Characterization of Microbial Communities, Disinfection and Removal of Human Pathogenic Bacteria in Arctic Wastewater Stabilization Ponds

by

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ABSTRACT

Municipal wastewater management in Arctic Canada is different than in Southern Canada, mostly due to climatic and infrastructural constraints. Most arctic communities use trucked sewage collection systems followed by treatment in passive systems including wastewater stabilization ponds (WSPs). The objectives of this thesis were to determine if treatment of municipal wastewater in arctic WSPs successfully removes fecal indicator bacteria (generic *Escherichia coli*) and selected human bacterial pathogens (pathogenic *eae*-positive *E. coli* including O157:H7, *Salmonella* spp., *Campylobacter* spp., and *Helicobacter pylori*, and the non-enteric *Listeria monocytogenes*) and investigate the size, composition, diversity and potential function of bacterial WSP communities in relation to the impact of the Arctic climate, especially low temperatures, and treatment processes. This 3-year study (2012-2014) was conducted in the Nunavut communities of Pond Inlet and Clyde River with one-cell and two-cell WSP systems, respectively.

Anaerobic conditions with an absence of algal blooms and constant pH values of 7.5-7.8 prevailed throughout the study period in the WSPs of both communities. The WSPs provided a primary disinfection treatment of the wastewater with a 2-3 log removal of generic *E. coli.* The bacterial pathogens *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes*, but not *Campylobacter* spp. and *H. pylori*, were detected in the treated wastewater, indicating human pathogens were not reliably removed. The bacterial population size and diversity was highly dependent on the treatment train and different geographic locations. However, the bacterial diversities in raw wastewater were not different between the communities. Seasonal and annual variations in temperature significantly ($p<0.05$) affected the disinfection efficiency, WSP bacterial diversities and potential functionalities. The best treatment effect in terms of disinfection and the removal of pathogen and nutrients was observed in the secondary pond of the two-cell WSP and in the middle of the treatment season.

Future research should involve a quantitative microbial risk assessment to determine if the release of low levels of human pathogens into the arctic environment poses a human health risk and a bench-scale study to clarify the effect and significance of each variable

(e.g., temperature, DO, pH and nutrients) to optimise the microbial functionality and removal of fecal bacteria.

LIST OF ABBREVIATIONS USED

AGI	acute gastrointestinal illness
AO	anaerobic/oxic
A^2O	anaerobic/anoxic/oxic
AOB	ammonia oxidizing bacteria
APHA	American Public Health Association
BOD	biochemical oxygen demand
bp	base pair
CCME	Canadian Council of Ministers of the Environment
CBOD5	carbonaceous biochemical oxygen demand at Day 5
COD	chemical oxygen demand
Ct	threshold cycle
DGGE	denaturing gradient gel electrophoresis
DO	dissolved oxygen
F/M	food/microorganisms ratio
HRT	hydraulic residence time
IMR	Integrated Microbiome Resource
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOs	Kyoto Encyclopedia of Genes and Genomes Orthologs
LOD	limit of detection
LOQ	limit of quantification
MBRs	membrane bioreactors
min	minutes
MLSS	mixed-liquid-suspended-solids
NGS	next-generation sequencing
NSTI	Nearest Sequenced Taxon Index
NWQL	Northern Water Quality Laboratory
OTUs	Operational Taxonomic Units
PCR	polymerase chain reaction
PCoA	Principal Coordinate Analysis

PE	paired-end
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	quantitative polymerase chain reaction
RDP	Ribosome Database Project
ROX	Reliable Oxygen Sensor
rRNA	ribosomal ribonucleic acid
s	seconds
SRB	sulphate reducing bacteria
SRT	sludge retention time
STAMP	statistical analysis of taxonomic and functional profiles
TN	total nitrogen
TP	total phosphorus
t-RFLP	terminal restriction fragment length polymorphism
TSS	total suspended solids
WSER	Wastewater Systems Effluent Regulations
WSPs	wastewater stabilization ponds
WWTPs	wastewater treatment plants

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to extend my gratitude to the people in Pond Inlet and Clyde River, and to the Nunavut Research Institute for providing laboratory space in Iqaluit.

Chapter 1 Introduction

Currently, the new Wastewater Systems Effluent Regulations (WSER) implemented by Environment Canada do not apply to wastewater treatment facilities in the Canadian Far North (Nunavut, the Northwest Territories and northern regions of Quebec and Newfoundland and Labrador), because Environment Canada recognizes that the climatic, geographic, social and economic constraints in Arctic communities create difficulties in complying with the new regulations. The Canadian Council of Ministers of the Environment (CCME) endorsed the Canada-wide Strategy for the Management of Municipal Wastewater Effluent on February 17, 2009. The CCME strategy sets out a harmonized framework to manage the effluents from wastewater facilities in Canada, many of which are currently in need of repair and upgrading. In this strategy, it is explicitly stated that little information exists on the performance of current wastewater treatment systems operating in the Canadian Far North and the potential risks they pose to human health and the environment. It is challenging to design, construct, and operate wastewater treatment systems in the Canadian Far North because of continuous permafrost conditions, long winters lasting up to nine months, short and cool summers lasting about three months, unpredictable environmental conditions, high costs of electricity, fuel and transportation, as well as a settlement pattern with limited accessibility, especially in the remote Arctic communities. Between Arctic communities, inter-community road connections do not exit. While communities are accessible by air transportation, weather permitting, throughout the year, access by ship is only possible during the short summer season when the ice conditions are favourable. Thus, communities receive major consumer and infrastructural supplies and equipment through

one or two annual sea lifts during the short ice-free period. These constraints cause challenges to construction, operation and maintenance of infrastructure as the spare or supporting equipment resources are limited in these remote Arctic communities. Specialized equipment is most likely to be obtained externally by the high cost of air transport year-round or by the economic cost of ship only during the short ice-free period.

Therefore, the CCME strategy was to establish a five-year research period (ending in 2014) to allow investigations into the performance of wastewater treatment systems in Northern Canada for the purpose of creating baseline information that could aid in setting realistic effluent targets in these regions. A team from the Centre of Water Resource Studies at Dalhousie University has taken part in the research into a) understanding the current performance of wastewater treatment systems in the Canadian Territory of Nunavut, b) investigating environmental or operational factors that affect their performance, and c) determining suitable approaches to upgrade the operation of wastewater treatment systems or defining appropriate effluent standards in these regions.

Wastewater treatment systems in Nunavut primarily consist of wastewater stabilization ponds (WSPs) (Kelly and Mayr 2011). The WSPs are completely exposed to environment and rely on natural environmental conditions to degrade or stabilize the wastewater. Operation of the existing mechanical wastewater treatment plants were observed to encounter performance challenges in Nunavut, for example in Iqaluit and Pangnirtung, due to a mixture of climatic, geographic and economic factors, leading to both systems currently being upgraded. As a result, the use of WSPs, a passive treatment system, has been the most common treatment solution to manage municipal wastewater and is currently implemented in 22 out of 25 Nunavut communities. WSPs operate as

storage lagoons during the 9-10 month period with ice coverage and no discharge. The sewage therefore stays frozen in the WSPs for up to nine months of the year, then is gradually thawed from the middle of June and stays liquid until freeze-up starts in early September. This short summer season is called the "treatment season" due to the sewage's physical phase-transition, above freezing-point temperatures and extended daylight period, which potentially yields high solar radiation levels and algae blooms in the facultative or aerobic pond. In early September before the freeze-up the treated sewage is decanted into the ocean or to a natural tundra wetland. In arctic communities, due to the harsh climate and continuous permafrost conditions, the wastewater transportation system relies on trucks and not on septic fields and piped distribution systems, which are common solutions in southern regions. Households in the arctic communities have inside holding tanks for generated wastewater. On a regular basis, trucks come to pick up the wastewater and to transport it to the WSPs. Raw wastewater is characterized by high organic and nutrient concentrations, because of the low per capita water use in these arctic communities (Smith 1986). Therefore, discharging untreated or inadequately treated wastewater into the receiving arctic environment could cause a potential risk to human health and the environment. Due to the low temperature and low biological activity in the arctic ecosystem, the ecosystem has a high vulnerability to manage environmental contaminants (Gunnarsdottir et al. 2013). Therefore, proper treatment of the wastewater before discharging into the receiving environment is becoming an important societal task in the Arctic.

In arctic communities, many members of the indigenous inhabitants still practise traditional activities, such as fishing, hunting and foraging, in their daily life (Fleming et

al. 2006; Suk et al. 2004). These traditional activities increase the risks that people are prone to be exposed to wastewater effluent directly when the activities take place near wastewater treatment areas or indirectly when consuming the food carrying infectious agents from the wastewater effluents (Daley et al. 2017). Moreover, overcrowded housing conditions can easily lead to person-to-person spread of infectious agents (Goldfarb et al. 2013). As a result, the exposure can cause acute gastrointestinal illness, severe infectious enteric disease, and long-term chronic illness (Ashbolt 2004; Prüss et al. 2002). It is believed that the burden of waterborne- and sanitation-related illness in Arctic communities is higher than in other parts of Canada (Harper et al. 2011; Harper et al. 2015a; Harper et al. 2015b; Thomas et al. 2013). Therefore, from a public health perspective, it is important to investigate whether current WSPs treatment achieves compliance with targets for disinfection and removal of human bacterial pathogens.

Microbial communities present in biological wastewater treatment plants (WWTPs) are responsible for most of the carbon and nutrient removal from sewage and therefore play a key role in the biological treatment process in every WWTP (Wagner and Loy 2002). Operating and environmental parameters of wastewater treatment influence the formation of complex microbial communities, their diversity and function. The composition, diversity and function of microbial communities can influence the quality of treated wastewater (Cydzik-Kwiatkowska and Zielińska 2016). Since biological activity is likely to be reduced in WWTPs operated in cold climates, this can result in limited biological removal of nutrients in discharged effluents, which can be potentially hazardous to the receiving environment. Therefore, a systematic understanding of bacterial community size, composition, diversity and function along the WSP treatment

train could help us to contribute knowledge that could potentially be used to enhance biological wastewater treatment under cold temperatures in an operational cost- and energy-wise manner.

At the present time, very limited research has been focused on disinfection treatment efficiency, removal of human bacterial pathogens, and the composition, diversity and function of bacterial community in WSPs which have been operated as passive wastewater treatment systems under harsh climate of the Canadian Arctic over the past 30-50 years. To fill in this knowledge gap, the objectives of this thesis are described in Chapters 3 and 4 and are briefly summarized below:

1) Determine if treatment of municipal wastewater in arctic WSPs successfully removes fecal indicator bacteria (generic *Escherichia coli*, i.e. all *E. coli* including commensal and pathogenic strains) and selected human bacterial pathogens (pathogenic *eae*-positive *E. coli* including O157:H7, *Salmonella* spp., *Campylobacter* spp., and *Helicobacter pylori*, and the non-enteric *Listeria monocytogenes*). The disinfection treatment efficacy was investigated over three years (2012-2014) in two remote communities in Nunavut, Pond Inlet one-cell (Figure 1.1) and Clyde River two-cell (Figure 1.2) WSPs, respectively. We hypothesized that the disinfection treatment efficacy and removal of human bacterial pathogens was affected by a) the time of the sampling during the summer treatment season and annual variations in temperature and b) the treatment type (two-cell vs one-cell), respectively. This is further described in Chapter 3.

2) Investigate the size, composition, distribution, diversity and potential function of bacterial WSP communities in relation to how the arctic climate, especially low temperatures, and the treatment processes affect them. This study was conducted over

three years (2012-2014) in two remote communities in Nunavut, Pond Inlet and Clyde River, where the municipal wastewater treatment is performed using one-cell (Figure 1.1) and two-cell (Figure 1.2) WSPs, respectively. We hypothesized that the bacterial community size, composition, distribution and diversity would be affected by a) the WSP treatment train, b) the time of the sampling during the summer treatment season, and c) the sampling years, respectively. It was hypothesized that the bacterial diversity in raw wastewater would not be different, as the communities resemble each other in regards to life style, diet, lack of industry and agriculture. Due to differences in micro-climates and WSP designs between two communities, it was also hypothesized that the treated wastewater would differ across the two geographic locations. Finally, it was hypothesized that the potential functionality of bacterial communities would be influenced by the time of the sampling during the summer treatment season, annual variations in temperature and the treatment type. This is further described in Chapter 4.

Figure 1.1 Aerial photo of the one-cell WSP in Pond Inlet (Google Maps 2017a).

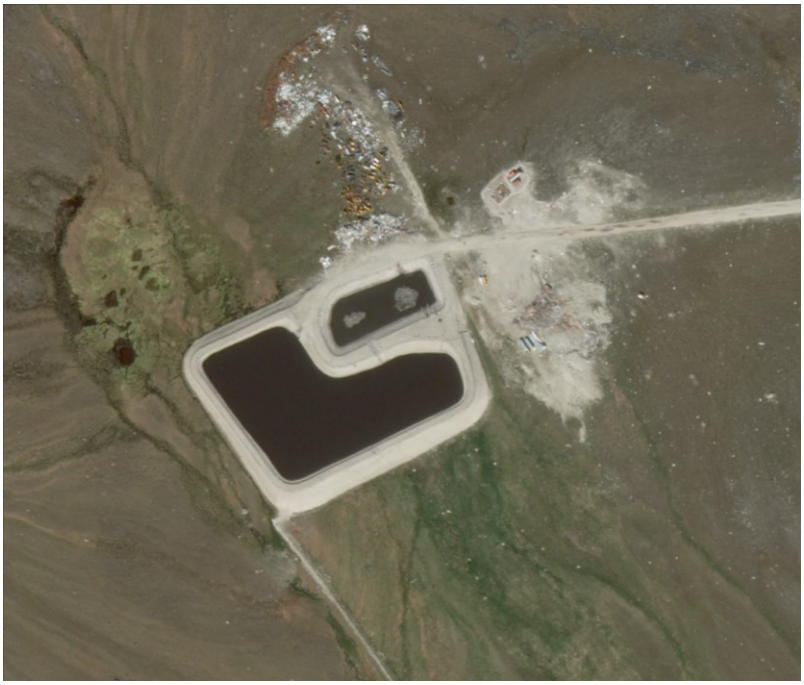

Figure 1.2 Aerial photo of the two-cell WSP in Clyde River (Google Maps 2017b).

Chapter 2 Literature Review

As described in the introduction, passive wastewater treatment systems are the preferred method for treating municipal wastewater in the Canadian Arctic. The following literature review will look at the characteristics and performance of these systems in the context of nutrient and pathogen removal and microbial processes which affect the treatment in WSPs.

2.1 Pond Design and Operation as A Passive Wastewater Treatment Method

Wastewater stabilization ponds are shallow engineered natural or manmade water basins that are used to detain liquid waste from industries or municipalities for treatment before discharging to the environment (Shilton and Walmsley 2005). They are able to treat wastewater to meet the needs of agriculture, industry, cities and remote communities, and therefore are considered as one of the most common pond treatment technologies (Shilton and Walmsley 2005). In order to eliminate or minimize the environmental impact of wastewater effluent, WSPs make use of biological processes to remove or reduce biological and chemical contaminants in the wastewater prior to its release into the environment.

Human and animal excrements, especially in high concentrations, are more likely to present a potential health hazard due to the potential spread of pathogens from the excrement to the environment (Droste 1997). Also, high concentrations of human and animal excrements may release high levels of nutrients to the environment, resulting in adverse environmental consequences, such as eutrophication and ammonia toxicity (Droste 1997). Water is used to be a means of transporting human excrements away from many settlements. It brings relatively large volume of wastewater that need to be

disposed to the environment. To eliminate or reduce the adverse environmental and public health impacts, wastewater effluent should be treated to reach the desired levels of nutrients and pathogen removal. WSPs are applied to treat human wastewater by using natural processes. Prior to discharging wastewater to the environment, the goal of WSPs is not to remove all nutrients or pathogens from wastewater, but to reduce their contents to an acceptable level as required by national and local water quality standards (Ramalho 1977).

WSPs are generally designed as a series of 2 to 5 ponds. The configuration of ponds in series results in better hydraulics and improved water quality from the first pond to the last pond (Shilton and Walmsley 2005). As the goals of the water quality standards and current treatments change, the pond design changes along with the series of ponds to optimize treatments. Fresh wastewater entering the WSPs are generally considered to be dominated by anaerobic microorganisms, because the proportion of obligate anaerobic bacteria is usually greater in very fresh wastewater due to the predominantly anaerobic nature of the intestinal tract microflora in warm-blooded animals (Shilton and Walmsley 2005). During the stay in the WSPs, the microflora in the wastewater will gradually become more aerobic, because biological, physical, and chemical processes tend to aerate the wastewater. Therefore, aeration will result in increasing oxygen concentrations in the wastewater, decreasing pollutant concentrations, and decreasing oxygen demand.

The main advantage of WSPs systems is that they are simple to build and operate. These systems are therefore often termed as “low-level technology” (Shilton and Walmsley 2005). However, the mechanisms involved in the way WSPs treat and stabilise wastewater are complex. In the study of biodegradation in WSPs, Thirumurthi (1974)

mentioned that “the biology and biochemistry involved are the most complex of all the engineered biodegradation systems known to man.” In WSPs systems, all aspects of conventional treatment are involved. For example, the treatments include settlement of solids, organics removal, disinfection, and nutrients and heavy metals removal.

2.2 Pond Types and Oxygen Concentration

2.2.1 Anaerobic

Based on the oxygen concentration, pond types are usually classified as anaerobic, facultative, and aerobic. But it is not practical to set up an absolute classification for any one pond, because the dissolved oxygen concentrations in WSPs are dynamic. Annual or seasonal variations in climate or changes in pond loading may result in changes to dissolved oxygen concentrations in WSPs and their classification. It is still worth to look at each pond’s function based on the oxygen concentrations, because oxygen is an important factor in the biological and chemical reactions occurring in WSPs.

When designing a series of ponds, an anaerobic pond is usually built first. The incorporation of an anaerobic pond as a first pond can substantially decrease the size of the following ponds, because wastewater is pre-treated in the first anaerobic pond, resulting in substantial land and cost savings (Shilton and Walmsley 2005). In cases with high organic loadings, the anaerobic pond is good at removing large proportions of the organic load. In an anaerobic pond, there is normally absence of dissolved oxygen and no significant algal population. An anaerobic pond functions well in warm climates, but in cold climates, the major function is primary settling. In a study of arctic WSPs in Nunavut, Canada (Ragush et al. 2015), the WSPs in Pond Inlet and Clyde River were observed to be anaerobic during the 2012-2014 summer treatment seasons. The removal

of nutrient as measured by $CBOD_5$ values from raw wastewater to the water in WSPs at the start of the summer treatment seasons was attributed to settling processes, because little or no biological activity occurred in the ponds when temperatures were close to 0 ºC. In warmer climates, the organic load can be reduced by 40 to 70% during relatively short retention times (just a few days) in an anaerobic pond (Shilton and Walmsley 2005).

2.2.2 Facultative

Frequently a facultative pond follows after an anaerobic pond. In a facultative pond, the bottom layer has a similar function as an anaerobic pond. The bottom layer usually contains an anaerobic sludge layer overlaid with an anaerobic zone in the water column. At higher levels in the water column, there is an aerobic zone formed due to the presence of algae. Compared to the depth of an anaerobic pond (2 to 5 m), a facultative pond is commonly relatively shallow (typically 1.5 m) with retention times measured in weeks. The rationale for the shallow depth is that a facultative pond contains algae that needs sunlight, and therefore is designed on an area basis. In contrast, an anaerobic pond is designed on a volume basis due to the requirement for the absence of oxygen (Shilton and Walmsley 2005).

2.2.3 Aerobic

Aerobic ponds are typically inserted after facultative ponds in the WSP series. The aerobic ponds usually receive a low organic loading after previous treatments and will continue to reduce the organic content. Generally, aerobic ponds work as a series of smaller ponds rather than a single large pond, because this design provides good hydraulic efficiency and more importantly ensures good pathogen removal (Shilton and Walmsley 2005).

2.3 Physical and Chemical Environments

2.3.1 The Dynamic Environment

The design and management of a WSP is conducted in a similar way as the design and management of a small lake. Although both of them seem to have the bodies of water with a constant state of flux, every single physical and chemical parameter changes with the seasonal and diurnal changes in sunlight, wind, and temperature and the changes in the influent quantity and quality (Paterson and Curtis 2005). Therefore, any successful design and management of a WSP is not created based on certain fixed conditions, but is created considering a range of conditions compatible with environmental changes and the changes in the influent quantity and quality. When people monitor pond performance and conditions, they must have proper background knowledge about WSPs. For example, they should be aware of the dynamic nature of WSP to make sure that they obtain representative and repeatable measurements of the WSPs characteristics (Paterson and Curtis 2005).

In WSPs, the physical and chemical environment is not only dynamic, but also complex. For example, light affects temperature and initiates the growth of algae, subsequently resulting in the increase of the pH levels, but at the same time, oxygen-producing algae can block light penetration (Paterson and Curtis 2005). Therefore, for most practicable purposes, the pond performance can be related to four important factors, which are light, dissolved oxygen, pH, and temperature. Those four important factors can largely affect all other physicochemical factors. Each of the four important factors will be briefly introduced in the following section.

2.3.2 Light

For WSPs, light plays an important role in two ways: 1) light enables photosynthesis

by algae, thus resulting in the production of oxygen and increasing pH level and 2) the UV segment of the light kills pathogens (Paterson and Curtis 2005). Light can vary within one day (daytime or night time), vary with the season (summer or winter), the weather (sunshine or overcast), and vary within one pond (the bottom layer versus the upper layer in the water column). Therefore, the variation in the amount of light, which is one of the important climatic variables, influences the performance of WSPs.

Light initiates photosynthesis by algae. Algae produce oxygen, and then oxygen results in the aerobic bacterial degradation of organic waste to reduce odour emission and nutrient levels. Those reactions provide conditions (such as high pH) for improved removal of both pathogens and gaseous ammonia (NH_3). The relationship between light intensity and photosynthesis has generated many models aimed at predicting algal production (Marra 1978), and also fundamental for understanding algal ecology (Neale and Marra 1985). In Arctic WSPs, Ragush and other researchers (2017) measured a light attenuation coefficient of 14 m^{-1} in Pond Inlet WSP, and this was subsequently simulated in bench-scale model WSP experiments. They found that only 1% of the light penetrates to a depth of 30 cm. The light attenuation was likely attributed to the highly concentrated wastewater that these WSPs receive, and would lead to a limited depth of the algal productive layer.

Light can kill pathogens. The mechanism involved is called photo-oxidation (Paterson and Curtis 2005). In photo-oxidation, visible and UV light firstly emit energy. A sensitizer, such as humic substances, can absorb the energy. The sensitizer then reacts with oxygen to produce singlet oxygen. The singlet oxygen finally kills pathogens (Curtis et al. 1994). The sensitizer can be located inside or outside the cell. Previous research

indicated that humic substances, which are found outside the cell, can function as sensitizers. Therefore, the humic substances can absorb light energy emitted from all visible and UV wavelengths (Curtis et al. 1992). It also found that even red light has enough energy to form singlet oxygen to kill pathogens, but it only happens in environments with high pH exceeding 9 and oxygen concentrations above 5 mg/L (Curtis et al. 1992).

2.3.3 Dissolved Oxygen

In WSPs, oxygen functions to control odour, and increase disinfection efficiency (Paterson and Curtis 2005). Oxygen plays an important role in reducing odour by facilitating the oxidation of sulphides and other smelly chemical compounds in the sediments at the bottom of the pond. Oxygen also assists in pathogen removal. Several studies indicated that dissolved oxygen and pH significantly affect the sunlight inactivation of faecal microorganisms (Curtis et al. 1992; Davies-Colley et al. 1999). Their results indicated that dissolved oxygen concentrations directly affect sunlight disinfection, suggesting that a photo-oxidative process is involved. Depending on the amount of light, the photosynthetic activity of algae will increase dissolved oxygen concentrations and pH levels to some extent (Curtis et al. 1994).

In most WSPs, oxygen primarily comes from photosynthesis, and secondarily comes from aeration that may occur at the wastewater surface (Ellis and Mara 1983). However, for production of oxygen, the surface aeration may play a more significant role than photosynthesis, during the winter in temperate climates with longer nights and shorter days. Thus, the importance of surface aeration depends on the extent of the photosynthesis (Ellis and Mara 1983). In addition, in heavily organic loaded ponds,

dissolved oxygen levels are relatively low, because increasing organic load not only uses up oxygen, but also decreases algae's variety and photosynthetic activity. Thus, increasing organic load decreases dissolved oxygen levels (Paterson and Curtis 2005).

Photosynthesis can simply be described as the process where algae utilise the energy of sunlight to produce carbohydrates from carbon dioxide and water. Although many complex mechanisms are involved, the overall process can be described as: $CO_2 + 2H_2O \rightarrow CH_2O + H_2O + O_2$ (Paterson and Curtis 2005). Algae not only produce oxygen, but also consume oxygen during respiration (Reynolds and Irish 1997). Respiration is the process by which fixed carbon is consumed by algae to produce carbon dioxide. Therefore, the measurement of dissolved oxygen changes over time can be used to determine the relationship between the changes of photosynthetic and respiration rates (Reynolds and Irish 1997).

2.3.4 pH

pH plays an important role in removing pathogen, nutrients, and odour. Studies of the survival of bacterial pathogens in WSPs have indicated that when pH values are above 9, the levels of bacterial pathogens are reduced (Paterson and Curtis 2005). Also, as previously mentioned increasing pH levels can improve pathogen removal by enhancing photo-oxidation (Paterson and Curtis 2005). The studies of nutrient removal in WSPs demonstrated that increasing pH levels enhance volatisation of ammonia and precipitation of phosphorus. For controlling odour, pH is also important, because pH affects the disassociation of H_2S that contributes to bad odour. The sulphide ion exists in three forms: H_2S, HS^-, S^{2-}. When pH levels are below 7.5, H_2S predominates among those three forms. Thus, increasing pH levels can control odour in WSPs.

Changes in photosynthesis and organic load produce changes in pH. In WSPs, pH levels are lowest at night and then gradually increase during the day. During the daytime, pH levels may increase to be more than 9 in a moderately loaded pond in warm weather. When photosynthesis occurs, pH levels are usually high at the upper level where is close to the surface of the pond, and then gradually decrease as light penetration decreases toward the bottom of the pond. In temperate climates, the pH levels are usually lower in winter with shorter daytime than in summer with longer daytime. pH levels are usually lower in ponds receiving higher organic loads than in ponds receiving lower organic loads (Paterson and Curtis 2005).

2.3.5 Temperature

Temperature plays two important roles in WSPs. Firstly, temperature of the wastewater significantly affects the rate of the biological processes. Temperature is also a good guide to test performance, because it is related to the amount of sunshine penetration into the water column. It is therefore that when designing a pond, the temperature factor is usually considered as an important parameter in design equations (Paterson and Curtis 2005). Secondly, temperature affects the hydraulic properties of the water. For example, stratification occurs when the sun is shining on the surface of the pond, because the sun causes an increase of the temperature in the upper layers, thus becoming less dense than the cooler water in the bottom layers. Stratification typically does not occur at night when the pond surface is cool. It is possible that if the pond surface cools fast, then the cooler and denser surface layer may sink and cause the bottom layers to rise. This process is known as turnover. Therefore, temperature plays an important role for the hydraulic pond properties and mixing of the water (Paterson and

Curtis 2005).

2.4 Removal of Human Infectious Disease Pathogens

2.4.1 Bacteria, Parasites and Viruses

Disinfection, which is the removal of pathogenic (i.e., disease-causing) microorganisms, is an important and desirable outcome of wastewater treatment processes. WSPs are well known to efficiently and effectively remove different types of pathogenic microorganisms, such as fecal enterococci, *Cryptosporidium*, *Giardia*, helminth eggs (for example, *Taenia*, *Ascaris*, and *Trichuris*) (Davis-Colley et al. 2000; Anceno et al. 2007; Reinoso and Bécares 2008; Tyagi et al. 2008). Their high efficiencies of removing pathogens and relatively low costs are two of the main reasons that make WSPs very popular in the developing world (Davis-Colley et al. 2000).

