Analysis of the Efficacy of a Constructed Wetland in Treating Human Fecal Contamination

By

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ABSTRACT

The efficiency of a system of constructed wetlands in treating non-point source pollution, particularly, human fecal contamination, was evaluated by collecting and analyzing water samples using both conventional culture-based methods to enumerate indicator bacteria and quantitative polymerase chain reaction (qPCR) method to quantify the human host-specific *Bacteroides* 16S rRNA genetic marker. Constructed wetlands behave as sinks and transform pollutants to improve the quality of surface runoff. The Alexandra Canal constructed wetland in Singapore is a system of four wetlands, each having different retention times, where water flows in series from a sedimentation bay to a surface flow wetland, thereafter passing through a floating aquatic wetland, and finally through a subsurface wetland.

Measured concentrations of total coliform ranged between 300 and 40,000 MPN/100 mL, *E. coli* between 1 and 1000 MPN/100 mL, enterococci between 1 and 750 MPN/100 mL, and the HF marker between 2.7×10^3 and 4.6×10^5 CE/100 mL. The overall removals of total coliform, *E. coli*, and enterococci were negligible. HF marker cells were present at higher concentrations than the indicator bacteria. The concentrations of indicator bacteria were found to decrease through the wetland system until they reached the subsurface wetland, where the concentrations increased. This may be attributed to bacterial growth in the subsurface environment in the absence of sunlight which would otherwise cause bacterial die-off. The HF marker increased as water flowed through the system; however the increase was within the range of measurement variability. No significant statistical correlations were found between microbiological indicators and the HF marker. Overall, the constructed wetland is effective in that the concentrations of indicator bacteria decreased, although further research is recommended to understand the decay mechanisms of HF marker in wetlands. Also, a better understanding of the persistence of the HF marker as compared to indicator bacteria is required.

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Title: Senior Lecturer of Civil and Environmental Engineering

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1. Background Information

Sections 1.1 to 1.6 were written in collaboration with Janhvi Manoj Doshi, Laurie Kellndorfer, and Suejung Shin.

1.1. Active, Beautiful, and Clean Waters Program

The Singapore Public Utility Board (PUB) wishes to expand recreational activities at Singapore's reservoirs. Singapore has limited land area for recreation, and making use of selected waterways and waterbodies is an integral part of PUB's plan to meet public recreational needs. Singapore has been working to enhance the accessibility, usability, and aesthetics of green spaces and parks, especially near waterways and drainage (Soon et al., 2009). PUB wishes to open more of Singapore's surface waters to recreational activities under the Active, Beautiful, and Clean Waters Program (ABC Waters). The goals of the ABC Waters program are to bring the people of Singapore closer to their water resources by providing new recreational space and developing a feeling of ownership and value. The program aims to develop surface waters into aesthetic parks, estates, and developments. This plan will minimize pollution in the waterways by incorporating aquatic plants, retention ponds, fountains, and recirculation to remove nutrients and improve water quality (PUB, 2011). One of the greatest areas of concern with this plan is microbial pollution.

Disease-causing pathogens pose the greatest immediate threat to human health in polluted surface waters. Humans can come into contact with waterborne pathogens through drinking water supply and through recreation in contaminated surface waters. Infection in humans can be caused by ingestion of, contact with, or inhalation of contaminated water (Hurston, 2007). While the exact total number of waterborne pathogens is unknown, it is estimated that over 1,000 viral and bacterial agents in surface waters can make humans sick. Diseases from waterborne pathogens can range from mild to life threatening forms of gastroenteritis, hepatitis, skin and wound infections, conjunctivitis, respiratory infection, and other general infections. In order to open surface waterways and reservoirs for recreation, PUB must minimize pathogenic pollution in surface waters and keep the public safe.

1.2. Singapore's Water History

Water use and water resources have been of great concern to Singapore throughout its history. After over a century under British rule and Japanese occupation during World War II, Singapore and Malaysia became one independent nation in 1963 (Evans & Scrivers, 2008). Singapore separated from Malaysia two years later and became its own independent nation in 1965. Although Singapore had gained political independence, Singapore had no adequate source of fresh drinking water for its citizens. Singapore has been dependent on Malaysia for freshwater for its entire history as an independent country.

To date, Singapore and Malaysia have signed four water agreements—one each in 1927, 1961, 1962, and 1990 (Chew, 2009). Two of these agreements have already expired, but the 1962 Johor River Water Agreement and a 1990 agreement between PUB and the Johor State Government allow Singapore to use freshwater from Malaysia until 2061. With price increases

from the Malaysian government and fear of future conflicts, the government of Singapore constantly is currently working toward water independent.

1.3. Innovative Water Resource Management

With a dense population inhabiting a small island, Singapore is forced to be innovative with its water management practices. PUB attributes its success in water management to the separation of storm water and wastewater, incorporation of technological developments, and strict regulation and legislation. About 20 percent of Singapore's water supply comes from rainfall, about 40 percent is imported from Malaysia, about 30 percent comes from reclaimed wastewater, and about 10 percent comes from desalination (PUB, 2011).

Singapore keeps its stormwater and wastewater streams completely separate. Stormwater is collected in a network of drains, rivers, canals, ponds, and reservoirs. All collected water, even from urban catchments is collected and treated for drinking water. Singapore aims for sustainable stormwater management practices and has been using Best Management Practices (BMPs) to treat stormwater before it enters rivers and reservoirs. Many BMPs in Singapore include bioretention and vegetated swales, bioretentive basins, rain gardens, sedimentation basins, constructed wetlands, and cleansing biotopes (PUB, 2011).

Singapore also has the largest desalination capacity in Southeast Asia. Currently, Singapore treats 30 million gallons per day (MGD) of sea water for drinking water. By 2060, PUB hopes to expand this capacity to meet 30 percent of Singapore's drinking water supply (PUB, 2011).

All sewage and wastewater is collected and treated. Wastewater is reclaimed after secondary treatment, dual-membrane filtration, and ultraviolet treatment technologies through the NEWater program. NEWater reclaimed wastewater is of drinking water quality but is mostly used for industrial and commercial water supply. Its purity is higher than most tap water, making it ideal for industries such as semiconductor manufacturing requiring ultrapure water (Tortajada, 2006). Currently there are four NEWater plants in Singapore that contribute to approximately 30 percent of Singapore's water needs. PUB plans to expand NEWater to 50 percent of Singapore's water needs by 2050 (PUB, 2011).

1.4. Population Growth and Water Use in Singapore

Singapore is highly urbanized, with an ever growing population living on a 700 km^2 island. As of 2010, 100 percent of the population lives in urban areas (Central Intelligence Agency, 2011). Despite Singapore's increasing population (Figure 1), per-capita water consumption has decreased due to successful demand management practices (Figure 2). These include a progressive tariff structure, a water conservation tax, and a water-borne fee. The tariff charges 117 cents per m^3 for 1-20 m^3 of water used per month, with progressively higher rates for 20-40 m^3 used and above 40 m^3 used. The water conservation tax charges 30 percent upon consumption 40 m^3 and under, and 45 percent for consumption above 40 m^3. The water-borne fee charges 30 percent for all consumption blocks (Tortajada, 2006). These charges and taxes reflect great increases from original tariffs and fees implemented prior to 1997 and are attributed to the decline in per capita water use.

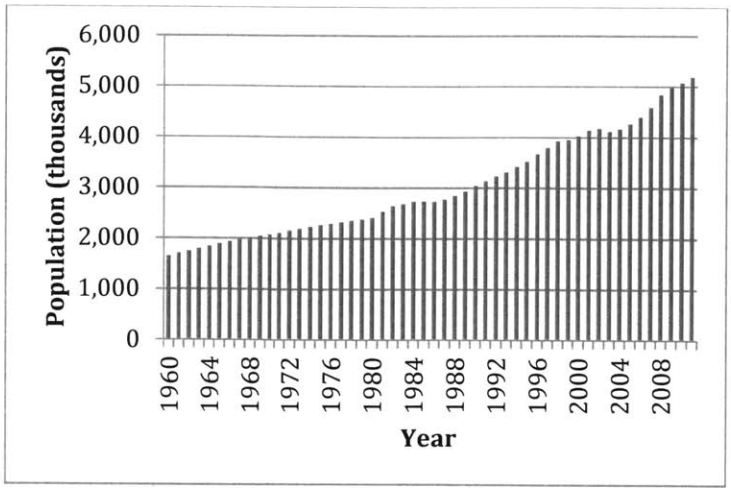

Figure 1 - Singapore population growth over time (Singapore Department of Statistics, 2011)

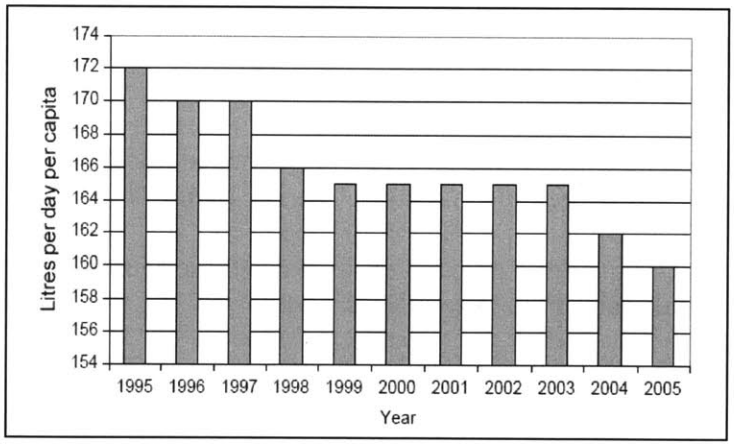

Figure 2 - Per-capita domestic water consumption (Tortajada, 2006)

1.5. Economics of the Water Industry in Singapore

In addition to implications of future water independence, recent technological developments for water collection, treatment, reclamation, and desalination have significant economic implications for Singapore. Overall, these projects require large initial capital investments, but the water industry in Singapore has a clear economic benefit.

Economic investments in research and development have led to an increased supply of self-sufficient water sources within Singapore. In 2009, Singapore invested S$6 billion in the water related research and development to promote the growth of this industry. The water industry added S$590 million to the economy since 2005 and 2,300 new domestic professional and skilled jobs. Singapore companies have also secured S$8.4 billion in water related business overseas (Nie, 2011).

In 2008, around S$1.47 billion capital expenditure was set for water infrastructure development between 2008 and 2013. Some of this capital contributed to 87 km of expansion of water delivery lines, which cost about S$400 million. Additional water infrastructure projects include the construction of water reclamation facilities. Currently, 30 percent of the demand for water in Singapore is being met by reclaimed wastewater (Evans & Scrivers, 2008). Singapore spent approximately S$200 million for a reclamation plant at SingSpring and S$380 million for a plant in Ulu Pandan (MEWR, 2008). Singapore has also recently invested S$7 billion in a sewer system expansion called the Deep Tunnel Sewerage System in an effort to meet the wastewater demand of a growing population. While this project may seem expensive, it is estimated to save Singapore S$5.2 billion in wastewater management costs in the future (Soon et al., 2009). Singapore also has monitoring programs to reduce losses in their water delivery system due to leaks and illegal draw-off. After the implication of these monitoring systems, losses in the water delivery system dropped from 9.5% to 4.4%, saving around S$200 million. Continued monitoring is expected to save an additional S$24 million in the future (Soon et al., 2009).

1.6. Overview of the Geology and Soils of Singapore

Singapore's geology and soils have a large impact on hydrologic processes, including above-ground and below-ground transport of water and contaminants. This section contains a brief overview of the island's geology and soil types and properties.

The solid rock foundation below Singapore is generally divided into four main series (Sharma et al., 1999): Bukit Timah granite and Gombak norite (igneous rocks), Jurong Formation (sedimentary rocks), Old Alluvium (Quaternary deposits), and Kallang Formation (recent, alluvium, marine clay). The geologic map below includes these four rock types, along with two others that make up a small part of the island (Figure 3).

The space limitations of the island coupled with the drive for infrastructural development on it has meant that soil studies rarely impact the decision to develop a plot of land. If the original soil is deemed unsuitable, the project is built all the same on modified, additionally supported or replaced soil (Rahman, 1991). The island's geology makes the extraction of groundwater unfeasible (Rahman, 1993), though Pitts (Pitts, 1985) reported that in the low-lying areas of the island the groundwater table is only 1.5 m below the ground surface. Because of the lack of general interest there are only a handful of cited studies on the soils in Singapore. Most of these studies look at the impact of soils on construction rather than the hydrogeology. However, since the 1980s there have been several studies aimed at classifying soils around the island and estimating values of permeability. Given the highly varied geology, these studies report that the soil is extremely heterogeneous and the reported hydraulic conductivity values range is over multiple orders of magnitude. The hydraulic conductivity of a highly productive aquifer can range from 10^{-4} (gravels, sands) to as much as 1 m/s (gravels). In comparison, the hydraulic conductivity values of the soils of Singapore are extremely low and as a result, groundwater is not regarded part of the island's resources.

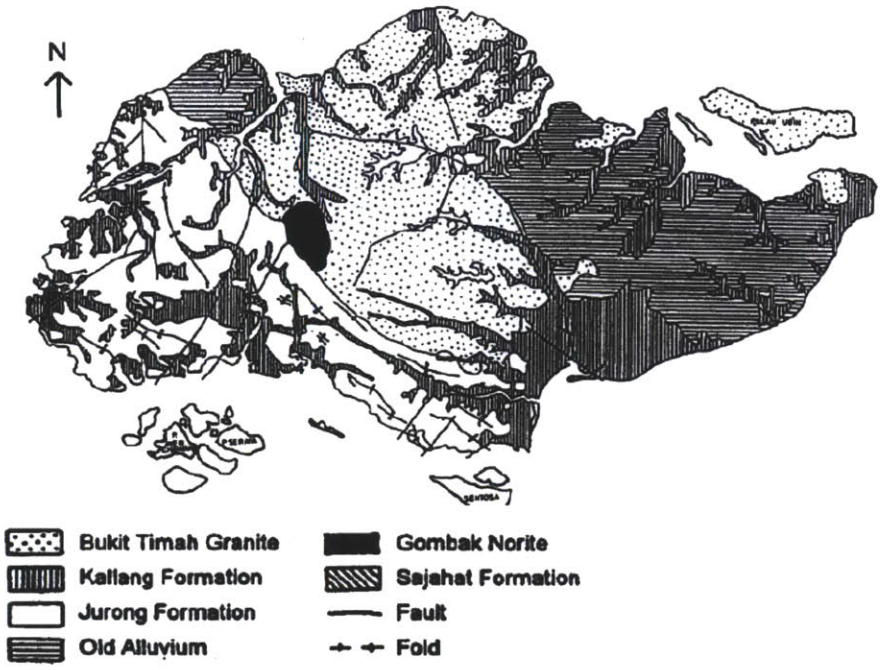

Figure 3 - Geologic rock types found in Singapore (Sharma et al., 1999). Originally published by Public Works Department, Singapore in 1974.

1.7. Site Characterization of the Alexandra Canal Wetland

Singapore's Public Utilities Board (PUB) launched the Active, Beautiful, and Clean (ABC) Waters Programme in 2007 (AsiaOne, 2011). All the projects completed by ABC Waters until May, 2012 can be seen in Figure 4; they propose to complete more than 100 projects in the next 15-20 years. The 14th project since ABC Waters' commencement is the Alexandra Canal project.

Alexandra Canal, a concrete canal, is a part of the Marina Catchment area and is constructed on the Singapore River (He, 2011). The 1.2-kilometer stretch of the canal from Tanglin Road to Delta Road and Prince Charles Crescent was transformed into a beautiful yet functional waterway (CH2M HILL, 2011). The location map can be viewed in Figure 5. It now serves as a natural habitat for fishes, dragonflies, water birds, and other wildlife (AsiaOne, 2011). The Singapore government's effort to reach out and educate its citizens regarding the importance of water is reflected in this project. One of the prime focuses of this undertaking was on transforming attitudes and behavior of people towards stormwater and waterways.

