 4700 | 0 | 1.383 | 103.739 | | |
| 1/9/2009 | 22.9-44.3-C | Pang Siang | | | 90 | 3 | 0 | | 9000 | 0 | 1.383 | 103.739 | | |
| 1/7/2009 | 22.9-44.3-C | Pang Siang | TNTC | TNTC | 28 | | | | 2800 | 0 | 1.383 | 103.739 | | |
| 1/9/2009 | 22.9-44.3-B | Pang Siang | | | 88 | 9 | 1 | | 9000 | 0 | 1.383 | 103.739 | | |
| 1/7/2009 | 22.9-44.3-B | Pang Siang | TNTC | TNTC | 43 | | | | 4300 | 0 | 1.383 | 103.739 | | |
| 1/7/2009 | 22.9-44.3-A | Pang Siang | TNTC | TNTC | 43 | | | | 4300 | 0 | 1.383 | 103.739 | | |
| 1/9/2009 | 22.9-44.3-A | Pang Siang | | | 29 | 7 | 1 | | 7000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-9 | Pang Siang | | | 13 | | 0 | 0 | 1300 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-8 | Pang Siang | | | 57 | | error | 0 | 5700 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-7 | Pang Siang | | | TNTC | | 10 | 0 | 100,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-6 | Pang Siang | | | 78 | | 2 | 0 | 20,000 | 0 | 1.383 | 103.739 | | |

| Date | Names | Sub-catchment | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | Averaged *E. coli* | DNA | Latitude | Longitude | DNA Results | DNA ID |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dilutions** | | | **1** | **10** | **100** | **1000** | **10000** | **100000** | | | | | | |
| 1/22/2009 | 22.9-44.3-5 | Pang Siang | | | TNTC | | 6 | 0 | 60,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-4 | Pang Siang | | | 26 | | 0 | 0 | 2600 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-3 | Pang Siang | | | TNTC | | 1 | 0 | 10,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-23 | Pang Siang | | | 74 | | 4 | 0 | 40,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-22 | Pang Siang | | | 107 | | 2 | 0 | 20,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-21 | Pang Siang | | | 43 | | 1 | 0 | 4300 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-20 | Pang Siang | | | TNTC | | 1 | 0 | 10,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-2 | Pang Siang | | | 54 | | 3 | 0 | 30,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-19 | Pang Siang | | | 10 | | 1 | 0 | 1000 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-18 | Pang Siang | | | 23 | | 1 | 0 | 2300 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-17 | Pang Siang | | | 5 | | 2 | 0 | 500 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-16 | Pang Siang | | | 20 | | 0 | 0 | 2000 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-15 | Pang Siang | | | 35 | | 6 | 1 | 3500 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-14 | Pang Siang | | | 28 | | 6 | 3 | 2800 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-13 | Pang Siang | | | 90 | | 4 | 1 | 40,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-10 | Pang Siang | | | 16 | | 0 | 0 | 1600 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-1 | Pang Siang | | | TNTC | | 15 | 0 | 150,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-0 | Pang Siang | | | TNTC | | 6 | 0 | 60,000 | 0 | 1.383 | 103.739 | | |
| 1/16/2009 | 22.9-43.8-D | Pang Siang | | | TNTC | | 145 | 7 | 700,000 | 0 | 1.383 | 103.731 | | |
| 1/16/2009 | 22.9-43.8-C | Pang Siang | | | 141 | | 4 | 0 | 40,000 | 0 | 1.383 | 103.731 | | |
| 1/16/2009 | 22.9-43.8-B | Pang Siang | | | TNTC | | TNTC | 21 | 2,100,000 | 0 | 1.383 | 103.731 | | |
| 1/22/2009 | 22.9-44.3-20 | Pang Siang | | | TNTC | | 1 | 0 | 10,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-2 | Pang Siang | | | 54 | | 3 | 0 | 30,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-19 | Pang Siang | | | 10 | | 1 | 0 | 1000 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-18 | Pang Siang | | | 23 | | 1 | 0 | 2300 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-17 | Pang Siang | | | 5 | | 2 | 0 | 500 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-16 | Pang Siang | | | 20 | | 0 | 0 | 2000 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-15 | Pang Siang | | | 35 | | 6 | 1 | 3500 | 0 | 1.383 | 103.739 | | |
| 1/21/2009 | 22.9-44.3-14 | Pang Siang | | | 28 | | 6 | 3 | 2800 | 0 | 1.383 | 103.739 | | |