There are three main types of pathogens that are present in wastewaters (Davis-Colley et al. 2000). These are bacteria, viruses, and parasites including protozoan parasites and worm parasites. Bacterial pathogens include bacteria such as *Salmonella*, *Shigella*, *Vibrio cholerae*, pathogenic strains of *E. coli* and several bacteria that cause zoonotic (harboured by domestic or wild animals) diseases. *Campylobacter* spp. is one of those bacteria that cause zoonotic diseases. Studies have indicated that *Campylobacter* spp. including the common human-pathogenic *C. jejuni*, are effectively removed in WSPs (Davis-Colley et al. 2000). *C. jejuni* is one of the most important causes of waterborne gastroenteritis in the developed world (Davis-Colley et al. 2000). Oragui and other researchers (1986) indicated that *Campylobacter* spp. were completely removed in deep WSPs in northeast Brazil. One year later, Pearson and other researchers (1987) indicated that *C. jejuni* along with *Salmonella* spp. was removed more rapidly than *E. coli* in a WSP system. Bacterial

diseases cause many thousands of people to die every year, especially in the developing world where public health engineering is not well established. Therefore, WSPs play a key role in reducing the incidence of bacterial diseases and avoiding epidemic outbreaks (Mara 2001).

The enteric viruses cause waterborne enteric diseases in both developed and developing worlds. There are at least 140 types of waterborne enteric viruses reported including Hepatitis A virus and rotavirus (Davies-Colley et al. 2000). For people who have healthy immune systems, viral diseases do not have lethal effects, but some viral diseases, such as hepatitis A can have life-long effects. Some viruses are resistant to standard disinfectants, such as chlorine. However, they can be efficiently removed in WSPs by sunlight, adsorption/sedimentation of solids and increased pH levels (Davies-Colley et al. 2000).

Protozoan parasites including *Giardia* and *Cryptosporidium* have been reported to cause public health concerns (Bitton 1999; Robertson et al. 1999). Those parasites can cause diarrhoea, abdominal pain, and nausea. Even though the symptoms are rarely fatal, they can last for many months. The cysts of *Giardia* and *Entamoeba*, and oocysts of *Cryptosporidium* in the infective stages are robust in waters and wastewaters. And they are resistant to standard disinfectants, such as chlorine. Therefore, ultraviolet disinfection is commonly applied in developed countries, because those parasites are found to be susceptible to ultraviolet (Bitton 1999). WSPs can efficiently remove these parasites because of the occurrence of sedimentation and exposure to ultraviolet under sunlight in WSPs (Davies-Colley et al. 2000).

Worm parasites (helminths) can cause severe symptoms and sometimes death (Bitton

1999). At least 50% of the world's population may be infected with one or more helminth species (Davies-Colley et al. 2000). Like protozoan parasites, worm parasites can also be efficiently removed in WSPs for the same reasons mentioned above.

2.4.2 Factors Affecting Pathogens Removal

There are several factors affecting disinfection in WSPs. The factors are listed in Table 2.1. And each factor is briefly discussed in the following section.

Table 2.1 Climatic and operational factors with potential effects on disinfection in WSPs (adapted from Davies-Colley et al. 2000).

Factor	Possible mechanism(s)	Microorganisms affected[1]	The types of ponds[2]
Temperature	Affects rates of removal processes	B,V, P, and H	An, F, A
Hydraulic residence time (HRT)	Affects extent of removal (time for operation)	B,V, P, and H	An, F, A
Algal toxins	Algal exudates are toxic to certain bacteria	Mainly B	F, A
Sedimentation	Settlement of infectious agent (e.g., ova, cysts)	H	An, F, A
	Settlement of aggregated solids including infectious agents	P, H, possibly B and V	An, F, A
Biological disinfection	Ingestion by antagonistic organisms (protozoans)	B,V, and possibly P	F, A
Sunlight	DNA damage by solar UV-B radiation, pH (when the algae pushes the pH to values above 10)	B,V, and P	F, A
	Photo-oxidation	B and possibly P	F, A

1. Microorganisms: B-bacteria, V-viruses, P-protozoan parasites, H-helminth worms.
2. Ponds: An-anaerobic, F-facultative, A-aerobic.

Several studies have indicated that temperature is the primary factor to predict the disinfection efficiency as it affects the rates of pathogen removal (Bartsch and Randall 1971; Lettinga et al. 2001). Hydraulic residence time (HRT) controls the time available for operation of removal processes such as sunlight, sedimentation, or ingestion by antagonistic organisms (Davies-Colley et al. 2000). The wastewater in poorly constructed or maintained WSPs will not stay in the ponds for the required amount of HRT to undergo proper treatment. Some algae in WSPs are found to inactivate faecal bacteria, because the extracellular materials they produce are toxic to faecal bacteria (Oufdou et al. 2001). The study pointed out that cyanobacteria in WSPs were toxic to *E. coli*, *Salmonella*, and other bacteria. Sedimentation in WSPs is considered to be the dominant mechanism responsible for removing helminth worms (Maynard et al. 1999). Protozoan parasites are also efficiently removed in WSPs by sedimentation. They aggregate with settleable solids, and then the aggregated solids including parasites settle down to the bottom of the pond. It should, however, be noted that since parasite eggs/oocysts are able to survive for long periods in pond sludge, any disturbance in sludge may cause these parasites to remobilise and resuspend in wastewaters. If bacteria and viruses are absorbed onto settleable solids, they are theoretically expected to be removed by sedimentation as well (Davies-Colley et al. 2000). There is little information on the removal of bacteria or viruses by sedimentation. However, wastewater solids in WSPs were found to absorb coliphages under aerobic conditions (Ohgaki et al. 1986). This finding suggested that there is a potential for viral removal by sedimentation.

There are different types of micro-fauna (consisting of heterotrophic protists and micro-metazoa) living in WSPs. Those micro-fauna obtain nutrition from wastewater

solids including microorganisms. Therefore, the micro-fauna may cause bacteria, viruses, and possibly parasites oocysts to become inactivated. Even if excreted microorganisms by micro-fauna are not inactivated, the infectivity of those microorganisms is likely to be reduced and those microorganisms are possibly removed by sedimentation (Davies-Colley et al. 2000). The disinfection of micro-fauna has been studied in constructed wastewater wetlands (Decamp and Warren 1998). Manage and other researchers (2002) found that virus-like particles were removed by ingestion by flagellates in a hyper-eutrophic urban pond. This may indicate that there could be similar processes occurring in WSPs. Therefore, micro-fauna in WSPs through their predation may become an important mechanism to remove bacteria and viruses, especially when there is less sunlight exposure within WSPs, such as at night and in the deep level of the wastewater column in WSPs. Sunlight has traditionally been considered as the important factor to inactivate pathogens in WSPs (Davies-Colley et al. 2000). There are three main mechanisms related to sunlight-mediated disinfection (Table 2.2).

Table 2.2 The three main mechanisms by which sunlight inactivates pathogens in WSPs (adapted from Davies-Colley et al. 2000).

Mechanism	Wavelengths (nm)	Absorbed by	Primary target	Oxygen-dependent	pH-dependent	Repairable
1. Photo-biological	UV-B (300-320 nm)	DNA	DNA	No	No	Yes
2. Photo-oxidative	UV-B and some longer-wavelength UV-A	DNA and other cell constituents	DNA	Yes	No	Yes
3. Photo-oxidative	UV-A (320-400 nm) and visible light (400 - 550 nm)	Humic substances	Cell membrane and possible capsid proteins	Yes	Yes	No

In mechanism 1, DNA absorbs energy from solar UV-B irradiated (300–320 nm), which in turn damages the DNA (or RNA) by formation of pyrimidine dimers (Jagger 1985). This process is independent of oxygen and pH in the medium. However, the damaged DNA in bacteria can be repaired under enzymatic processes (Jagger 1985).

In mechanism 2, DNA and other cellular constituents absorb energy from solar UV-B and some longer-wavelength UV-A rays. The activated photo-sensitizers react with oxygen to form reactive photo-oxidising compounds. Then those photo-oxidising compounds damage DNA and/or viral RNA. This mechanism is dependent on oxygen in the medium (Davies-Colley et al. 2000). However, the damaged DNA in bacteria could also be repaired as mentioned above.

In mechanism 3, humic substances (light absorbing materials) in wastewater absorb solar UV-A (320-400 nm) and visible light (400-550 nm) to form reactive photo-oxidising compounds, which can lead to unrepairable damaged of bacterial membranes or host-binding proteins in viral particles. This mechanism is dependent on oxygen and pH as well. pH alone is not toxic to faecal indicator bacteria, except at extremely high levels of pH which does not normally occur in WSPs (Curtis et al. 1992). However, when pH interacts with sunlight, the combination of the two factors can disinfect pathogens. Davies-Colley and other researchers (2000) found that when faecal coliforms were exposed to sunlight, the level of faecal coliforms decreased significantly with increasing pH at the same level of sunlight.

2.5 Pond Microbiology

The microbiology of wastewater treatment in WSPs includes aerobic and anaerobic processes and involves a broad range of microorganisms. The goal of wastewater treatment technologies is to optimize the conditions for microbial growth and therefore optimize the treatment processes. These treatment processes will lead to better removal of organic carbon, nutrients, and pathogens, and thus produce wastewater effluent suitable for discharge into the environment (Pearson 2005). Compared to conventional and electro-mechanical wastewater treatment plants, there is less control of the environmental conditions in WSPs, thus the rate of microbial growth and the efficiency of the WSP treatment process can be relatively slow. It may therefore be that WSPs require longer treatment processing times and larger land areas to provide acceptable treatment. Also, since the microbial metabolic rate basically doubles for every 10 ºC rise in temperature, WSPs systems are more efficient and thus require a smaller land area in tropical climates

than in cold climates (Bartsch and Randall 1971; Lettinga et al. 2001). Even though WSPs have a slower treatment rate than conventional and electro-mechanical wastewater treatment plants, WSPs can provide conditions for better pathogen removal (Pearson 2005). Aspects of the microbiology of WSPs including anaerobic, aerobic, and photosynthetic processes and the composition and diversity of wastewater bacterial communities will be briefly introduced in the following subsections.

2.5.1 Anaerobic Processes in Ponds

Anaerobic digestion occurs after sedimentation. For stabilisation of organic carbon in WSPs, anaerobic digestion is considered the principal mechanism in anaerobic ponds and an important mechanism in facultative ponds. The organic carbon is usually determined by measuring the biochemical oxygen demand (BOD) or chemical oxygen demand (COD) (Pearson 2005). Picot and other researchers (2003) studied the mass balance of carbon in an anaerobic pond. They found that 74% of the removed organic carbon was converted to methane, 13% into dissolved inorganic carbon and 15% was stored in sludge. Their results suggested the importance of the methanogenesis process in terms of organic carbon removal in WSPs. When the methane gas is released during the methanogenesis process, this reaction will stir the wastewater and cause re-suspension of microorganisms that are bound with sediments. Thus, it helps to bring the microorganisms in better contact with the wastewater, and subsequently improves treatment efficiency (Pearson 2005).

During anaerobic digestion, the first step involves the hydrolysis and solubilisation of the constituent proteins, fats, and polysaccharides by fermentative bacterial genera such as *Escherichia* and *Aerobacter* (Pearson 2005). These bacteria contain hydrolytic exo-

enzymes. These enzymes are exported by the periplasmic membrane and released into the surrounding environment. These enzymes assist in the hydrolysis of the organic compounds. The hydrolysis process will result in production of soluble molecules of amino acids, long chain fatty acids, and mono and disaccharides. Those soluble molecules are then assimilated by the same bacteria for their metabolism and also by other fermentative species that are not able to hydrolyse the original polymeric materials. This is followed by the production of organic acid anions known as the acetogenic phase. In this stage, the soluble products produced by fermentation are converted into a combination of short chain fatty acids, ethanol and other alcohols, organic acids (such as lactate), H_2 and CO_2. And then those products are fermented to acetate, CO_2, and H_2 by different types of obligate hydrogen-producing acetogenic bacteria. Acetate, CO_2, and H_2 are the important substrates for methanogenesis. This is finally followed by the production of methane. In this final stage, different types of methanogenic bacteria produce methane gas by either one of the two following processes. The first process is called the acetoclastic reaction. In the acetoclastic reaction, methanogens, such as *Methanosarcina barkeri* convert acetic acid to methane. The acetoclastic reaction is shown in the following equation:

$$CH_3COOH \longrightarrow CH_4 + CO_2$$

The second process is called the CO_2 reducing reaction. In this reaction, methanogens, such as *Methanosarcina hungatei* produce methane from H_2 and CO_2. The CO_2 reducing reaction is shown in the following equation: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$

The anaerobic WSPs present a risk of creating bad odour with the emission of H_2S (Picot et al. 2003). In anaerobic WSPs, the sulphate reducing bacteria (SRB) are

responsible for producing H_2S, such as members from the genera *Desulfovibrio* and *Desulfobacter*. The SRB are obligate anaerobic bacteria and can be found in the anaerobic layer and sediments of facultative and anaerobic ponds. During the process of sulphate reduction and bad odour production, the SRB require organic material (such as organic acids) or hydrogen as their source of reductant. Also they require sulphate, sulphur, or sulphite as the terminal electron acceptor to re-oxidise their electron transport chains under anaerobic conditions during the production of energy (ATP) required for their growth. The following equations are presented as an example to show how H_2S is produced by SRB (Pearson 2005).

$$CH_3COO^- + SO_4^{2-} + 3H^+ \longrightarrow 2CO_2 + H_2S + 2H_2O$$

$$4H_2 + SO_4^{2-} + H^+ \longrightarrow HS^- + 4H_2O$$

Both sulphate and organic material overloading will enhance the growth and activity of SRB. It will result in production of bad odour. The preferred growth conditions of SRB are acidic conditions (pH <6) or alkaline conditions (pH>8). Those conditions will boost the growth of SRB over that of methanogens which are obligate anaerobes and require very strict environmental conditions for their growth. They need conditions with an optimum pH between 7 and 8 and require a negative redox potential (E_0) that is less than -0.24 V. When pH level is lower than 6 or greater than 8 in anaerobic ponds, SRB compete with methanogens for the same organic material, especially acetate and hydrogen. This phenomenon results in the production of more H_2S (Pearson 2005).

In the anaerobic WSPs, researchers found that the bottom sludge layer plays an important role in the microbiological activity (Parker and Skerry 1968; Parker et al. 1950). They found that the anaerobic ponds with no sludge performed less well than the ones

with an active sludge layer. The reason is that methanogens are more biologically active and thus grow more quickly when in contact with solid surfaces (Parker and Skerry 1968; Parker et al. 1950). Paing and other researchers (2000) indicated that some spatial separation of the processes of anaerobic degradation occurs in the sludge layer of an anaerobic pond. Their results showed that the greater rates of acidogenesis and higher levels of volatile fatty acid were found near the inlet where pH values were less than 6.6; however, higher concentrations of potential methanogenesis were found near the outlet where pH values were suitable for the growth of methanogens. Parker and Skerry (1968) also indicated that in the sludge layer of an anaerobic pond, high levels of volatile fatty acid were measured near the inlet. Based on those findings, Paing and other researchers (2000) suggested that the observed sequential distribution of microbiological activity in the sludge layer of an anaerobic pond probably resulted in increasing the efficiency of anaerobic digestion compared to septic tanks.

2.5.2 Aerobic Processes in Ponds

There is a wide range of aerobic chemo-organotrophic bacterial genera present in aerobic WSPs, including *Pseudomonas*, *Achromobacter*, *Flavobacterium*, and *Bacillus* (Gann et al. 1968). Less is known about the activity of these saprophytes compared to the photosynthetic organisms. In the facultative ponds, the bacteria present in the aerobic layers also include *Beggiatoa*, *Sphaerotilus*, and *Alcaligenes*. The microbial degradation of organic material in WSPs is similar to other biological wastewater treatment systems, but the biomass concentrations in WSPs are much lower. Such lower active biomass concentrations in aerobic WSPs lead to the requirement for larger pond volumes and longer retention times to achieve effective treatment (Pearson 2005).

2.5.3 Photosynthetic Processes and Algal Diversity in Ponds

In WSPs, pond algae play an important role in photosynthetic oxygen production. It has been estimated that at least 80% of the dissolved oxygen are produced from the photosynthetic activity of the phytoplankton population and only secondarily through surface mass transfer. Some studies elucidated the relationship between molecular oxygen released and algal material synthesized. The studies indicated that the ratio of molecular oxygen released to algal material synthesized varies with algal species, the age of the algal cells, and the available nutrients source especially nitrogen. It was measured that for algae having an average age of 3 to 6 days and using ammonia as the nitrogen source, the ratio of oxygen released to algal material synthesized is between 1.5 and 1.6. It basically means that for the synthesis of every 1 g of algae (ash-free dry weight), between 1.5 and 1.6 g of oxygen is released from the wastewater (Oswald 1988). Thus, the maintenance of a healthy algal population is fundamentally important for bacteria to efficiently oxidize the organic material in WSPs.

Koenig (1984) reported an inverse relationship among surface organic loading, algal biomass concentration, and oxygen production per m^2 of pond surface in facultative pond and showed that if the algal biomass concentration is too low (less than 300 μg chlorophyll α/L), there is a risk of the facultative pond turning anoxic. The reason is that in this case, the net oxygen production only meets oxygen demand. Thus, at a water temperature of 24°C, the maximum acceptable BOD_5 surface loading should be approximately 400 kg BOD_5/ha/day. This value agrees with the suggested one for designing equations for facultative ponds in tropical regions (Mara 1987; Mara et al. 1992). Acceptable loading values for arctic WSPs are unknown at this time; however, a model study by Ragush et al. (2017) revealed these systems likely to be more sensitive to

surface organic loading rates to a degree where the acceptable loading would be much lower than in other systems.

The study of the depth profile of algal photosynthesis indicated that dissolved oxygen concentrations vary with pond type and organic loading (Pearson 2005). The levels of oxygen can be super saturation in the surface layers of ponds during the hours of maximum photosynthesis. In the cleaner and less cloudy aerobic ponds, photosynthetic activity can extend down to 60 cm or more and thus the entire water column may be aerobic during daylight hours, if not for the whole 24 hours. However, in facultative ponds having more organic loading than aerobic ponds, the photosynthetic activity may only extend down to 20-30 cm from the surface with dissolved oxygen only measureable in the top 20 cm during daylight hours and with the complete water column turning anoxic at night. Photosynthetic activity also varies with the time of day. It usually increases when increasing levels of solar radiation incident occur upon the pond surface. The specific levels of solar radiation on the pond surface can also inhibit photosynthetic activity, but the micro-algae can adjust their position in the water column by using flagellar movements or altering their buoyancy in response or order to absorb light for photosynthesis. This mechanism can result in maximum photosynthetic activity occurring some 20 cm below the water surface during periods of high light intensities.

A wide diversity of algae has been found in WSPs (such as: *Euglenophyta*, *Chlorophyta* and *Chrysophyta* genera). The algal genera and species that predominate in a pond depend on the surface organic loading (Pearson 2005). For example, when organic loadings are high, algal diversity decreases. Consequently, there is less algal diversity found in facultative ponds when compared to aerobic ponds. Flagellated genera appear to

predominate in facultative ponds, while non-flagellated genera prefer aerobic ponds (Pearson 2005).

Many factors control algal dominance in WSPs. High concentrations of ammonia and sulphide, which are associated with high organic loadings, control algal biomass concentration and algal dominance (Pearson 2005; Athayde 2001). Both ammonia and sulphide in the non-ionic forms in relation to water pH are able to quickly enter into algal cells where the compounds will be toxic to the algae. Thus, water pH and the concentrations of ammonia and sulphide are important factors that control algal dominance. Total algal biomass concentration, which can be determined by the chlorophyll α concentration, is usually higher in facultative ponds than in the subsequent aerobic ponds of the same series. The reason for this difference is that the reduced available nutrients and the increased grazing pressures by the zooplankton population are more likely to occur in aerobic ponds rather than in facultative ponds (Pearson 2005).

2.5.4 Composition and Diversity of Bacterial Communities in Municipal Wastewater Treatment Plants

The biological treatment of municipal wastewater in municipal WWTPs relies on the self-assembly of the bacterial community, which is responsible for most of the carbon and nutrient removal from sewage (Wagner and Loy 2002). From the perspective of microbial ecology, an in-depth understanding of the bacterial community is needed to uncover factors that influence the efficiency and stability of the biological treatment in WWTPs. Thus, the knowledge could help engineers develop promising strategies to improve the treatment performance in WWTPs. Conventionally, bacterial communities in WWTPs were analysed by the application of microscopic or cultivation-dependent techniques. These techniques are not able to capture the big picture of bacterial

communities, because not all bacteria are able to grow on microbiological media in laboratories and the complete bacterial composition therefore could not be identified. In the past decade, cultivation-independent approaches have increasingly been used to study bacterial communities in WWTPs. These studies have demonstrated that most of the cultured microorganisms are of minor importance while in contrast the uncultured bacteria play an essential role for most key processes in WWTPs (Loy et al. 2003).

Initially, the culture-independent approaches to study microbial communities in wastewater treatment systems were highly dependent on PCR methodologies for the analysis of the conserved regions in microbial genomes (for example, the 16S ribosomal ribonucleic acid (rRNA) gene). Amplicons were initially used to create clone libraries and in denaturing gradient gel electrophoresis (DGGE) but more recently advanced high-throughput sequencing techniques, such as 454-pyrosequencing and Illumina Miseq and Hiseq sequencing, are being used. Additionally, PCR-independent techniques, which are used to perform shutgun sequencing on all DNA or RNA in a microbial community, to derive the metagenomic (genes, genomes) and metatranscriptomic (transcribed RNAs, gene expression) composition of the studied microbial community. One important limitation for metagenomics and metatranscriptomics techniques is that most of the information is obtained from the most abundant groups of the community. Studies of wastewater bacterial communities where those two PCR-independent techniques are applied, are still rare (Ferrera and Sánchez 2016). On the other hand, important limitations for the PCR-dependent techniques are found in PCR amplification and primer biases (Ferrera and Sánchez 2016). Compared to the time consuming and lower coverage of the microbial ecology that cloning and PCR-DGGE-based methods resulted in, PCR-

dependent advanced high-throughput sequencing techniques have been become increasingly popular as reliable, cost- and time-effective methods to explore bacterial communities deeply at genera and/or species levels in environmental samples (Caporaso et al. 2012; Vanwonterghem et al. 2014). Therefore, the advanced sequencing techniques allow researchers to understand more about how microbial communities respond to WWTPs operational conditions, such as oxygen levels, pH, temperature and nutrient concentrations.

In municipal WWTPs located in Denmark, Belgium and China, the composition of bacterial communities has been intensively explored by clone library construction, PCR-DGGE and 454-pyrosequencing techniques (Nielsen et al. 2010; Nguyen et al. 2011; Wan et al. 2011; Hu et al. 2012; Wang et al. 2012). These studies showed that the most abundant phylum in wastewater was Proteobacteria with the relative abundance ranging from 21 to 65%, and the most numerous Proteobacterial class was Betaproteobacteria which was responsible for organic and nutrient removal. The sub-dominant phyla were Bacteroidetes, Acidobacteria, and Chloroflexi. A study, which used a high-throughput sequencing technique to amplify *16S rRNA* gene amplicons (V1-3 region) and encompassed 20 WWTPs, found that the most abundant bacterial genera were *Tetrasphaera*, *Trichococcus*, *Candidatus*, *Microthrix*, *Rhodoferax*, *Rhodobacter*, *Hyphomicrobium*, p-55-a5 belonging to the phylum Firmicutes and P2CN44 and B45 belonging to the phylum Chloroflexi (McIlroy et al. 2015). The core bacterial phyla were in two studies identified in effective activated sludge in WWTPs from different geographic locations in China, Singapore, Canada and the United States (Zhang et al. 2012; Xia et al. 2010), with results indicating that Proteobacteria dominated with

Firmicutes, Actinobacteria and Bacteroidetes being sub-dominant phyla. The lower abundance of those phyla was thought to explain the malfunctioning of the wastewater treatment performance in the studied activated sludge systems (Zhang et al. 2012; Xia et al. 2010). Zhang and other researchers (2012) found that a comparison of the distribution of bacterial communities at deeper taxonomic levels revealed geographic differences among the 14 WWTPs being compared. For example, *Flavobacterium*, were present with the relative abundance levels ranging from 1.83 to 7.44% in the three samples collected from North America. However, they were the minor groups with the relative abundance less than 1% in all the samples obtained from China and Singapore. Furthermore, the three samples from North America contained higher levels of *Rhodobacter* (1.43 to 3.72%) than the Asian samples from China and Singapore (0.32 to 0.99%). They also found that the distribution of some dominant genera across the geographical locations was temperature-dependent. The psychrotolerant genus *Trichococcus* was found at higher relative abundance levels ranging from 1.55 to 5.53% in the samples from colder areas (10 ºC at time of the sampling) whereas relative abundance levels of 0-0.96% were detected in the wastewater samples collected from sub-tropical or tropical areas (Ju and Zhang 2015). Observed geographic variations in bacterial community characteristics in 14 Chinese WWTPs could similarly be attributed to the effect of temperature (Wang et al. 2012).

In municipal WWTPs, the diversity of bacterial communities was shown to be affected by wastewater characteristics, bacterial interactions, bioreactor size and treatment processes (Wang et al. 2012; Ju and Zhang 2015; Valentín-Vargas et al. 2012; Hu et al. 2012). Further to the study of the bacterial diversity in wastewater samples from 14

WWTPs in China (Wang et al. 2012), the results revealed that the variation in bacterial communities correlated strongly with wastewater characteristics including wastewater temperature, conductivity, pH and dissolved oxygen (DO) content. Then other factors including operational parameters and geographical locations influenced the bacterial diversity as well. Beside wastewater characteristics and operational conditions, bacterial interactions were also found to be the dominant drivers in determining the assembly of bacterial communities in WWTPs (Ju and Zhang 2015). The size of the biological reactor can influence the diversity of bacterial community (Valentín-Vargas et al. 2012). They observed during their one-year study that the larger activated sludge (CAS) biological reactor had a less dynamic but more efficient and diverse bacterial community compared to the smaller conventional reactors (Valentín-Vargas et al. 2012). In addition, the treatment process affected the bacterial community composition. In the study by Hu et al. (2012), it was shown that the anaerobic/oxic (AO) and anaerobic/anoxic/oxic (A^2O) systems contained a more diverse and even bacterial community composition than membrane bioreactors (MBRs) or oxidation ditches. The low diversity and evenness in MBRs was more likely caused by the long sludge retention time (SRT) and low food/microorganisms ratio (F/M) or high availability of biodegradable organics (Hu et al. 2012; Wan et al. 2011).

At the present time, to the best of our knowledge, advanced high-throughput sequencing techniques have not been used to study microbial communities in WSPs. So far, there was one study that used the DGGE technique with PCR-amplified *16S rRNA* gene fragment technique to investigate the microbial communities in WSPs operated in a tropical climate, with an emphasis on elucidating the diversity of the sulfate-reducing and

the sulfur-oxidizing bacteria occurring during ‘red-water’ events (Belila et al. 2013). The results from that study showed that Proteobacteria, Chlorobi, Bacteroidetes and Cyanobacteria were present as the major phyla. During the ‘red-water’ events, the presence of sulfate-reducing bacteria and sulfur-oxidizing bacteria was confirmed with detection of sulfate-reducing bacteria belonging to the deltaproteobacterial class and sulfur-oxidizing bacteria belonging to the Chlorobi and Proteobacteria phyla. The results indicated that the different metabolic processes occurred in WSPs during the observed ‘red-water’ events.