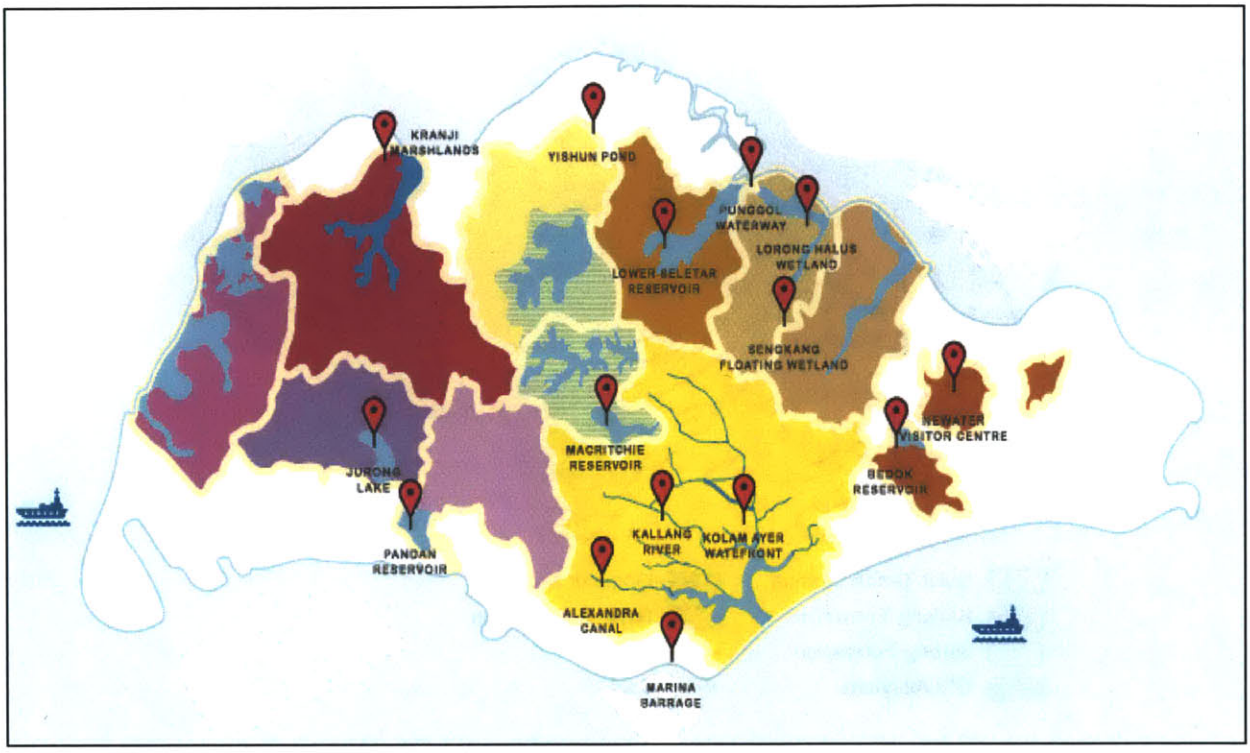

Figure 4 - ABC Waters projects (PUB, 2012)

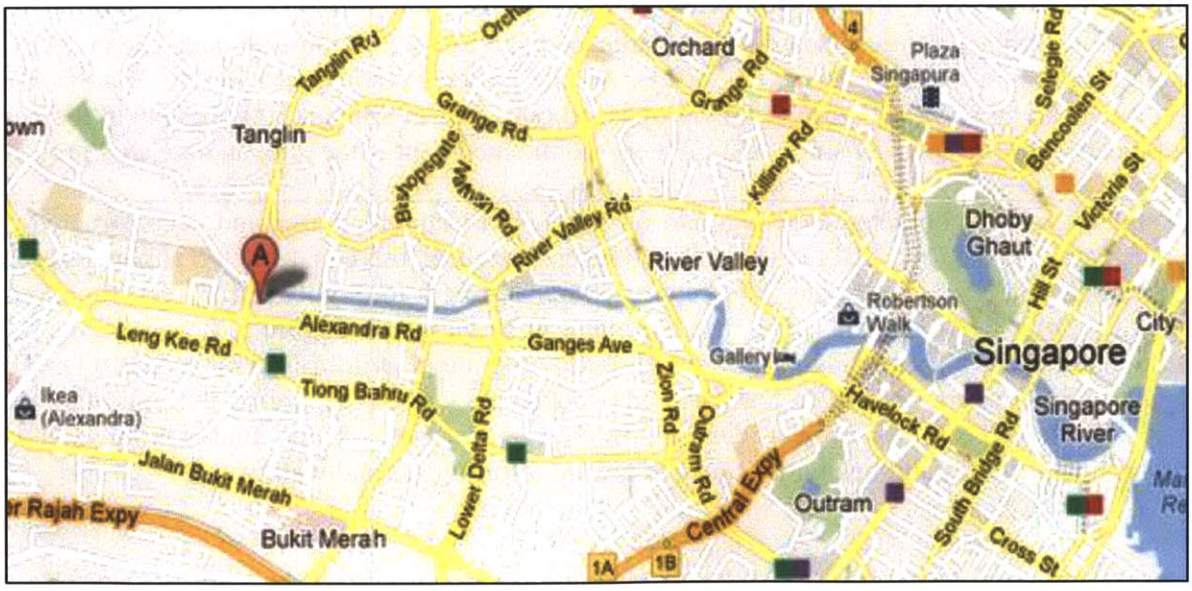

Figure 5 - Alexandra Canal Wetland location (Google Maps, 2012)

CH2M HILL partnered with PUB to design and deliver the Alexandra Canal Wetland (CH2M HILL, 2011). The total cost of the project was S$34 million (AsiaOne, 2011). The entire project was completed in a span of 23 months and was inaugurated by Minister Mentor Lee Juan Yew on March 19, 2011 (AsiaOne, 2011; World Architecture News, 2011). The wetland project was created on a deck constructed over the pre-existing canal. Figure 6 gives a panoramic view of the newly constructed Alexandra Canal Wetland. As the canal was constructed in a highly urbanized area, it led to several design restrictions. This problem was overcome by using innovative structural design and hydraulics modeling (CH2M HILL, 2011). This project shows how one can incorporate sustainable stormwater designs with minimal negative impacts to the hydrologic cycle and aquatic ecology (CH2M HILL, 2011).

The main design features of the canal are a shallow stream, four different types of educational wetlands, and dragonfly sculptures (CH2M HILL, 2011). An elevated lookout deck is what one would find when entering the project from Tanglin Road (World Architecture News, 2011). This deck slopes down and a mild water cascade flows down to create a shallow stream (see Figure 7) which serves as a play area for children (World Architecture News, 2011). The shallow stream is followed by an educational hut where posters developed by students from Crescent Girls' School in collaboration with CH2M HILL and PUB are exhibited (He, 2011).

The water from the Alexandra Canal is pumped into the wetlands, gets treated through the system, and is then allowed to flow back into the underlying canal through an outlet. The four types of urban educational wetlands are as follows (CH2M HILL, 2011).

1. Sedimentation bay
2. Surface flow wetland
3. Floating aquatic wetland
4. Subsurface wetland

The canal water enters the wetland system at the sedimentation bay. The effluent of the sedimentation bay is the influent of the surface flow wetland. The effluent of the surface flow wetland is the influent of the floating aquatic wetland and the effluent of the floating aquatic wetland is the influent of the subsurface wetland. Finally, the canal water exits the wetland system as the effluent of the subsurface wetland. However, the retention times of the canal water through each of these wetlands vary. The retention time in the sedimentation bay is 3 days, in the surface flow wetland is 2.9 days, in the floating aquatic wetland is 7 days, and in the subsurface wetland is 2 days. A flow chart of the canal water flow through the wetland system is shown in Figure 8. Photographs of the sedimentation bay (Figure 9), surface flow wetland (Figures 10 and 11), floating aquatic wetland (Figure 12), and subsurface wetland (Figure 13) are shown.

Figure 6 - Panoramic view of Alexandra Canal Wetland with Tanglin Road in foreground (World Architecture News, 2011)

Figure 7 - Shallow stream / play area (World Architecture News, 2011)

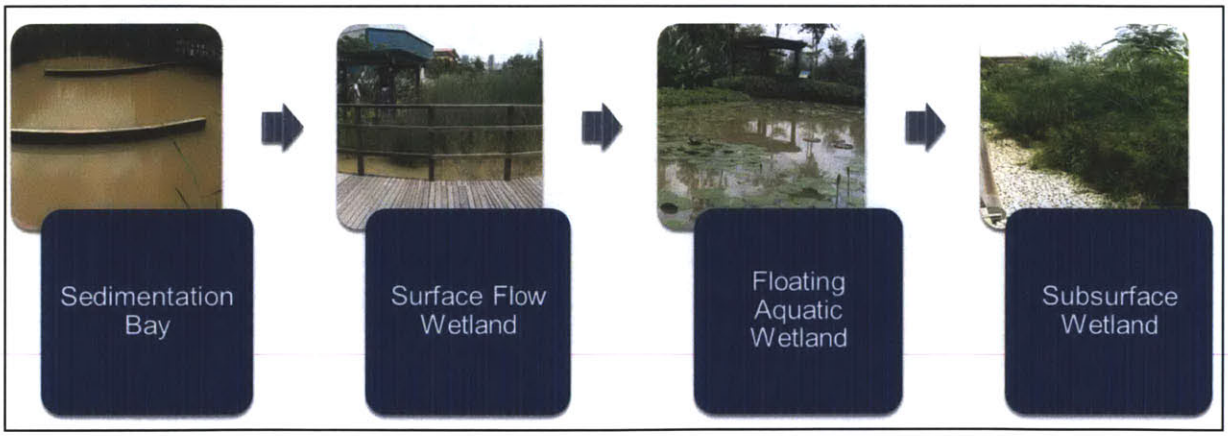

Figure 8 - Flow chart of the water flow through the system of wetlands

Figure 9 - Sedimentation bay

Figure 10 - Surface flow wetland (Photo: Laurie Kellndorfer)

Figure 11 - Surface flow wetland (World Architecture News, 2011)

Figure 12 - Floating aquatic wetland (Photo: Laurie Kellndorfer)

Figure 13 - Subsurface wetland

2. Literature Review

2.1. Introduction

Precipitation from rain and snowmelt events generates runoff flows over land or impervious surfaces. This type of runoff is called stormwater runoff (US EPA, 2011). The sources of pollutants that contribute to stormwater runoff are point sources and non-point sources. A measurable portion of the pollutants which come from urbanized areas originate from non-point sources. These non-point sources (or diffuse pollutants) contribute in significant amounts to polluting the surface-water bodies (Novotny and Olem, 1994). Wash-off of dust, dirt, and leaves from roadways, sewage inputs, overflow from stormwater drains and combined sewers, construction works, fertilizers, runoff from lawns, pet wastes, and atmospheric deposition from vehicles and industry are some of the common non-point sources (Novotny and Olem, 1994; US EPA, 2005).

Singapore is a highly urbanized country (Figure 14) and therefore the impervious surfaces have increased to a great extent. The degree of imperviousness is directly proportional to the coefficient of runoff. The coefficient of runoff is the ratio of the volume of runoff to the volume of rain (Novotny and Olem, 1994). The degree of impervious cover can increase the pre-development runoff from 2 to 16 times (US EPA, 2005). When the impervious cover is greater than 25% of the total area, the stability of the receiving stream and its water quality are affected, and loss of habitat and decrease in biodiversity also occur (CT DEP, 2004). Figure 15 is a pictorial representation of the extent to which the impervious cover affects the runoff.

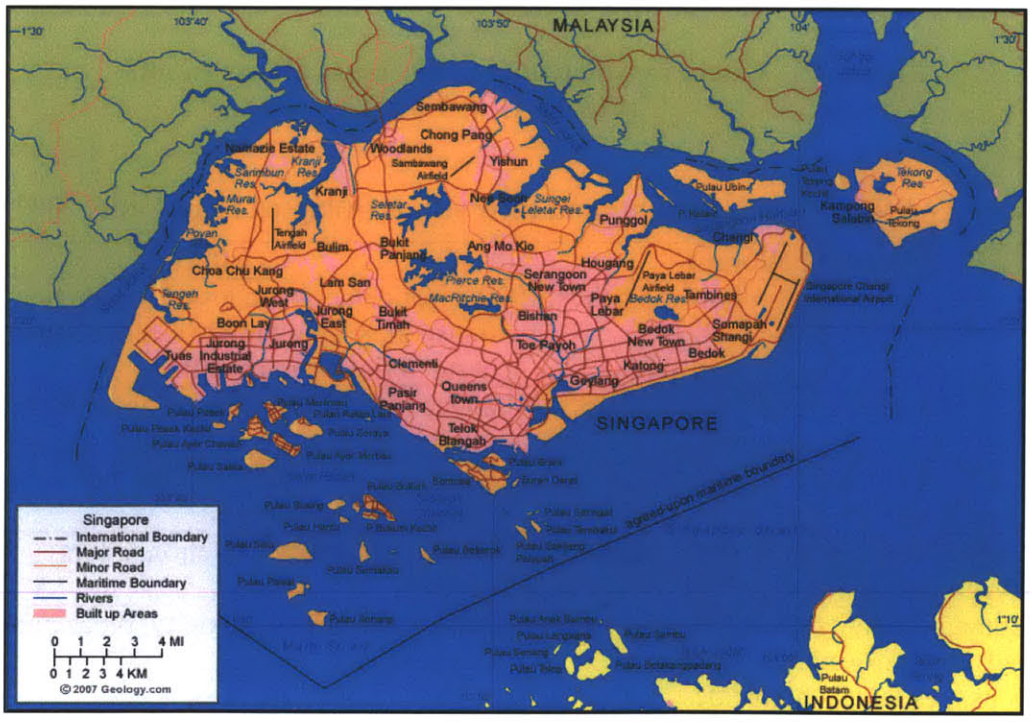

Figure 14 - Singapore map (Geology.com, 2007)

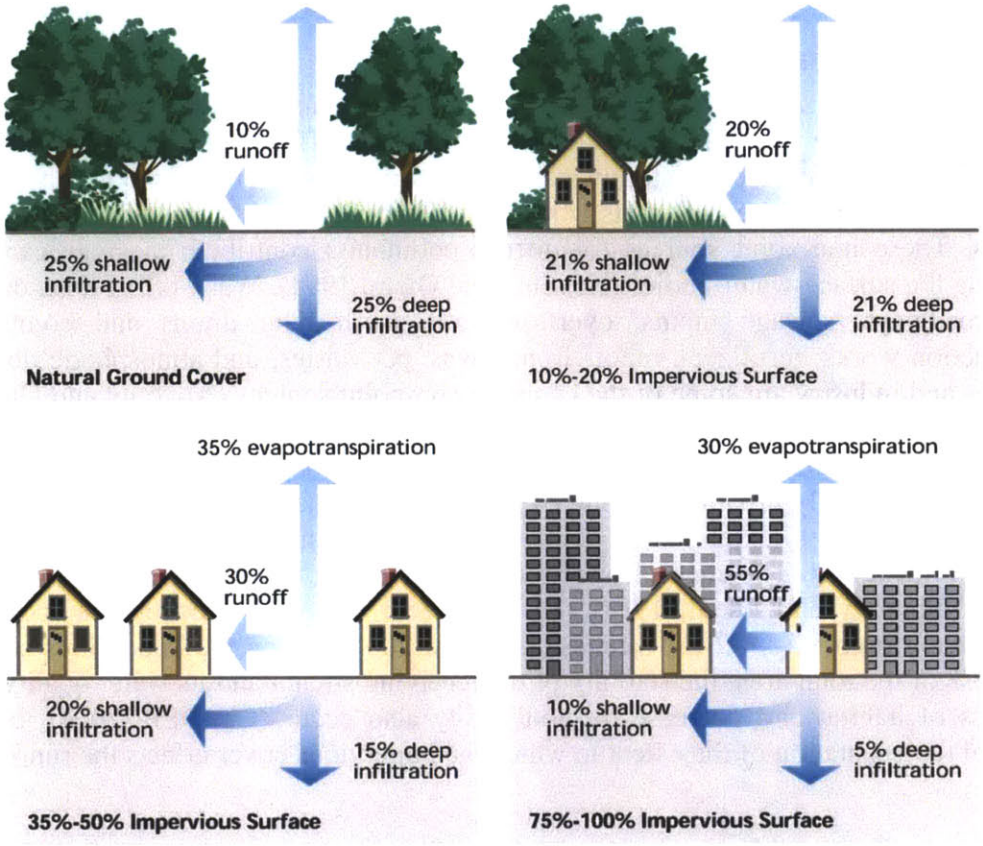

Figure 15 - Extent to which impervious cover affects runoff (FISRWG, 1998)