| Date | Names | Sub-catchment | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | Averaged *E. coli* | DNA | Latitude | Longitude | DNA Results | DNA ID |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dilutions** | | | **1** | **10** | **100** | **1000** | **10000** | **100000** | | | | | | |
| 1/22/2009 | 22.9-44.3-1 | Pang Siang | | | TNTC | | 15 | 0 | 150,000 | 0 | 1.383 | 103.739 | | |
| 1/22/2009 | 22.9-44.3-0 | Pang Siang | | | TNTC | | 6 | 0 | 60,000 | 0 | 1.383 | 103.739 | | |
| 1/16/2009 | 22.9-43.8-D | Pang Siang | | | TNTC | | 145 | 7 | 700,000 | 0 | 1.383 | 103.731 | | |
| 1/16/2009 | 22.9-43.8-C | Pang Siang | | | 141 | | 4 | 0 | 40,000 | 0 | 1.383 | 103.731 | | |
| 1/16/2009 | 22.9-43.8-B | Pang Siang | | | TNTC | | TNTC | 21 | 2,100,000 | 0 | 1.383 | 103.731 | | |
| 1/16/2009 | 22.9-43.8-A | Pang Siang | | | 125 | | 1 | 1 | 12,500 | 0 | 1.383 | 103.731 | | |
| 1/21/2009 | 22.8-45.5-C | Pang Sua | | | 10 | | 1 | 1 | 1000 | 1 | 1.381 | 103.759 | P | KC7.5 |
| 1/21/2009 | 22.8-45.5-B | Pang Sua | | | 5 | | 0 | 0 | 500 | 1 | 1.381 | 103.759 | I | KC7.4 |
| 1/21/2009 | 22.8-45.5-A | Pang Sua | | | 70 | | 21 | 5 | 500,000 | 0 | 1.381 | 103.759 | | |
| 1/21/2009 | 22.8-45.5-A | Pang Sua | | | 21 | | 7 | 4 | 400,000 | 1 | 1.381 | 103.759 | A | KC7.3 |
| 1/9/2009 | 22.8-44.7 | Pang Siang | | | 90 | 8 | 0 | | 9000 | 0 | 1.380 | 103.745 | | |
| 1/14/2009 | 22.7-44.9-D | Pang Siang | | | 2 | 0 | 0 | | 200 | 0 | 1.379 | 103.749 | | |
| 1/12/2009 | 22.7-44.9-C | Pang Siang | | | TNTC | TNTC | 76 | | 760,000 | 0 | 1.379 | 103.749 | | |
| 1/14/2009 | 22.7-44.9-C | Pang Siang | | | 79 | 10 | 0 | | 7900 | 1 | 1.378 | 103.749 | A | KC2.5 |
| 1/12/2009 | 22.7-44.9-B | Pang Siang | | | TNTC | TNTC | 158 | | 1,580,000 | 0 | 1.379 | 103.749 | | |
| 1/12/2009 | 22.7-44.9-B | Pang Siang | | | TNTC | 17 | 3 | | 30,000 | 0 | 1.379 | 103.749 | | |
| 1/14/2009 | 22.7-44.9-B | Pang Siang | | | 10 | 1 | 0 | | 1000 | 0 | 1.379 | 103.748 | | |
| 1/14/2009 | 22.7-44.9-B | Pang Siang | | | 7 | 0 | 0 | | 700 | 0 | 1.379 | 103.748 | | |
| 1/12/2009 | 22.7-44.9-A | Pang Siang | | | error | error | error | | 0 | 0 | 1.379 | 103.749 | | |
| 1/14/2009 | 22.7-44.9-A | Pang Siang | | | 9 | 13 | 2 | | 20,000 | 0 | 1.379 | 103.748 | | |
| 1/12/2009 | 22.7-44.8-B | Pang Siang | | | 79 | 43 | 7 | | 70,000 | 0 | 1.379 | 103.747 | | |
| 1/12/2009 | 22.7-44.8-A | Pang Siang | | | TNTC | TNTC | 52 | | 520,000 | 0 | 1.379 | 103.747 | | |
| 1/12/2009 | 22.6-44.9-B | Pang Siang | | | 1 | 0 | 0 | | 100 | 0 | 1.378 | 103.749 | | |
| 1/12/2009 | 22.6-44.9-A | Pang Siang | | | 50 | 15 | 3 | | 30000 | 0 | 1.378 | 103.749 | | |
| 1/14/2009 | 22.6-44.9-A | Pang Siang | | | 11 | 0 | 0 | | 1100 | 1 | 1.378 | 103.749 | P | KC2.6 |
| 1/13/2009 | 22.6-44.4-E | Pang Siang | | | 0 | 0 | 0 | | 0 | 0 | 1.378 | 103.741 | | |
| 1/13/2009 | 22.6-44.4-D | Pang Siang | | | 0 | 0 | 0 | | 0 | 0 | 1.378 | 103.741 | | |
| 1/13/2009 | 22.6-44.4-C | Pang Siang | | | 18 | 1 | 1 | | 1800 | 0 | 1.378 | 103.741 | | |
| 1/13/2009 | 22.6-44.4-B | Pang Siang | | | 10 | 0 | 0 | | 1000 | 0 | 1.378 | 103.741 | | |