Chapter 3 Disinfection and Removal of Human Pathogenic Bacteria in Arctic Waste Stabilization Ponds

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3.1 Abstract

Wastewater stabilization ponds (WSPs) are commonly used to treat municipal wastewater in Arctic Canada. The biological treatment in the WSPs is strongly influenced by climatic conditions. Currently, there is limited information about the removal of fecal and pathogenic bacteria during the short cool summer treatment season. With relevance to public health, the objectives of this paper were to determine if treatment in arctic WSPs resulted in the disinfection (i.e., removal of fecal indicator bacteria, *Escherichia coli*) and removal of selected human bacterial pathogens from the treated effluent. The treatment performance, with focus on microbial removal, was assessed for the one-cell WSP in Pond Inlet (NU) and two-cell WSP in Clyde River (NU) over three consecutive (2012-2014) summer treatment seasons (late June-early September).

The WSPs provided a primary disinfection treatment of the wastewater with a 2-3 log removal of generic indicator *E. coli.* The bacterial pathogens *Salmonella* spp., pathogenic *E. coli*, *Listeria monocytogenes*, but not *Campylobacter* spp. and *Helicobacter pylori*, were detected in the untreated and treated wastewater, indicating human pathogens were not reliably removed. Seasonal and annual variations in temperature significantly ($p<0.05$) affected the disinfection efficiency. Improved disinfection and pathogen removal was

observed for the two-cell system in Clyde River as compared to the one-cell system in Pond Inlet. A quantitative microbial risk assessment should be performed to determine if the release of low levels of human pathogens into the arctic environment poses a human health risk.

Keywords: Wastewater treatment, Arctic Canada, municipal wastewater, disinfection, fecal indicator bacteria, bacterial pathogens, wastewater temperature

3.2 Introduction

In Canada's Arctic regions (Nunavut, Nunavik and Northwest Territories), WSPs continue to be the most common wastewater treatment solution to manage municipal wastewater. Since there is no need to add chemical flocculants and install mechanical equipment to aerate and mix the wastewater, the WSPs can be easily operated and maintained despite a limited capital and operational budget. However, WSPs, which are completely reliant on un-aided natural biological processes to treat wastewater, may experience treatment limitations due to the harsh arctic climate and not be able to achieve treatment goals set out by Canada-wide strategy for the management of municipal wastewater in the new Wastewater System Effluent Regulation (Environment Canada 2015).

In the majority of Nunavut's 25 small and remote communities, WSPs operate as retention lagoons with no discharge during the winter period. During the nine months of winter the perimeter of the ponds (surface, walls and floor) are frozen while the interior liquid hovers around the freezing point. In June the ponds begin to thaw, and the entire volume remains liquid until freeze-up starts in September. This period of 2-3 months is called the "treatment season", as it characteristically has higher biological activity

(phytoplankton and bacteria) due to warmer air temperatures and extended daylight yielding elevated water temperatures. In September before the freeze-up, the contents of the ponds are discharged either directly into the aquatic receiving environment or to a natural tundra wetland for further polishing.

Release of inadequately treated wastewater with a content of human infectious pathogens into the environment may pose a potential human health risk. People living in Nunavut communities may particularly be at risk as their diets are reliant on the local harvest of food from traditional sources (Daley et al. 2014). In addition, recreational activities often take place near wastewater effluent discharging areas (Harper et al. 2011, Daley et al. 2017). Finally, the overcrowded housing in these communities may be exacerbating the frequency of inter-person spread of infectious agents (Goldfarb et al. 2013, Harper et al. 2011). From a public health perspective, it is important to investigate whether current WSPs in Nunavut achieves compliance with disinfection goals and removal of human bacterial pathogens to minimize the pathways for the transmission of infectious diseases.

Many bacterial pathogens have been associated with waterborne diseases, including the enteric pathogenic *Escherichia coli* serotypes such as O157:H7, *Salmonella* spp., *Campylobacter* spp., *Helicobacter pylori* and the non-enteric, environmental *Listeria monocytogenes*. Outbreaks of enterohemorrhagic *E. coli* (EHEC) O157:H7 have occurred in Canada's northern communities. While the original source of infection was not identified, person-to-person transmission was in both cases suggested as a significant risk factor (Rowe et al. 1994, Orr et al. 1994). Goldfarb et al. (2013) tested 86 stool specimens, which had been obtained from patients with diarrhea at the hospital in Iqaluit (NU), for

the presence of 50 different bacterial, viral and parasitic pathogens. They detected *Salmonella* spp. and *Campylobacter* spp. in 47% of the specimens, indicating that outbreaks of *Salmonella* spp. and *Campylobacter* spp. may have occurred. *H. pylori* infections have arisen as an emerging health concern in communities in the Canadian Arctic with the detection of the bacterium in community water supplies in Chesterfield Inlet (NU) and Repulse Bay (Naujaat, NU) (Lefebvre et al. 2013, Goodman et al. 2008, McKeown et al. 1999). The cold-tolerant *L. monocytogenes* is mainly associated with foodborne outbreaks such as the large outbreak in 2008 in Ontario, Canada (Weatherill et al. 2009). This environmental bacterium can be readily isolated from fresh water in Southern Canada (Stea et al. 2015, Lyautey et al. 2007) while its presence in the arctic environment is unknown.

At the present time, there is a lack of information regarding the removal of fecal indicator bacteria (i.e., disinfection) and human bacterial pathogens in WSPs in Nunavut. To close this knowledge gap, the objectives of the present study were to determine if treatment of municipal wastewater in arctic WSPs successfully removes fecal indicator bacteria (*E. coli*) and selected human bacterial pathogens (pathogenic *E. coli* serotypes (e.g., O157:H7), *Salmonella* spp., *Campylobacter* spp., and *H. pylori*, and the non-enteric *L. monocytogenes*). The treatment efficacy was investigated over three years (2012-2014) in the two remote communities in Nunavut, Pond Inlet and Clyde River, which are serviced by WSP treatment systems consisting of a single cell and two cells, respectively.

3.3 Materials and Methods

3.3.1 Study Sites

From September, 2012 to September, 2014, seven sampling trips were made to Pond

Inlet (latitude 72° 41′ 57″ N, longitude 77° 57′ 33″ W; population: 1549 [Statistics Canada 2012]) and another six sampling trips to Clyde River (latitude 70° 28′ 26″ N, longitude 68° 35′ 10″ W; population: 934 [Statistics Canada 2012]). Both Pond Inlet and Clyde River are remote fly-in communities that are located on the eastern shore of Baffin Island, in Nunavut's Qikiqtani region. Both communities have polar arctic climates with long cold winters and short cool summers. Based on 1981 to 2010 Canadian climate normals, February is the coldest month with daily average temperatures of -34 °C in Pond Inlet and -30 °C in Clyde River, while July is the warmest month with daily average temperatures of 6 °C in Pond Inlet and 5 °C in Clyde River (Environment Canada 2014). Most of people living in these communities are Inuit, who follow a traditional lifestyle of hunting and fishing.

Pond Inlet employs a one-cell engineered WSP, which was commissioned in 2005 and is located approximately 1.4 km to the east of the hamlet. All wastewater generated is delivered by trucks to the WSP. The treated wastewater is discharged from the WSP once annually, usually over a three week period starting in September and ending in early October just prior to freeze-up. The wastewater effluent exits the WSP over the berm and travels through a ditch and then down a steep hill (500 m) before arriving in the ocean receiving environment (Eclipse Sound). The WSP was designed to be a facultative pond with a surface area of approximately 4 ha and an average depth of approximately 1.9 m during the summer. The estimated volume of wastewater effluent discharged is 8.0×10^7 L. Traditional uses of the ocean receiving environment include fishing and hunting. During the summer season, especially in July and August, schools of Arctic char migrate past the wastewater effluent discharge point. The timing of the annual decant is therefore

timed to coincide with the departure of the Arctic char from the area. In addition, during the August and September sampling trips, hunting of narwhals and seals in the nearshore environment surrounding the community was observed.

Clyde River recently expanded their WSP system to include a larger, secondary pond in 2011. The original WSP, the primary pond, had due to the increasing population become too small to accommodate the annual wastewater volume. Therefore, the secondary pond was built to increase the wastewater holding capacity. The expected annual wastewater volume is 3.1×10^7 L using the assumption that each of the 934 inhabitants produces 90 L of wastewater per day. The intended use of the two-cell WSP system is a scheme where the raw wastewater is dumped into the primary pond to enable settling and precipitation processes. At regular intervals, pre-processed wastewater should then be transferred from the primary pond into the secondary pond to receive further treatment before the annual decant from the secondary pond, where treated wastewater is passed through a vegetated filter strip before going into the ocean receiving environment (Patricia Bay).

3.3.2 Sampling Strategy

The same sampling strategy was used in Pond Inlet and Clyde River during visits from September 2012 to September 2014, where representative samples were obtained of raw wastewater from trucks, wastewater in different parts and depths of the WSPs and treated wastewater just prior to the decant (Clyde River) or during the decant (Pond Inlet). In Pond Inlet, outfall samples, i.e., the effluent just prior to entry into the ocean receiving environment, were also collected. In addition, the ocean samples from the immediate vicinity of the outfall point, were collected before and during the decant event in Pond

Inlet. Specifically, four outfall samples were collected in both 2013 (two samples) and 2014 (two samples) during the decant event. Six ocean samples from the immediate vicinity of the outfall point, were also collected before (three samples) and during the decant event (three samples) in Pond Inlet in September 2014.

The first trip to Pond Inlet and Clyde River was the end of the summer treatment season in 2012. The WSP in Pond Inlet was sampled at the start, middle, and end of the summer treatment season, including decant events, in 2013 and 2014. Both the primary and secondary ponds in Clyde River were sampled at the start, middle, and end of the summer treatment season in 2013, while in 2014 the ponds were sampled at the start and end of the summer just prior to the decant event. The sampling events representative of the start, middle, and end of the summer treatment season took place late June/early July, late July/early August, and early/middle September, respectively.

3.3.3 Continuous WSPs Monitoring Parameters Collection

Deployment of multi-parameter sondes (YSI Inc., Yellow Spring, OH) allowed for *in-situ* measurements of wastewater temperature, pH, and DO. During the first sampling trip in each year, the sondes were installed in the WSPs to record the parameters hourly until the sondes were retrieved at the end of the treatment season. In addition, HOBO temperature/light pendants, temperature/water level loggers and ROX DO probes (Onset Computer Corporation, Cape Cod, MA) were installed at various depths of the WSPs to capture parameters and also to validate the continuous recording measured by the *in-situ* sondes.

3.3.4 Degree Days Calculation

'Degree days' is a concept used in the agricultural field to indicate the accumulative effect of temperature on the growth potential of plants in a specific geographical site. Use of degree days also allows for comparison of biological activity in wastewater treatment carried out in different geographical sites with different climates (Ragush et al. 2015). To calculate degree days in order to study how temperature influenced the disinfection performance of WSPs in Pond Inlet and Clyde River, the surface wastewater temperatures were used. The calculation of degree days involves averaging temperature measurements for each day and then subtracting the reference temperature. In this study, the reference temperature was chosen as 5 °C. For example, if the average temperature at a specified day 1 was 10 ºC then this would lead to a degree day value for that day of 5 (10 ºC - 5 ºC = 5 ºC). In this calculation, only days with average temperature above 5 ºC are considered, meaning that on days where, for example, the average temperature is 2 ºC, the degree day value would be recorded as 0. To obtain the total degree day values for a certain number of days, the number of degrees for each day is summed up for the specified period, i.e., the degree days for a period of three days would be 6 (5+1+0) if the degree days were recorded as follows on day 1=(10-5 ºC)=5, day 2=(6-5 ºC)=1, day 3=(2-5 ºC)=0.

3.3.5 Microbiological Sample Analysis

Wastewater samples were obtained in sterile 1 L or 500 mL containers (Nalgene, Fisher Scientific, Nepean, ON, Canada), stored in a cooler (4 °C) and flown to the Northern Water Quality Laboratory (NWQL) at the Nunavut Research Institute in Iqaluit, NU. Upon arrival to NWQL, the analysis for the content of fecal indicator bacteria (*E.*

coli) was performed immediately. Samples were also flown to Halifax, NS for the commencement of the selective enrichment for pathogenic bacteria within 24 to 48 hours. DNA was also extracted from wastewater samples within 24 hours of the original sampling event. The immediate processing was done to minimize changes in the microbiology of the samples due to the transportation time.

3.3.6 Enumeration of Fecal Indicator Bacteria (*E. coli*)

Fecal indicator bacteria (*E. coli*) were enumerated using the American Public Health Association (APHA) Standard Method 9223 (American Public Health Association 1998). Samples were processed using Colilert®-18 and Quanti-Trays/2000® following the manufacturer's procedure (IDEXX Laboratories, Inc., Westbrook, ME, USA). The result was log transformed and expressed as Log MPN/100 mL.

3.3.7 Kinetics of *E. coli* Removal

First order rate constants were calculated to estimate *E. coli* removal rates based on the assumption of a completely mixed batch reactor, an assumption which was supported by water quality results from both Pond Inlet and Clyde River WSPs. This first order rate constant was a conservative estimate of *E. coli* removal rates because of the limited sample size. The impact of not including the continuous addition of raw wastewater was expected to be small, because the additional wastewater being added during the treatment period (ranging from 31-34 days in Pond Inlet (start to middle of the treatment season) and 64-74 days in Clyde River (start to end)) only represented $1/12^{th}$ of the annual wastewater volume in Pond Inlet, and $1/6^{th}$ of the annual wastewater volume in Clyde River. Therefore, the actual rate constant would be expected to be higher than the conservative rate constant.

The first order rate constant for *E. coli* removal was calculated as follows:

$$K = -Ln(C_t/C_0)/t$$

Where:

K is the first order rate constant (1/day)

C_0 is *E. coli* concentration (Log MPN/100 mL) at the beginning season in the WSP

C_t is *E. coli* concentration (Log MPN/100 mL) at the middle season for Pond Inlet and at the end season for Clyde River WSPs

t is the time interval between the two treatment seasons (days)

3.3.8 Detection of Bacterial Pathogens Presence/Absence and Concentrations by TaqMan Quantitative Polymerase Chain Reaction (qPCR) Assays

Duplicate wastewater samples (10 mL each) were subjected to an initial pathogen enrichment step in Fraser, Bolton, Rappaport-Vassiliadis, buffered peptone water for *L. monocytogenes*, *Campylobacter, Salmonella* and pathogenic *Escherichia coli* serotypes, respectively. The enrichment steps were carried out using previously published protocols (Stea et al. 2015a and 2015b). Following enrichments, 2 mL from each of the enrichment broths were combined and added into a 15 mL sterile test tube and were centrifuged at 3200 x g for 10 minutes to obtain cell pellets for DNA extractions. DNA was extracted from cell pellets using the PowerSoil MoBio kit (MoBio, Carlsbad, CA, USA) following the manufacturer's instructions with a final volume of 100 µL. Each qPCR reaction (25 µL) consisted of 7.7 µL of DNase-free water (Fisher Scientific), 12.5 µL of TaqMan master mix (Applied Biosystems Fast Advanced 2X, Applied Biosystems), 0.3 µL each

of 10 μM forward and reverse primers, 0.2 μL of 10 μM TaqMan hydrolysis probes, and 4 μL of sample DNA. The qPCR primers, TaqMan hydrolysis probes, running conditions for *Campylobacter* spp., *Salmonella* spp., pathogenic *E. coli* and *L. monocytogenes* were described in Lund et al. (2004), Cheng et al. (2008), Ibekwe et al. (2002), Rodriguez-Lazaro et al. (2004) and Stea et al. (2015b), respectively, and also listed in the supplemental material (Table S1) together with details of the qPCR conditions. Bioinformatic analysis revealed that the Ibekwe et al. (2002) method, which targets the eae gene (intimin), detected enterohemorrhagic and enteropathogenic *E. coli* (e.g., O157:H7, O145:H28, O55:H7 and O111:H7, see the full list and *eae* amplicon alignment in the supplemental material, Figure S1). Positive controls contained DNA that were extracted from *Salmonella* Typhimurium (ATCC 14028, Manassas, VA, USA), *E. coli* O157:H7 (strain EC 961019, kindly provided by H. Schraft, Lakehead University, Thunder Bay, ON, Canada), *Campylobacter jejuni*, *C. lari*, and *C. coli* strains (kindly provided by L. Waddington, Canada Food Inspection Agency, Dartmouth, NS, Canada), and *L. monocytogenes* 568 (serogroup IIa). Negative controls consisted of DNA extracted from sterilized enrichment media. Each qPCR run contained positive, negative, and non-template controls, and samples. The qPCR detection was performed in a StepOne Plus system (Applied Biosystems). The results were reported as the presence/absence of each pathogen in 10 mL of wastewater originally used in the pathogen enrichment protocols.

To quantify pathogen cell numbers in each sample, 100 mL wastewater volumes were centrifuged at 3200 x g for 10 minutes to harvest microbial cells. DNA was extracted from the cell pellets using the PowerMax Soil DNA isolation MoBio kit (MoBio, Carlsbad, CA, USA) following the manufacturer's instructions. The final volume for each

DNA extract was 100 μL. In addition to detection of the pathogens mentioned above, the presence/absence of *H. pylori* was also analyzed following a protocol based on that from He and other researchers (2002) using DNA extracted from *H. pylori* 26695 (ATCC 700392D-5) as the positive control (see Table S1 for details on the method).

Standard curves, which allow for the quantification of each pathogenic bacterium in samples collected from 2013 to 2014 treatment seasons, were created. DNA was extracted from ten-fold dilution series of positive control cultures (10^8 to 10^0 CFU/mL) in tryptic soy broth (TSB, BD-Difco). Prior to the DNA extraction, 10-mL volumes of each dilution of the positive control samples were pelleted at 3200 x g for 10 minutes followed by DNA extraction using the PowerSoil MoBio kit (MoBio, Carlsbad, CA, USA), with a final elution volume of 100 μL. The TaqMan qPCR assays were performed as described above. The obtained standard curves for all pathogenic bacteria had qPCR efficiencies ranging from 82% to 108%, with R^2 values ranging from 0.986 to 0.998. Two technical replicates were run for all standards, samples, negative controls (DNA extracted from 10 mL of sterile TSB), non-template controls and the difference of the threshold cycle (Ct) value between the replicates was less than 0.5. The limit of detection (LOD) of the qPCR assay was determined to be 1 CFU/mL for *Salmonella* spp., 1 CFU/mL for *C. jejuni*, *C. lari* and *C. coli*, respectively, 10 CFU/mL for *L. monocytogenes*, and 10^3 CFU/mL for pathogenic *E. coli* serotypes. Quantity of approximate cell numbers for each pathogenic bacterium was reported as Log CFU/100 mL. The absence of PCR inhibitors in the DNA extracts was confirmed in experiments with each positive bacterial strain spiked into wastewater samples (data not shown).

3.3.9 Confirmation of The Presence of Pathogenic *Campylobacter* spp. by Triplex Polymerase Chain Reaction (PCR)

The TaqMan assay (Lund et al. 2004) was designed to detect six species of *Campylobacter*. Samples that tested positive for *Campylobacter* spp. in the TaqMan qPCR assay were further analyzed for the presence of *C. jejuni*, *C. lari*, or *C. coli* in a triplex PCR method (Khan and Edge 2007). PCR reactions (25 µL) contained 12.5 µL of master mix (Taq 2X Master Mix, New England Biolabs), 0.5 µL of each 10 µM forward and reverse primers, 1 µL of sample DNA and 8.5 µL of Dnase-free water. The triplex PCR reactions contained the following forward and reverse primers: J-UP/J-DN for detection of *C. jejuni* (349 bp), L-UP/L-DN for detection of *C. lari* (279 bp), and C-UP/C-DN for detection of *C. coli* (72 bp) and was performed in a T-Gradient thermocycler (Biometra). The PCR condition had initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 45.6 °C for 30 seconds, extension at 68 °C for 45 seconds, and had a final extension at 68 °C for 5 minutes. Each PCR run contained positive controls (DNA from *C. jejuni*, *C. lari*, and *C. coli*), samples and non-template controls. PCR products were detected by 2% agarose gel electrophoresis. For the TaqMan assay to detect *Campylobacter* spp., the detection limit for the enriched sample was 1 CFU/10 mL (enriched to at least 50 CFU/mL of Bolton enrichment broth), while for the triplex PCR, the detection limit was 1 CFU/mL for *C. jejuni, C. coli*, *C. lari*, respectively.

3.3.10 Statistical Analysis

The normality of the data presented in this paper was checked by D'Agostino-Pearson omnibus normality test in Prism 7 (version 7.0b, Graph Pad Software, Inc., La Jolla, CA,

USA). The test result showed that the data did not follow a normal distribution. Differences between two groups were therefore tested with the non-parametric t-test (Mann-Whitney test) while differences among three groups were tested using the non-parametric one-way ANOVA test (Kruskal-Wallis test). The Spearman rank correlation test was used to assess the correlation between the concentrations of *E. coli* and other related wastewater parameters. All the tests mentioned above were performed in Prism 7 (version 7.0b, Graph Pad Software, Inc., La Jolla, CA, USA). Differences among Spearman rank correlation coefficients (r_s) were considered significant if $p<0.05$.

3.4 Results and Discussions

3.4.1 Pond Environment and Wastewater Quality in The One-cell and Two-cell Arctic WSPs

The pond surface temperature, pH and DO profiles obtained from the Pond Inlet and Clyde River WSPs during the 2012, 2013 and 2014 treatment seasons are presented in Figures 3.1 and 3.2, respectively.

In Pond Inlet, the temperatures gradually increased from the beginning to the middle of the treatment season (2012: 13.1 to 16.9 °C; 2014: 11.1 to 17.8 °C) followed by a decrease to 4.3-5.4 °C at the end of the season in 2012 and 2014 (Figure 3.1a). A similar trend was seen in 2013, except the temperature fluctuated during the last part of the season from 15.9 °C to 8.8 °C, followed by an increase to 14.6 °C and then a gradual decrease to 2.0 °C at the end. In 2012, temporal spikes in pH-values were observed in the WSP where pH rose from 7.5 to 8.1 mid-season (Figure 3.1b), suggesting algal growth. The pH stayed at about 7.7 for the remainder of the treatment season. In 2013, however,

the pH gradually increased from 7.2 to 7.6 over the treatment season with no apparent spikes. In 2014, the pH gradually increased from 7.6 to 8.0 mid-season and followed by a slow decrease to 7.7. Interestingly, the constant low levels of DO close to or below 0.2 mg/L through the entire summer season in 2012 (Figure 3.1c) contradicted the presence of algal growth that was indicated by pH measurements that year.

In Clyde River, the pond surface temperatures similarly peaked mid-season (Figure 3.2a). For example, during the sampling trip in 2013, the highest temperature of 13.7°C was observed in mid-July compared to 6.2°C in the late June and 2.9°C in September. Figure 3.2b shows pond pH-values in 2014 exhibited a small peak going from 7.4 to 7.8 around mid-season after which the pH stabilized at 7.4-7.5 for the remainder of the season. Similarly to observations in Pond Inlet WSPs, pH-values in Clyde River gradually increased from 7.3 to 7.6 during the 2013 treatment season. DO levels consistently remained below the detection limit (0.2 mg/L) in both 2013 and 2014 (data not shown), suggesting that the secondary pond remained anaerobic during the summer treatment seasons for two consecutive years.

An assessment of efficiency of the wastewater treatment offered by the one-cell Pond Inlet and two-cell Clyde River WSPs revealed that the anaerobic ponds effectively removed total suspended solids (TSS) to approach the Canadian municipal wastewater standards (25 mg/L), however, removal of carbonaceous biochemical oxygen demand ($CBOD_5$) was limited due to low temperatures and anaerobic environments within the WSPs (Ragush et al. 2015). Taken together, it appeared that the WSPs only delivered limited primary treatment when it comes to the removal of nutrients. While WSPs in both communities were intended to operate as facultative ponds, this was not consistently the

case, likely due to the cool arctic summers.

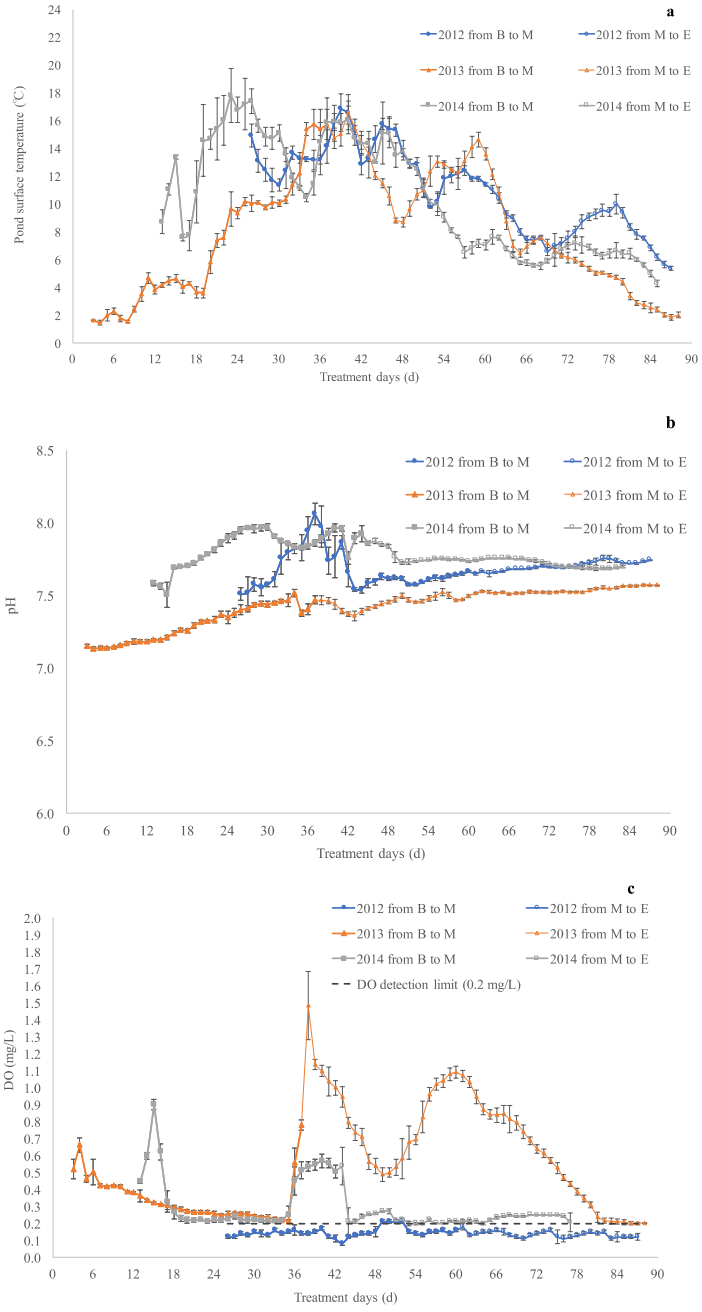


Figure 3.1 Environment in the Pond Inlet waste stabilization pond during the treatment seasons of 2012, 2013 and 2014 shown by a) the surface pond temperature, b) wastewater pH and c) DO concentrations. Each data point represents daily averages of hourly measurements (n=24, mean ± standard deviation). Closed symbols indicate values

obtained between the beginning (B) and middle (M) of the treatment season while the open symbols indicate values obtained between M and the end (E) of the treatment season.