Table 1 – Stormwater pollutants and their effects (Adapted from Lulla, 2007)

Stormwater Pollutant	Effects
Sediments: Suspended solids, dissolved solids, turbidity	Recreation/aesthetic loss, contaminant transport, stream turbidity habitat changes, harm to finfish and shellfish
Nutrients: Nitrate, nitrite, ammonia, organic nitrogen, phosphate, total phosphorous	Algae blooms, eutrophication, ammonia and nitrate toxicity, recreation/aesthetic loss
Microbes: Total and fecal coliforms, fecal streptococci, viruses, *E. coli*, enterococci	Intestinal infections, shellfish bed closure, recreation/aesthetic loss
Organic Matter: Vegetation, sewage, other oxygen oxygen-demanding material	Dissolved oxygen depletion, odors
Toxic Pollutants: Heavy metals (cadmium, copper, lead, zinc), organic chemicals, hydrocarbons, pesticides/ herbicides	Human and aquatic toxicity, bioaccumulation in food chain

Urban stormwater runoff usually contains five main categories of pollutants which are "suspended solids, nutrients, litter and refuse, bacteria and pathogens, and toxicants such as pesticides and heavy metals" (Table 1) (Lulla, 2007). Suspended solids form the most critical pollutant because they tend to cause three main problems, one of which is that pollutants tend to get adsorbed on-to them. They also increase the turbidity which prevents sunlight from entering the waters, thus affecting photosynthesis which in turn is harmful to plant and aquatic life. Another detrimental effect caused by suspended solids is that they clog the gills of fish and thus inhibit the exchange of CO_2 and O_2. Nutrients are necessary for aquatic life to survive although excess presence of nitrogen and phosphorous leads to eutrophication. Eutrophication promotes excess growth of algae and plants which depletes the DO level in the water body. It also leads to changes in phytoplankton, algae, benthic, periphyton, and fish communities (Lulla, 2007; US EPA, 2005). Litter reduces aesthetic appeal and is a potential health hazard to aquatic species. Toxicity in water is contributed by heavy metals such as copper, zinc, lead, etc. These toxic metals accumulate in sediments and bio-accumulate in the food chain, thus becoming a threat to humans along with aquatic life. Automobiles are suspected to be the largest source for the release of heavy metals (US EPA, 2005). Chesapeake Bay is the largest estuary in the United States and studies have shown that 6% of the total cadmium and 19% of the total lead in the estuary is from non-point sources (US EPA, 2005).

Conventional stormwater management mainly focuses on the collection of runoff and transferring this runoff through gutters and curbs to larger water bodies (Prince George's County, 2000a). It addresses the probable problems of downstream flooding and streambank erosion by construction of ponds and detention basins at the lower elevation points of the area. However, this method fails to consider the loss of storage volume due to factors such as rainfall abstraction and loss of groundwater recharge.

In order to address the problems of high concentrations of pollutants and high runoff rates, in highly urbanized areas like Singapore, different methods are adopted. Low-Impact Development (LID) is a different approach to conventional stormwater management. LID emulates the natural hydrological regime and creates an artificial, functional landscape that reduces potential flooding and erosion (Prince George's County, 2000b). It improves the aesthetic value while being functional at the same time. LID techniques address some of the problems encountered by conventional stormwater management methods. Precipitation events may appear to be random but over a long period of time, there occurs a statistical pattern. LID techniques consider these precipitation patterns in order to implement and design a better management system. LID techniques also account for the losses in runoff due to rainfall abstractions and groundwater recharge as opposed to conventional stormwater management. Rainfall abstraction represents that depth of water over the total area of the site which does not contribute to surface runoff. It includes interception of rainfall by vegetation, evaporation from land surfaces, transpiration by plants, and infiltration of water into soil surfaces. LID therefore focuses on maintaining natural drainage courses, minimizing clearing, reducing imperviousness, conserving natural resources, and increasing wildlife habitat. It also strives to educate and encourage the public to use pollution prevention methods. The above mentioned aspects are achieved by implementing techniques such as retention storage, which allows for a reduction in the post-development volume and the peak runoff rate, and detention storage, which provides additional storage, if required, to maintain the same peak runoff rate and/or prevent flooding. Another simple yet

effective advantage of these LID techniques is that infiltration occurs throughout the retention and detention phases and not only at the edge of the development like it happens in conventional stormwater management.

2.2 Best Management Practices

Best management practice (BMP) is "a practice or combination of practices that are the most effective and practicable (including technological, economic, and institutional considerations) means of controlling point or nonpoint source pollutants at levels compatible with environmental quality goals" (Prince George's County, 2000a). BMPs include LID techniques, and are one of the most effective means to either completely remove or help reduce pollutants from runoff before runoff enters rivers, streams, etc. (US EPA, 2005).

BMPs can be classified into two main categories: nonstructural practices and structural practices (US EPA, 2005). Nonstructural practices manage runoff and reduce potential pollutants right at the source while "Structural practices are engineered to manage or alter the flow, velocity, duration, and other characteristics of runoff by physical means" (US EPA, 2005). Structured BMPs can improve water quality by controlling peak discharge rates; they can also help reduce downstream erosion, promote groundwater recharge, provide flood control measures, and improve infiltration. Thus they overcome the problems encountered in conventional stormwater management techniques mentioned in Section 2.1.

There are various types of BMPs in practice. A general overview of some of these practices is provided in this section. In places where there are roads and low-density development, grassed swales are used (Prince George's County, 2000a). These are grassed channels that transport stormwater runoff away from roadways, provide a certain amount of infiltration, and reduce stormwater impacts by trapping sediment and sediment-bound pollutants. Infiltration trenches are another kind of BMP that are used in highly restricted areas. These trenches are backfilled with stone to form a sub-surface basin and stormwater runoff can be directed there to be treated and stored for a period of several days. Rooftop runoff can be managed using rain barrels, cisterns, and dry wells. Rain barrels are low-cost systems that are effective and easily maintainable retention devices. They can be used in residential, industrial, and commercial areas where the runoff water can be re-used in lawn and garden watering. Cisterns on the other hand provide retention storage volume in underground storage tanks. Dry wells consist of small excavated pits backfilled with aggregate, usually pea gravel or stone. They function as infiltration systems where mechanisms such as adsorption, trapping, filtering, and bacterial degradation take place. An interesting fact is that dry wells and infiltration trenches have 60-80% bacterial removal efficiencies whereas the bacterial removal efficiencies of the other BMPs have not yet been determined (Prince George's County, 2000a). Some of the other BMPs are detention ponds, retention ponds, constructed wetlands, rain gardens, and bioswales.

2.3 Constructed Wetlands

Wetlands are ecosystems with unique soil systems, vegetation, wildlife, and are invaluable as they behave as "sources, sinks, and transformers of a multitude of chemical, biological, and genetic materials" (Mitsch & Gosselink, 2007). They are often called "kidneys of the landscape" (Mitsch & Gosselink, 2007). There exist several definitions for a wetland depending on whether a wetland scientist is using it or if a wetland regulator is. In context to my project, the most

appropriate definition is the U.S. Fish and Wildlife Service definition (Mitsch & Gosselink, 2007): "Wetlands are lands transitional between terrestrial and aquatic systems where the water table is usually at or near the surface or the land is covered by shallow water." This is represented in Figure 16. Wetlands are capable of recharging aquifers and can simultaneously eliminate pollutants from the water and protect shorelines.

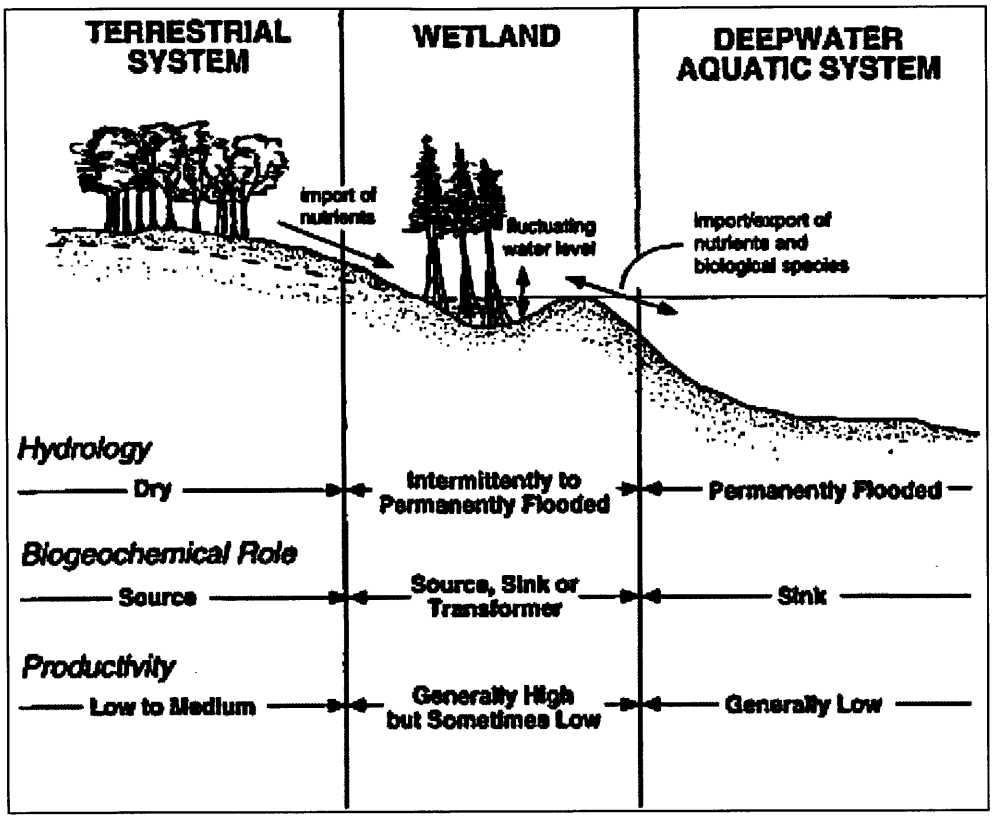

Figure 16 - Wetlands being a continuum between terrestrial and deep-water aquatic systems (Mitsch & Gosselink, 2007)

Treatment wetlands focus mainly on improving the quality of water. These wetlands can be used as a sink for varied chemicals and scenarios but in the case of this project, we are interested in wetlands that treat pollutants from non-point sources. Maintenance of wildlife and mosquito control is very important in wetlands. Treatment wetlands are classified into three types. There are natural wetlands, surface constructed wetlands, and subsurface constructed wetlands (Mitsch & Gosselink, 2007). Constructed wetlands are systems capable of treating runoff (US EPA, 2005). Surface constructed wetlands mimic natural wetlands; they have standing water while there is no standing water in subsurface constructed wetlands. Instead, in the subsurface constructed wetland, the water passes through a porous medium (Mitsch & Gosselink, 2007). Subsurface constructed wetlands are generally preferred in areas where the availability of land is less.

Physical, chemical, and biological processes are used to remove pollutants in wetlands (Schueler, 1992). The physical mechanism that occurs is sedimentation by gravitational settling. Removal of metals, and some nutrients and hydrocarbons occurs by adsorption onto surfaces of sediments, vegetation, detritus, etc. in the wetland system. Physical filtration by plants is one of the basic and effective processes that occur. Another mechanism by which pollutants that have settled in the sediments can be removed is uptake by wetland plants which mostly happens through roots. Soluble nutrients like phosphate, nitrate, and ammonia are removed by planktonic/benthic algae. These get converted to biomass and then get settled into the sediment. Algal mats are found on the sediment surface sometimes and these effectively remove nutrients as well. The mechanisms of the transformations of chemical compounds and biological processes in a wetland are yet to be understood completely. Thus, in this thesis, an attempt is being made to understand the patterns of bacteria loading removal by a constructed wetland. A generalized diagram of carbon transformation in a wetland is shown in Figure 17.

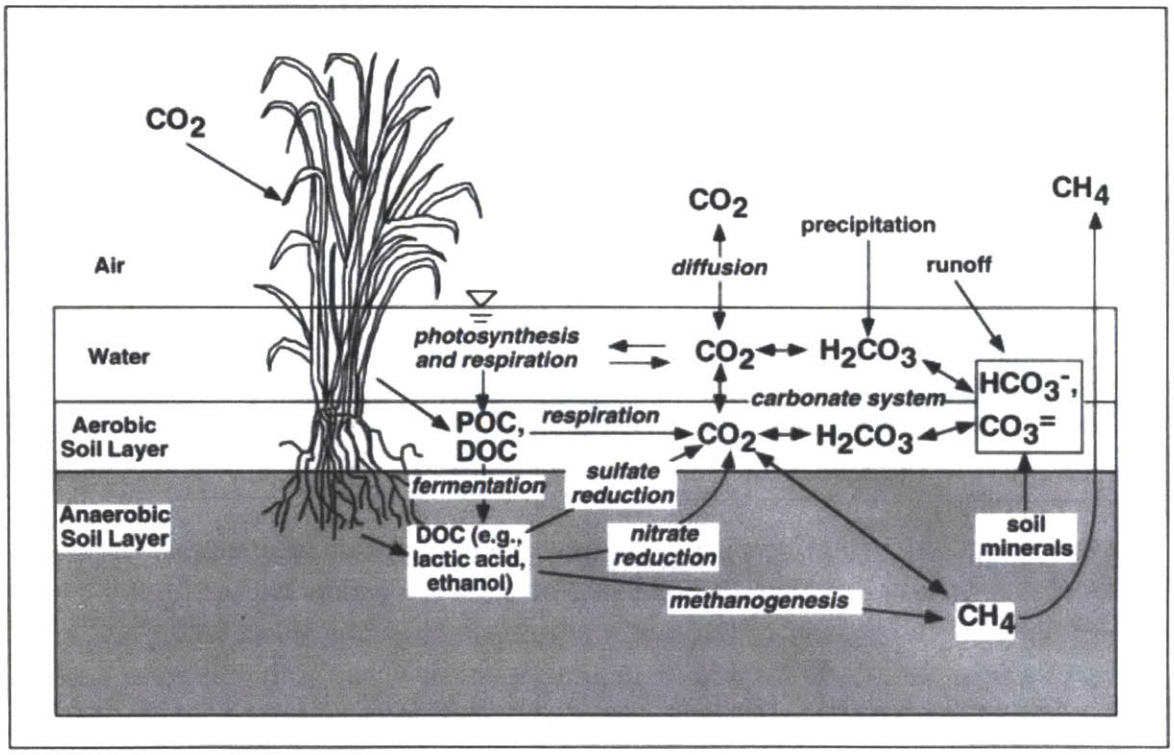

Figure 17 – A generalized diagram of carbon transformation in a wetland (Mitsch & Gosselink, 2007)

The performance of a constructed wetland can be assessed microbiologically by learning the quality of the influent and effluent stormwater. However, it is of utmost importance to choose the appropriate indicator in order to calculate the microbiological parameters and understand the efficacy of the system.