| Date | Names | Sub-catchment | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *E. coli* | Averaged *E. coli* | DNA | Latitude | Longitude | DNA Results | DNA ID |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dilutions** | | | **1** | **10** | **100** | **1000** | **10000** | **100000** | | | | | | |
| 1/13/2009 | 22.6-44.4-A | Pang Siang | | | 11 | 2 | 2 | | 2000 | 0 | 1.378 | 103.741 | | |
| 1/16/2009 | 22.6-44.4-A | Pang Siang | | | 10 | | 1 | 0 | 5500 | 0 | 1.378 | 103.741 | | |
| 1/13/2009 | 22.6-44.3 | Pang Siang | | | 22 | 5 | 0 | | 5000 | 0 | 1.378 | 103.740 | | |
| 1/7/2009 | 22.6-44.0 | Pang Siang | 87 | 7 | 0 | | | | 87 | 0 | 1.377 | 103.735 | | |
| 1/15/2009 | 22.5-44.1-C | Pang Siang | | | error | error | error | | 0 | 0 | 1.376 | 103.736 | | |
| 1/15/2009 | 22.5-44.1-B | Pang Siang | | | 2 | 0 | 0 | | 200 | 0 | 1.376 | 103.736 | | |
| 1/19/2009 | 22.5-44.1-B | Pang Siang | | | 0 | | 0 | 0 | 0 | 1 | 1.376 | 103.736 | A | KC1.2 |
| 1/15/2009 | 22.5-44.1-A | Pang Siang | | | 3 | 0 | 0 | | 300 | 0 | 1.376 | 103.736 | | |
| 1/19/2009 | 22.5-44.1-A | Pang Siang | | | 0 | | 0 | 0 | 0 | 1 | 1.376 | 103.736 | A | KC1.1 |
| 1/7/2009 | 22.3-42.9 | Tengah | error | error | 0 | | | | 0 | 0 | 1.373 | 103.715 | | |
| 1/22/2009 | 22.1-42.7-B | Tengah | | | 1 | | 0 | 0 | 0 | 1 | 1.369 | 103.712 | I | KC4.3 |
| 1/22/2009 | 22.1-42.7-A | Tengah | | | 0 | | 0 | 0 | 0 | 1 | 1.369 | 103.712 | I | KC4.2 |
| 1/15/2009 | 21.7-44.5 | Pang Siang | | | 37 | 4 | 0 | | 4000 | 0 | 1.362 | 103.743 | | |
| 1/15/2009 | 21.7-44.5 | Pang Siang | | | 30 | 8 | 0 | | 8000 | 0 | 1.362 | 103.743 | | |
| 1/22/2009 | 24.7-43.7 | Reservoir | | | | | | | | 1 | 1.413 | 103.729 | A | R2/ResC |
| 1/22/2009 | 25.9-44.5 | Reservoir | | | | | | | | 1 | 1.432 | 103.742 | P | R6/ResB |
| 1/22/2009 | 24.7-43.5 | Reservoir | | | | | | | | 1 | 1.412 | 103.725 | A | R3/ResD |
| 1/22/2009 | 26.1-44.2 | Reservoir | | | | | | | | 1 | 1.435 | 104.476 | A | R1/ResA |