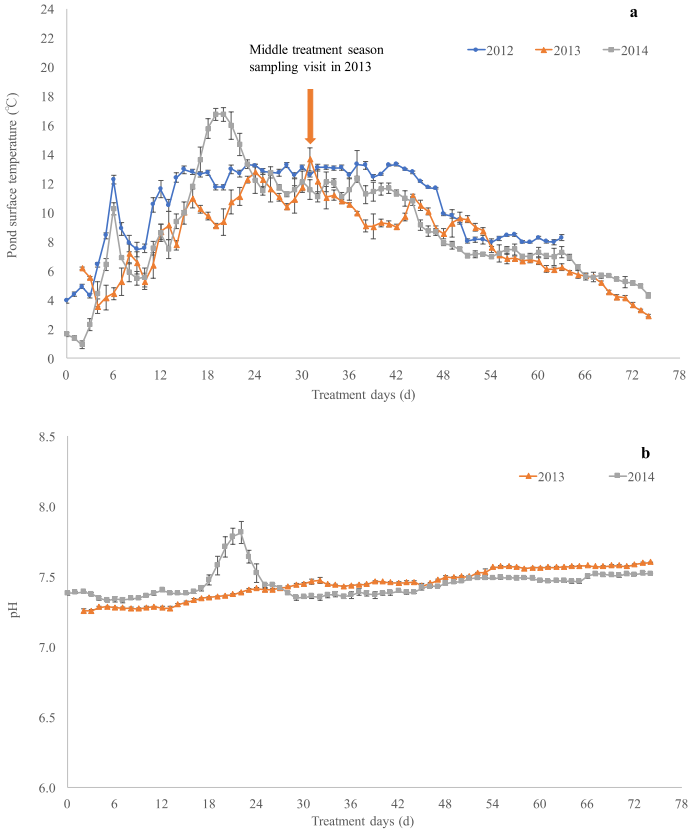


Figure 3.2 Environment in the secondary waste stabilization pond in Clyde River during the treatment seasons of 2013 and 2014 shown by a) the surface pond temperature (also 2012) and b) wastewater pH. Each data point represents daily averages of hourly measurements (n=24, mean ± standard deviation). The DO levels consistently remained below the detection limit (0.2 mg/L) and the data is therefore not shown.

3.4.2 Disinfection Treatment in Arctic WSPs

3.4.2.1 Removal of *E. coli*

In Pond Inlet, *E. coli* levels were on average reduced by 1.5 Log MPN/100 mL as raw wastewater levels of 7.2-7.5 Log MPN/100 mL were reduced to final *E. coli* levels averaging 5.8 Log MPN/100 mL in the effluent (Figure 3.3), which was within the permitted 4-6 Log MPN/100 mL in the current territorial effluent standards (Nunavut

Water Board 2014). In the early to mid-season of 2012 and 2013, $CBOD_5$ and TSS levels exhibited a strong relationship with the reduction of *E. coli* concentrations to the lowest levels of 5.3 Log MPN/100 mL, as indicated by the Spearman Rank Correlation coefficients (r_s) of 0.64 and 0.75 ($p<0.05$), respectively. However, in the later part of the treatment season, i.e., from late July/early August to early/middle September, *E. coli* levels rose significantly ($p<0.05$) from 5.3 to 5.9 Log MPN/100 mL. Taken together, it appeared that the disinfection (i.e., *E. coli* removal) and removal of suspended solids (TSS) and nutrients ($CBOD_5$) (Ragush et al. 2015) were optimal in the middle of the treatment season.

In Clyde River, just prior to decant in September, *E. coli* similarly reached levels in the secondary pond that met the current territorial effluent standard (Nunavut Water Board 2014). *E. coli* concentrations in the raw wastewater in Clyde River ranged from 6.7 to 7.3 Log MPN/100 mL with no significant differences ($p>0.05$) among sampling events (Figure 3.4). Treatment in the primary pond removed an average 1.1 Log MPN/100 mL from the raw wastewater resulting in average *E. coli* concentrations of 5.9 Log MPN/100 mL in wastewater samples from the primary pond. Within the secondary pond, there was a significant ($p<0.05$) reduction of 1.5 Log MPN/100 mL seen from initial levels in June of 5.5 Log MPN/100 mL to 4.0 Log MPN/100 mL in September, yielding an overall 3 log reduction in the *E. coli* concentration during the 2012-2014 treatment seasons. Reductions in TSS levels correlated ($p<0.05$) with the reduction of *E. coli* concentrations in the secondary pond as indicated by the Spearman Rank Correlation coefficient values (r_s) of 0.75 and 0.74 in 2012 and 2014, respectively. In 2013, there was a weak correlation relationship ($r_s = 0.45$) between the reduction of TSS and *E. coli* levels in the

secondary pond, which may be due to the direct discharge of raw wastewater into this pond observed during sampling trips in 2013.

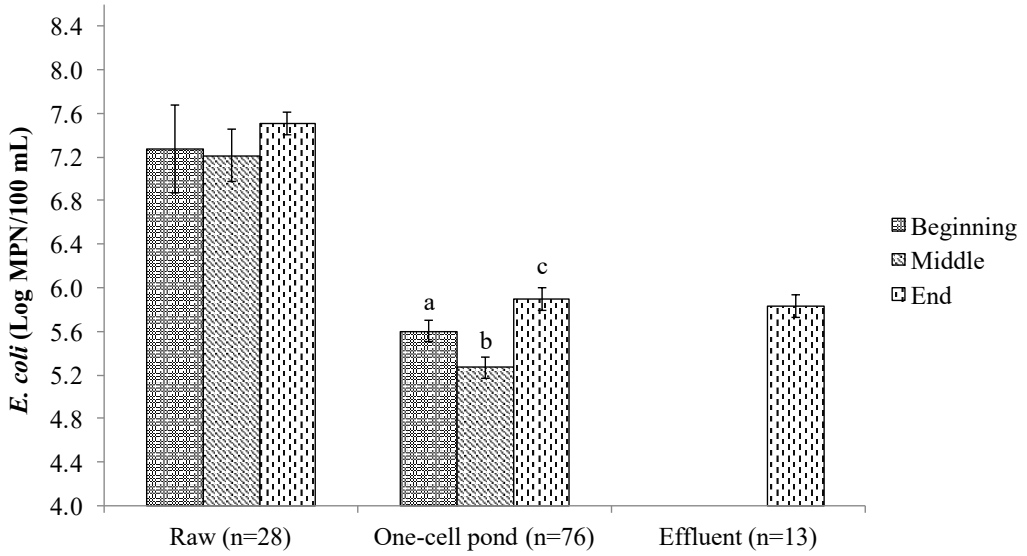


Figure 3.3 The average *E. coli* levels (Log MPN/100 mL) measured in raw (untreated), one-cell pond and effluent wastewater samples in Pond Inlet in the beginning, middle and end of the 2012 to 2014 treatment seasons. Error bars indicate the standard deviation. Different letters within the same sampling site indicate significant differences ($p<0.05$) as determined by the Kruskal-Wallis test.

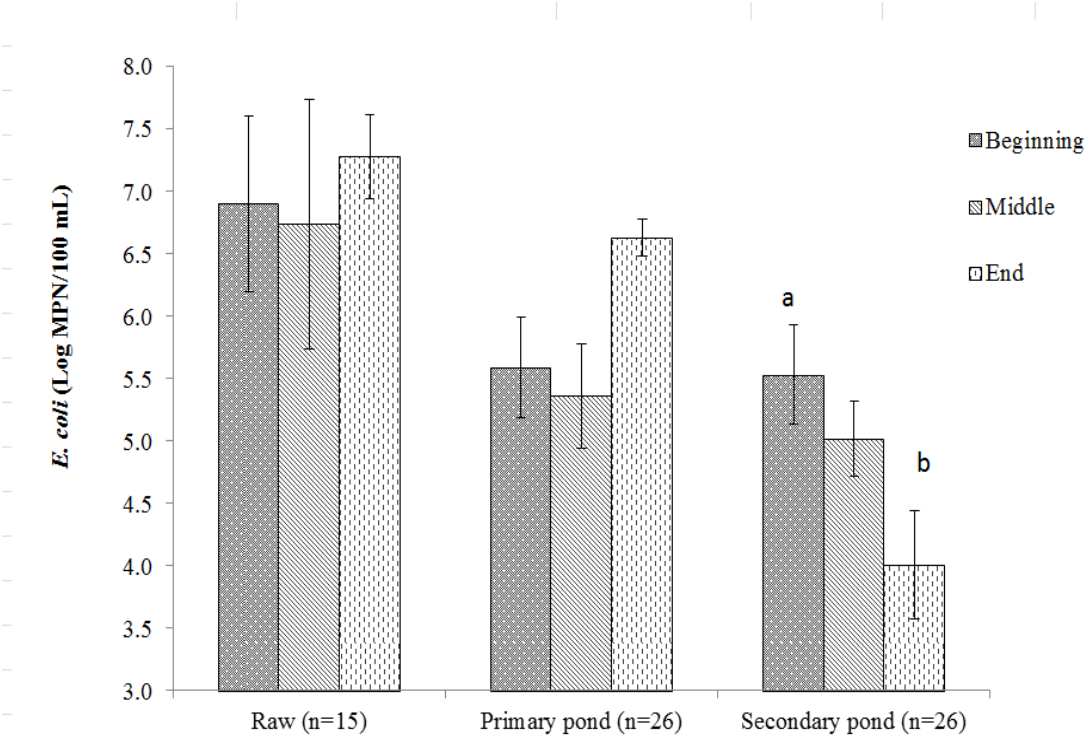


Figure 3.4 The average *E. coli* levels (Log MPN/100 mL) in raw (untreated) wastewater and samples from the primary and secondary ponds in Clyde River obtained during the 2012 to 2014 treatment seasons. Error bars indicate standard deviations. Different letters within the same sampling site indicate significant differences ($p<0.05$) as determined by the Mann-Whitney test.

3.4.2.2 Kinetics of *E. coli* Removal

The kinetics of the removal of *E. coli* over the different treatment seasons was compared by calculating the first order rate constants in the two WSP systems (Table 3.1). It should be noted that the first order rate constants were only computed from time periods where the levels of *E. coli* were decreasing.

Table 3.1 The first order rate constants (k) for *E. coli* removal in Nunavut WSPs.

Location	Year	K (1/day)	Duration of summer treatment (days)	Degree days above 5 °C
Pond Inlet	2012	$1.4 \times 10^{-4A} \pm 2.5 \times 10^{-6a}$	33	251
	2013	$1.7 \times 10^{-3B} \pm 5.1 \times 10^{-5}$	34	280
	2014	$3.3 \times 10^{-3C} \pm 1.7 \times 10^{-5}$	31	308
Clyde River (Secondary pond)	2012	$5.6 \times 10^{-3C} \pm 8.1 \times 10^{-5b}$	64	324
	2013	$3.7 \times 10^{-3A} \pm 1.6 \times 10^{-5}$	72	246
	2014	$4.6 \times 10^{-3B} \pm 3.3 \times 10^{-5}$	74	300

A-C: different letters in the same column for each community indicated that significant differences ($p<0.05$) were detected by the Kruskal-Wallis test.

[a]: average of calculated k values in Pond Inlet between two sampling events from 14 biological replicates with two technical duplicates (mean ± standard deviation)

[b:] average of calculated k values in Clyde River between two sampling event from eight biological replicates with two technical duplicates (mean ± standard deviation)

Pond Inlet exhibited significantly different ($p<0.05$) first order rate constants for *E. coli* removal from the beginning to the middle of the treatment season in each of the study years (Table 3.1) with the highest first order rate constant occurring in 2014, followed by 2013 and then 2012. Since previous studies found that temperature plays an important role in inactivation of *E. coli* in WSPs (Curtis et al. 1992, Davies-Colley et al. 2000, Klock 1971, Marais 1974), the degree days above 5 °C were calculated for these time periods. The trend of degree days above 5 °C indicated that the pond in 2014 (308 degree days above 5 °C) experienced a relatively warmer environment than in 2013 (280 degree days above 5 °C) and 2012 (251 degree days above 5 °C) and offers a possible

explanation for observed differences in the first order rate constants for *E. coli* removal over the three study years. This finding agreed with past WSP studies in non-arctic regions, which also showed the importance of temperature in *E. coli* die-off kinetics (Marais 1974, Polprasert et al. 1983, Klock 1971). The seasonal and annual variations in pH and DO (Figures 3.1b and 3.1c) also appeared to relate to disinfectant treatment efficiencies, for example, in 2014 a drop in *E. coli* levels coincided with increased pH (7.6 to 8.0) and DO (0.2 to 0.6 mg/L) levels. Previous studies have shown that pH values exceeding 9, and increased DO levels, effectively removed fecal coliforms including *E. coli* in WSPs (Curtis et al. 1992, Parhad and Rao 1974, Pearson et al. 1987).

In Clyde River, the first order rate constant for *E. coli* removal was highest in 2012 and lowest in 2013, which again appeared linked to a comparatively warmer environment in 2012 compared to the other years (Table 3.1). For this community, however, pH levels were relatively stable and DO levels were constantly below the detection limits, indicating that algae were unlikely to grow (Figure 3.2). Overall, the observed differences in *E. coli* removal kinetics indicated annual variations in disinfection treatment performance within the same passive treatment system and geographical location, which may in part be due to local climatic fluctuations.

3.4.3 Removal of Human Bacterial Pathogens in Arctic WSPs

3.4.3.1 WSP Temperature and Removal of Pathogens

The presence of human bacterial pathogens in the Pond Inlet WSP during the 2014 treatment season is depicted in Figure 3.5. It should be noted that a similar trend was seen in 2013. The non-enteric environmental pathogen *L. monocytogenes* was consistently present in 100% of the samples throughout the treatment season. Results showed that in

late June, the enteric pathogens *Salmonella* spp. and pathogenic *E. coli* serotypes were present in 88% and 100% of the samples, respectively. However, mid-season only 55% of samples contained *Salmonella* spp. while 72% of samples tested positive for pathogenic *E. coli* serotypes. On the last visit in conjunction with the annual decant, these numbers rose back up to 79% and 100% of the samples testing positive for *Salmonella* spp. and pathogenic *E. coli* serotypes, respectively. The other pathogens, *C. jejuni*, *C. lari*, *C. coli*, and *H. pylori*, were not detected in any samples, indicating that their levels remained below the detection limit.

The seasonal temperature variation had no impact on the presence of *L. monocytogenes*, which is a cold-adapted environmental bacterium previously associated with soil, water, and wastewater (Linke et al. 2014). A study of sludge from Swedish sewage treatment plants similarly showed that *L. monocytogenes* persisted in raw sludge samples (Sahlström et al. 2004). Improved removal of *Salmonella* spp. and pathogenic *E. coli* serotypes was observed mid-season coinciding with the highest environmental temperatures. Therefore, similar to the findings for the fecal indicator *E. coli* removal kinetics, it appeared that the higher WSP temperature measured mid-season in late July/early August (average 13.5 ˚C) improved the removal of *Salmonella* spp. and pathogenic *E. coli* serotypes. Taken together, this indicates the importance of temperature (degree days) measurements to gauge the disinfection efficiency (i.e., removal of fecal indicator bacteria) and removal of selected human pathogens in the arctic WSPs.

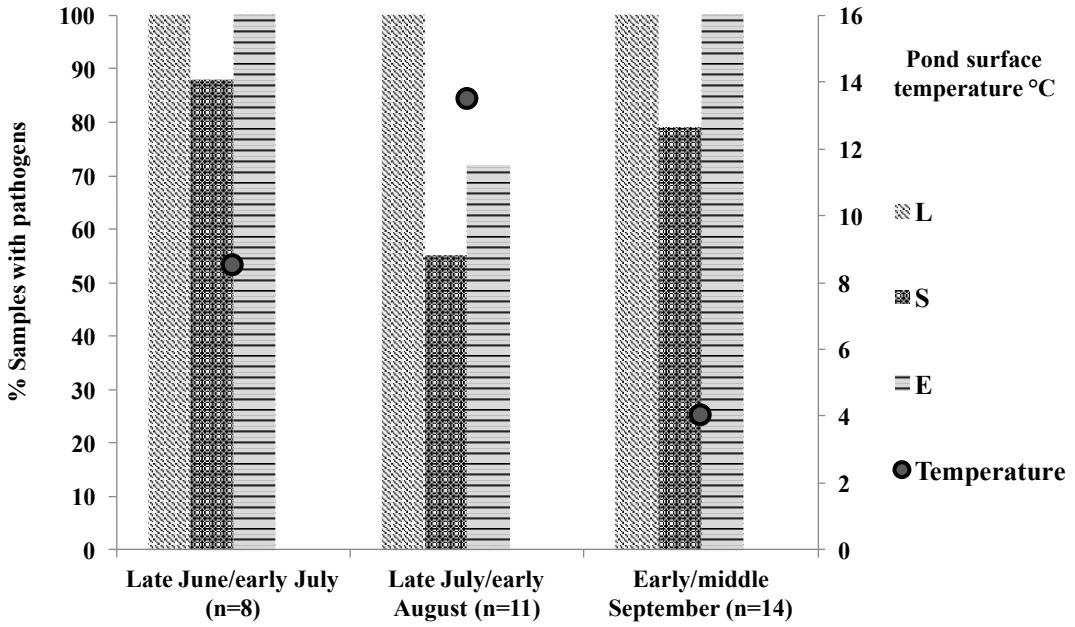


Figure 3.5 WSP surface temperature and percentage of WSP samples testing positive for the presence of human pathogens during the 2014 treatment season in Pond Inlet. Legend: L - *L. monocytogenes*, S - *Salmonella* spp., and E – pathogenic *E. coli* serotypes.

3.4.3.2 Removal of Human Bacterial Pathogens Along the Arctic WSP Treatment Train

The percentage of the samples testing positive for the presence of as well as the direct counts of human pathogens in raw and treated wastewater samples from the WSP systems in Pond Inlet and Clyde River are shown in Tables 3.2 and 3.3, respectively.

Table 3.2 Percentage of samples testing positive and quantity of human pathogens in raw and treated wastewater samples from the 2013 and 2014 treatment seasons in Pond Inlet.

Year	Wastewater samples (no. enriched samples)	Positive samples (%) following enrichment			Log CFU/100 mL following direct detection (no. positives/total sample no.)		
		L[a]	S[b]	E[c]	L	S	E
2013	Raw (8)	100	88	88	$3.8^{Ad}\pm0.3$ (2/4)	$4.1^{A}\pm0.2$ (1/4)	$5.5^{A}\pm0.5$ (1/4)
	WSP (23)	100	78	87	$3.4^{A}\pm0.2$ (7/7)	$3.6^{B}\pm0.1$ (3/7)	$4.8^{B}\pm0.2$ (3/7)
	Effluent (6)	100	83	83	$3.5^{A}\pm0.3$ (4/4)	$3.4^{B}\pm0.3$ (2/4)	$4.6^{B}\pm0.3$ (2/4)
2014	Raw (9)	100	89	100	$4.2^{A}\pm0.4$ (4/4)	$4.5^{A}\pm0.3$ (2/4)	$5.5^{A}\pm0.4$ (3/4)
	WSP (27)	100	74	89	$3.5^{A}\pm0.5$ (7/7)	$3.7^{B}\pm0.1$ (4/7)	$4.5^{B}\pm0.3$ (5/7)
	Effluent (4)	100	75	75	$3.6^{A}\pm0.4$ (4/4)	$3.6^{B}\pm0.2$ (3/4)	$4.5^{B}\pm0.2$ (3/4)

[a]L: *L. monocytogenes*

[b]S: *Salmonella* spp.

[c]E: Pathogenic *E. coli* serotypes

[d]Different capital letters in the same column indicate that there were significant differences ($p<0.05$) detected by the Kruskal-Wallis test (mean ± standard deviation).

In Pond Inlet, all three pathogens were detected at levels ranging from 1,000-10,000 copies/100 mL in the September decant (effluent) samples (Table 3.2). *L. monocytogenes*

was consistently present in all raw (untreated), WSP (treated), and effluent samples at unchanged levels, suggesting that this bacterium was not removed in the Pond Inlet WSP. *Salmonella* spp. were present in 88-89% of raw and 74-78% of treated samples in 2013 and 2014, indicating a consistent presence. The concentration of *Salmonella* spp. fell significantly ($p<0.05$) by 0.5-0.8 Log CFU/100 mL from raw to treated/effluent wastewater samples, indicating some removal in the WSP. Depending on the year, 88 to 100% of raw wastewater samples tested positive for pathogenic *E. coli* serotypes. While the level of positive samples stayed high in the treated samples, the quantitative analysis revealed that the pathogenic *E. coli* population was reduced by 0.7-1.0 Log CFU/100 mL. The three major *Campylobacter* pathogens (*C. jejuni*, *C. lari*, and *C. coli*) were not detected in neither the enriched samples nor by direct enumeration, indicating a low prevalence in the Pond Inlet wastewater treatment system.

Table 3.3 Percentage of samples testing positive and quantity of human pathogens in raw and treated wastewater samples obtained in Clyde River in September of 2013 and 2014.

Year (September)	Wastewater samples (no. samples)	Positive samples (%) following enrichment			Log CFU/100 mL following direct detection (no. positive samples)		
		L[a]	S[b]	E[c]	L	S	E
2013	Raw (4)	100	100	100	$4.6^{Ad}\pm0.3$ (4)	$5.1^{A}\pm0.2$ (3)	$5.2^{A}\pm0.5$ (3)
	Primary (4)	100	100	100	$4.4^{A}\pm0.2$ (4)	$4.9^{A}\pm0.1$ (3)	$4.9^{A}\pm0.2$ (3)
	Secondary (4)	100	100	100	$4.5^{A}\pm0.3$ (4)	$4.6^{A}\pm0.3$ (3)	$4.7^{A}\pm0.3$ (3)
2014	Raw (4)	100	75	100	$4.5^{A}\pm0.3$ (4)	$4.8^{A}\pm0.3$ (3)	$4.9^{A}\pm0.4$ (3)
	Primary (4)	100	100	100	$4.2^{A}\pm0.2$ (4)	$4.4^{B}\pm0.1$ (3)	$4.3^{B}\pm0.3$ (3)
	Secondary (4)	75	50	75	$3.5^{B}\pm0.3$ (4)	$3.6^{C}\pm0.2$ (2)	$3.4^{C}\pm0.2$ (2)

[a]L: *L. monocytogenes*

[b]S: *Salmonella* spp.

[c]E: Pathogenic *E. coli* serotypes

[d]Different capital letters in the same column indicate that there were significant differences ($p<0.05$) detected by the Kruskal-Wallis test (mean $\pm$ standard deviation).

In Clyde River in 2013 all three pathogens were detected in all raw sewage and grab samples from both the primary and secondary pond during the September sampling visit,

shortly before the annual decant event (Table 3.3). In 2013, the levels of all three pathogens remained unchanged ($p>0.05$) along the treatment system. To improve the performance of the two-cell WSP system in Clyde River, it was proposed based on the treatment suggestions by Dawson and Grainge (1969) and Heinke et al. (1991), that the community use the system in a manner where the smaller primary pond is utilized as a primary treatment cell followed by the transfer of pre-settled wastewater from the primary pond to the secondary, larger pond. Clyde River followed this suggestion in 2012, but returned to dumping raw wastewater into the secondary pond from mid-August to early September in 2013 due to the lack of holding capacity in the primary pond. It may be that this caused the poor disinfection and removal of pathogen performance in 2013; however, 2013 was also a year characterized by lower temperatures (Table 3.1).

In 2014, Clyde River was able to operate the system according to the recommendations, which led to a reduction of pathogens in treated wastewater samples from the secondary pond. In absolute numbers, this resulted in reductions of one log for *L. monocytogenes*, 0.8 log for *Salmonella* spp., and 0.9 log for pathogenic *E. coli* serotypes. In line with past observations of a relationship between TSS and pathogen removal (Bitton 2011), the current observation of pathogen removal coincided with a significant reduction of TSS observed in the secondary pond (Ragush et al. 2015).

Campylobacter spp. and *H. pylori* were not detected in any of samples during sampling events in Pond Inlet and Clyde River. While their presence might have been expected in the raw sewage (Goldfarb et al. 2013), one possible reason for the absence of *Campylobacter* spp. in the WSP samples is their thermophilic nature, making them vulnerable to the cold arctic climate. It has previously been reported that viable and

culturable *Campylobacter* spp. numbers quickly decreased following the discharge of untreated sewage into coastal waters (Jones et al. 1999a and 1999b). The same study also found that *Campylobacter* spp. suspended in the effluent became unculturable after only 15 minutes of exposure to direct sunlight. During the study period, a high level of incident solar radiation was measured during sunny days with clear sky in Pond Inlet (Ragush et al. 2015), which may have aided in the inactivation of *Campylobacter* spp.

In terms of the detection of bacterial pathogens in the adjacent environment during the September decant in Pond Inlet, all four outfall samples contained *L. monocytogenes* (average 3.2 Log CFU/100 mL), *Salmonella* spp. (average 2.2 Log CFU/100 mL) and pathogenic *E. coli* serotypes (average 4.1 Log CFU/100 mL). Prior to the decant event, all ocean samples tested negative for the pathogens. However, during the decant event, two pathogens (*L. monocytogenes* and *Salmonella* spp.) were detected in all three ocean samples at average levels of 2.1 and 1.5 Log CFU/100 mL, respectively.

The presence of pathogens in the effluent may pose a risk to human health through various exposure pathways but this will obviously depend on the number and survival of the pathogens being released into the arctic environment and the human and wild-life interactions with impacted areas (Harper et al. 2011, Daley et al. 2017). The predicted infectious dose for pathogenic enterohemorrhagic *E. coli* serotypes such as O157:H7 is only 10 to 100 bacteria (Theron and Cloete 2002), while for *L. monocytogenes* it is 10^7-10^8 CFU in healthy hosts and 10^5-10^7 CFU in susceptible individuals (Farber et al. 1996). The infectious dose is 10^3-10^5 CFU for non-typhoidal *Salmonella* spp. (Bronze and Greenfield 2005, Ray and Sherris 2004). The prevalence of acute gastrointestinal illness (AGI) is reported to be higher in Arctic Canada compared to other parts of the country

(Harper et al. 2015a and 2015b), however, the cause of this remains uncertain. Goldfarb et al. (2013) investigated the presence of a range of bacterial, parasitic, and viral agents in patients with diarrhea in Nunavut and commented on their inability to track the source of the observed infectious agents. The source of food and waterborne infectious agents can be local, as in present in locally harvested foods (mammals and fish) or drinking water, or imported. The latter appeared to have been the case in the *E. coli* O157:H7 outbreak in Arviat (NU) where imported frozen hamburger patties were a likely source (Orr et al. 1994). Few studies exist on the prevalence of pathogens in local food sources. Gauthier et al. (2010) reported that 129 samples of arctic mammals, fowl, fish and community freezers in Nunavik tested negative for the presence of *E. coli* O157:H7 and *Salmonella* spp. While it must be assumed that agents of AGI end up in the municipal wastewater treatment systems, little is known about the potential attributions to human disease and wild-life carriage of pathogens being released with (un)treated wastewater in the arctic. A survey of the release of pathogens into the Antarctic Ocean due to wastewater disposal from Antarctic research stations found that microorganisms released from wastewater remained viable for prolonged periods and thus available for transmission to the local fauna (Gröndahl et al. 2009). Earlier studies had reported the presence of human pathogens (*Salmonella* Enteritidis, *Salmonella* Typhimurium, *Campylobacter jejuni*, and *Pasteurella multocida*) in antarctic seal and bird populations leading the researchers to speculate that the presence of these pathogens could presumptively be attributed to human activity (Broman et al. 2000, Palmgren et al. 2000). Clearly, future studies are needed to uncover whether the release of human pathogens from the discharge of untreated and treated wastewater from arctic communities constitute a human health

hazard.