2.4 Need for Indicator Bacteria

Pathogens are transmitted by polluted water to humans who come in contact with that water through drinking, swimming, fishing, wading, etc. (Schueler, 1992). They are not only harmful to aquatic life but can be harmful to humans also when humans consume seafood like fish, prawns, etc. Sources of these pathogens are waste from pets, wildlife, and waterfowl (CT DEP, 2004). Other sources of pathogens are leakage of combined sewer systems, septic system failures, and illegal cross connections between storm drains and sewers. Table 2 shows the types of human pathogens that can potentially be present in raw domestic wastewater.

The United States recognized the problems mentioned above early-on and the United States Environmental Protection Agency (EPA) was established in 1972 to implement the 'Clean Water Act,' a federal law that required that every state meet the minimal standard of wastewater treatment (Byappanahalli, 2000). The EPA conducted elaborate studies in both marine waters of Boston Harbor, New York, Lake Pontchatrain in New Orleans, and in freshwater beaches in Pennsylvania and Oklahoma. The EPA found that analyzing the water directly for the pathogenic organisms is neither feasible nor practical as the fecal-borne pathogens in sewage are too numerous to count and it would be too expensive and time consuming to routinely monitor pathogens. As a result of the studies mentioned above, the EPA adopted the strategy of analyzing and monitoring the hygienic quality of water by measuring the concentrations of fecal indicators. They are named fecal indicators because they indicate the possible presence of sewage-borne pathogens in water. Also, the amount of indicator bacteria can quantitatively be related to the health hazard (Ekklesia, 2011).

The criteria for fecal-pathogen indicator bacteria were indicated in (Hazen, 1988) as follows:

1. The indicator must be present whenever pathogens are present.
2. It must be present only when the presence of pathogenic organisms is an imminent danger.
3. It must occur in much greater numbers than the pathogens.
4. It must be more resistant to disinfectants and to aqueous environments than the pathogens.
5. It must grow readily on relatively simple media.
6. It should preferably be randomly distributed in the sample to be tested.

Table 2 - Potential human pathogens present in raw domestic wastewater (Byappanahalli, 2000)

Organism	Disease	Symptoms
Bacteria		
Escherichia coli (enteropathogenic)	Gastroenteritis	Diarrhea
Legionella pneumophila	Legionellosis	Acute respiratory illness
Leptospira (150 spp.)	Leptospirosis	Jaundice and fever
Salmonella typhi	Typhoid fever	High fever, diarrhea, and ulceration of small intestines
Salmonella (1700 spp.)	Salmonellosis	Food poisoning
Shigella (4 spp.)	Shigellosis	Bacillary dysentery
Vibrio cholerae	Cholera	Extremely heavy diarrhea and dehydration
Yersinia enterolitica	Yersinosis	Diarrhea
Protozoa		
Balantidium coli	Balantidiasis	Diarrhea and dysentery
Cryptosporidium	Cryptosporidiosis	Diarrhea
Entamoeba histolytica	Amebiasis (amoebic dysentery)	Prolonged diarrhea with bleeding and abscesses of the liver and small intestine
Giardia lamblia	Giardiasis	Mild to severe diarrhea, nausea, and indigestion
Viruses		
Adenovirus	Respiratory disease	
Enterovirus	Gastroenteritis, heart anomalies, and meningitis	
Hepatitis A	Infectious hepatitis	Jaundice and fever
Norwalk agent	Gastroenteritis,	Vomiting
Reovirus	Gastroenteritis,	
Rotavirus	Gastroenteritis,	
Helminths		
Ascaris lumbricoides	Ascariasis	Roundworm
Enterobius vericularis	Enterobiasis	Pinworm
Hymenolepis nana	Hymenolepiasis	Dwarf tapeworm
Taenia solium	Taeniasis	Pork tapeworm
Trichuris trichiura	Trichuriasis	Whipworm

2.5 Indicator Bacteria

The different types of fecal indicator bacteria used are (Byappanahalli, 2000):

1. Total coliform
2. Fecal coliform
3. Fecal streptococci
4. Enterococci
5. Clostridium perfringens
6. *Bacteroides spp.*
7. Bifidobacteria
8. Total heterotrophic bacteria
9. Bacteriophages

Reliance on one kind of indicator is not advisable, therefore different types of indicator bacteria are used for different types of water (Byappanahalli, 2000). Three groups of bacteria—coliforms, fecal streptococci, and gas-producing clostridia—were argued to be the best indicators of recent fecal pollution as they were usually found in the feces of warm-blooded animals (Hazen, 1988). As a part of the coliforms, total coliform and *Escherichia coli* are used as fecal indicators, and as a part of the fecal streptococci, enterococci is used as a fecal indicator.

The coliform bacterium can be classified as an aerobic and facultative anaerobic, gram-negative, non-spore forming, rod-shaped bacterium that ferments lactose to produce gas within 48 hours at 35°C (Byappanahalli, 2000). The genera included in total coliform are *Escherichia, Klebsiella, Citrobacter,* and *Enterobacter* (Ekklesia, 2011). Total coliform bacteria are used for indicating the microbiological quality of drinking water (Rivera et al., 1988).

Fecal coliforms are a subset of the total coliform bacteria that ferment lactose and produce acid and gas at 44.5°C within 24 hours (Byappanahalli, 2000). The two genera of fecal coliforms include *Escherichia coli (E. coli)* and *Klebsiella* but because *E. coli* represent the majority (90-95%), it is usually used synonymously with fecal coliform (Ekklesia, 2011). *E. coli*, also known as thermotolerant coliform, has good characteristics for a fecal indicator; it is normally non-pathogenic and is present at higher concentrations than the pathogens it predicts (Ekklesia, 2011; Lulla, 2007). The correlation between gastrointestinal illness and *E. coli* was the highest among the coliforms and they are also the most abundantly available coliforms in humans and warm-blooded animals (Ekklesia, 2011). Thus fecal coliforms are used regularly to indicate the quality of recreational water (Rivera et al., 1988).

Enterococci are gram-positive cocci with spherical or ovoid cells arranged in pairs (sometimes in chains), are non-spore forming, facultative anaerobic, homofermentative bacteria that are also known to be nutritionally complex (Byappanahalli, 2000). This bacterium has the ability to grow at both 10°C and 45°C at a pH of 9.6 in the presence of 6.5% NaCl, and to reduce 0.1% methylene blue in milk. Classical enterococci are *E. faecalis* and *E. faecium.* Enterococci are more resistant to disinfection and environmental stress, making it a more reliable indicator than total coliform and *E. coli* (Ekklesia, 2011). Also, because enterococci are directly correlated to swimmer-associated gastroenteritis and have an inactivation rate that is much slower than total

coliforms, enterococci are generally used as indicators for recreational waters (Byappanahalli & Fujioka, 1998). This indicator is also well suited for indicating sediment contamination because they die more slowly than fecal coliform bacteria in sediments (Byappanahalli & Fujioka, 1998). Table 3 indicates the approximate concentrations of these indicator bacteria in raw sewage.

Table 3 - Concentrations of indicator bacteria in raw sewage (Byappanahalli, 2000)

Type of indicator bacteria	Concentration (CFU/100mL)
Total Coliform	$10^7 - 10^9$
Escherichia coli	$10^6 - 10^7$
Enterococci	$10^4 - 10^5$

The decay rate of indicator bacteria is an important factor to consider when choosing the right indicator. Several factors such as sunlight, pH, temperature, and turbidity affect the inactivation of bacteria in receiving water (Ekklesia, 2011). There is a net die-off of bacteria during the day because the rate of deactivation is much faster than rate of growth and vice-versa during the night.

2.6 Drawbacks of Indicator Bacteria

Although using indicator bacteria seems like a reliable solution to identifying fecal-pathogens in water, they have a few drawbacks. The natural habitat of these indicator bacteria is usually the gastrointestinal tract of humans and warm blooded animals. Thus, they are not expected to survive or multiply in the environment. One of the major reasons why these indicators are not applicable is because they were originally measured in temperate climates, therefore are not very reliable in tropical climates. The extreme variations in survival times with climate can be viewed in Table 4. (Hazen, 1988) mentions experiments conducted in tropical countries like Ceylon, India, Egypt, and Singapore where they found that densities of *E. coli* did not coincide with known sources of fecal contamination. Growth and survival of coliforms for several months was reported in tropical waters in India (Hazen, 1988). In Nigeria, Hawaii, New Guinea, Puerto Rico, Sierra Leone, and the Ivory Coast, high densities of *E. coli* were found in the complete absence of any known fecal source i.e., no pathogens (Hazen, 1988).

Table 4 - Survival rates of *E. coli* in temperate and tropical climates (Hazen, 1988)

Climate	Initial Density	Survival time (hours)*
Temperate Climate	10^9	50
	10^5	30.6
	10^8	24
Tropical Climate	10^7	294
	10^6	206

*Survival time: time to reach 90% reduction of initial cell density

Total coliform bacteria fail to meet a few of the ideal criteria for indicator organisms as indicated by (Hazen, 1988). One of the most important limitations of using this indicator is that while *Escherichia and Klebsiella* have fecal origins, *Citrobacter,* and *Enterobacter* have no fecal origin, which makes these bacteria not very representative of fecal contamination (Byappanahalli, 2000). Another major criterion that this indicator fails to meet is that they multiply under environmental conditions. They are also sensitive to disinfection which is another limiting criterion. Researchers found that plants and soils contain these coliform bacteria (Ekklesia, 2011) which prompted the need to find a more representative indicator. Enterococci multiply much less than coliform in water, but they still do multiply.

Indicator bacteria are not supposed to grow or be found in the environmental individually without fecal contamination (Byappanahalli, 2000). Byappanahalli and Fujioka (1998) found that the indicator bacteria were present in freshwater, streams, and in soils and that apart from that, the indicator bacteria seemed to have the potential to multiply. In Hawaii, bacteria were readily recoverable from soil even in the absence of any sewage which proved that the soil contained enough moisture, and nutrients to support the growth of *E. coli.* In the tropical island of Oahu, indicator bacteria are naturally found in soil environments where fecal bacteria became a part of the soil biota. The growth and multiplication of indicator bacteria in natural soils are dependent on available nutrients (particularly carbon), moisture, and competing microorganisms. Also, *E. coli* and enterococci were found in abundance in swash-zone sand at freshwater beaches (Alm et al., 2003). In tropical environments, enterococci may naturally exist in soils and water, as well as multiply.

E. coli was isolated from water accumulated from leaf axilea of epiphytic flora in a tropical rain forest in Puerto Rico (Rivera et al., 1988). Thus it was found that *E. coli,* once introduced, can remain and/or become a part of the normal flora. Another study conducted by (Litton et al., 2010) shows that these indicator bacteria regrow in river sediments. These indicator bacterial growths were documented also in estuaries, tidal creeks, and marine beaches (Litton et al., 2010). Fecal matter of birds and wild animals enter the water stream as non-point source pollutants (Byappanahalli, 2000). The pollution can be controlled to an extent but not completely because we cannot regulate wild animals like mongoose, most birds, stray cats, deer, etc.. Thus, the assumption that indicator bacteria do not occur in natural environments is being challenged and it raises questions as to the validity of using these indicators in tropical environments. Another reason why *E. coli* is not very reliable an indicator is because they have a natural tendency to get inactivated due to sunlight under environmental conditions (Whitman et al., 2004). On the other hand, in the absence of sunlight, if the nutritional requirements are met, growth in *E. coli* was observed (Fujioka & Unutoa, 2006).

2.7 Alternate Indicator

Bacteroides are strict anaerobic, non-spore forming, gram-positive bacteria, found in the gastrointestinal tract of humans and warm-blooded animals (Byappanahalli, 2000). They are the most common genus in the human intestines and outnumber the coliforms by three orders magnitude, approximately 10^{12} CFU/g feces (Ekklesia, 2011). They are present in high concentration, have no environmental sources, do not multiply, and concentrations are actually good indicators to predict the concentrations of pathogens (Byappanahalli, 2000). The two main

concerns are that they neither survive in the environment for long periods of time, nor do they survive the usual cultural methods used to detect indicator bacteria (Byappanahalli, 2000). Therefore alternate methods, such as the fluorescent antiserum test and genetic probe assay, are used to detect the concentration of this bacterium (Byappanahalli, 2000). Molecular-based approaches using specific 16S primers have been developed by (Bernhard & Field, 2000a). Bernhard and Field (2000a) identified two human-host-specific 16S rDNA genetic markers that were found in *Bacteroides-Prevotella* and *Bifidobacterium*. *Bacteroides-Prevotella* is easier to detect and has a longer survival rate than *Bifidobacterium*. Polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) assays have been developed to identify host specific *Bacteroides-Prevotella* 16S rDNA and 16S rRNA gene markers in humans and in animals (Bernhard & Field, 2000a; Seurinck et al., 2005). Thus, recent developments in molecular biology have revolutionized microbiology (Srinivasan et al., 2011).

The qPCR method is a new method that directly measures genetic material with a wide detection range of (10^0 – 10^8 copies/reaction) (Converse et al., 2011; Srinivasan et al., 2011). In this method, amplification and quantification of the nucleic acids occurs simultaneously (Lavender & Kinzelman, 2009). It is therefore much faster as the incubation step that exists in the culture-based methods is eliminated (Converse et al., 2011). A qPCR test takes only two hours to complete, which is a huge improvement over the traditional methods which require 18-96 hours for results (Noble et al., 2010). These rapid methods can give us results in a few hours which means beach managers can give out warnings on the same day as the contamination is detected rather than having to wait for results to come in a couple of days (Lavender & Kinzelman, 2009). Further, the qPCR method has the ability to detect fresh sewage, independent of the type of water present (e.g., freshwater, seawater, or distilled water) (Ahmed et al., 2009).

One of the most commonly used parameters to quantify the performance of these host-specific markers is specificity (Ahmed et al., 2009). 'Host-specificity' is the probability of detecting a source when the source is not present while 'sensitivity' is the probability of detecting a source when it is present. There are five sewage-associated host-specific *Bacteroides* markers: HF183, BacHum, HuBac, BacH, and Human-Bac. (Ahmed et al., 2009) found through their experiments in Australia, that the host-specificity of HF183 is 98% and sensitivity is 100%. Table 5 compares the specificities of various *Bacteroides* markers. The specificity of HF183 human-specific markers was tested and the results for various countries are shown in Table 6.

Table 5 - Comparison of specificities of various *Bacteroides* markers (Ahmed et al., 2009)

***Bacteroides* marker**	**Specificity (%)**
HF183	99
BacHum	94
HuBac	63
BacH	94
Human-Bac	79

Table 6 - Specificity of HF183 markers in various countries (Ahmed et al., 2009)

Sewage-associated markers	Geographical region	Specificity (%)
HF183	Australia	100
HF183	France	94
HF183	France	91
HF183	Ireland	100
HF183	Portugal	96
HF183	UK	100
HF183	Belgium	100
HF183	Belgium	100
HF183	USA	85
HF183	USA	100
BacHum	USA	98
HuBac	USA	33
BacH	Austria	99

Specificity of the HF183 marker has to be verified at the location where research is being conducted (Ahmed et al., 2009). (Nshimyimana, 2010) confirmed through his thesis research that we could apply the HF183 assay to detect human fecal contamination in Singapore, thus validating the basis of using the HF183 marker for my research. One of the limitations of using human factor (HF) is that it cannot differentiate between different sources of fecal pollution (point sources, non-point sources, untreated sewage) (Ahmed et al., 2007). Another limitation of qPCR is the inability to differentiate between viable and non-viable cells (i.e. live and dead cells), which may lead to an overestimation in concentrations (Noble et al., 2010; Srinivasan et al., 2011). In the context of my project, I used the HF marker (as a conservative marker) and included indicator bacteria analysis because a combination of using both types of indicators will reduce the margin of error in detecting the human fecal pollution and hopefully, if one indicator fails to detect the pollution at a certain point, the second indicator would do so instead.