## Appendix F: Field Data Sheets: January 2009 and Field Sheets July 2009

## Appendix F1: Field Data Sheet: DNA sampling Locations January 2009

1

Date: 20/01/09

Sample Name: 24.0-42.0-B

Latitude: 1°24.033'

Longitude: 103°42.016'

Sub-Catchment: KC6

Notes: DNA

Location Sketch

See 24.0-42.0-A

N

| Sampling | |
|---|---|
| Time Sampled | 1:10PM |
| Sample Volume | 500ml bottle 500ml bag |
| Sampled By | JP |
| A/W Temp | 34/30 |
| pH | |
| Conduc. | |
| DO | |
| Turbidity | |

| Analysis | | | | |
|---|---|---|---|---|
| Dilution | $10^6$ | $10^4$ | $10^2$ | DNA |
| Volume Analyzed | 100ml | → | → | 323ml Vol Pump |
| Incubator Time | 2:11PM | 2:11PM | 2:11PM | 10:30AM Pump |
| Read Date | 21/01/09 | 21/01/09 | 21/01/09 | 20/01/09 Date pumped |
| Read Time | 3.51pm | 3.51pm | 3.53pm | |
| Read By | LJ | LJ | LJ | |
| # TC | 0 | 4 | ~~52~~ | |
| # EC | 0 | 0 | 0 | |

2

Date: 20/01/09

Sample Name: 24.0-42.0-C

Latitude: 1°24.070'

Longitude: 103°42.005'

Sub-Catchment: KC6

Notes: DNA

Location Sketch

N

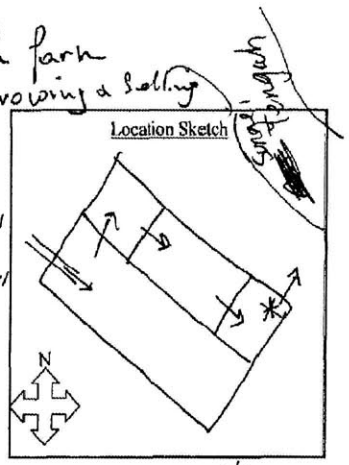
| Sampling | |
|---|---|
| Time Sampled | 2:35PM |
| Sample Volume | 500mL bottle 500mL bag |
| Sampled By | JP |
| A/W Temp | 34/32 |
| pH | |
| Conduc. | |
| DO | |
| Turbidity | |

| Analysis 20/01/09 | | | | |
|---|---|---|---|---|
| Dilution | $10^6$ | $10^4$ | $10^2$ | DNA |
| Volume Analyzed | 1ml | → | → | 1liter Volume pumped |
| Incubator Time | 1:39PM | → | → | 2:35 PM |
| Read Date | 21/01/09 | 21/01/09 | 21/01/09 | 9338AM Pump |
| Read Time | 3.43pm | 3.43pm | 3.43pm | 20/01/09 Pumped |
| Read By | LJ | LJ | LJ | |
| # TC | 1 | 0 | 2 | |
| # EC | 0 | 0 | 0 | |

2

Date: 20/1/09

fish farm
Evovoing & Selling




Sample Name: 23.2-43.3

Latitude: ~~1°23'~~ 01°23.177'

Longitude: 103°43.285'

Sub-Catchment:

Notes: septic tank drains directly to Sungei Tengah

| Sampling | |
|---|---|
| Time Sampled | 5:15p |
| Sample Volume | 100ml bag bottle for DNA |
| Sampled By | JP |
| Temp | |
| pH | |
| Conduc. | |
| DO | |
| Turbidity | |

| Analysis | | | | |
|---|---|---|---|---|
| Dilution | $10^6$ | $10^4$ | $10^2$ | DNA |
| Volume Analyzed | 100 | → | → | 186ml/bottle |
| Incubator Time | 11:50a | → | → | 20/01/09 5:15PM |
| Read Date | 22/01/09 | 22/01/09 | 22/01/09 | 21/01/09 |
| Read Time | 12:15AM | 12:15AM | 12:15AM | 9:30AM |
| Read By | JP | JP | JP | |
| # TC | 2 | 16 | TNTC | |
| # EC | 0 | 1 | 15 | |