3.5 Conclusions

The study investigated the disinfection and removal of human pathogens in arctic WSPs treating municipal wastewater in Pond Inlet and Clyde River, Nunavut, Canada. The results revealed that WSPs in both communities reduced the content of *E. coli* to levels that are in compliance with the Nunavut Water Board (2014) regulatory limits. The seasonal pond temperatures appeared to influence the treatment efficiency. The single-cell WSP in Pond Inlet was able to significantly remove *Salmonella* spp. (0.7-0.9 log) and pathogenic *E. coli* serotypes (~1.0 log) but not *L. monocytogenes* from raw to effluent wastewater. The two-cell Clyde River WSP provided better treatment in regards to disinfection and removal of bacterial pathogens with reductions of 1.0-1.5 log, provided the primary pond was used as the only recipient of raw wastewater which then after a settling period was transferred to the secondary pond for further treatment. The best removal of fecal indicator bacteria and pathogens was achieved mid-season in Pond Inlet, likely due to the warmer water temperatures, however, due to the traditional and important harvest of seafood at that time of year, the treated wastewater was not released until just prior to freeze-up in September. In spite of the WSP treatment, it should be noted that the bacterial pathogens were still present in levels of 2-4 Log CFU/100 mL in the treated wastewater being discharged into the receiving environment. In summary, arctic WSPs achieved a modest removal of fecal and pathogenic bacteria from municipal raw sewage with some local, seasonal and year-to-year variations. From a public health perspective, it may be prudent to assess the potential risks that the wastewater effluents pose to human health.

Chapter 4 Bacterial Communities in Arctic Wastewater Stabilization Ponds as Affected by Environmental and Treatment Processes

4.1 Abstract

In Arctic Canada, wastewater stabilization ponds (WSPs) are commonly used to treat municipal wastewater. However, the biological treatment processes in passive WSPs are strongly influenced by climatic conditions. There is limited knowledge about the bacterial community in the WSPs operated in the harsh arctic climate. The objective of this chapter was to investigate the population size, composition, distribution, diversity and functional content of bacterial communities in arctic WSPs treating municipal wastewater in Pond Inlet and Clyde River, Nunavut, Canada over three consecutive (2012-2014) summer treatment seasons.

Anaerobic conditions with an absence of algal blooms were seen and pH stayed constant between 7.5 to 7.8 in both WSPs. The bacterial population size was significantly ($p<0.05$) influenced by the treatment process and the sampling time during the summer treatment season. The alpha- and beta-diversities analyses showed that the bacterial communities in raw wastewater samples were significantly ($p<0.05$) different from communities in pond and effluent samples. The results also showed that the bacterial community structure was significantly different ($p<0.05$) during the course of the summer treatment season, between sampling years and the two geographical locations. However, the bacterial diversity in raw wastewater was not significantly different ($p>0.05$) between the communities. The predicted gene functionalities from analysis with PICRUSt confirmed that the middle of the treatment season was the optimal time for removal of nutrients, represented by $CBOD_5$ values, observed in Pond Inlet, and the treatment in the

secondary pond in Clyde River constituted a better treatment for $CBOD_5$ removal than the primary pond. Also, the predicted gene content supported the observation in both Pond Inlet and Clyde River WSPs that there was an absence of bacterial ammonia removal (oxidation) in the anaerobic pond environments during the 2012-2014 study period. Future research will perform a bench-scale study to clarify the effect and significance of each variable (e.g., temperature, DO, pH and nutrients) on the WSP bacterial community population size, composition, diversity and potential function.

4.2 Introduction

The majority of communities in Arctic Canada (Nunavut, Nunavik and Northwest Territories) use WSPs to treat municipal wastewater. The WSPs are passive treatment systems that are strongly affected by the climate conditions (Heinke et al. 1991). Due to the simplicity in design and economical operation, the WSPs have become a common solution to treat municipal wastewater in many remote communities in Arctic Canada. Past studies have shown that WSPs can effectively remove nutrients, oxygen demanding matters, suspended solids and pathogens (Shilton and Walmsley 2005). New WSER that stipulate that effluents should contain less than 25 mg/L carbonaceous biochemical oxygen demand at Day 5 ($CBOD_5$), 25 mg/L total suspended solids (TSS), 1.25 mg/L un-ionized ammonia, and 0.02 mg/L total residual chlorine), have been implemented for Southern Canada (Environment Canada 2015). Due to the unknown impact of climatic conditions in Arctic Canada on the current wastewater treatment practices, research is needed into factors that affect the performance of the current wastewater treatment systems and provide strategies to create WSERs that are realistic for Arctic WSPs operated under harsh climatic conditions.

The microbial community plays an important role in biological wastewater treatment (Wagner et al. 2002) and has been studied for many years by both traditional culturing methods (Neilson 1978) and molecular methods, such as PCR-DGGE (Muyzer et al. 1993), terminal restriction fragment length polymorphism (t-RFLP) (Liu et al. 1997), cloning (Schuppler et al. 1995), and fluorescent *in situ* hybridization (Erhart et al. 1997). Increasingly, next-generation sequencing (NGS) techniques, such as Illumina Miseq sequencing (Bartram et al. 2011), is replacing these methods. This NGS technique can generate multimillion-sequence *16S rRNA* gene libraries to rapidly and reproducibly assess and compare the taxonomic diversity present in complex microbial communities, while also providing access to unculturable organisms and/or those present at low relative abundances (Bartram et al. 2011). This new and affordable approach has been extended to study the human microbiome (Kuczynski et al. 2012) and bacterial biogeography of the human digestive tract (Stearns et al. 2011). Recently, a wastewater treatment study used DGGE to study the microbial community in activated sludge samples in two wastewater treatment plants (WWTPs) of Västerås (59° 36′ 58″ N, 16° 33′ 10″ E) and Eskilstuna (59° 21′ 59″ N, 16° 30′ 30″ E) in Sweden (Caballero 2011). These two facilities faced difficulties in maintaining the required rates of nitrogen removal during winter time due to low temperatures (approximate wastewater average temperature: 5°C). The study showed that treatment performance in biological WWTPs could be related to the diversity and structure of the bacterial community. For example, nitrogen removal efficiency was related to the shift of a *Nitrosomonas ureae*-like bacterial cluster belonging to the ammonia oxidizing bacteria (AOB) community. The higher ammonia removal performance was related to the higher diversity of AOB community. A recent

study used pyrosequencing to investigate bacterial diversity in 14 wastewater treatment systems in China and reported that the bacterial community variance correlated most strongly with water temperature, conductivity, pH, and DO (Wang et al. 2012). Also, the statistical results indicated that wastewater characteristics COD, total nitrogen (TN), total phosphorus (TP), pH, and conductivity had the greatest contribution to the bacterial community variance, explaining 25.7% of the variance of bacterial communities independently, followed by operational parameters (DO, temperature, SRT, mixed-liquid-suspended-solids (MLSS) (23.9%) and geographic locations (14.7%). In the field of wastewater treatment study, the local climatic conditions, differences in treatment systems and wastewater characteristics, as the compositions of wastewater vary significantly among different places and sources (Henze 1997), are likely to yield differences in WWTPs microbial communities.

The objectives of the present study were to use Illumina Miseq technology to investigate the composition, distribution, diversity and potential function of bacterial WSP communities in relation to how the arctic climate, especially low temperatures, and the treatment train affect them. Information about the microbiology of the systems can help in optimizing the passive, biological wastewater treatment systems in Arctic Canada. This study was conducted over three years (2012-2014) in two remote communities in Nunavut, Pond Inlet and Clyde River, where municipal wastewater treatment is performed using one-cell and two-cell WSPs, respectively.

4.3 Materials and Methods

4.3.1 Study Sites

The two study communities, Pond Inlet (latitude 72° 41′ 57″ N, longitude 77° 57′ 33″

W; population: 1549 [Statistics Canada 2012]) and Clyde River (latitude 70° 28′ 26″ N, longitude 68° 35′ 10″ W; population: 934 [Statistics Canada 2012]), are located on the eastern shore of Baffin Island in Nunavut's Qikiqtani region and accessible by plane year-round and seasonal ship traffic. Both communities have a polar arctic climate, which is characterized by long cold winters (approximately nine months from middle of September to May) and short cool summer (about three months from June to middle of September). Based on the 1981 to 2010 Canadian climate normal, the warm season in Pond Inlet lasts from June to the middle of September with an average daily high temperature of just above 2 °C, while cold season lasted from December to April with an average daily high temperature below -22 °C. Similar to Pond Inlet, Clyde River had warm season average daily temperatures above 2 °C, while the cold season (December to March) had an average daily temperature below -19 °C. July was the warmest month in Pond Inlet and Clyde River with the daily average temperatures of 6 and 5 °C, respectively. February was the coldest month in Pond Inlet and Clyde River with the daily average temperatures of -34 and -30 °C, respectively (Environment Canada 2014). Degree days above 0 and 5 ºC for the ambient air temperature were previously calculated using data from 1981 to 2010 and this comparison showed that Pond Inlet (degree days above 0 ºC: 473; degree days above 5 ºC: 99) experiences a warmer climate than Clyde River (degree days above 0 ºC: 382; degree days above 5 ºC: 64) (Ragush et al. 2015).

4.3.2 Sampling Strategy

From September 2012 to September 2014, seven sampling trips were made to Pond Inlet and another six sampling trips to Clyde River. The first trip to Pond Inlet and Clyde River was at the end of the summer treatment season in 2012. Efforts were made to

sample the WSP in Pond Inlet at the start, middle, and end of the summer treatment seasons including the decant events in 2013 and 2014. The primary and secondary ponds in Clyde River were sampled at the start, middle, and end of the summer treatment season in 2013. In 2014, both ponds in Clyde River were sampled at the start and end of the summer treatment season, just prior to the decant event. The beginning, middle, and end of the summer treatment season indicated that the sampling trips took place in late June/early July, late July/early August, and early/middle September, respectively.

The single cell WSP in Pond Inlet consists of an approximately pentagon-shaped pond with a surface area of approximately 4 ha and an average depth of approximately 1.9 m during the summer. It has been in use since 2005 and was initially designed to be a facultative pond. The detail of sampling sites is schematically depicted in Figure 1. The wastewater samples were sampled at the surface level from Sites 1 to 5. At Site 5, wastewater was additionally sampled at the middle depth (approximately 1.1 m away from the surface level) and at the bottom level of the water column (Figure 4.1). During each of the 2014 sampling trips, sludge samples were also collected from Sites 1 to 5 and combined into one composite sample. All wastewater generated in Pond Inlet is delivered by trucks to the WSP. Raw wastewater samples were collected from a minimum of two trucks per sampling trip at Site T (Figure 4.1). The treated wastewater is decanted from the WSP once annually, usually over a three-week period starting in early/middle September and ending in early October just prior to freeze-up. The wastewater effluent exits the WSP over the berm and travels through a ditch and then down a steep hill (500 m) before arriving in the ocean receiving environment (Eclipse Sound). The effluent samples were collected every four hours during the decant event at Site E (Figure 4.1),

for 12 hours in 2012 (three samples in total), for eight hours (two samples in total) in 2013 and for 24 hours (six samples in total) in 2014.

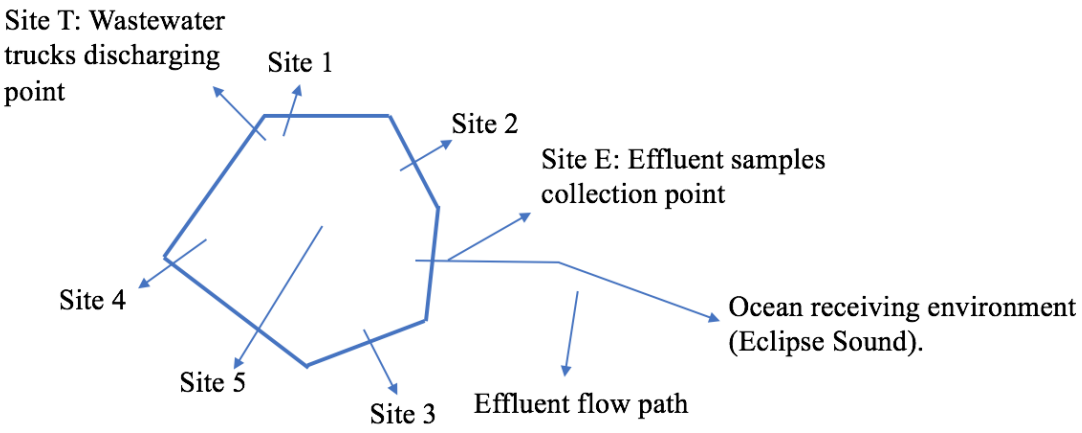


Figure 4.1 Sampling strategy for the single-cell WSP system in Pond Inlet, NU.

The Clyde River WSP system consists of two cells in series. The first one, referred to as the primary pond, was constructed in 1976. Due to the increasing population, the primary pond (surface area of 0.6 ha) became unable to accommodate the annual wastewater volume. Therefore, the larger second cell, referred to as the secondary pond, was built in 2011 with a surface area of 1.5 ha. The sampling strategy is schematically depicted in Figure 4.2. The intended use of the two-cell WSP system is an arrangement where the raw wastewater is firstly discharged into the primary pond to enable settling and precipitation processes. And then at regular intervals, pre-processed wastewater should be transferred from the primary pond into the secondary pond to receive further

treatment before the annual decant from the secondary pond, where the treated wastewater is passed through a vegetated filter strip before going into the ocean receiving environment (Patricia Bay) (Figure 4.2). Similar to Pond Inlet, all wastewater generated in Clyde River is delivered to the WSP by trucks. The raw wastewater samples were collected from trucks at Site T1 during the beginning and middle of the 2013 treatment season and during the beginning and end of the 2014 treatment season. However, at the end of the 2013 treatment season, the raw wastewater was directly discharged into the secondary pond as the primary pond was full. Therefore, the raw samples were collected at Site T2 (Figure 4.2). The wastewater samples in both primary and secondary ponds were collected at the surface level from Sites 1 to 3. At Site 3 in both ponds, the wastewater samples were also collected at the bottom of the water column (Figure 4.2). The sludge samples were not collected in Clyde River due to logistical constraints.

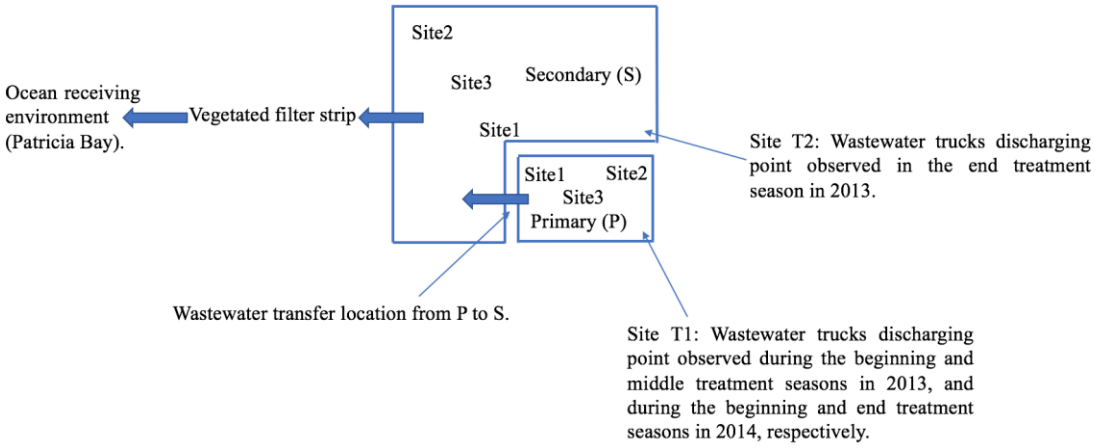


Figure 4.2 Sampling strategy for the two-cell WSP system in Clyde River, NU.

4.3.3 Continuous WSPs Monitoring Parameters Collection

The wastewater temperature, pH, and DO in WSPs were measured with *in-situ* multi-parameter sondes (YSI Inc., Yellow Spring, OH). During the first visit in each year, the

sondes were installed in the WSPs to record the parameters hourly until the end of the treatment season. In addition, HOBO temperature/light pendants, temperature/water level loggers and ROX (Reliable Oxygen Sensor) DO probes (Onset Computer Corporation, Cape Cod, MA) were used in WSPs to measure the parameters at other depths and also to validate the continuous measurements recorded by the *in-situ* sondes.

4.3.4 DNA Extraction

Wastewater samples were collected in 1 L or 500 mL sterile containers (Nalgene, Fisher Scientific, Nepean, ON, Canada) and then stored in a cooler (4 ºC). Within 24 hours of the original sampling event, samples were flown to the NWQL at the Nunavut Research Institute in Iqaluit, NU. Upon arrival to the NWQL, the extraction of genomic DNA from samples were performed immediately. Microbial community genomic DNA was extracted from three biological replicates (separate samples) per sampling site whenever available with two technical replicates per sample. Prior to the DNA extraction, 10 mL of each sample was added to a 15 mL sterile test tube and then centrifuged at 3200 x *g* for 10 minutes to obtain cell pellets for DNA extractions. After discarding the supernatant, the cell pellet was resuspended using the residual liquid (250 µL) and subjected to genomic DNA extraction using the MO BIO PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Within 24 to 48 hours, all DNA samples were flown to Halifax, NS in a cooler (4 ºC). DNA concentrations were then quantified with a QuantiFluor@ ds DNA kit (Promega Corporation, Madison, WI, USA) and a Quantus Fluorometer (Promega). Most of samples (75%) had DNA concentrations ranging from 1-20 ng/µL, while some samples (22%) had DNA concentrations ranging from 0.1 to 1.0 ng/µL, and few samples (3%) had

DNA concentrations above 20 ng/µL. All DNA samples were stored at -20°C until further analyses.

4.3.5 *16S rRNA* Copy Number Determination

To determine the bacterial population size, the copy numbers of bacterial *16S rRNA* genes were detected using a qPCR assay with the BACT 2 primer set (1369F CGGTGAATACGTTCYCGG; 1492R GGWTACCTTGTTACGACTT, Suzuki et al. 2000). The qPCR amplification was performed on a Bio-Rad CFX96 Touch Real-Time PCR system (Bio-Rad, Hercules, CA, USA) in 20-µL total reaction volumes consisting of 4.0 µL of template DNA, 4.4 µL of sterile and nuclease-free water (Fisher Scientific, ON, Canada), 0.8 µL of each primer (10 µM), and 10 µL of 2x Power SYBR Green PCR master mix (Applied Biosystems, Life Technologies, Canada) using the following thermocycler program: 10 minutes (min) of initial denaturation at 95 °C, followed by 40 cycles of 15 seconds (s) denaturation at 94 °C, 30 s annealing at 55 °C, and 30 s extension at 72 °C. Melt curve analysis was also performed to confirm presence of the *16S rRNA* gene PCR amplicon with a melting temperature of 80.7 °C ± 0.4 °C.

A plasmid construct containing the partial region of the bacterial *16S rRNA* gene was kindly provided by Dr. Yost (University of Regina, personal communication) and was used to create a standard curve (10-fold serial dilutions, 6.86×10^0 to 6.86×10^8 copies/µL) to enable quantification of *16S rRNA* copy numbers in samples. Two technical replicates were run for all standards, samples, non-template controls and the difference of the threshold cycle (Ct) value between the replicates were less than 0.5. The qPCR assay efficiency was 102%, with an R^2 value of 0.999. The LOD is 68.6 copies/mL wastewater. The limit of quantification (LOQ) for the *16S rRNA* gene is 6.86×10^2 copies/mL

wastewater. Quantity of *16S rRNA* gene copy numbers was reported as Log_{10} gene copies/mL in each wastewater sample.

4.3.6 Illumina Miseq Sequencing

Amplicon library preparation and sequencing were performed at Dalhousie University's Integrated Microbiome Resource (IMR; http://cgeb-imr.ca). The amplicon library preparation and sequencing were performed following by the established protocol (Comeau et al. 2017) at IMR. The sequencing primers (Forward ACGCGHNRAACCTTACC; Reverse ACGGGCRGTGWGTRCAA) were used to amplify the V6-V8 regions of the *16S rRNA* gene in bacteria. The generation of paired-end (PE) sequencing reads of the *16S rRNA* gene PCR amplicons with multiple barcodes were processed on the Illumina MiSeq machine. The resulting PE sequencing reads had approximately 400 to 500 base pair (bp) and the theoretical length of the demultiplexed read for each forward and reverse read was 301 bp (Comeau et al. 2017). The demultiplexed PE reads in fastq format were obtained from IMR for further bioinformatics analysis.

4.3.7 Bioinformatics Sequence Processing and Analysis

The general overview of the bioinformatics workflow is presented in Figure 4.3. The detailed information for each analytic step is described in the following sections (3.7.1 to 3.7.3).

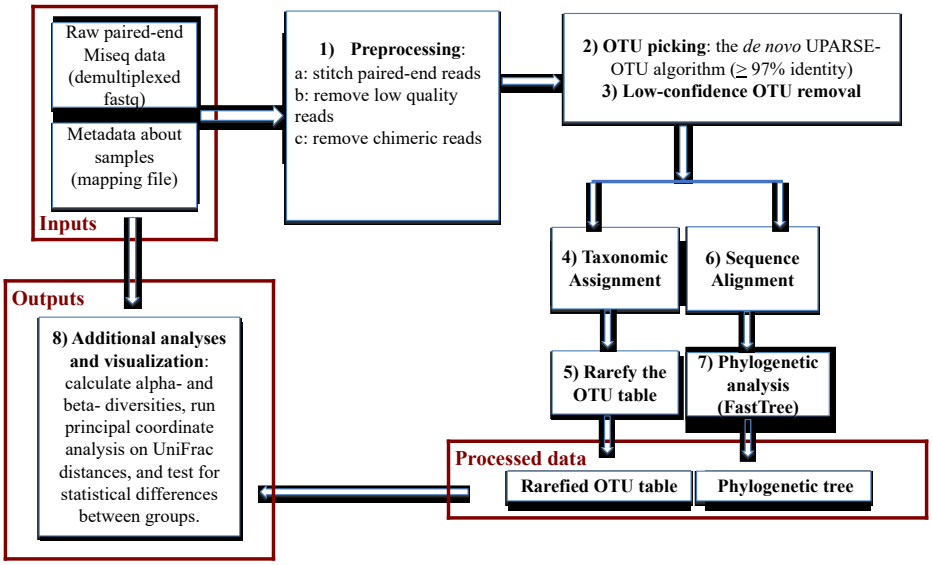


Figure 4.3 The bioinformatics workflow for analyzing Miseq paired-end sequencing reads.

4.3.7.1 Preprocessing

The sequencing analysis was performed in three steps (Figure 4.3) to preprocess the raw PE MiSeq sequencing reads by following the 16S rRNA analysis standard operating procedure (https://github.com/mlangill/microbiome_helper/wiki/16S-Bacteria-and-Archaea-Standard-Operating-Procedure) in the bioinformatics pipeline called Microbiome Helper at IMR (Comeau et al. 2017). The first step was to stitch PE reads together in PEAR (Zhang et al. 2014). The vast majority (98.3 to 99.6%) of PE reads were successfully stitched for all samples. And then the stitched reads were filtered to remove low quality reads (<400 bp, low quality scores, content of "Ns"). After filtering low quality reads, the majority (61.9 to 86.0%) of the stitched reads with an average size of 440 bp remained. The last step was to remove chimeric sequences using VSEARCH with version 1.11.1 (Rognes et al. 2016). After removing chimeras, the majority (49.6 to 94.6%) of reads were kept as the final high quality sequences for the following

bioinformatics analysis.

4.3.7.2 OTU Picking, Low-confidence OTU Removal, Taxonomic Assignment and Phylogenetic Tree Building

The final high quality sequences in the fasta format were then clustered into Operational Taxonomic Units (OTUs) at an identity level of ≥97% using the *de novo* USPARSE-OTU algorithm in the USEARCH pipeline (Edgar 2013), in the OTU picking step (Figure 4.3). OTUs making up <0.1% of the total number of sequences, which is the maximal expected bleed-through between MiSeq runs based on information from Illumina (Illumina, 2013), were removed in the Microbiome Helper pipeline, resulting in a total of 4.2 million sequences being clustered into 2,093 OTUs. Subsequent analyses were carried out using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline version 1.9.1 (Caporaso et al. 2010b), unless otherwise noted. The representative sequence from each of the OTUs (from UPARSE) was classified with the Ribosome Database Project (RDP) classifier version 2.2 (Wang et al. 2007) based on the Greengenes database in its most recent version 13_8. The cut-off value to define a sequence's taxonomy was set at 60% to provide at least 95% accurate taxonomy assignment at the genus level (Claesson et al. 2009; Liu et al. 2008). Representative OTU sequences were then aligned using the default and template guided alignment method (PyNAST) (Caporaso et al. 2010a). This alignment was used to build a phylogenetic tree in FastTree with version 2.1.10 (http://www.microbesonline.org/fasttree/) (Price et al. 2010). FastTree infers approximately-maximum-likelihood phylogenetic trees. Using FastTree, a phylogenetic tree was constructed from the multiple sequence alignment employing the generalized time-reversible model of nucleotide evolution. The CAT model in FastTree was used to account for the varying rates of evolution across the

nucleotide site, i.e., these are columns in the multiple sequence alignment (Stamatakis 2006; Price et al. 2010).

4.3.7.3 OTU Table Summarization, Alpha- and Phylogenetic Beta-diversity Analyses

Prior to performing alpha and beta diversity analyses, the OTU table was normalized per sample by subsampling to a minimal number of reads (10,539 sequences) (Figure 4.3). The resulting normalized OTU table contained 1,924 OTUs and associated taxonomic classification of each OTU in each sample. This table was used to summarize the relative abundance of each OTU from taxonomic phylum to genus level for each sample. A heatmap was used to visualize the summarization of the OTU table, where each row corresponds to the relative abundance of an OTU and each column corresponds to a sample.

To test whether there was a core bacterial community in raw wastewater samples between the two communities, the compute_core_microbiome function within QIIME, that requires the core OTUs to be present in 100% of the samples in the normalized OTU table, was used. The definition of core bacteria is that the bacterium is present in at least 95% of all samples (Huse et al. 2012). Alpha and beta diversity analyses were conducted based on the normalized OTU table (Figure 4.3). The alpha-diversity analysis, as in the number of the observed OTUs, Chao1, the Shannon-Wiener diversity and the Simpson evenness indexes, were performed to characterize species diversity within a given sample. The phylogenetic beta-diversity was quantified using the weighted UniFrac metric (Lozupone and Knight 2005) to indicate whether different wastewater treatments or treatment seasons caused the relative abundance of taxonomic groups to change. Also, the phylogenetic beta-diversity was calculated using the unweighted UniFrac metric

(Lozupone and Knight 2005) to reveal whether different wastewater treatments or treatment seasons caused the presence of taxonomic groups to change. An exploratory multivariate statistics method, Principal Coordinate Analysis (PCoA), was then performed on the weighted UniFrac beta-diversity distance matrix to identify trends between different treatments or seasons. Unweighted UniFrac analyses gave similar results as weighted UniFrac results shown in the PCoA plot and are therefore not presented.

4.3.7.4 Functional Content Prediction in Bacterial Communities

To explore the functional profiles of the bacterial communities, PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) with version 1.1.0 in Microbiome Helper pipeline (https://github.com/mlangill/microbiome_helper/wiki/PICRUSt-workflow) was used to predict gene contents based on *16S rRNA* gene surveys. The PICRUSt workflow is illustrated in Figure 4.4.

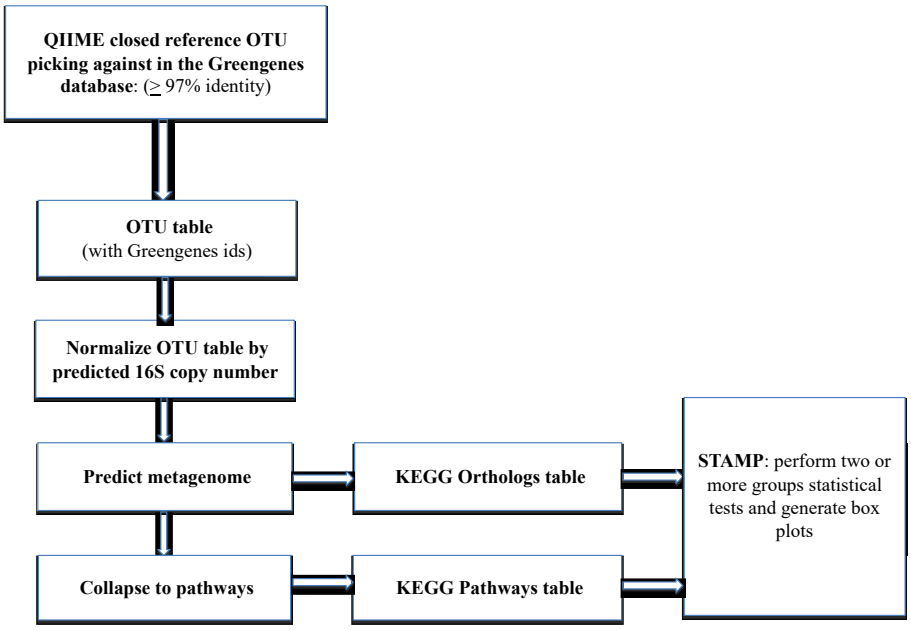


Figure 4.4 The PICRUSt workflow for predicting gene contents in bacterial communities.