3. Methods Used for Laboratory Analysis

3.1. Collection of Water Samples

Water samples were collected on January 25, 2012 at 07:00, 09:00, 11:00, and 13:00 at the Alexandra Canal wetland. Samples were collected at the inlets (as shown in Figure 18) in Whirl-Pak® bags (Nasco, Fort Atkinson, WI, USA) (Figure 19) while grab samples were collected in accordance with methods specified by US EPA (2000) at the outlets at each of the four wetland systems explained in Section 1.7. The inlet of the subsurface wetland had no flow throughout the sampling session and therefore samples were collected only at the outlet. Collected samples were immediately sealed and chilled on ice and transported back to the laboratory in a cooler within 3 hours of collection.

Figure 18 - Inlet of wetland systems

Figure 19 - Whirl-Pak® (Nasco, 2012)

Blank samples were prepared to ensure that cross-contamination did not occur within the ice-cooler. Upon arrival in the laboratory, all samples intended for fecal indicator bacteria analysis were held at 4°C. A total of seventeen samples were analyzed for the above mentioned indicator bacteria on the 25th January while the remaining 20 samples were analyzed on the following day. The samples intended for DNA analysis were passed through 0.22-micron Millipore Sterivex™ filter units (EMD Millipore Corporation, Billerica, MA, USA) (Figure 13) and subsequently stored at -80°C. They are stored at such low temperatures because if the DNA extracts are not analyzed within one week of extraction, they tend to lose a significant amount of target DNA unless stored at very low temperature, thus becoming unsuitable for analysis (Lavender & Kinzelman, 2009).

Figure 20 - Millipore Sterivex™ filter unit (Amazon, 2012)

3.2. Laboratory Analysis

To assess the microbiological quality of water, two types of analysis were conducted:

1. Culture-based microbiological analysis
2. DNA-based analysis

3.2.1. Culture-based microbiological analysis

The Most Probable Number (MPN) method was used for microbiological analysis. IDEXX Quanti-Tray®/2000 product (IDEXX, 2012b) was used in combination with the reagents Enterolert® (IDEXX, 2012a) and Colilert® (IDEXX, 2012a) to test the water samples for enterococci and for total coliform and *E. coli*, respectively. The general principle behind this method is that a selective culture medium is applied to grow the fecal indicator bacteria and within that medium is a reagent that creates some detectable change in color or fluorescence. The substrate that total coliform uses to turn the sample yellow is *ortho*-nitrophenyl- β-D-galactopyranoside (ONPG) while *E. coli* uses 4-methyl-umbelliferyl-β-D-glucuronide (MUG) to turn the sample yellow and create a blue-white fluorescence. Enterococci use 4-methyl-umbelliferyl-β-D-glucoside to create blue-white fluorescence (Ekklesia, 2011). Water samples are incubated with growth media in Quanti-Trays®.

Enumeration of the indicator bacteria is done by counting the positive wells on the Quanti-Tray® after incubation and thereafter applying the Most Probable Number (MPN) method. The wells from the Colilert® test that turned yellow are a positive indication of total coliform being present in the sample while wells that did not turn yellow indicated the absence of total coliform (Figure 21). A 6-watt 366-nm UV light is placed within five inches of the Quanti-Tray® and wells that are yellow and fluoresce under the UV light indicate the presence of *E. coli* (Figure 22). The wells from the Enterolert® test that fluoresce under UV light indicate the presence of enterococci (similar to Figure 22). The wells that do not fluoresce indicate the absence of *E. coli* and enterococci. The Most Probable Number is an estimate of the average number of microorganisms in a given sample.

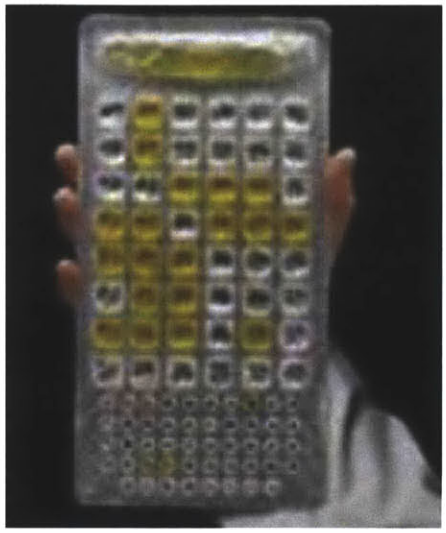

Figure 21 - Interpretation of Quanti-Tray® results; yellow wells indicate presence of total coliform (IDEXX, 2012b)

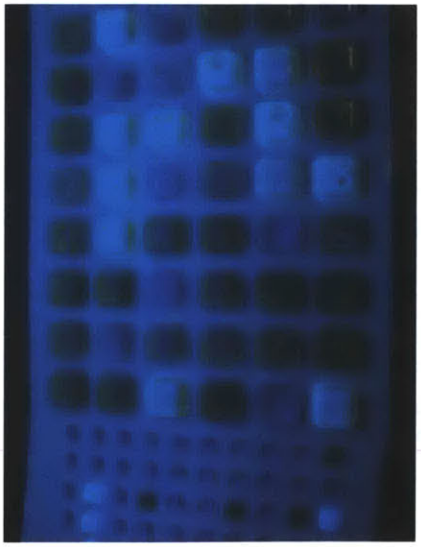

Figure 22 - Interpretation of Quanti-Tray® results; fluorescent wells indicate presence of *E. coli* and enterococci (NYC Water Trail News, 2012)

3.2.1.1. Procedure

The analyses were completed in the Environmental Engineering Laboratory II at the Nanyang Technological University (NTU), Singapore. The Whirl-Pak® bags with samples were shaken slightly to suspend the bacteria that may have settled. The counting range with the IDEXX MPN method has a maximum of 2,419 and samples with higher bacterial counts must be diluted in order to lower the count to below this limit. Therefore, dilutions of 1:1, 1:100, and 1:10,000 were used. Three 250-mL glass bottles are used. A 100-mL aliquot of the sample was poured into one glass bottle using a glass graduated cylinder. A second glass bottle was filled with 99 mL of sterile water to which 1 mL of water from the first glass bottle was transferred using a 1 mL sterile tip and an Eppendorf Research Pipette® (Eppendorf AG, Hamburg, Germany). Following this step, the third bottle was filled with 99 mL of sterile water and 1 mL of water is transferred from the second to the third bottle. Colilert® (IDEXX, 2012a) and Enterolert® (IDEXX, 2012a) substrates were added, shaken thoroughly and poured into the Quanti-Tray® by gently squeezing the tray to separate the foil backing of the tray and the wells. This tray was fed into the Quanti-Tray® sealer on a rubber holder. After sealing, we ensured that the samples were evenly distributed among the 49 large and 48 small wells. Labeling was done and the total coliform/*E. coli* test trays were incubated at 35°C, while the enterococci samples were incubated at 44.5°C. The samples were read after 24 to 28 hours of incubation.

3.2.2. DNA-based Analysis – qPCR Method

3.2.2.1. DNA Extraction

As mentioned above, the water samples that were collected for DNA analysis were passed through 0.22-micron Millipore Sterivex™ filter units. Samples were shipped on dry-ice (approximately -80°C) from Singapore to the Thompson Laboratory at the Parsons Laboratory in the Department of Civil and Environmental Engineering, MIT. The filters were broken open using a metal hammer and the filter membrane present inside was split into two equal parts; one half of which was stored as a back-up sample while the other half was processed for DNA extraction.

The DNA extraction was conducted using the UltraClean® Plant DNA Isolation Kit Protocol (MO BIO Laboratories, 2012a). The filter membrane was placed in a 2-mL bead solution tube containing 550 µL of bead solution to facilitate the removal of genetic material from the membrane. The solutions and the respective quantities used in the procedure are summarized in Table 7. To first lyse the cells, 60 µL of Solution P1 was added to the bead solution tubes and (MO BIO Laboratories, 2012b) briefly in order to homogenize the material (MO BIO Laboratories, 2012a). The tubes were then placed in a water bath (65°C) for 10 minutes after which they were vortexed at maximum speed for 10 minutes. The tubes were centrifuged at a speed of 13,000 rpm (the same speed is applied throughout the extraction procedure) for 30 seconds to remove unwanted debris. At this point the tube contains a debris-free supernatant.

Table 7 - Solutions used in DNA extraction (MO BIO Laboratories, 2012a)

Solution	Quantity	Role
Bead solution	550 µL	Facilitates genetic removal from filter membrane
P1	60 µL	Cell lysis
P2	250 µL	Protein precipitation reagent: Removes unwanted proteins
P3	1 mL	Binding salt: Enables the DNA to bind to the spin filter membrane
P4	300 µL	Wash buffer: Removes residual salt and cleans the DNA
P5	50 µL	Tris buffer: Allows bound DNA to be released from spin filter membrane

Solution P2 is a protein precipitation reagent that enables in removing any unwanted proteins. The supernatant containing the DNA was transferred to a clean 2-mL tube after centrifugation. If the quantity of supernatant was in the range of 400 to 500 µL, then 250 µL of Solution P2 was added. If the amount of supernatant exceeded 500 µL, the excess above 500 mL was transferred to another clean tube and a required proportional amount of P2 was added. During the extraction procedure in the laboratory, I found that, for these environmental samples, the supernatant obtained was sometimes 500 µL while it exceeded 500 µL in a good number of samples. The tubes containing supernatant and P2 were then vortexed for 5 seconds, then incubated for 5 minutes at 4°C, after which they were centrifuged for 1 minute. After centrifuging, 500 µL of the supernatant was transferred to a clean 2-mL tube to which 1 mL of Solution P3 was added and vortexed for 5 seconds.

Thereafter this solution containing Solution P3 and the supernatant is passed through a spin filter. A spin filter is a tube that contains a filter membrane inside it. The spin filters were used to make the latter stage of DNA purification easier, more reliable, and to avoid any possible variability in testing. Solution P3 is a binding salt that enables the DNA to bind to the membrane in the spin filter. The solution in the tube was added to the spin filter in three loads (approximately 650 µL per load) and each load was centrifuged for 30 seconds. The liquid that passes through the filter was discarded after each load but the filtered solids were retained and accumulated through all three loads. After the three loads were centrifuged, 300 µL of Solution P4 was added to the spin filter to remove any residual salts present in the membrane. The tubes were centrifuged for 30 seconds to remove Solution P4 and the flow-through was discarded. The spin filter was centrifuged again for another minute to remove the residual traces of Solution P4. The spin filter was carefully transferred to a clean 2-mL tube. The DNA bound to the membrane in the spin filter was released by adding 50 µL of Solution P5 with care to the center of this filter after which the tube was centrifuged again to separate the DNA from the membrane. After this, the spin filter membrane was discarded. Once the spin filter membrane was discarded, the solution remaining in the tube was the total DNA extracted. This is the last step of the DNA extraction procedure.

3.2.2.2. qPCR Assay

qPCR was used to quantify the HF183 marker using the instrument LightCycler® 480 (Roche Applied Sciences, 2012). The HF183 marker was amplified using the forward and reverse primers indicated in Table 8. The constituents of each qPCR reaction mixture (20 µL) were as follows: 10 µL of KAPA SYBR® FAST qPCR Master Mix (2X), 0.4 µL of forward primer, 0.4 µL of reverse primer, 1 µL of template (DNA sample) or positive control, and 8.2 µL of distilled water. The key components of KAPA™ SYBR® FAST qPCR Master Mix (2X) are SYBR® Green I fluorescent dye (which binds to the double-stranded DNA), $MgCl_2$, deoxynucleotide triphosphates (dNTPs), antibody-mediated hot start, and stabilizers (Kapa Biosystems, 2012). Each DNA sample was analyzed in triplicate. In our experiment, a plate consisting of 96 wells was used wherein the positive control was placed in the wells marked in red in Figure 23 and the environmental samples were placed in the rest of the wells.

Table 8 - The forward and reverse primers used in qPCR assay, with their sequences

Primer	Target	Sequences (5'–3')	Reference
HF183 (Forward)	HF marker (*B. dorei* 16S rRNA)	ATCATGAGTTCACATGTCCG	(Bernhard & Field, 2000b)
Bac242 (Reverse)	HF marker (*B. dorei* 16S rRNA)	TACCCCGCCTACTATCTAATG	(Dick et al., 2010; Seurinck et al., 2005)

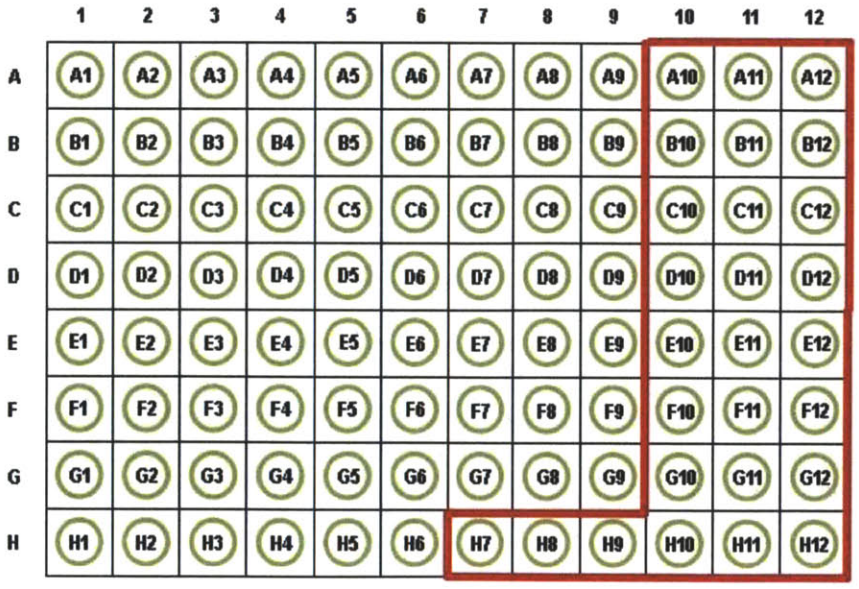

Figure 23 - qPCR array layout (96-Well) (Adapted from eENZYME, 2012)

The qPCR assay includes a pre-incubation step followed by amplification. The temperatures and durations at which the samples were held in the machine are summarized in the flow chart in Figure 24. Previously isolated HF183 positive control (DSM 17855 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) was used to prepare standards for HF183 markers. A 10-fold serial dilution was prepared from this plasmid DNA ranging from 10^0 – 10^8 copies/µL. Preparation of the positive control was based on Equation 1 (Dixon et al., 2009).

$$\frac{Measured\ positive\ control\ concentration}{Mass\ of\ one\ plasmid\ copy} = \frac{Number\ of\ copies}{\mu L\ of\ the\ positive\ control} \quad (1)$$

The mass of one plasmid copy = 4.88×10^{-18} ng/copy (Dixon et al., 2009). Positive control dilutions were prepared at environmentally relevant copy concentrations (Table 9).

The qPCR assay is capable of amplifying DNA sequences and simultaneously measuring the concentration (Sambrook & Russel, 2001). While the amplification is taking place, the fluorescent signal is plotted versus the cycle number to create an amplification plot, from which one can measure the concentration (Sambrook & Russel, 2001). In qPCR, the amount of fluorescence is a direct measure of the gene amplification (Applied Biosystems, 2012a). The baseline for the amplification plot is defined as the initial cycles of qPCR during which there is little change in fluorescence (Applied Biosystems, 2012a). The threshold line is the level of detection i.e., it is the point at which the reaction reaches a certain fluorescent intensity that is greater than the background (Applied Biosystems, 2012b). The threshold cycle (C_t) is the cycle at which the sample reaches the threshold line (Applied Biosystems, 2012b). Figure 25 represents the amplification plot with the threshold line and C_t. The threshold cycle is lesser if the initial concentration of the target DNA sequences in the sample is larger, because the number of cycles required to achieve a particular yield of the amplified product is lesser (Sambrook & Russel, 2001).