24/1 JY

Date: 20/1/09

Sample Name: 23.0-43.6

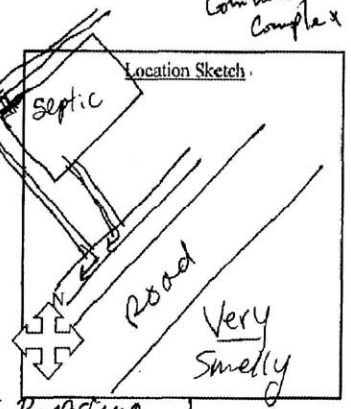
Latitude: 01°23.005'

Longitude: 103°43.569'

Sub-Catchment:

Notes: Farm A septic drains directly through concrete drain on side of St. to Sungei Pengstang

of Fishing & Restaurant Commercial Complex




| Sampling | |
|---|---|
| Time Sampled | 5:55p |
| Sample Volume | 100ml Bag bottle DNA |
| Sampled By | JP |
| Temp | 31/26 |
| pH | |
| Conduc. | |
| DO | |
| Turbidity | |

| Analysis | | | | |
|---|---|---|---|---|
| Dilution | $10^6$ | $10^4$ | $10^2$ | DNA |
| Volume Analyzed | 100ml | → | → | 70ml vol |
| Incubator Time | 12:00A | 12:00A | 12:00A | 21/01/09 |
| Read Date | 22/01/09 | 22/01/09 | 22/01/09 | 9:45AM |
| Read Time | 12:10AM | 12:10AM | 12:10AM | |
| Read By | JP | JP | JP | |
| # TC | TNTC | TNTC | TNTC | |
| # EC | TNTC | TNTC | TNTC | |

**Appendix F2: Field Data Sheets of Environmental DNA Water Samples Collected July 2009**

7/12/2009

| Names of Samples | Time collected | Time setup | Date read | ~~pH~~ water filtered | Time ~~ready~~ filtering | Total Coliform (Yellow) # Large positive | Total Coliform (Yellow) # Small positive | Total Coliform (Yellow) MPN (CFU/100ml) | E.coli (Yellow and Fluorescent) # Large Positive | E.coli (Yellow and Fluorescent) # Small Positive | E.coli (Yellow and Fluorescent) MPN (CFU/100ml) | Field Notes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PB002 (1) DNA Sample KC1 KC1 Main Drainage | left 10:38A → 10:36A | PB002 | | 1L | 9 min | | | | | | | Tray DNA sampling Left hand KC1-A PB002 |
| PB003 (2) DNA KC1-M-Right (A) KC1-1 | 10:48A → 11:00A | 500ml | | 1L | 11 min | | | | | | | 11 ml 650 (KC1-B) PB003 KC1-B-1 |
| (B) | S-11:09A E-11:10A | 400ml | | | 3 min | | | | | | | 2 minutes KC2-B-2 14 min |
| (3) KC2-A - upstream ackl PS002 Chog chu kang loop | S-11:42A E-11:43A | | | 1L | 5 min | | | | | | | Chog chu kang loop upstream KC2 Across blk 342-341 PS003 Natural drainage |
| (4) PS005 | | | | | | | | | | | | blk 296, Drainage in front of the road entrance |
| (5) PS005 | | | | | | | | | | | | blk 296, Drainage in front of main entrance |
| NB :- The Ecoli & Total coliform tests were not performed, because it was Friday and we were not allowed to use the laboratory. | | | | | | | | | | | | |