The PICRUSt analysis works from the observation that there is a good correlation between the phylogenetic information contained in *16S rRNA* marker gene sequences and genomic content when related reference genomes are available (Langille et al. 2013). Thus, using 16S information, PICRUSt can accurately predicts the abundance of gene families in host-associated and environmental communities (Langille et al. 2013). Prior to the analysis, OTUs were closed-reference picked against the Greengenes database (version 13_5) at the 97% of similarity threshold by using QIIME version 1.9.1 (Caporaso et al. 2010b) (Figure 4.4). In the closed-reference OTU table, the proportion of reads that mapped to reference sequences during the OTU picking was 74.5%. In the PICRUSt analysis workflow (Figure 4.4), the first step was to correct the OTU table based on the predicted *16S rRNA* copy number for each microorganism in the OTU table, thus OTU abundances more accurately reflect the true abundance of the underlying microorganisms (Langille et al. 2013). And then the corrected OTU table was used to

perform functional predictions by looking up the precalculated genome content for each OTU, multiplying the normalized OTU abundance by each KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthologs (KOs) abundance in the genome and summing these KO abundances together per sample. Therefore, the functional prediction generated a table of KO abundances for each sample in the OTU table (Langille et al. 2013). Finally, the predicted KOs were grouped into functional categories based on KEGG pathway predictions. The step yielded a KEGG pathways table for samples (Langille et al. 2013). The accuracy of metagenome predictions depends on how closely related the microorganisms in a given sample are to the representative microorganisms with sequenced genomes, as measured by the Nearest Sequenced Taxon Index (NSTI), with lower values indicating a closer relationship and availability of closely related reference genomes (Langille et al. 2013). Low NSTI values of 0.07 ± 0.01 and 0.08 ± 0.02 were calculated for wastewater samples from Pond Inlet and Clyde River, respectively. For comparison, Langille and other researchers (2013) found that human-associated samples had the lowest (best) NSTI values (0.03 ± 0.02). Other mammalian guts had a higher mean NSTI value (0.14 ± 0.06), and diverse communities such as soil also had a much higher NSTI value (0.17 ± 0.02). Thus, arctic municipal wastewater samples appeared to constitute a suitable data set to derive functional predictions from PICRUSt.

4.3.8 Statistical Analysis

In the study of *16S rRNA* copy number determination and alpha diversity measures, differences at a 5% significance level between two groups were tested with the non-parametric t-test (Mann-Whitney test) while differences at a 5% significance level among three groups were tested using the non-parametric one-way ANOVA test (Kruskal-Wallis

test). The tests mentioned above were performed in Prism 7 (version 7.0b, Graph Pad Software, Inc., La Jolla, CA, USA). In the study of phylogenetic beta-diversity measures, the ANOSIM approach was performed in the QIIME pipeline (Caporaso et al. 2010b) to test whether there were statistically significant differences among the trends that were observed in PCoA plots. In the study of inferring functional content in the bacterial communities (Figure 4.4), significant differences at 5% level between two groups were tested using the Welch's t-test with a multiple test correction Benjamini-Hochberg FDR while significant differences at 5% level among three groups were tested using the Kruskal-Wallis H-test with a multiple test correction Benjamini-Hochberg FDR. The two tests were conducted in STAMP (statistical analysis of taxonomic and functional profiles) version 2.1.3 (Parks et al. 2014).

4.4 Results

4.4.1 Quantification of Bacterial Population Size Measured by *16S rRNA* Copy Numbers in The One-cell and Two-cell Arctic WSPs

The quantification of bacterial population size as *16S rRNA* gene copy numbers during the 2014 treatment season in Pond Inlet is depicted in Figure 4.5a. It should be noted that a similar trend was seen in 2013. Figure 4.5a showed that there was an averaging 2.03 Log *16S rRNA* copies/mL reduction along the WSP treatment process from raw wastewater (averaging 9.08 Log *16S rRNA* copies/mL) to effluent (averaging 7.05 Log *16S rRNA* copies/mL). The bacterial population size in raw wastewater stayed at a constant ($p>0.05$) level ranging from 9.07 to 9.10 Log *16S rRNA* copies/mL during the summer treatment seasons. From the beginning to the middle of the treatment season, the WSP bacterial population size increased significantly ($p<0.05$) from 8.24 to 8.85 Log *16S rRNA* copies/mL. However, at the end of treatment season, the size decreased

significantly ($p<0.05$) from 8.85 to 6.95 Log *16S rRNA* copies/mL (Figure 4.5a). The population size of bacterial community in sludge samples kept increasing from 8.85 Log *16S rRNA* copies/mL at the beginning to 10.15 Log *16S rRNA* copies/mL at the end of the treatment season in 2014. The trend of bacterial population size and the WSP temperature profile in Pond Inlet during both the 2013 and 2014 summer treatment seasons is illustrated in Figure 4.5b. In 2014, the temperature gradually increased from 11.1 to 17.8 °C at the middle of the treatment season followed by a decrease to 5.4 °C at the end of the season. The bacterial population size followed the increases in pond temperature, and also, when the temperature decreased in the later part of treatment season, the bacterial population size dropped as well. A similar trend for bacterial population size and temperature changes was seen in 2013 (Figure 4.5b), except the temperature fluctuated during the last part of the season from 15.9 °C to 8.8 °C, followed by an increase to 14.6 °C and then a gradual decrease to 2.0 °C at the end. The bacterial population size in 2014 was significantly ($p<0.05$) higher than in 2013. Ragush and other researchers (2015) found that the WSP environment was warmer in 2014 than in 2013, as indicated by the degree days above 5 °C in pond surface water temperature profile (degree days above 5 °C was 386 and 313 for 2014 and 2013, respectively).

In Clyde River, the bacterial population size along the treatment process and the pond surface temperature was determined in 2013 and 2014 (Figures 4.6a and 4.6b, respectively). The bacterial population levels in the raw wastewater ranged from 8.92 to 9.03 Log *16S rRNA* copies/mL with no significant ($p>0.05$) differences among sampling events in both years. In 2013 (Figure 4.6a), there was a significant ($p<0.05$) reduction in the bacterial population levels from the raw wastewater to the secondary pond, resulting

in a 3.33 and 2.24 Log *16S rRNA* copies/mL reduction in the beginning and the middle of the treatment season, respectively. At the end of the treatment season, treatment in the primary pond significantly ($p<0.05$) reduced bacterial populations in raw wastewater by an average of 2.88 Log *16S rRNA* copies/mL to result in average bacterial population concentrations of 6.04 Log *16S rRNA* copies/mL in the primary pond. However, in the secondary pond, bacterial populations were significantly increased ($p<0.05$) by 2.23 Log *16S rRNA* copies/mL, as compared to the primary pond, to levels of 8.27 Log *16S rRNA* copies/mL. This is likely due to the direct discharge of raw wastewater into the secondary pond, which was observed during the sampling trip at the end of the treatment season. Within the primary pond during the summer treatment season (Figure 4.6a), the highest and lowest bacterial population levels (average 8.51 and 6.04 Log *16S rRNA* copies/mL, respectively) coincided with the highest and lowest pond surface temperatures (averaging 11.50 and 3.02 °C, respectively). Within the secondary pond in Figure 4.6a, the bacterial population levels followed the similar pattern as seen in the primary pond during the early treatment season. When the pond temperature rose from 3.41 to 11.50 °C, there was a significant ($p<0.05$) growth of bacterial population from 5.66 to 6.79 Log *16S rRNA* copies/mL. However, the bacterial population kept increasing in the later part of the treatment season while the pond temperature declined from 11.50 to 3.02 °C, probably due to the direct discharge of the raw wastewater into the secondary pond mentioned above. In 2014 (Figure 4.6b), during the two-cell WSP treatment process in the summer treatment season, the bacterial population levels in raw wastewater were significantly ($p<0.05$) reduced following treatment in the primary pond and then in the secondary pond, resulting in final reductions of 1.34 and 1.64 Log *16S rRNA* copies/mL in the beginning

and end of the treatment season, respectively. Within both primary and secondary ponds in 2014 from the beginning to end treatment seasons (Figure 4.6b), the decreasing temperature (from 6.42 to 4.33 °C) likely caused the significant ($p<0.05$) reduction of bacterial population concentrations by 0.37 and 0.34 Log *16S rRNA* copies/mL in the primary and secondary pond, respectively. Despite the observed enrichment of bacterial population in the secondary pond probably caused by the flexible use of the two-cell WSP, the reduction level of bacterial population along the treatment process from the raw wastewater to the secondary pond was smaller in 2014 (ranging from 1.34 to 1.64 Log *16S rRNA* copies/mL) than in 2013 (ranging from 2.24 to 3.33 Log *16S rRNA* copies/mL). According to the degree days calculations (Ragush et al. 2015), the secondary pond had relatively warmer environment in 2014 (degree days above 5 °C: 300) than in 2013 (degree days above 5 °C: 246), probably causing the observed differences of the reduction level for bacterial population along the treatment process between 2013 and 2014.

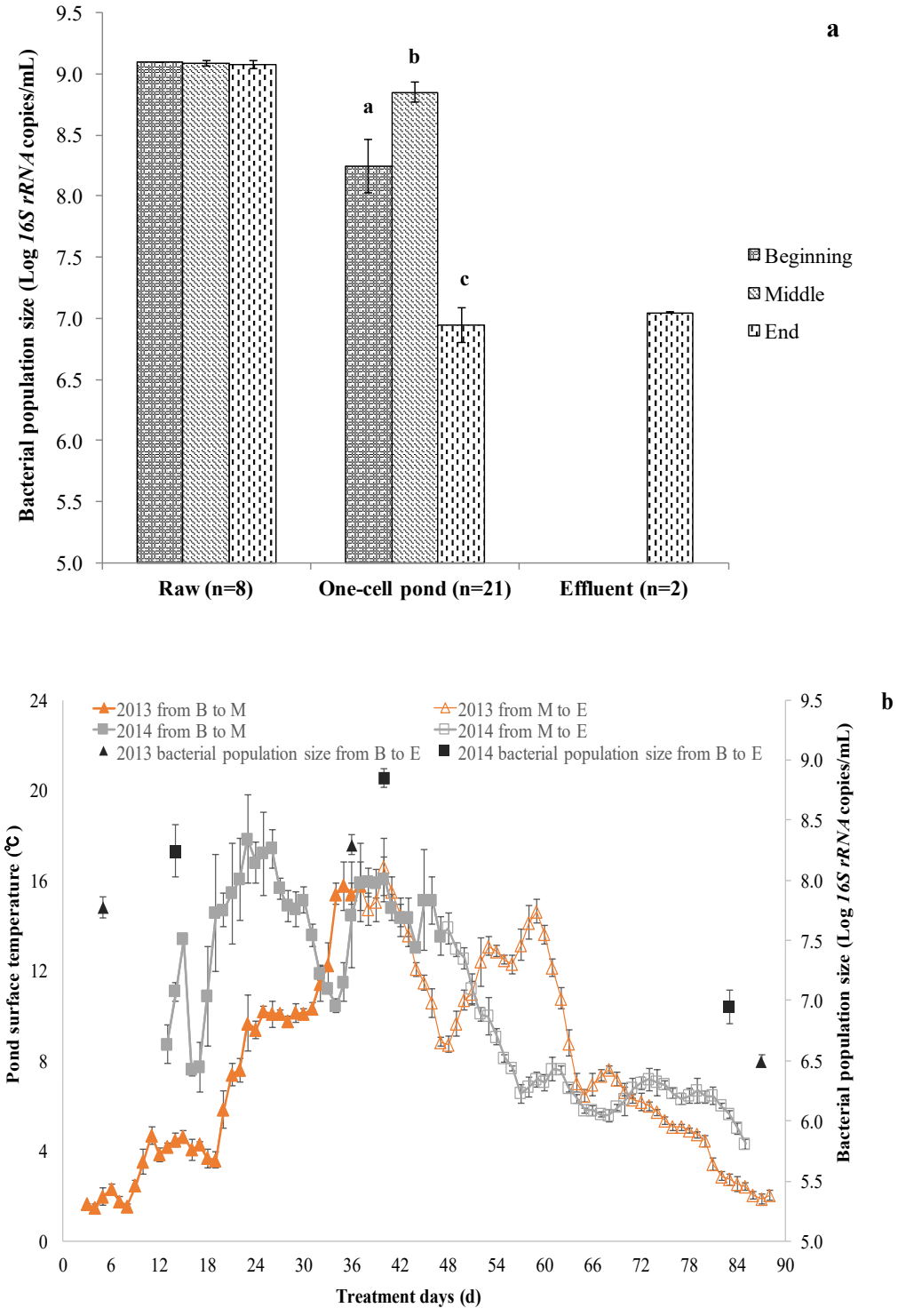
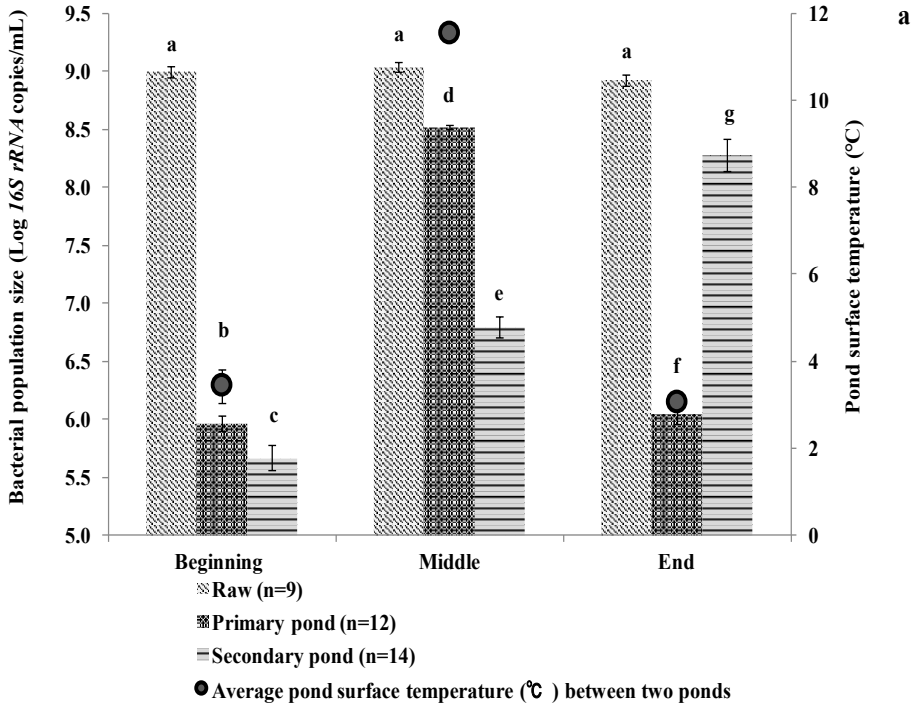


Figure 4.5 The average bacterial population levels (Log *16S rRNA* copies/mL) and the surface pond temperature profile in Pond Inlet during the treatment seasons of 2013 and 2014. a) The average bacterial population levels (Log *16S rRNA* copies/mL) measured in

raw (untreated), one-cell pond and effluent wastewater samples at the beginning, middle and end of the 2014 treatment season. Error bars indicate the standard deviation. Different letters within the same sampling site indicate significant differences ($p<0.05$) as determined by the Kruskal-Wallis test. b) The average bacterial population levels (Log *16S rRNA* copies/mL) and the average pond surface temperature measured in the one-cell pond at the beginning, middle and end of the 2013 and 2014 treatment seasons. Error bars in the data points of bacterial population size indicate the standard deviation. In the temperature profile, each data point represents daily averages of hourly measurements (n=24, mean $\pm$ standard deviation). Closed symbols indicate values obtained between the beginning (B) and middle (M) of the treatment season while the open symbols indicate values obtained between M and the end (E) of the treatment season.



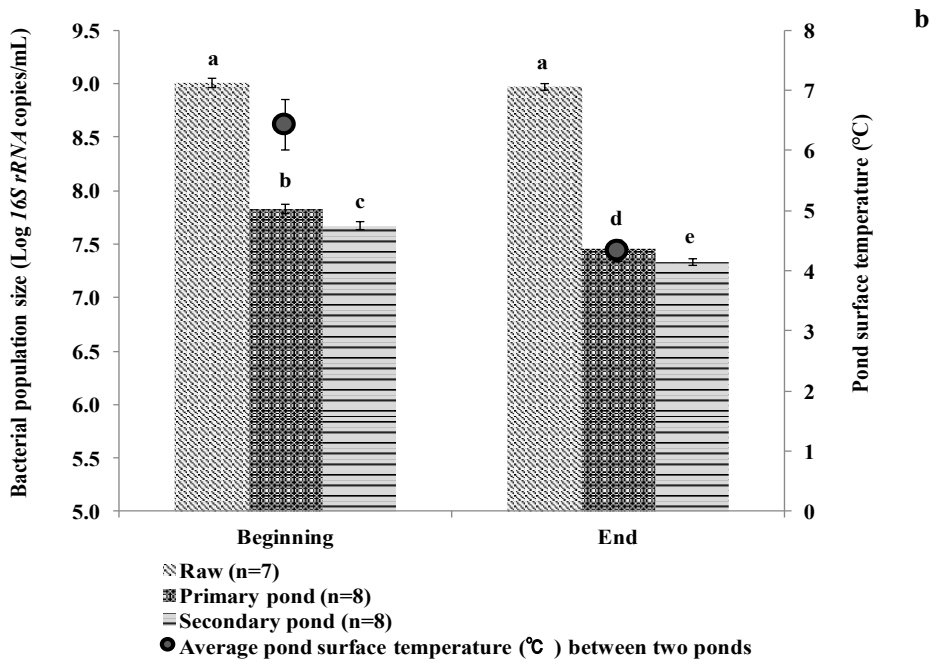


Figure 4.6 The average bacterial population size (Log *16S rRNA* copies/mL) in raw (untreated) wastewater and samples from the primary and secondary ponds and the average surface pond temperature between two ponds obtained in Clyde River during the treatment seasons of 2013 a) and 2014 b). Error bars indicate standard deviations. Different letters within the same treatment season indicate significant differences ($p<0.05$) as determined by the Kruskal-Wallis test.

4.4.2 The Diversity, Composition and Distribution of Bacterial Communities in Arctic WSPs Across Two Communities in Nunavut

4.4.2.1 Alpha Diversity Measures

Alpha diversity was calculated to characterize the diversity of the bacterial community within each wastewater sample during 2012 to 2014 treatment seasons in Pond Inlet (Table 4.1) and Clyde River (Table 4.2).

Table 4.1 Alpha diversity indices within wastewater samples in Pond Inlet.

Sampling year	Sampling season	Sample type (sample size)	Observed_OTUs	Chao1	Shannon-Wiener	Simpson_e
2012	End	Raw (n=4)	$327^{A}\pm6.26^{a}$	$418^{A}\pm2.72$	$4.87^{A}\pm0.04$	$0.05^{A}\pm0.002$
		Pond (n=4)	$194^{B}\pm3.54$	$262^{B}\pm9.48$	$4.07^{B}\pm0.07$	$0.04^{A}\pm0.002$
		Effluent (n=3)	$195^{B}\pm4.95$	$264^{B}\pm1.19$	$3.53^{C}\pm0.03$	$0.03^{A}\pm0.020$
2013	Beginning	Raw (n=2)	$324^{A}\pm3.94$	$414^{A}\pm4.16$	$4.86^{A}\pm0.16$	$0.05^{A}\pm0.021$
		Pond (n=5)	$217^{C}\pm1.52$	$316^{C}\pm7.7$	$3.91^{B}\pm0.10$	$0.04^{A}\pm0.014$
	Middle	Raw (n=3)	$322^{A}\pm6.56$	$416^{A}\pm6.68$	$5.01^{A}\pm0.18$	$0.04^{A}\pm0.013$
		Pond (n=7)	$250^{D}\pm1.60$	$351^{D}\pm6.51$	$4.49^{D}\pm0.02$	$0.03^{A}\pm0.012$
	End	Raw (n=2)	$320^{A}\pm3.03$	$414^{A}\pm9.82$	$5.04^{A}\pm0.14$	$0.04^{A}\pm0.002$
		Pond (n=7)	$192^{B}\pm0.71$	$298^{E}\pm7.81$	$3.60^{C}\pm0.09$	$0.03^{A}\pm0.002$
		Effluent (n=6)	$190^{B}\pm4.95$	$265^{B}\pm1.77$	$3.43^{E}\pm0.04$	$0.02^{A}\pm0.010$
2014	Beginning	Raw (n=3)	$322^{A}\pm6.16$	$413^{A}\pm8.12$	$5.03^{A}\pm0.10$	$0.06^{A}\pm0.030$
		Pond (n=7)	$218^{C}\pm1.31$	$319^{C}\pm4.61$	$4.10^{B}\pm0.09$	$0.03^{A}\pm0.010$
	Middle	Raw (n=3)	$319^{A}\pm8.49$	$418^{A}\pm2.78$	$5.05^{A}\pm0.18$	$0.05^{A}\pm0.008$
		Pond (n=7)	$276^{E}\pm1.24$	$374^{F}\pm3.71$	$4.59^{F}\pm0.03$	$0.03^{A}\pm0.010$
	End	Raw (n=2)	$324^{A}\pm4.55$	$415^{A}\pm1.93$	$4.82^{A}\pm0.15$	$0.04^{A}\pm0.001$
		Pond (n=7)	$199^{B}\pm1.73$	$326^{G}\pm4.47$	$4.08^{B}\pm0.18$	$0.02^{A}\pm0.010$
		Effluent (n=2)	$195^{B}\pm1.71$	$267^{B}\pm6.38$	$3.56^{C}\pm0.15$	$0.03^{A}\pm0.010$

A-G: different letters in the same column indicated that the significant differences ($p<0.05$) were detected by the Kruskal-Wallis test.

[a]: average of calculated values in biological replicates with two technical duplicates (mean ± standard deviation).

No significant ($p>0.05$) differences were detected in terms of the alpha diversity

values for pond samples obtained in the same sampling event in Pond Inlet, suggesting that there were no spatial or vertical differences in the alpha diversity of the bacterial communities within the one-cell WSP. Therefore, the seven WSP samples were grouped together. Also, for raw wastewater sampled from different trucks (at least two trucks per trip), there were no significant ($p>0.05$) differences in the alpha diversity. Therefore, raw wastewater samples were pooled for each sampling trip. A similar trend was seen for the effluent samples obtained every four hours during each decant event (2012-2014), and these samples were therefore pooled as well. In Pond Inlet, as shown in Table 4.1, the wastewater treatment and seasonal factors showed a significant ($p<0.05$) relationship to the number of observed OTUs, the richness estimator (Chao1) and Shannon-Wiener diversity index, respectively, but not the evenness measure as shown by Simpson_e index. During the beginning and middle of the summer treatment season in 2013 and 2014, the number of observed OTUs in the raw wastewater samples was significantly ($p<0.05$) higher than that in the pond samples. During the end of the summer treatment seasons including decant events from 2012 to 2014, the raw wastewater samples had significantly ($p<0.05$) higher observed OTUs numbers than the pond and effluent samples. The number of observed OTUs between the pond and effluent samples were not significantly ($p>0.05$) different. Different parts of summer treatment seasons in 2013 and 2014 appeared linked to the number of observed OTUs in pond samples, where the number of observed OTUs was highest mid-season followed by the beginning and end of the season. When comparing 2013 and 2014, the mid-season pond samples contained significantly ($p<0.05$) higher observed OTUs in 2014 than in 2013, while no differences ($p>0.05$) were detected at the beginning and end of the summer treatment seasons. Regardless of

summer treatment seasons (from beginning to end) or sampling years (2012, 2013 and 2014), the numbers of observed OTUs in the raw wastewater samples did not vary significantly ($p>0.05$). For the richness estimator (Chao1), at the both beginning and middle of summer treatment seasons in 2013 and 2014, the Chao1 index indicated that the bacterial community was significantly ($p<0.05$) richer in the raw wastewater than in pond samples. In later part of summer treatment seasons during the decant events in both 2013 and 2014, the Chao1 index was highest in the raw wastewater samples and lowest in the effluent, suggesting that bacterial community richness decreased along the treatment train. However, this trend was only partially observed during the decant event in 2012, where the richness of the raw wastewater was significantly ($p<0.05$) higher than in the pond and effluent samples (similar Chao1 indices, Table 4.1). Similar to the trend for the number of observed OTUs in the raw wastewater samples, the Chao1 index in the raw wastewater stayed at a constant level ($p>0.05$), showing that the richness of bacterial community in raw wastewater showed no seasonal or annual differences. However, the richness of bacterial community in the WSP varied during the summer treatment seasons in 2013 and 2014, where the mid-season samples harboured the highest richness. The Shannon-Wiener diversity index similarly showed that diversities in raw wastewater samples were significantly ($p<0.05$) higher than in pond and effluent samples, suggesting that the bacterial community was the most diverse in raw wastewater. By only looking at the raw wastewater sampled over three years, the diversity of the bacterial communities was likely to be stable as the Shannon-Wiener index did not differ significantly ($p>0.05$). Similarly to the OTU richness and Chao1 indices, the Shannon-Wiener index revealed that the diversity of bacterial communities varied in the pond during the different

treatment seasons with the mid-season samples showing the highest bacterial communities diversity. For the evenness of bacterial communities, all wastewater samples had similar ($p>0.05$) Simpson_e index values, regardless of the sample type or year.

For the sludge samples collected in 2014, all four alpha diversity indices kept increasing from the beginning to the end of the treatment season, showing that the richness, Shannon diversity and Simpson evenness of bacterial communities increased during the 2014 summer treatment season.

Table 4.2 Alpha diversity indices within wastewater samples in Clyde River.