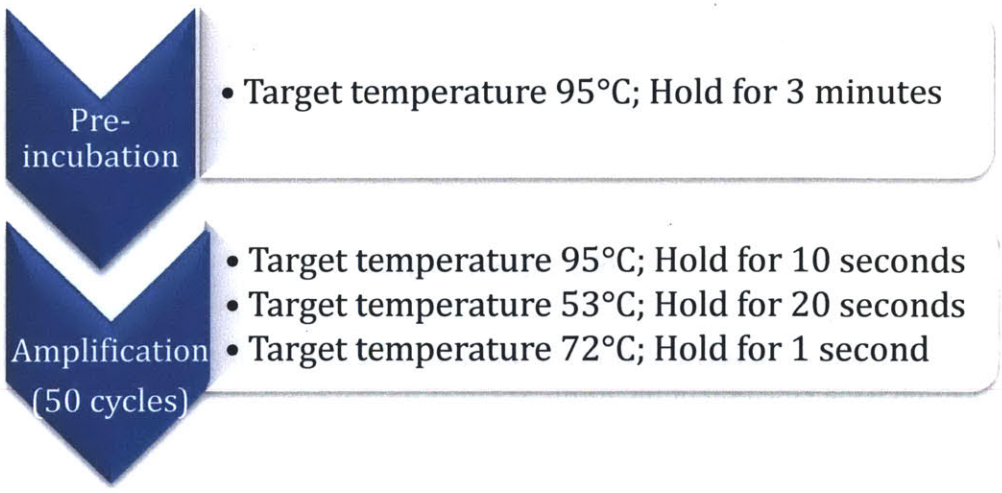

Figure 24 - Flow chart of pre-incubation and amplification stages of qPCR assay

Table 9 - Positive control dilutions at environmentally relevant copy concentrations

Dilution Stock (ng/µL)	Number of Copies per Micro-liter (copies/µL)
1:100	5.97×10^8
1:1000	5.97×10^7
1:10,000	5.97×10^6
1:100,000	5.97×10^5
1:1,000,000	5.97×10^4
1:10,000,000	5.97×10^3
1:100,000,000	5.97×10^2
1:1,000,000,000	5.97×10^1
1:10,000,000,000	5.97×10^0

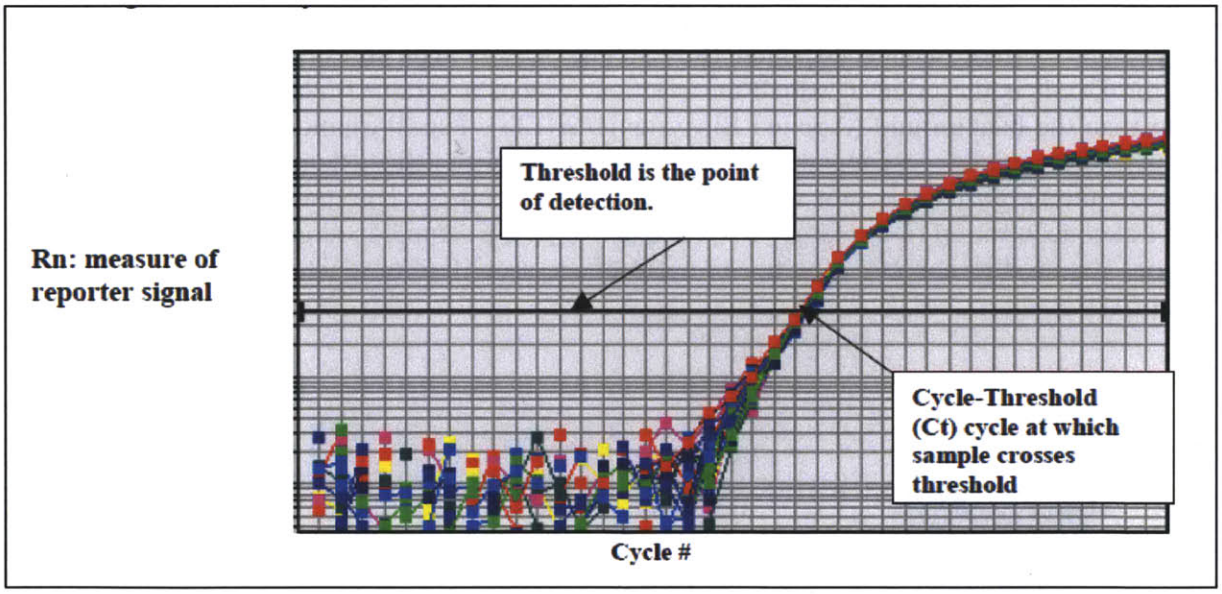

Figure 25 - Amplification plots: Threshold line and threshold cycle (C_t) (Applied Biosystems, 2012b)

The so-called "standard curve" is a straight-line plot of C_t versus $\log_{10}$ of copies/qPCR. It is a regular slope-intercept equation of the form:

$$y = mx + c \tag{2}$$

where, y is the measure of the reporter signal and corresponds to C_t, x is $\log_{10}$(copies/µL), m is the slope, and c is the intercept. Each time the machine is run, the slope and the intercept of the

standard curve change regardless of the samples. Therefore, once we know the C_t value from the amplification curve and both the slope and the intercept from the standard curve, we can calculate x, the copies/qPCR of the environmental samples using Equation 3:

$$x = \frac{(Ct-c)}{m} \tag{3}$$

DNA amplification gets inhibited when high-molecular-weight compounds in the source water (such as humic acids and complex carbohydrates) combine with metal ions and prevent amplification (Noble et al., 2010). Therefore, to avoid having a bias in our quantification, an inhibition analysis was conducted. This inhibition analysis entailed running the qPCR assay with duplicate samples. However, the constituents of the qPCR reaction mixture (20 µL) for the inhibition analysis vary slightly. The reaction mixture was as follows: 10 µL of KAPA SYBR® FAST qPCR Master Mix (2X), 0.4 µL of forward primer, 0.4 µL of reverse primer, 1 µL of template (DNA sample), 1 µL of standard, and 8.2 µL of distilled water. The standard is a reference gene of known concentration. Therefore, if the resultant qPCR value is greater than the reference gene concentration, it means that no inhibition occurred. However, if the resultant qPCR value is lower than the reference gene concentration, it would confirm the presence of strong inhibitors in the sample. The samples are reanalyzed with dilutions (1:10 to 1:100) if inhibition occurs with the expectation that dilution of the inhibitors will reduce or eliminate their effect. Also, the reproducibility of the qPCR assay was assessed by determining intra-assay repeatability by conducting a variability analysis. This analysis gives an indication of the variability in the analysis due to error in the methodology of the qPCR assay or error in laboratory techniques. The variability analysis was conducted by quantification of a spiked sample (template spiked with a standard). The variability is defined as the difference between the measured concentration and the standard concentration divided by the standard concentration. High variability should be avoided and therefore, if the variability was found to be above 35%, then the sample was diluted (1:10) and reanalyzed.

4. Results

4.1. MPN Analysis Results

A total of 28 samples were collected on 25 January, 2012 and analyzed for total coliform, *E. coli*, and enterococci in the laboratory in two days, 25 January, 2012 and 26 January, 2012. The IDEXX Colilert® Quanti-Tray®/2000 (IDEXX, 2012a) and IDEXX Enterolert® Quanti-Tray®/2000 (IDEXX, 2012a) methods were used for analysis to generate MPN/100 mL concentrations for total coliform and *E. coli*, and for enterococci respectively. The least value of concentration detectable by this procedure is 1 MPN/100 mL. Therefore, some of the samples whose concentrations were less than this limit of 1 MPN/100 mL were assumed to be 1 MPN/100 mL. The concentrations of total coliform ranged between 300 and 40,000 MPN/100 mL and *E. coli* ranged between 1 and 1000 MPN/100 mL, while enterococci ranged between 1 and 750 MPN/100 mL. Table 10 presents the calculated concentrations of total coliform, *E. coli*, and enterococci in MPN/100 mL.

4.2. DNA Extraction Results

DNA was extracted from the 28 environmental samples collected; they were all of good quality after going through the extraction procedure. The DNA extraction results, i.e. the genome concentrations of the samples, are presented in Table 11. The qPCR assay was conducted to quantify the HF183 marker in the samples collected, as explained in Section 3.2.2.2. The inhibition analysis described in Section 3.2.2.2 showed that no inhibition occurred in any of the samples. Further, the variability analysis showed that of the samples analyzed, 50% were identified to have variability below 65%. Thus, these samples were diluted (1:10) and reanalyzed.

In the analysis presented in Section 4.3 of this thesis, HF concentrations above the detection limit are used for comparative analysis while concentrations below the detection limit (BDL) are not. Concentrations and detection limits are shown in Table 12. The detection limit is defined as the lowest concentration that the procedure can detect below which the reproducibility of the procedure is not dependable. The detection limit is different for every sample and is calculated using Equation 4:

$$\frac{HF\left(\frac{copies}{qPCR}\right)\times B.dorei\left(\frac{cells}{copy}\right)\times Volume\ DNA\ suspended\ (\mu L)}{\begin{matrix}Volume\ of\ template\times Volume\ of\ sample\ filtered\ through\ Sterivex^{TM}\\ \times Efficiency\ factor\times Factor\end{matrix}} \quad (4)$$

Table 10 - Concentrations of total coliform, *E. coli*, and enterococci in MPN/100 mL

Sample Name	Date Sampled	Time Sampled	Total Coliform (MPN/100mL)	*E. coli* (MPN/100mL)	Enterococci (MPN/100mL)
SBI1	1/25/2012	7:00	38,730	200	579
SBO1	1/25/2012	7:00	13,960	308	201
SI1	1/25/2012	7:00	27,550	579	150
SO1	1/25/2012	7:00	12,910	150	125
FAI1	1/25/2012	7:00	13,340	141	86
FAO1	1/25/2012	7:00	4,890	130	1
SSO1	1/25/2012	7:00	20,140	194	102
SBI2	1/25/2012	9:00	30,760	980	727
SBO2	1/25/2012	9:00	18,600	816	186
SI2	1/25/2012	9:00	18,600	308	204
SO2	1/25/2012	9:00	9,090	82	130
FAI2	1/25/2012	9:00	21,870	134	59
FAO2	1/25/2012	9:00	5,810	308	13
SSO2	1/25/2012	9:00	14,670	137	104
SBI3	1/25/2012	11:00	48,840	980	613
SBO3	1/25/2012	11:00	21,410	276	119
SI3	1/25/2012	11:00	24,890	687	79
SO3	1/25/2012	11:00	8,200	387	47
FAI3	1/25/2012	11:00	7,710	192	39
FAO3	1/25/2012	11:00	6,200	110	26
SSO3	1/25/2012	11:00	13,540	488	59
SBI4	1/25/2012	13:00	23,590	649	579
SBO4	1/25/2012	13:00	2,420	461	76
SI4	1/25/2012	13:00	326	166	96
SO4	1/25/2012	13:00	5,300	260	13
FAI4	1/25/2012	13:00	5,690	101	20
FAO4	1/25/2012	13:00	4,250	69	28
SSO4	1/25/2012	13:00	4,570	291	25

Table 11 - DNA analysis results – genome concentration in ng/μL

Sample Name	Date Sampled	Time Sampled	Volume Filtered (mL)	Date of DNA Extraction	Genome Concentration (ng/μL)
SBI1	1/25/2012	7:00	288	4/1/2012	33.7
SBO1	1/25/2012	7:00	200	4/1/2012	35.6
SI1	1/25/2012	7:00	200	4/1/2012	30.5
SO1	1/25/2012	7:00	365	4/1/2012	23.0
FAI1	1/25/2012	7:00	210	4/1/2012	26.6
FAO1	1/25/2012	7:00	200	4/1/2012	23.6
SSO1	1/25/2012	7:00	290	3/1/2012	44.7
SBI2	1/25/2012	9:00	240	4/1/2012	30.4
SBO2	1/25/2012	9:00	340	3/1/2012	41.0
SI2	1/25/2012	9:00	200	4/1/2012	23.9
SO2	1/25/2012	9:00	370	3/1/2012	42.7
FAI2	1/25/2012	9:00	270	3/1/2012	41.4
FAO2	1/25/2012	9:00	310	4/1/2012	32.3
SSO2	1/25/2012	9:00	200	4/1/2012	21.7
SBI3	1/25/2012	11:00	200	4/1/2012	23.8
SBO3	1/25/2012	11:00	250	4/1/2012	30.8
SI3	1/25/2012	11:00	380	4/1/2012	37.0
SO3	1/25/2012	11:00	350	4/1/2012	35.2
FAI3	1/25/2012	11:00	250	3/1/2012	58.8
FAO3	1/25/2012	11:00	420	3/1/2012	32.6
SSO3	1/25/2012	11:00	335	4/1/2012	46.9
SBI4	1/25/2012	13:00	390	3/1/2012	40.2
SBO4	1/25/2012	13:00	270	4/1/2012	29.6
SI4	1/25/2012	13:00	230	3/1/2012	30.1
SO4	1/25/2012	13:00	430	3/1/2012	42.4
FAI4	1/25/2012	13:00	550	3/1/2012	43.9
FAO4	1/25/2012	13:00	400	4/1/2012	30.6
SSO4	1/25/2012	13:00	330	4/1/2012	23.7

Table 12 - qPCR results in Cell Equivalents/100 mL (CE/100 mL) including detection limits
BDL = Below detection limit

Sample Name	Volume Filtered (mL)	qPCR concentration (CE/100ml)	Detection Limit CE/100ml (for 100 copies/QPCR)	Is result > detection limit?
SBI1	288	7,050	18,500	
SBI2	200	45,100	11,100	BDL
SBI3	200	18,300	26,700	
SBI4	365	2,690	13,700	
SBO1	210	64,000	26,700	BDL
SBO2	200	5,150	15,700	
SBO3	290	41,900	21,300	BDL
SBO4	240	34,800	19,800	BDL
SI1	340	26,800	26,700	BDL
SI2	200	114,000	26,700	BDL
SI3	370	16,600	14,000	BDL
SI4	270	5,900	23,200	
SO1	310	43,100	14,600	BDL
SO2	200	33,600	7,210	BDL
SO3	200	139,000	10,700	BDL
SO4	250	30,700	6,200	BDL
FAI1	380	268,000	12,700	BDL
FAI2	350	30,900	9,880	BDL
FAI3	250	16,300	6,350	BDL
FAI4	420	4,120	4,850	
FAO1	335	166,000	13,300	BDL
FAO2	390	76,400	8,600	BDL
FAO3	270	38,000	7,960	BDL
FAO4	230	171,000	6,670	BDL
SSO1	430	12,700	18,400	
SSO2	550	23,700	26,700	
SSO3	400	99,500	5,560	BDL
SSO4	330	461,000	8,080	BDL

4.3. Correlations between E. coli, Enterococci, and HF marker

4.3.1. Pearson Coefficient

To measure the linear relationship between the $\log_{10}$ concentrations of the indicators, I used the Pearson coefficient (Srinivasan et al., 2011). The Pearson coefficient is a statistic that quantifies the strength of the relationship between two variables, in this case two indicators. It is represented by the variable R. This factor can be calculated easily in Microsoft Excel; it can also be calculated as the square root of R^2 in a linear curve-fit. Table 13 represents the log of the concentrations used to calculate the Pearson coefficient between *E. coli* and enterococci, HF and *E. coli*, and HF and enterococci. As explained in Section 4.2, only HF values above the method detection limits are used in computing correlation coefficients. The Pearson coefficients for the indicators are presented in Table 14.