(1) Reservoir & Sample Incubation sheet 13/7/09

| Names of Samples | Time collected | Time setup | Date read | ~~pH~~ Volume | Time read | Total Coliform (Yellow) # Large positive | Total Coliform (Yellow) # Small positive | Total Coliform (Yellow) MPN (CFU/100ml) | E.coli (Yellow and Fluorescent) # Large Positive | E.coli (Yellow and Fluorescent) # Small Positive | E.coli (Yellow and Fluorescent) MPN (CFU/100ml) | Field Notes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6, 1/1000 Res006 | | 3:12 pm | 07/14 | ①250ml ②~~100ml~~ 350ml | 3:37 PM | 0 | 0 | <1 | 0 | 0 | <1 | |
| 6, 1/10,000 Res006 | | 3:40 pm | 07/14 | | 3:44 pm | 0 | 0 | <1 | 0 | 0 | <1 | |
| 6, undiluted Res006 | | 3:43 pm | 07/14 | | 3:41 pm | 49 | 29 | 579.4 | 2 | 0 | 2.0 | |
| 4, undiluted Res004 | | 3:39 pm | 07/14 | 180ml | 4:27 pm | 49 | 45 | 1732.9 | 2 | 1 | 3.0 | |
| 4, 1/1000 Res004 | | ~~3:48~~ 4:09 pm | 07/14 | | 4:31 PM | 0 | 0 | 0 | 0 | 0 | 0 | |
| 4, 1/10,000 Res004 | | 3:56 pm | 07/14 | | 4:29 pm | 0 | 0 | 0 | 0 | 0 | 0 | |
| 11, undiluted Res011 | | 3:50 PM | 07/14 | 250ml | 3.47 pm | 49 | 48 | >2419.6 | 4 | 0 | 4.1 | |
| 11, 1/1000 Res011 | | 3:57 PM | 07/14 | ~~350ml~~ PM | 3:57 pm | 0 | 0 | 0 | 0 | 0 | 0 | |

(9)

7/13/09

| Names of Samples | Time collected | Time setup | Date read | ~~pH~~ Volume | Time read | Total Coliform (Yellow) # Large positive | Total Coliform (Yellow) # Small positive | Total Coliform (Yellow) MPN (CFU/100ml) | E.coli (Yellow and Fluorescent) # Large Positive | E.coli (Yellow and Fluorescent) # Small Positive | E.coli (Yellow and Fluorescent) MPN (CFU/100ml) | Field Notes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11, 1/10,000 Res 11 | | 4:08 PM | | | 4:25 pm | 0 | 0 | 0 | 0 | 0 | 0 | |
| ✱ 11, Duplicate Res 11 | | 9:20 PM | | | 4:24 pm | 49 | 47 | 2419.6 | 2 | 1 | 3.0 | |
| 3, undiluted Res 3 | | 4:20 pm | | | | 45 | 13 | 1483 | 0 | 0 | 0 | |
| 3, 1/100 Res 3 | | 4:27 pm | | 200ml | | 0 | 0 | 0 | 0 | 0 | 0 | |
| 3, 1/10,000 Res 3 | | 4:39 pm | | | 4:50 PM | 0 | 0 | 0 | 0 | 0 | 0 | |
| 12, undil Res 12 | | 4:27 PM | | 400ml | 5:10 PM | 49 | 48 | >2419.6 | 10 | 0 | 11.0 | |
| 12, 1/1000 Res 12 | | 4:32 PM | | | 5:09 PM | 0 | 0 | 0 | 0 | 0 | 0 | |
| 12, 1/10,000 Res 12 | | 4:40 PM | | | 5:08 PM | 0 | 0 | 0 | 0 | 0 | 0 | |

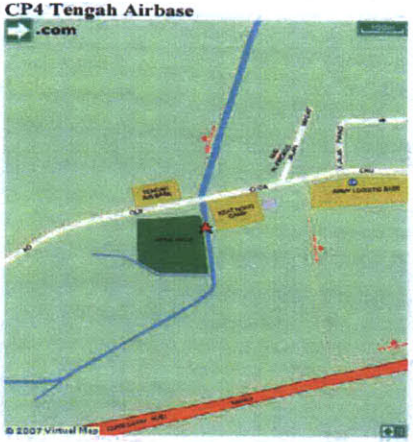
## Appendix G: Locations of Auto-samplers and Rainfall Gauges in Kranji Catchment

KRANJI CATCHMENT SAMPLING LOCATIONS

CP1 Bricklands Road

CP2 Choa Chu Kang Avenue 4


CP4 Tengah Airbase



CP6 Ama Keng Road

CP7 Choa Chu Kang Link