Sampling year	Sampling season	Sample type (sample size)	Observed_OTUs	Chao1	Shannon-Wiener	Simpson_e
2012	End	Primary pond (n=2)	$224^{A}\pm10.61^{a}$	$251^{A}\pm10.02$	$2.91^{A}\pm0.14$	$0.05^{A}\pm0.020$
		Secondary pond (n=2)	$218^{A}\pm6.16$	$233^{A}\pm10.45$	$2.97^{A}\pm0.05$	$0.03^{A}\pm0.010$
2013	Beginning	Raw (n=3)	$353^{B}\pm8.99$	$453^{B}\pm9.97$	$5.15^{B}\pm1.02$	$0.02^{A}\pm0.011$
		Primary pond (n=4)	$194^{C}\pm6.61$	$271^{C}\pm1.41$	$2.70^{C}\pm0.06$	$0.02^{A}\pm0.010$
		Secondary pond (n=4)	$139^{D}\pm7.07$	$218^{D}\pm4.39$	$2.66^{C}\pm0.03$	$0.02^{A}\pm0.008$
	Middle	Raw (n=2)	$358^{B}\pm6.90$	$461^{B}\pm10.3$	$5.27^{B}\pm0.92$	$0.06^{A}\pm0.020$
		Primary pond (n=4)	$302^{E}\pm5.96$	$341^{E}\pm10.3$	$4.21^{D}\pm0.53$	$0.04^{A}\pm0.016$
		Secondary pond (n=4)	$167^{F}\pm9.80$	$261^{A}\pm5.42$	$4.19^{D}\pm0.11$	$0.02^{A}\pm0.012$
	End	Raw (n=4)	$358^{B}\pm9.6$	$464^{B}\pm10.68$	$5.23^{B}\pm0.64$	$0.04^{A}\pm0.014$
		Primary pond (n=4)	$128^{G}\pm2.12$	$242^{A}\pm0.69$	$2.44^{E}\pm0.03$	$0.06^{A}\pm0.020$
		Secondary pond (n=6)	$198^{C}\pm9.21$	$293^{F}\pm10.57$	$3.97^{F}\pm0.16$	$0.04^{A}\pm0.006$

Sampling year	Sampling season	Sample type (sample size)	Observed_OTUs	Chao1	Shannon-Wiener	Simpson_e
2014	Beginning	Raw (n=3)	$353^{B}\pm2.83$	$464^{B}\pm7.09$	$5.28^{B}\pm0.64$	$0.03^{A}\pm0.010$
		Primary pond (n=4)	$245^{H}\pm10.51$	$261^{A}\pm6.71$	$3.06^{A}\pm0.15$	$0.06^{A}\pm0.030$
		Secondary pond (n=4)	$223^{A}\pm3.54$	$216^{D}\pm9.53$	$2.96^{A}\pm0.05$	$0.03^{A}\pm0.010$
	End	Raw (n=4)	$353^{B}\pm11.31$	$454^{B}\pm9.45$	$5.17^{B}\pm0.84$	$0.04^{A}\pm0.033$
		Primary pond (n=4)	$219^{A}\pm3.54$	$224^{D}\pm5.18$	$2.73^{C}\pm0.11$	$0.05^{A}\pm0.005$
		Secondary pond (n=4)	$206^{C}\pm8.49$	$195^{G}\pm8.17$	$2.76^{C}\pm0.08$	$0.05^{A}\pm0.020$

A-G: different letters in the same column indicated that the significant differences ($p<0.05$) were detected by the Kruskal-Wallis test.

[a]: average of calculated values in biological replicates with two technical replicates (mean ± standard deviation).

Similar to Pond Inlet, samples obtained from the different pond sampling sites in Clyde River (Table 4.2) did not show any significant differences ($p>0.05$) in alpha diversity, indicating the absence of spatial or vertical distribution differences within the primary and secondary ponds. Wastewater samples obtained from each pond in the same sampling event were therefore pooled together in the assessment of the alpha diversity. A similar approach could be used for the raw wastewater samples obtained from different trucks in 2013 and 2014. In Clyde River (Table 4.2), the two-cell WSP treatment process and the summer treatment season significantly ($p<0.05$) influenced the number of observed OTUs, the richness estimator (Chao1) and Shannon-Wiener diversity, respectively, but did not affect the Simpson_e evenness measure. In 2012, the alpha diversity indices were not significantly ($p>0.05$) different among the primary and secondary ponds, which may, however, be due to the limited sample size ($n=2$ for each pond). During the beginning and middle of the summer treatment season in 2013 and the summer treatment season in 2014, the number of observed OTUs and the value of Chao1 were highest in the raw wastewater samples and lowest in the treated wastewater samples in the secondary pond, indicating that the bacterial community was richest in the raw wastewater, following by the primary pond and then the secondary pond. However, at the end of the 2013 summer treatment season, a reduction in the two indexes was seen from the raw wastewater to the primary pond, while there was an increase in the indices from the primary to the secondary pond. The direct discharging of raw wastewater into the secondary pond seen during this sampling trip was most likely the cause of this enrichment of the bacterial community alpha diversity in the secondary pond. In 2014, with the proper use of the two-cell WSP system, the decreasing trend for the richness of

bacterial community along the treatment process was observed. Within the raw wastewater samples, the numbers of observed OTUs and Chao1 index did not vary significantly ($p>0.05$) among any of the 2013 and 2014 sampling events, indicating that the richness of bacterial community in the incoming raw wastewater was stable. Within the primary pond, the treatment season significantly ($p<0.05$) affected the bacterial community richness with it being highest mid-season 2013 and lower in the beginning and end of the summer treatment season. A similar trend was seen in 2014 where the bacterial community was richer in the beginning than in the end of the treatment season. Within the secondary pond, due to the flexible use of the two-cell system in 2013, the summer treatment season did not affect the richness of bacterial community in the same way as shown within the primary pond. Instead, the richness of bacterial community kept increasing from the beginning to the end of summer treatment season. However, when the proper use of the system was observed in 2014, the richness of bacterial community was higher in the beginning than in the end of summer treatment season as the similar tendency was observed in the primary pond.

For the Shannon-Wiener diversity measure, the untreated and treated wastewater process caused the bacterial community to have different diversity levels. During each treatment season except the end treatment season in 2013, the raw wastewater samples had a significantly ($p<0.05$) higher Shannon-Wiener diversity index than the treated wastewater sampled either in the primary pond or in the secondary pond. Between the primary and secondary ponds, the diversity index did not differ significantly ($p>0.05$). The treatment season significantly ($p<0.05$) influenced the diversity measure for the wastewater obtained either from the primary or the secondary ponds, but did not

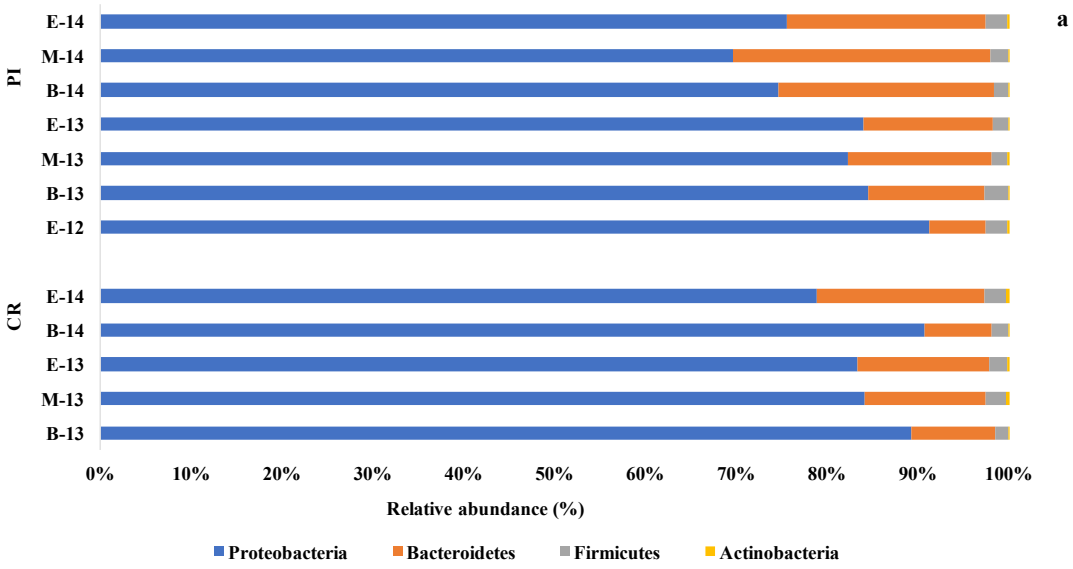
significantly ($p>0.05$) affect the diversity of bacterial communities in untreated wastewater samples, suggesting a stable bacterial community in raw wastewater across the two-year study period. Looking at the primary pond, the diversity level was the highest in the middle, followed by the beginning and end of summer treatment season. Within the secondary pond between the middle and end treatment season, the diversity index stayed at a similar level with no significant ($p>0.05$) change and was significantly ($p<0.05$) higher than that in the beginning of the season. In the summer treatment season of 2014 in both primary and secondary ponds, the diversity was significantly ($p<0.05$) higher in the beginning than in the end of the treatment season. For the evenness of bacterial communities measured by the Simpson_e index, there was no significant ($p>0.05$) difference among all wastewater samples regardless of the sampling site along the treatment process, seasons and year, suggesting that none of these factors affected the evenness of wastewater bacterial communities.

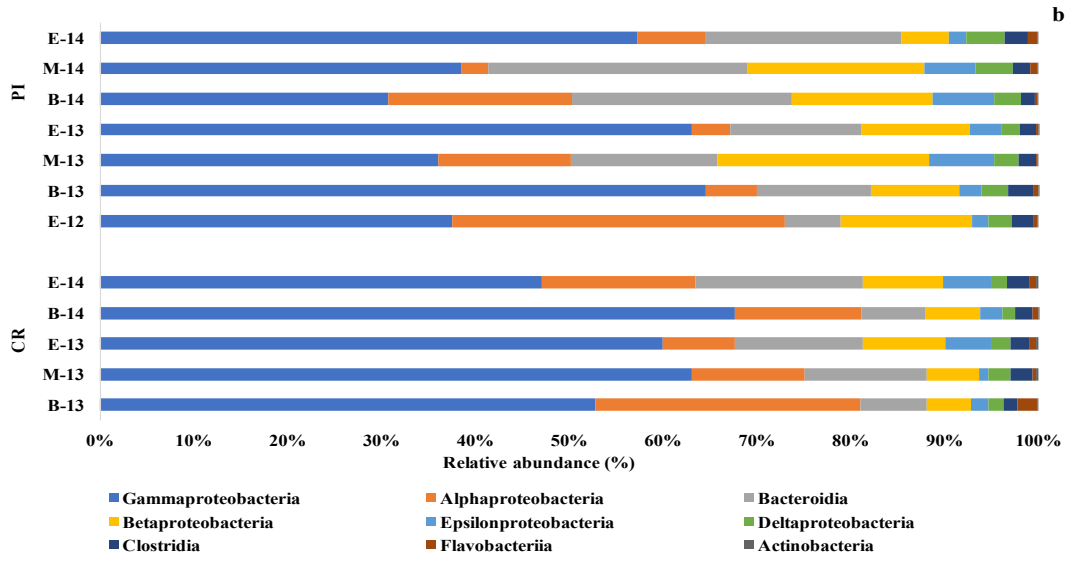
4.4.2.2 The Beta Diversity Measures and Core Microbiome Composition in Raw Wastewater

The beta diversity of bacterial communities in raw wastewater samples were compared between Pond Inlet and Clyde River. The weighted and unweighted UniFrac measures showed that the bacterial beta diversity was not significantly ($p>0.05$) different among raw wastewater samples from the two communities during the study period, showing quantitatively and qualitatively similarities in the composition of bacterial communities at two different geographical locations (Pond Inlet: latitude 72° 41′ 57″ N, longitude 77° 57′ 33″ W; population: 1549 and Clyde River: latitude 70° 28′ 26″ N, longitude 68° 35′ 10″ W; population: 934). The relative abundance of bacteria at the phyla, class and genera

levels in the raw wastewater samples (n=34 in total) are shown in Figure 4.7a-c. There were four bacterial phyla detected in 100% of the raw wastewater samples, with Proteobacteria being the predominant phylum, constituting from 70% to 91% of all OTUs in all wastewater samples (Figure 4.7a). Bacteroidetes, Firmicutes and Actinobacteria were the subdominant phyla, comprising 6.3 to 28.4%, 1.5 to 2.7%, 0.1 to 0.2%, respectively. Looking at the class level (Figure 4.7b), within Proteobacteria, Gammaproteobacteria was the largest group (30.7 to 67.6%), followed by Alphaproteobacteria (2.8 to 35.4%), Betaproteobacteria (4.8 to 22.5%), Epsilonproteobacteria (1.1 to 7.0%) and Deltaproteobacteria (1.4 to 4.2%). Within Bacteroidetes, two classes were present. Bacteroidia was the dominant group ranging from 6.0 to 27.6% of all raw wastewater samples while another class Flavobacteria was found in less than 5% of all samples with abundances ranging from 0.2 to 2.2%. Within Firmicutes, Clostridia was present with the relative abundance ranging from 1.5 to 2.7% in all wastewater samples. Lastly, Actinobacteria was found in low abundance (0.1-0.3%) in all raw samples. There were 31 genera shared by all of the 34 raw wastewater samples collected from Pond Inlet and Clyde River communities. The compositions of the 10 most abundant genera belonging to Proteobacteria and Bacteroidetes were summarized in Figure 4.7c. Within Gammaproteobacteria, there were five identified taxa. Unclassified genera belonging to the *Aeromonadaceae* family were the dominant group, comprising 13.59 to 34.0%. The following subdominant genera were *Tolumonas, Pseudomonas*, *Citrobacter* and *Serratia,* comprising 7.9 to 23.2%, 4.5 to 13.8%, 1.1 to 9.6% and 0.7 to 8.5%, respectively. One genus, belonging to Alphaproteobacteria, was identified as *Novispirillum* with its relative abundance ranging from 5.3 to 28.2% in raw wastewater

samples. Within Betaproteobacteria, *Comamonas* was present with the relative abundance of 1.0 to 18.1% in all wastewater samples, respectively. The relative abundance of *Arcobacter*, from Epsilonproteobacteria, ranged from 1.1 to 8.3% in the raw wastewater samples. Within Bacteroidia, the relative abundance of *Bacteroides* and unclassified genera belong to the *Porphyromonadaceae* family represented 4.0 to 16.6% and 2.1 to 9.9%, respectively.





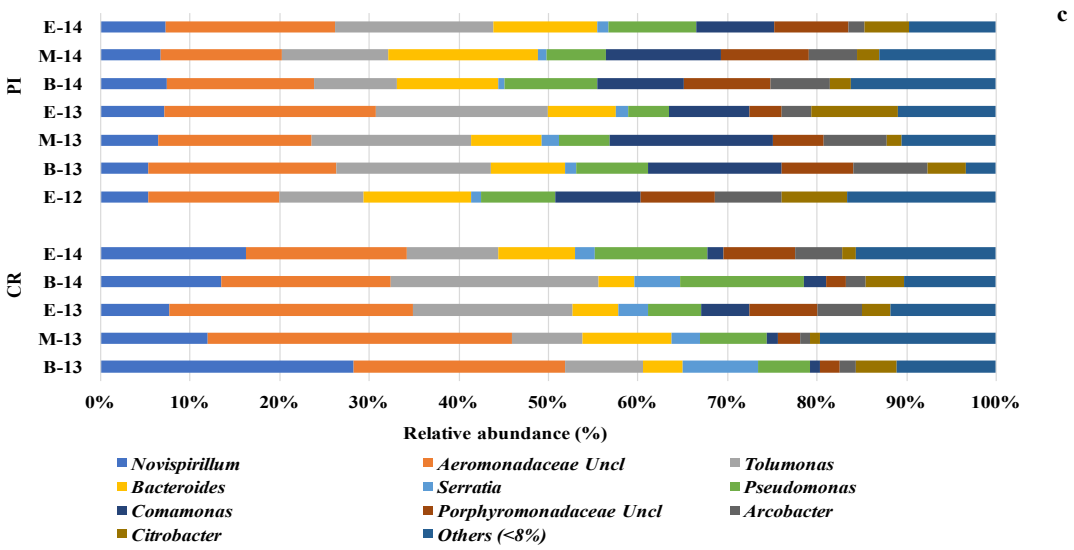


Figure 4.7 The composition of the core microbiome in all raw wastewater samples (n=34 in total) obtained from Pond Inlet (PI) and Clyde River (CR) over the 2012-2014 summer treatment seasons at the phyla a), class b) and genera c) levels, respectively. Legend: B – Beginning, M – Middle, E – End of summer treatment season. -12, -13 and -14 indicate 2012, 2013 and 2014 sampling years, respectively. Uncl: unclassified. Others (<8%) represent the bacterium including the unclassified with its relative abundance less than 8% was pooled into one group. The relative abundance of each phylum/class/genus for each treatment season was presented as the mean value of the number of raw wastewater samples collected.

4.4.2.3 Phylogenetic Beta Diversity in Samples from the One-Cell WSP in Pond Inlet, NU

The beta diversity in pond samples from the Pond Inlet WSP were assessed to study whether the diversity of bacterial community was affected by a) the WSP treatment train b) the sampling year and c) the time of sampling within the summer treatment season. The results of both weighted and unweighted UniFrac measures showed that the WSP treatment train significantly ($p<0.05$) affected the phylogenetic beta diversity of bacterial communities quantitatively and qualitatively. Also, results from 2013 and 2014 showed the time of sampling within the summer treatment season significantly ($p<0.05$)

influenced the diversity of bacterial community in both pond water and sludge samples. The distribution and composition of bacterial communities in 2014 based on the weighted UniFrac results are presented in Figure 4.8 as an example of this.

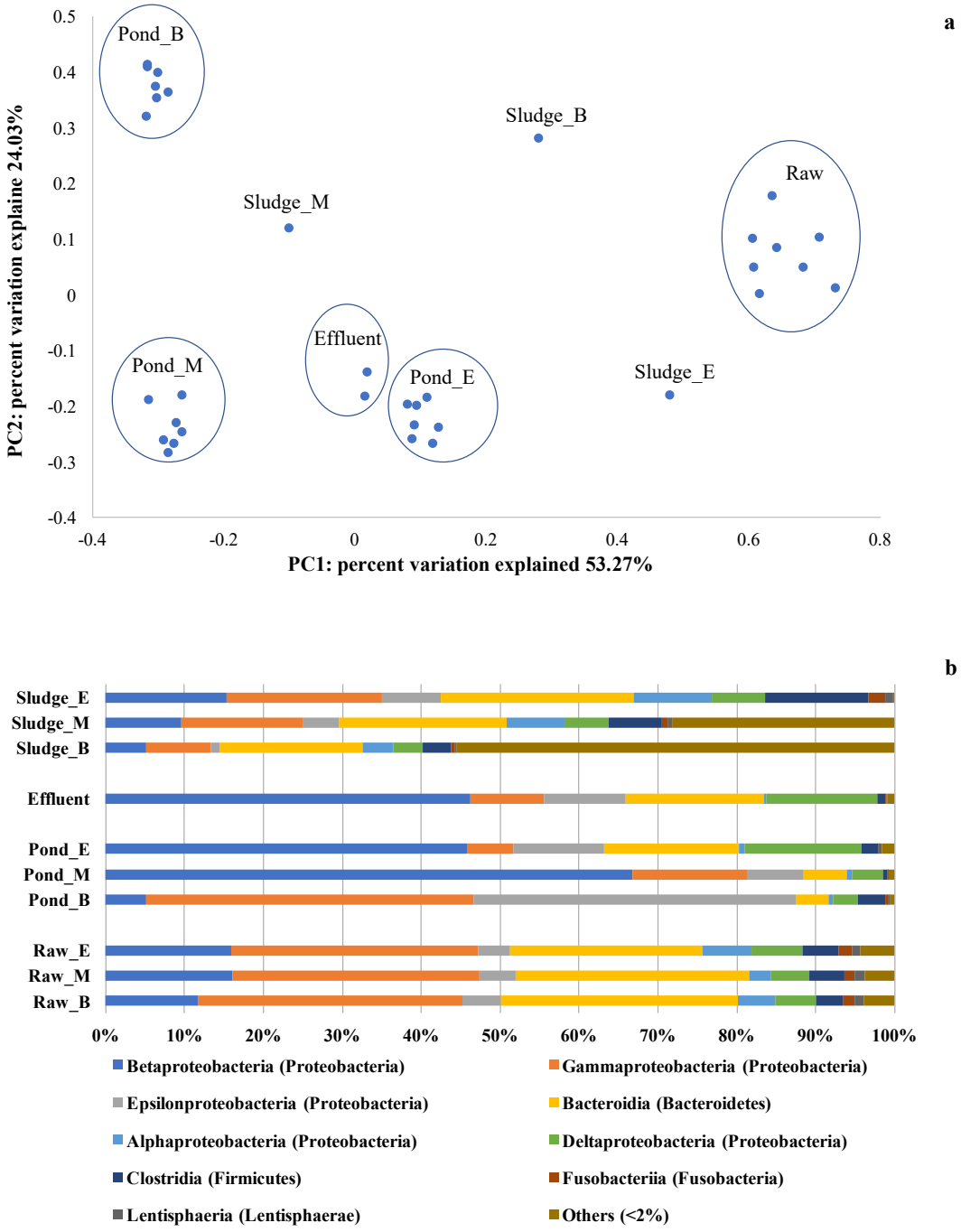


Figure 4.8 The diversity and distribution of bacterial communities along the one-cell WSP process in Pond Inlet during the 2014 summer treatment season. a) The phylogenetic beta diversity of bacterial community measured by the weighted UniFrac metric in the PCoA plot, and b) Relative abundance of bacterial classes. Legend: B – Beginning, M – Middle, E – End of summer treatment season, respectively. The bacterial taxonomic assignment is presented in the class level and the corresponding phylum is presented in the brackets after the class. Others (2%) indicate that the bacteria class including the unclassified with its relative abundance less than 2% was grouped into one bin. The relative abundance of each bacterial class for each treatment season was presented as the mean value of the number of wastewater samples collected, except sludge samples (only one composite sample was collected in each sampling event in 2014).

Also, the quantitative and qualitative diversity of the bacterial communities differed ($p<0.05$) in pond samples obtained at the same treatment stage in 2013 and 2014. A heat map of the distribution of the most abundant bacterial community members in pond samples from 2013 and 2014 is presented in Figure 4.9. The details of results shown in Figures 4.8 and 4.9 are described in following parts.

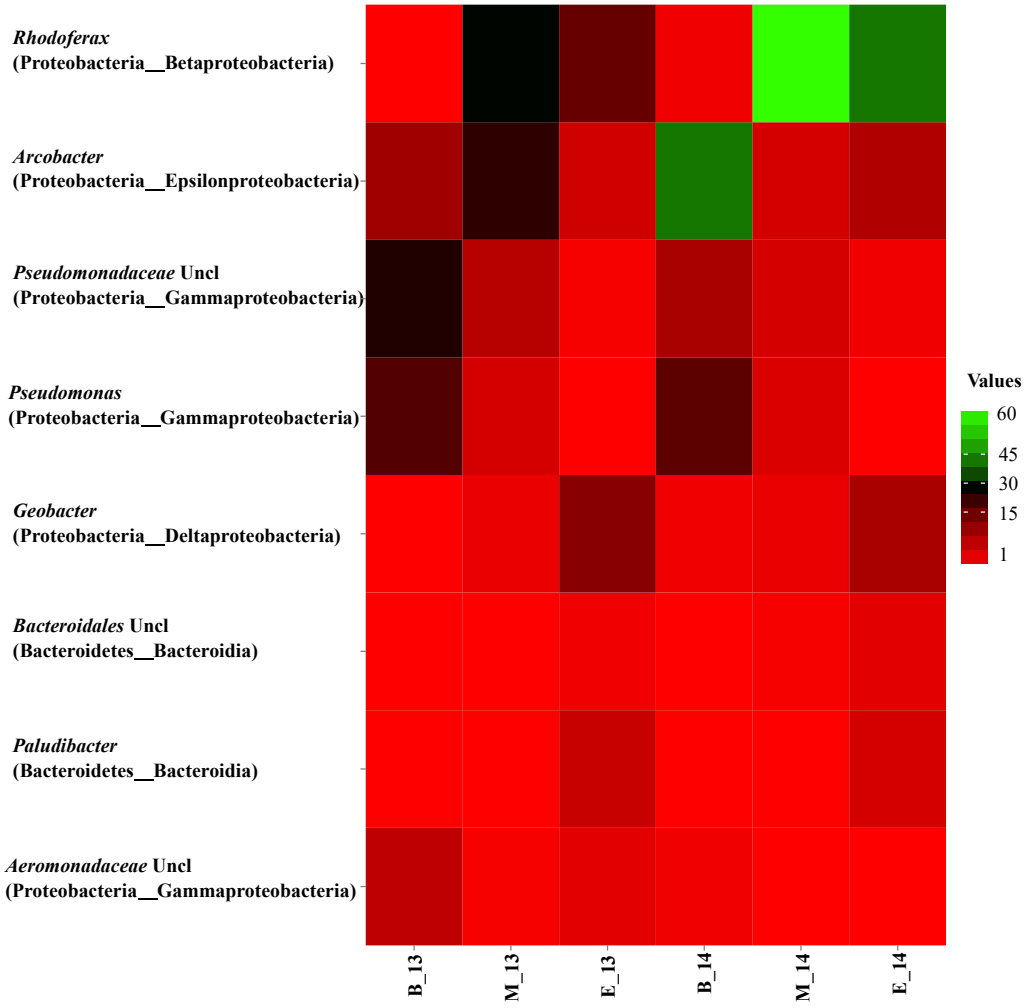


Figure 4.9 Heat map of the 8 most abundant genera in treated wastewater samples collected during the summer treatment seasons between 2013 and 2014 in Pond Inlet WSP. Legend: B – Beginning, M – Middle, E – End of summer treatment season, respectively. -13 and -14 indicate 2013 and 2014 sampling years, respectively. The bacterial taxonomic name is assigned in the genus level and the corresponding phylum and class label are presented in the brackets after the genus label. Uncl: unclassified. The relative abundance of bacterial genus for each treatment season in each year was presented as the mean value of treated wastewater samples collected from the Pond Inlet WSP.

Figure 4.8a shows that the weighted UniFrac measure in the PCoA plot for the dissimilarities in the bacterial diversity of wastewater samples obtained in 2014. The distribution and composition of bacterial communities in samples of raw wastewater,

pond, effluent and sludge are presented at the class level in Figure 4.8b. The PCoA plot shows that 53.27% of the variation in microbial diversity depended on the treatment process. Also, from the beginning to the end of treatment, the trends of dissimilarity of microbial diversity along the treatment train seemed to temporally resolve along PC2 (24.03% variation explained).

The classes (relative abundance > 2%) belonging to five phyla (Proteobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Lentisphaerae) were identified in all wastewater samples (Figure 4.8b). The composition of the raw wastewater samples did not change over the treatment season, as the abundance of each taxonomic group remained unaltered. Within Proteobacteria, Gammaproteobacteria constituted 31.3 to 33.5%, while the Beta-, Delta-, Alpha- and Epsilon-proteobacteria were the subdominant groups with relative abundances ranging from 11.7 to 16.0%, 4.9 to 6.5%, 2.7 to 6.2% and 4.1 to 4.9%, respectively. Within Bacteroidia, Bacteroidetes was present, comprising 24.4 to 30.0% of all raw wastewater samples. Clostridia (Firmicute) was present in a relative abundance of 3.5 to 4.6%. The Fusobacteriia class (Fusobacteria phylum) and the Lentisphaeria class (Lentisphaerae phylum) made up 1.3-1.6% and 1.1-1.3%, respectively, of the community. Among the pond samples, the distribution of subgroups with Proteobacteria varied during the summer treatment season. The relative abundance of the Betaproteobacteria class increased from 5.2 in the beginning to 66.8% in the mid-season and then decreased to 45.8% in the later part of the treatment season. The relative abundance of the Gammaproteobacteria class kept decreasing from initial levels of 41.4% to only 5.9% at the end treatment season. However, the relative abundance of the Deltaproteobacteria class kept increasing from the beginning (3.1%) to the end (14.8%)

of the treatment season. The decreasing trend for the relative abundance of Epsilonproteobacteria was seen from the beginning (40.8%) to the middle (7.1%) of the season and then an increasing trend was seen in the later part of treatment season (11.5%). The relative abundance of Alphaproteobacteria stayed at a low constant level (0.6 to 0.7%) over the treatment season. The relative abundance of Bacteroidia class rose from 4.2% in the beginning to 17.0% in the end treatment season. Low levels of Clostridia (0.6%) were seen in the middle of the season, down from initial levels of 3.4%. Members of the Fusobacteriia and Lentisphaeria classes remained at levels lower than 0.6% throughout the summer treatment season. Looking at the bacterial community in sludge samples, the relative abundance of each taxonomic group changed from the beginning to the end of the treatment season. At the end treatment season, the Bacteroidia class made up 24.3%, while Gammaproteobacteria, Betaproteobacteria and Clostridia represented 19.6, 15.3 and 13.1% of all sludge samples, respectively. The Alphaproteobacteria, Epsilonproteobacteria and Deltaproteobacteria were the minor groups, with the relative abundances of 9.9%, 7.6% and 6.7%, respectively. Again, Fusobacteriia and Lentisphaeria occurred in low abundances of 2.2% and 1.0%, respectively, in all sludge samples. The distribution and composition of bacterial community differed along the treatment process. For example, Gammaproteobacteria and Bacteroidia classes dominated in the raw wastewater samples. In sludge samples, the relative abundances of Gammaproteobacteria and Bacteroidia classes increased from the beginning to the end of the treatment season. In contrast in treated wastewater samples, the most two abundant groups were not always Gammaproteobacteria and Bacteroidia. For example, in the beginning of the summer treatment season, Gammaproteobacteria was the most dominant

group with the relative abundance of 41.4% with Epsilonproteobacteria that replaced Bacteroidia occurring at a similar relative abundance of 40.8%. The relative abundance of Bacteroidia dropped from 30.0% in raw wastewater samples to 4.2% in pond samples. Betaproteobacteria and Gammaproteobacteria were the most abundant groups mid-season, while Betaproteobacteria and Bacteroidia became the most dominant groups at the end of the treatment season. Effluent samples resembled the pond samples also obtained during the decant event in terms of the distribution and composition of bacterial community.