The linear correlation between indicators can also be observed through plotted graphs. As mentioned above, the square root of the R^2 values in the linear curve-fit graphs would give the Pearson coefficient. An R^2 value of one indicates perfect correlation and an R^2 value of zero indicates no correlation. The R^2 value for HF and *E. coli* is 0.039 (Figure 26), for HF and enterococci is 0.081 (Figure 27), and for *E. coli* and enterococci is 0.24 (Figure 28). All of these R^2 values are very weak, which indicates that there is essentially no correlation between the indicators used in this study.

Table 13 - $\log_{10}$ concentrations of *E. coli*, enterococci, and HF

Sample Name	$\log_{10}$ EC (MPN/ 100mL)	$\log_{10}$ ENT (MPN/ 100mL)	$\log_{10}$ HF (CE/ 100mL)
SBI1	2.3	2.76	
SBI2	2.99	2.86	4.65
SBI3	2.99	2.79	
SBI4	2.81	2.76	
SBO1	2.49	2.3	4.81
SBO2	2.91	2.27	
SBO3	2.44	2.07	4.62
SBO4	2.66	1.88	4.54
SI1	2.76	2.18	4.43
SI2	2.49	2.31	5.06
SI3	2.84	1.9	4.22
SI4	2.22	1.98	
SO1	2.18	2.1	4.63
SO2	1.91	2.11	4.53
SO3	2.59	1.67	5.14
SO4	2.42	1.12	4.49
FAI1	2.15	1.94	5.43
FAI2	2.13	1.77	4.49
FAI3	2.28	1.59	4.21
FAI4	2	1.31	
FAO1	2.11	0	5.22
FAO2	2.49	1.13	4.88
FAO3	2.04	1.41	4.58
FAO4	1.84	1.44	5.23
SSO1	2.29	2.01	
SSO2	2.14	2.02	
SSO3	2.69	1.77	5
SSO4	2.46	1.4	5.66

Table 14 - Pearson coefficients of indicators

Pearson Coefficient: EC and ENT	Pearson Coefficient: HF and EC	Pearson Coefficient: HF and ENT
0.49	-0.20	-0.28

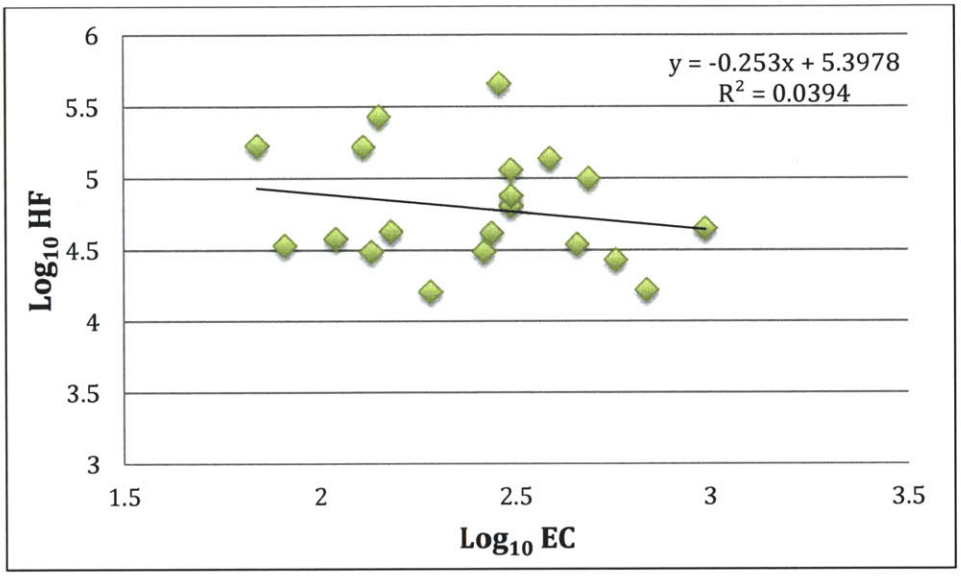

Figure 26 - Correlation between HF and EC

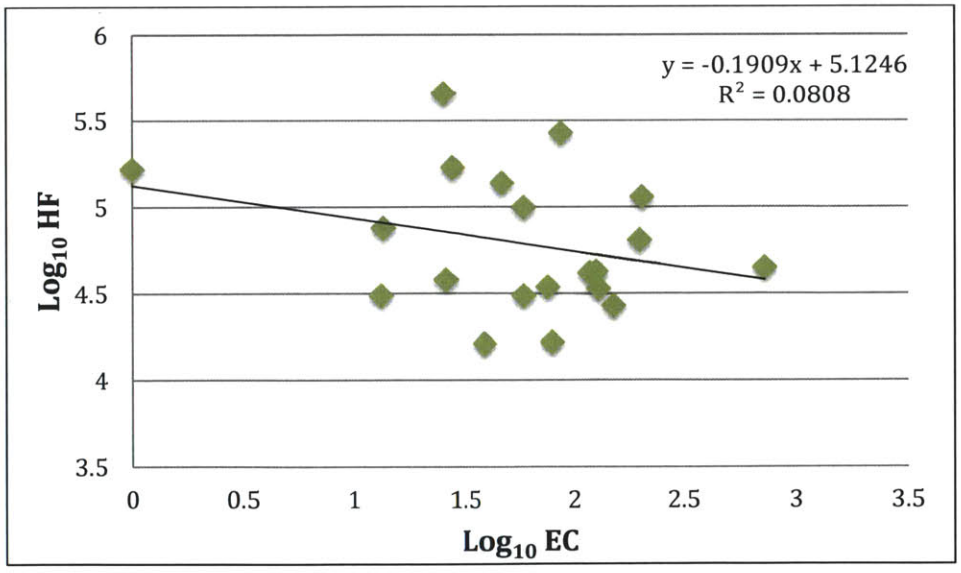

Figure 27 - Correlation between HF and ENT

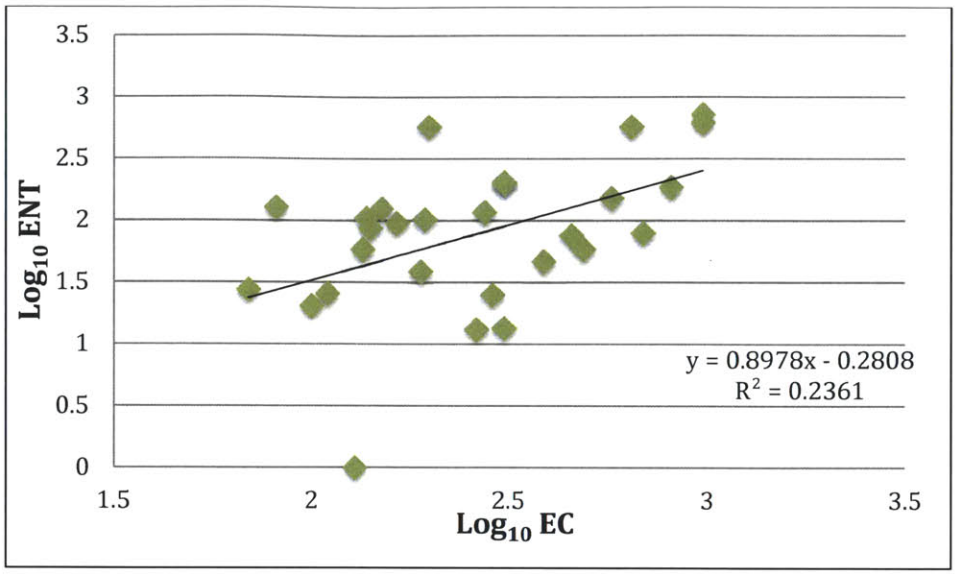

Figure 28 - Correlation between ENT and EC

4.3.2. Comparison of influent and effluent concentrations

Alexandra Canal water is pumped up into the wetland system and flow progresses from one wetland system to the next in series as shown in Figure 8. In order to compare the concentrations of the influent and the effluent, geometric means of the measured concentrations of total coliform, *E. coli*, enterococci, and HF marker are taken. The $\log_{10}$ of the geometric mean values are shown in Table 15. One of my findings is that the geometric mean concentrations of total coliform, *E. coli*, and enterococci decrease as the canal water progresses through the wetland system until it reaches the subsurface wetlands, where the bacterial concentrations increase instead of the expected decrease. Figures 29, 30, and 31 show the concentrations of indicators at the inlet and outlet of the sedimentation bay, surface flow wetlands, and floating aquatic wetland respectively. The bars show the geometric mean value of concentration with error bars indicating the total range between the maximum and minimum values measured. Figure 32 represents the concentration of indicators at the outlet of the floating aquatic wetland (essentially the inlet of the subsurface wetland) and the outlet of subsurface wetland. Figure 33 is a representation of the change in behavior of all indicators from the sedimentation bay to the subsurface wetland. Another counterintuitive result I found was that the HF marker increased very slightly as the canal water flows through the system (Figure 33). However, as evidenced by the error bars on the HF factor in Figures 29 through 32, the differences in HF in Figure 33 are within the measurement range for HF.

Table 15 - Log_{10} geometric means of concentrations

	Samples						
Indicators	**SBI**	**SBO**	**SI**	**SO**	**FAI**	**FAO**	**SSO**
TC (MPN/100mL)	2.53	2.53	2.40	1.93	2.03	1.72	2.07
EC (MPN/100mL)	2.77	2.63	2.58	2.27	2.14	2.12	2.39
ENT (MPN/100mL)	2.79	2.13	2.09	1.75	1.65	0.99	1.80
HF (CE/100mL)	4.05	4.42	4.37	4.70	4.44	4.98	4.79

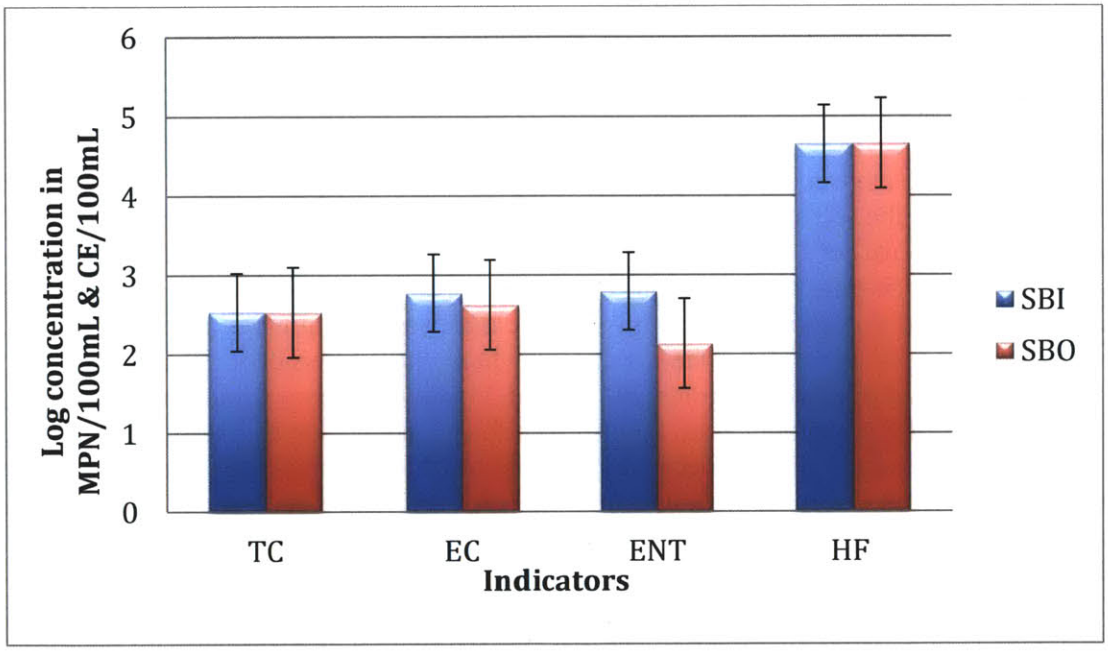

Figure 29 - Log_{10} concentration of indicators at the inlet (identified as SBI) and outlet (SBO) of sedimentation bay

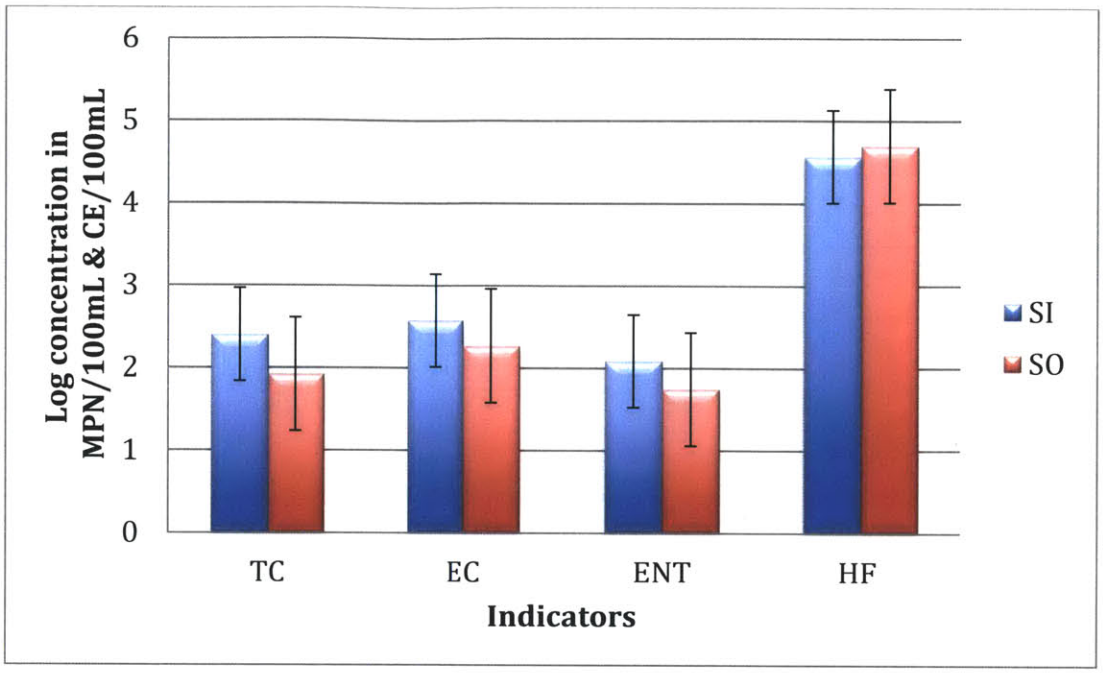

Figure 30 - Log_{10} concentration of indicators at the inlet (identified as SI) and outlet (SO) of surface flow wetland

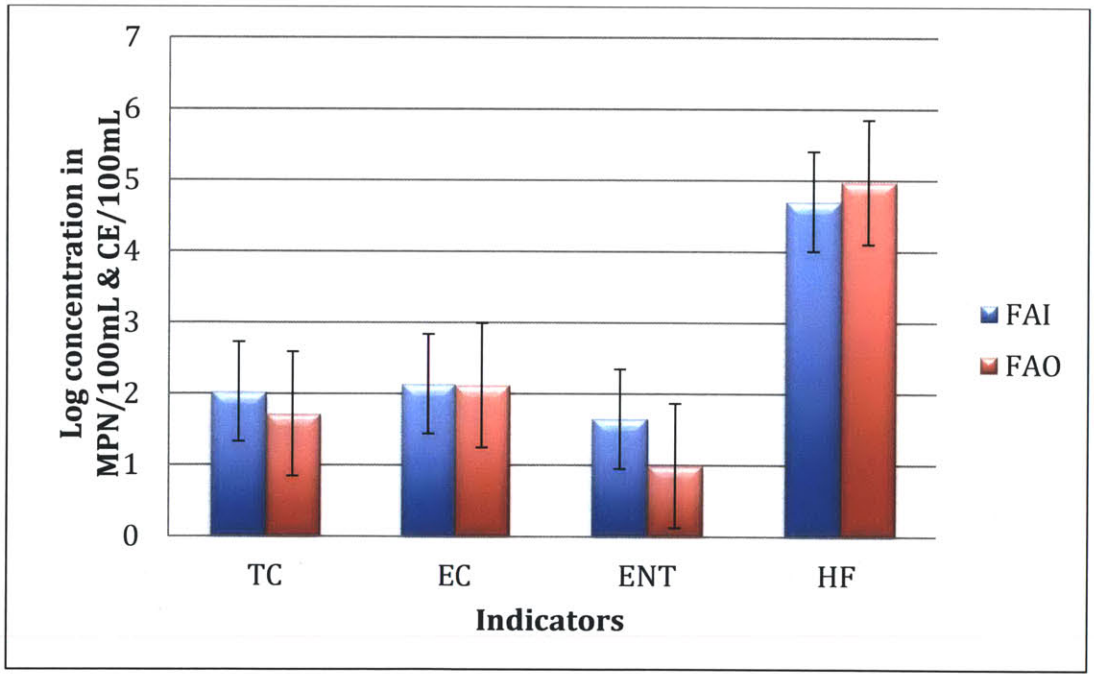

Figure 31 - Log_{10} concentration of indicators at the inlet (identified as FAI) and outlet (FAO) of floating aquatic wetland

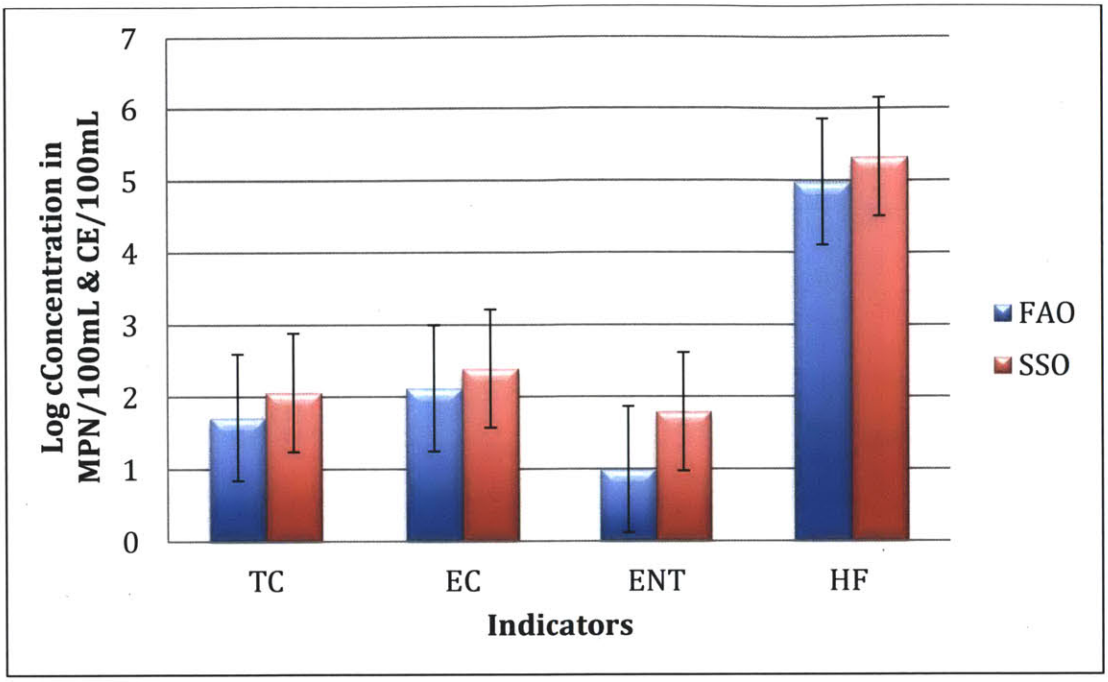

Figure 32 - Log_{10} concentration of indicators at the outlets of floating aquatic wetland (FAO) and subsurface wetland (SSO)

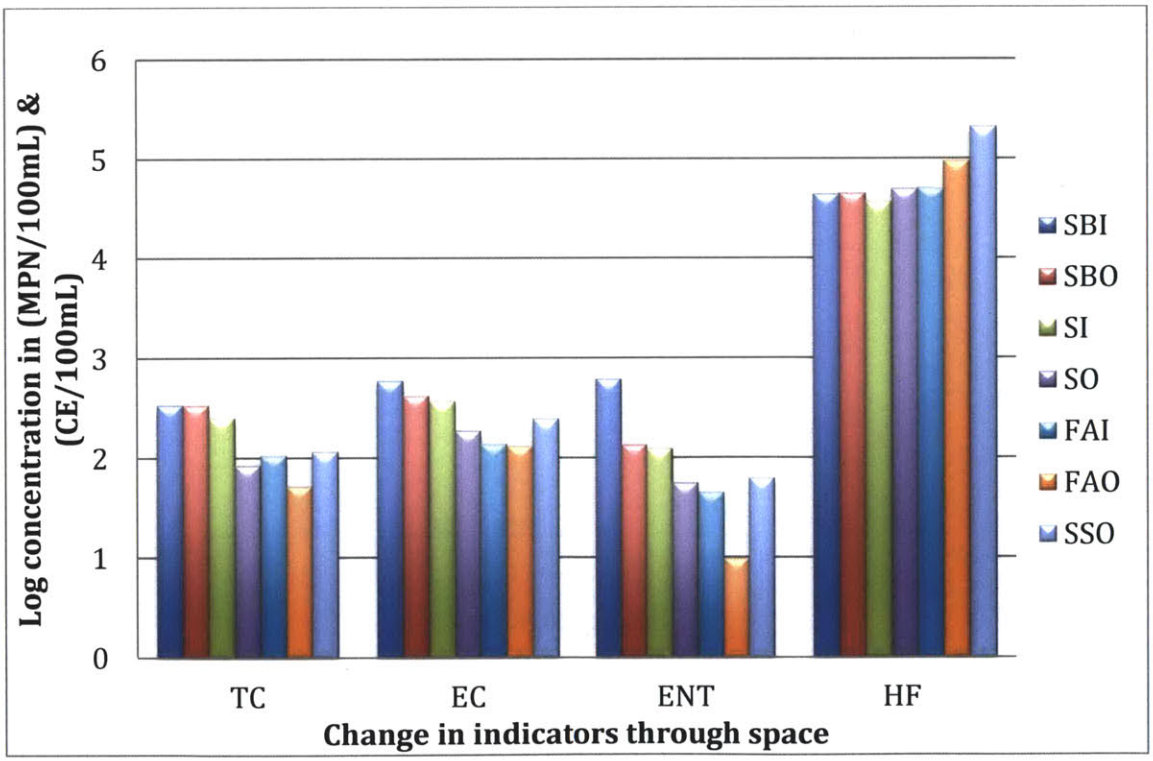

Figure 33 - Change in behavior of indicators through treatment system

5. Summary, Conclusions, and Recommendations for Additional Research

5.1. Summary

Urban stormwater runoff usually contains five main categories of pollutants which are suspended solids, nutrients, litter and refuse, bacteria and pathogens, and toxicants such as pesticides and heavy metals (Lulla, 2007). Constructed wetlands are one type of best management practice that behave as sinks and transform a multitude of pollutants to improve the quality of surface runoff. The Alexandra Canal constructed wetland was the fourteenth project since Singapore's Public Utilities Board (PUB) launched the Active, Beautiful, and Clean (ABC) Waters Programme in 2007 (AsiaOne, 2011). The Alexandra Canal wetland is a system of four wetlands, where water flows in series from the sedimentation bay to the surface flow wetland, thereafter passing through the floating aquatic wetland, and finally through the subsurface wetland.

This purpose of this study was to evaluate the efficiency of a system of constructed wetlands in treating non-point source pollution, particularly, human fecal contamination. The performance of a constructed wetland can be assessed microbiologically by learning the quality of the influent and effluent stormwater. Therefore, it is of utmost importance to choose the appropriate indicator. Reliance on one kind of indicator is not advisable, therefore different types of indicator bacteria are used (Byappanahalli, 2000). Total coliform, *Escherichia coli*, and enterococci are used as fecal indicator bacteria. However, in tropical climates similar to Singapore, it was found that the above mentioned indicator bacteria were present in the fecal matter of birds, that they grew in soil, fresh water, swash-zone sand, river sediments, estuaries, tidal creeks, marine beaches, and flora (Alm et al., 2003; Byappanahalli, 2000; Byappanahalli & Fujioka, 1998; Hazen, 1988; Litton et al., 2010; Rivera et al., 1988). Alternate, rapid molecular-based methods were developed for the first time using 16S primers by (Bernhard & Field, 2000a). The quantitative polymerase chain reaction (qPCR) assay was developed to identify human host specific *Bacteroides* 16S rRNA genetic markers (Bernhard & Field, 2000a; Seurinck et al., 2005). The qPCR method directly measures genetic material with a wide detection range of (10^0 – 10^8 copies/reaction) (Converse et al., 2011; Srinivasan et al., 2011). Further, the qPCR method is capable of measuring the contamination in less than 3 hours which means beach managers can give out warnings on the same day as the contamination is detected rather than having to wait for results by conventional culture-based methods (Lavender & Kinzelman, 2009).

In my field investigation of the Alexandra Canal wetlands, samples were collected at four times on January 25, 2012 and analyzed using both conventional culture-based methods and molecular methods (qPCR). The Most Probable Number (MPN) method was used for microbiological analysis. IDEXX Quanti-Tray®/2000 product (IDEXX, 2012b) was used in combination with the reagents Enterolert® (IDEXX, 2012a) and Colilert® (IDEXX, 2012a) to test the water samples for enterococci and for total coliform and *E. coli*, respectively. Enumeration of the indicator bacteria is done by counting the positive wells on the Quanti-Tray® after incubation.

The qPCR method is explained briefly in this paragraph. The samples meant for qPCR were shipped on dry-ice (approximately -80°C) from Singapore to the Thompson Laboratory at the

Parsons Laboratory of the Civil and Environmental Engineering Department, MIT. The DNA extraction was conducted using the UltraClean® Plant DNA Isolation Kit Protocol (MO BIO Laboratories, 2012a). qPCR was used to quantify the HF183 marker using the instrument LightCycler® 480 (Roche Applied Sciences, 2012). The qPCR reaction mixture includes the KAPA SYBR® FAST qPCR Master Mix (2X), primers, distilled water, and the template. The SYBR® Green I fluorescent dye, one of the key components of the Master Mix, binds to the double-stranded DNA and causes it to fluoresce. The primary advantage of qPCR assay is that it is capable of amplifying the DNA sequences and simultaneously measuring the concentration (Sambrook & Russel, 2001). In qPCR, the amount of fluorescence is a direct measure of the gene amplification (Applied Biosystems, 2012a). The threshold cycle (C_t) is the cycle on the qPCR amplification plot at which the sample reaches the level of detection. The so-called "standard curve" is a straight-line plot of C_t versus $\log_{10}$ of copies/qPCR of positive control measured, from which copies/qPCR of the samples are calculated. Converting copies/qPCR to cell equivalents/100 mL (CE/100 mL) gives us the HF marker concentrations.

The concentrations of total coliform ranged between 300 and 40,000 MPN/100 mL, *E. coli* between 1 and 1000 MPN/100 mL, enterococci between 1 and 750 MPN/100 mL, and the HF marker between 2.7×10^3 and 4.6×10^5 CE/100 mL. One of my findings was that the concentrations of total coliform, *E. coli*, and enterococci were found to decrease while traveling through the wetland system until they reached the subsurface wetland, where the concentrations increased. Another counterintuitive result I found was that the HF marker concentration increased gradually as the water flowed through the system. Further, relationships between the conventional and alternative indicators were evaluated and no significant correlations were found. The R^2 values for HF and *E. coli* was 0.04, for HF and enterococci was 0.08, and *E. coli* and enterococci was 0.24.

5.2. Conclusions

The results discussed above indicate that there are considerably higher concentrations of HF marker (10^3 to 10^5 CE/100 mL) than of the bacterial indicators (10^2 to 10^4 MPN/100 mL *E. coli* and 1 to 10^2 MPN/100 mL enterococci). This difference in cell magnitude may be due in part to an overestimation of HF concentrations by the qPCR method as it does not differentiate between viable and non-viable cells whereas the culture-based measurements of *E. coli* and enterococci do not count non-viable cells.

The concentrations of total coliform, *E. coli*, and enterococci were found to decrease as canal water passed through the sedimentation bay, the surface flow wetland, and the floating aquatic wetland, but the concentrations increased as it flowed through the subsurface flow wetland. Bacterial decay can be expected to occur in the initial three wetland systems as they are exposed to sunlight. Due to the absence of sunlight and its strong inactivation effect, bacterial growth may be occurring in the subsurface wetland and thereby an increase in every bacterial indicator is apparent. The literature cited in Section 2.6 indicates the plausibility of bacterial growth in the tropical climate of Singapore in the absence of sunlight.

In contrast to the bacteria, concentrations of the HF marker increased gradually as the water flowed through the system. This increase may simply be a happenstance since the measurement

range is much greater than the decrease within any single wetland. Another reason why this may be occurring would be due to the water being of different ages in the wetland system, as each system had variant retention times.

Relationships between the conventional and qPCR indicators were evaluated and no significant correlations were found. Low R^2 values were found for correlations between HF and *E. coli*, between HF and enterococci, and between *E. coli* and enterococci. This lack of correlation implies that the decay mechanisms of the conventional indicators and the HF markers in wetlands are very different. Although future research is necessary to understand the various growth and removal mechanisms occurring in the wetland and their relationship to the indicators being used, overall, I conclude that the constructed wetland is effective in that the concentrations of the indicator bacteria decreased.

5.3. Recommendations for Additional Research

The following are recommendations for future research:

1. The retention times of the four wetland systems are very different. Thus, the sampling program could be improved to collect more representative samples. For each wetland system, a sample could be collected at the inlet on a particular day; the effluent sample should then be collected after a delay in time equal to the retention period.
2. Further research is recommended to understand the decay mechanisms of HF marker in wetlands. Also, the current standards on indicator bacteria are culture-based (i.e. based on viable cells) whereas qPCR-based methods measure both viable and non-viable cells. These factors make the process of comparison between culture-based indicator bacteria and qPCR complicated. Therefore, a better understanding of the persistence of the HF marker as compared to indicator bacteria is required.
3. Future scientific research is required to determine if there is an overestimation bias related to qPCR or if there is in fact an underestimation bias with respect to the culture-based methods.
4. The processes in a wetland are complicated and measuring stormwater treatment effectiveness is a major challenge. Measurements could be made more efficient if a mathematical model of the wetland could be created. Some of the factors needed to be considered for the model are input pollutants, hydraulics, physico-chemical balance, and biota within the wetland.
5. Hazen (1988), Litton et al. (2010), and Byappanahalli and Fujioka (1998) indicate growth of indicator bacteria in soil sediments. An interesting project to undertake would be to examine the sediments from the bottom of the wetlands and correlate the patterns of bacterial contamination in water and soil sediments.

6. The variability analysis gives an indication of the variability in HF quantification due to error in the laboratory techniques or in the methodology of the qPCR assay. The variability was found to be 50% in this study, necessitating re-analysis of diluted samples. We know that every experiment conducted is attached to some amount of error, however much care is taken. Therefore, simplification of laboratory techniques is necessary. Another way of reducing human errors is using automated methods for DNA extraction.
7. To further understand the increase in the bacterial contamination shown by indicators in the subsurface wetlands, bench tests or pilot tests could be conducted.

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