The heat map in Figure 4.9 showed that for eight of 10 genera the distribution over the summer treatment season exhibited similar trends in both 2013 and 2014. For example, the relative abundances of *Pseudomonas* decreased from the beginning (21-22%) to the end (0.5-0.9%) of the season in both 2013 and 2014. In contrast, the relative abundance of *Geobacter* and *Paludibacter* increased in samples over the season. For instance, in 2013, the relative abundance of *Geobacter* and *Paludibacter* increased from 0.3 to 17.1% and from 0.1 to 9.0%, respectively. *Rhodoferax* were abundant in mid-season in both 2013 (30.0%) and 2014 (58.6%), up from a low relative abundance of 1-2% in the beginning of the season. However, the unclassified genera in the *Aeromonadaceae* family fell to low relative abundances mid-season (2.0% in 2013 and 0.5% in 2014) compared to a higher relative abundance in the beginning of the treatment season (9.6% in 2013 and 2.6% in 2014). The remaining two genera showed different distribution trends between 2013 and 2014. In 2013, the relative abundance of *Arcobacter* increased from initial levels of 13.4% to mid-season levels of 25.8% and then decreased to 7.5% at the end of the treatment season. However, in 2014, its relative abundance decreased from 40.1 to 7.1% during the early treatment season, which was followed by an increase to 11.4% in the

later part of the summer treatment season. The abundance and distribution of *Dechloromonas* was lower in 2013 compared to 2014. Early in the 2013 treatment season, the unclassified genera in the *Pseudomonadaceae* family and classified *Pseudomonas* were the first and second dominant groups, however, in 2014, *Arcobacter* was the first dominant group while *Pseudomonas* remained the second dominant group. Mid-season in both 2013 and 2014, *Rhodoferax* and *Arcobacter* were the most dominant groups in the bacterial community. However, at the end of the treatment season in both years, *Rhodoferax* and *Geobacter* became the most and second-most abundant genera. Therefore, the dominant genera were similar at the middle and end of the treatment seasons over the two study-years. In contrast, annual variations in the distribution of dominant bacterial genera were observed at the beginning of the season.

4.4.2.4 Phylogenetic Beta Diversity in the Two-Cell WSP in Clyde River, NU

The phylogenetic beta diversity measures in Clyde River two-cell WSP were investigated to study whether the bacterial diversity was affected by a) the wastewater treatment train, b) the sampling year and c) the time of sampling within the summer treatment season. The results based on the weighted and unweighted UniFrac distance matrices showed that the quantitative and qualitative structures of bacterial diversity were significantly ($p<0.05$) affected by all the three factors mentioned above. The distribution and composition of bacterial communities along the treatment train is presented in Figure 4.10a while the PCoA plot of bacterial beta-diversity measured by weighted UniFrac is illustrated in Figure 4.10b.

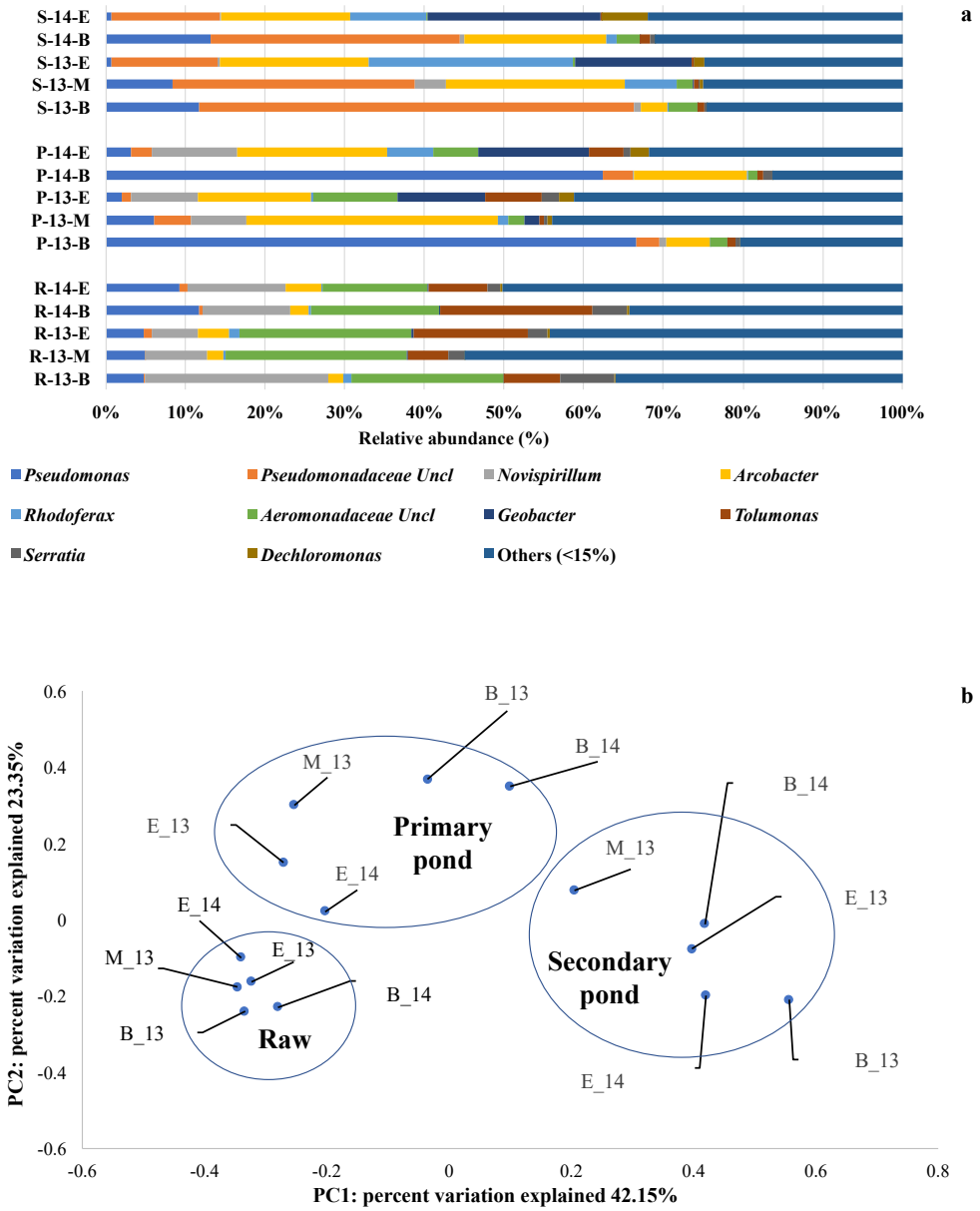


Figure 4.10 The diversity of bacterial community along the Clyde River WSP train. a) the distribution and composition of bacterial community and b) the PCoA plot of bacterial beta-diversity measured by weighted UniFrac metric. Legend: B – Beginning, M – Middle, E – End of the summer treatment season, respectively. -13 and -14 indicate 2013 and 2014 sampling years, respectively. R – raw wastewater, P – primary pond, S – secondary pond, respectively. Uncl: unclassified. Others (<15%) indicate that the bacterium including the unclassified with its relative abundance less than 15% was pooled into one group. The relative abundance of each bacterium was presented as the mean value of the number of wastewater samples collected.

The treatment effect was described using 2013 as an example to show that the composition of bacterial communities changed from the raw wastewater to the treated wastewater in the primary pond and then in the secondary pond (Figure 4.10a). When the summer treatment season started, *Novispirillum* and the unclassified genera in the *Aeromonadaceae* family were the top two dominant groups with relative abundances of 23.1% and 19.1%, respectively, while *Pseudomonas* was a minor group (4.7%) in the raw wastewater samples. Within the primary pond, the relative abundance of *Pseudomonas* increased to 66.7%, therefore becoming the most dominant group. In contrast, *Novispirillum* and the unclassified genera in the *Aeromonadaceae* family became reduced to be minor groups with relative abundances of 0.9% and 2.1%, respectively. In the secondary pond, *Pseudomonas* dropped to becoming the second most dominant group with a relative abundance of 11.7%. The unclassified genera belonging to the *Pseudomonadaceae* family became the most abundant group (54.6%). *Novispirillum* and the unclassified genera in the *Aeromonadaceae* family continued to be minor groups, representing 0.9% and 3.7%, respectively. During the mid-season, the unclassified genera in the *Aeromonadaceae* family was the largest group and the unclassified genera in the *Pseudomonadaceae* family was the smallest group, with the relative abundance of 22.6% and 0.5%, respectively, in the raw wastewater samples. In the primary pond, *Arcobacter* was the dominant group with the relative abundance of 31.6% and the relative abundance of the unclassified genera in the *Pseudomonadaceae* family increased to 4.6%. In the secondary pond, the unclassified genera in the *Pseudomonadaceae* family became the most abundant group and *Arcobacter* became the second most dominant group, showing the relative abundance of 30.4% and 22.6%, respectively. When the summer treatment

season ended prior to the freeze-up, the raw wastewater had 1.2% of *Rhodoferax* and 0.3% of *Geobacter* comprised 1.2% and 0.3%, respectively. In the primary pond, the relative abundance of *Rhodoferax* decreased to 0.3% while *Geobacter* increased to 11.0%. These genera became the most abundant groups in the secondary pond, representing 25.6% and 14.6%, respectively.

At the beginning of the 2013 treatment season, the primary pond was dominated by *Pseudomonas* however, *Arcobacter* became the dominant bacterial genus in both the middle and end of the season. Within the secondary pond, the relative abundance of *Pseudomonas* decreased from 11.7% in the beginning of the treatment season to 0.6% at the end of the season, while *Geobacter* rose from 0.1% to become to a noticeable group with the relative abundance of 14.6% at the end treatment season. Taken together, changes in the bacterial communities took place both along the treatment train and also in both the primary and secondary ponds, as the summer treatment season moved along.

The different sampling years also affected the bacterial distribution (Figure 4.10a). For example, within the secondary pond at the end sampling time, *Rhodoferax* was the dominant group with the relative abundance of 25.6% in 2013, however, *Geobacter* became the dominant group, representing 21.8% in 2014. The dissimilarities of bacterial diversity in the WSP train between 2013 and 2014 are demonstrated in the PCoA plot (Figure 4.10b), which shows that 65.5% of the variation in microbial diversity depended on treatment train based on the sum of PCs 1 (42.15% variation explained) and 2 (23.35% variation explained). Among wastewater samples from the primary and secondary ponds, a separation trend for the bacterial diversity was observed along the PCs 1 and 2 that corresponded to the assessment time in the treatment season and the sampling year,

respectively, confirming differences in bacterial communities along the treatment trains, assessment time in the treatment season and sampling year.

4.4.2.5 Phylogenetic Beta Diversity Between One-Cell Pond Inlet WSP and Two-Cell Clyde River WSP in NU

The results showed that bacterial diversity in treated wastewater differed significantly between the geographic locations. The dissimilarities of bacterial communities in treated wastewater between Pond Inlet WSP and Clyde River secondary pond is shown in the PCoA plot in Figure 4.11, which shows that there were 69.75% variation in total to explain the wastewater bacterial diversity between two locations. For example, mid-season 2013, the bacterial diversity in treated wastewater differed between Pond Inlet (PI_M_13) and Clyde River (CR_M_13) along the PC1 (52.84% variation explained). Along the PC2 (16.91% variation explained), for example, at the end of the 2014 treatment season, the phylogenic diversity of bacterial communities in treated wastewater from Pond Inlet (PI_E_14) and Clyde River (CR_E_14) differed. Therefore, the location and treatment trains in the two arctic communities influenced the bacterial diversity in treated wastewater.

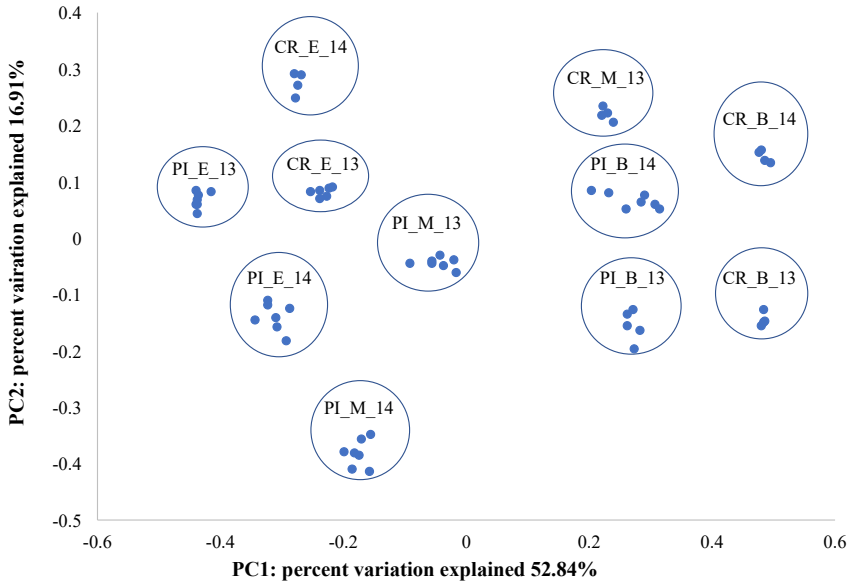


Figure 4.11 The PCoA plot of bacterial beta-diversity measured by weighted UniFrac in relation to the treated wastewater obtained from two different geographic locations (Pond Inlet: PI and Clyde River: CR) during the summer treatment seasons in both 2013 and 2014. Legend: B – Beginning, M – Middle, E – End of summer treatment season, respectively. -13 and -14 indicate 2013 and 2014 sampling years, respectively.

4.4.2.6 Functional Content Prediction in Bacterial WSP Communities

PICRUSt was used to infer the potential functional content of treated wastewater samples in terms of KEGG orthologs and pathways and to associate functional differences with seasonal, yearly and treatment differences in two communities. Results from these analyses are shown in Figures 4.12 and 4.13. During the 2014 summer treatment season, the two most significant ($p<0.05$) KEGG pathways that were present in the bacterial communities in Pond Inlet WSP samples were associated with carbohydrate and energy metabolism. Significant differences ($p<0.05$) in the abundance of predicted functions involved in the carbohydrate and energy metabolisms were observed along the summer treatment season with a mid-season peak and slightly and much lower levels at the beginning and end the treatment season, respectively (Figure 4.12a). The energy

metabolism followed the same trend (data not shown). Comparing mid-season pond samples from 2013 and 2014, the abundance of predicted functions responsible for carbohydrate and energy metabolisms were significantly ($p<0.05$) higher in 2014 than in 2013, as shown in Figure 4.12b for the carbohydrate metabolism as an example.

In Clyde River, the time in the summer treatment season significantly ($p<0.05$) affected the carbohydrate and energy metabolisms in each cell. For example, in Figure 4.13a, the boxplot of the predicted abundance of genes associated with carbohydrate metabolism in the secondary pond shows that the treated wastewater samples in the beginning of the treatment season had a higher activity than in the end of the season. The sampling year also significantly ($p<0.05$) affected the predicted carbohydrate and energy metabolisms in the secondary pond, with 2014 being higher, but not in the primary pond (data not shown). A significant ($p<0.05$) difference was also found for the predicted carbohydrate and energy metabolisms in the primary and secondary pond, with the activity being highest in the latter. In Figure 4.13b, an example of this is shown for bacterial communities in the primary and secondary pond at the end of the 2014 treatment season.

In both Pond Inlet and Clyde River WSPs, PICRUSt identified the presence of three KEGG pathways (K10944, K10945 and K10946) that encode ammonia monooxygenase, an enzyme which is involved in the microbial removal of ammonia. K10944 was contributed from the *Nitrosomonadaceae* family, while K10945 and K10946 originated from both the *Nitrosomonadaceae* and *Crenotrichaceae* families. The families were present in very low relative abundances (approximately 0.01%) in both WSPs, suggesting that the abundances of those KEGG pathways were unlikely to contribute substantially in

the removal of ammonia. This finding was supported by the previous observation by Ragush and other researchers (2015) that the ammonia removal did not occur in both WSPs during the study period.

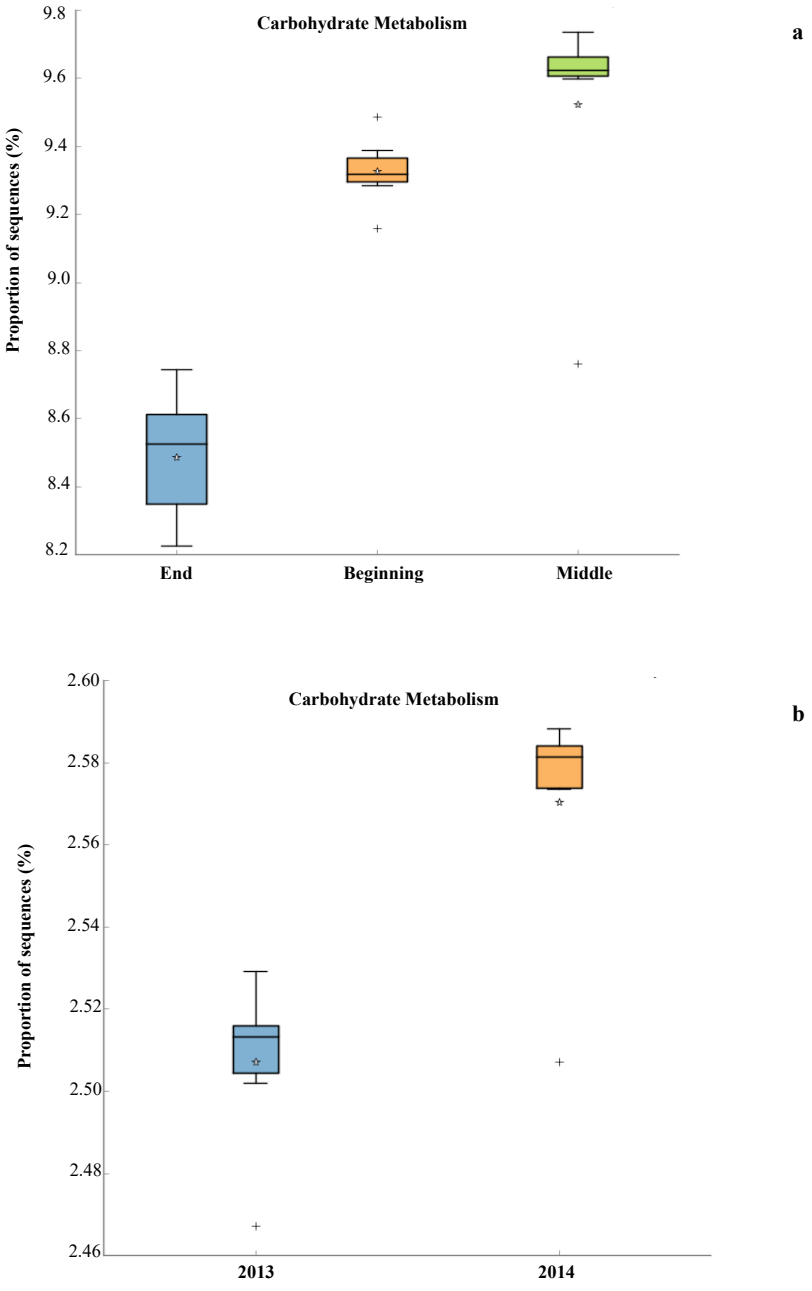


Figure 4.12 PICRUSt predicted abundance of genes belonging to carbohydrate metabolism associated KEGG pathways with significant differences ($p<0.05$) based on

the mean proportions and identities of *16S rRNA* genes in WSP samples in Pond Inlet a) from the beginning to end treatment seasons in 2014, and b) during the middle treatment seasons between 2013 and 2014 sampling years. The significant difference ($p<0.05$) was detected by the Kruskal-Wallis H-test with a multiple test correction Benjamini-Hochberg FDR in Figure 4.12a and was delectated by the Welch's t-test with a multiple test correction Benjamini-Hochberg FDR in Figure 4.12b, respectively.

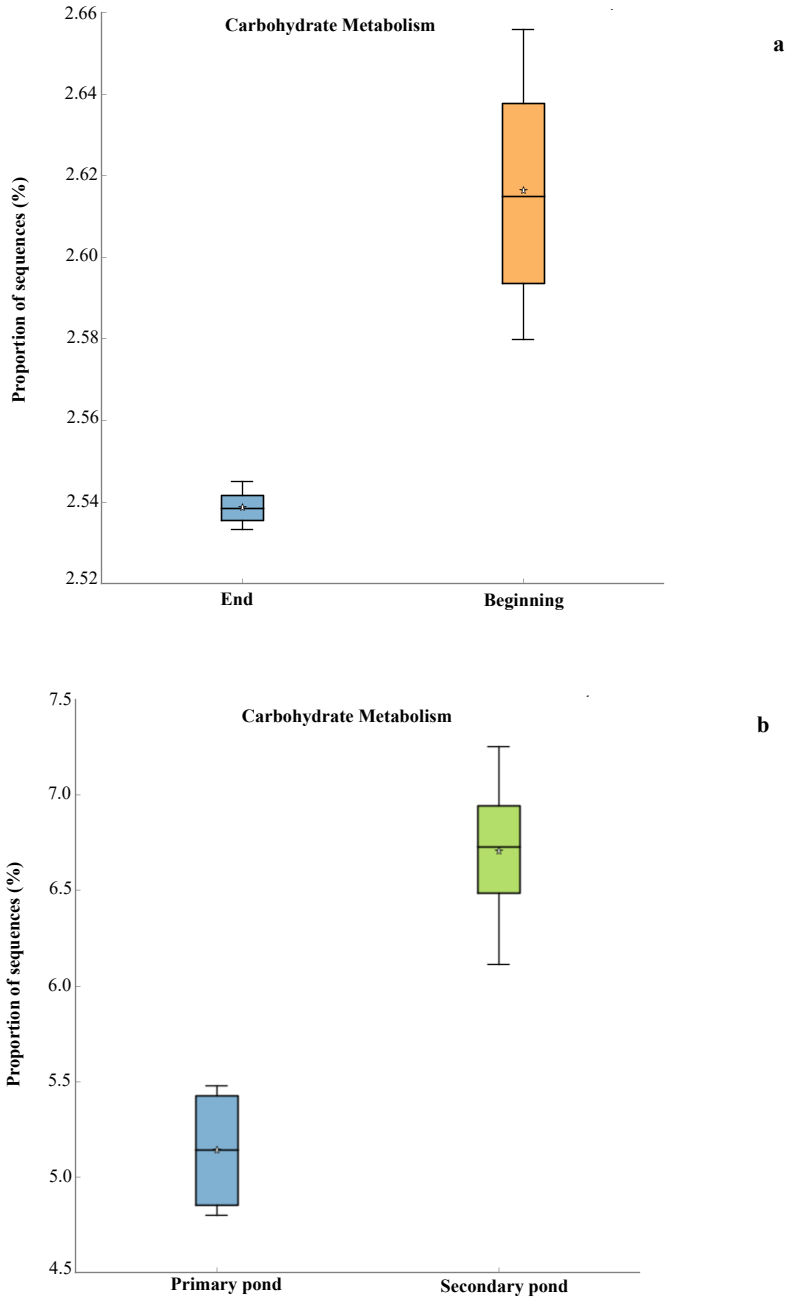


Figure 4.13 PICRUSt predicted abundance of genes belonging to carbohydrate metabolism associated KEGG pathways with significant differences ($p<0.05$) based on

the mean proportions and identities of *16S rRNA* genes in WSP samples a) in secondary pond in Clyde River during the summer treatment season in 2014, and b) between the primary and secondary pond at the end treatment season in 2014. The significant difference ($p<0.05$) was detected by the Welch's t-test with a multiple test correction Benjamini-Hochberg FDR.

4.5 Discussion

Past studies on WSP systems have focused on the design, operation and performance of the system to remove nutrients and pathogens by using micro-algae and their associated environmental conditions (Ludwig and Oswald 1952; Marais 1974; Mara et al. 1992; Shilton and Walmsley 2005). However, few studies have been conducted on the characterization of bacterial communities in WSPs (Shammas et al. 2009). Recently, a study investigating the diversity of bacterial communities with the emphasis on sulfur-reducing and -oxidizing bacteria in the WSPs operated under tropical climatic conditions in the city Mutuelleville in north-east of Tunisia (36˚49′N, 10˚10′E) (Belila et al. 2013). To the best of our knowledge, there has been no prior studies to explore bacterial communities found in WSPs operated in the hash arctic climate. Therefore, our study is the first to look at the microbiome of wastewater treatment in arctic WSPs located in Nunavut, Canada.

The present study provides a better understanding of the influence of temporal and environmental variables on the bacterial community size, composition, diversity and potential functionality in the one-cell WSP and the two-cell WSP of Pond Inlet and Clyde River, respectively.

The bacterial population size measured by *16S rRNA* copy numbers was greatly influenced by the treatment processed in both Pond Inlet and Clyde River, with the treatment trains causing a reduction in the total bacterial concentration from the untreated

wastewater to the final treated effluent. A similar observation was seen in the removal of the fecal bacterial levels measured by both as total coliform and *Escherichia coli* concentrations along the WSP treatment trains in Pond Inlet and Clyde River (Huang et al. 2017). The summer treatment season played an important role for the variation of the total bacterial concentration in both WSPs. Among the measured pond environmental parameters (temperature, pH and DO), temperature was more likely to affect the growth rate of bacterial populations through the course of the summer treatment season. When the wastewater temperature increased during the middle treatment season, the population size was increased as well in both locations. Previous studies have also indicated that temperature is one of the most important factors that influence the growth rate of bacterial population in WSPs (Bartsch and Randall 1971; Lettinga et al. 2001), activated sludge in WWTPs (Ding et al. 2013) and bioreactors (Wells et al. 2009). The pH in the WSPs were less likely to affect the level of bacterial population, because pH stayed constantly between 7.5 to 7.8 in both Pond Inlet and Clyde River during the summer treatment seasons in 2013 and 2014 (Ragush et al. 2015; Huang et al. 2017). In both WSPs, the pond had an anaerobic environment during the two-year study period. In Clyde River, the DO levels consistently remained below the detection limit (0.2 mg/L). In Pond Inlet, the overall DO levels stayed approximately between 0.2 to 1.3 mg/L during the summer treatment seasons of 2013 and 2014, except that there was a sharp peak from 0.2 to 1.5 mg/L during four days in the middle of the 2013 treatment season, and an increase from 0.2 to 0.5 mg/L and stayed at 0.5 mg/L for one week in the middle of the 2014 treatment season (Ragush et al. 2015; Huang et al. 2017). The mid-season increased DO levels may possibly have had an impact on the increased bacterial population size,

higher bacterial alpha diversities, and increases in the carbohydrate and energy metabolisms related to gene abundances measured in the middle treatment seasons. A previous study of activated sludge showed that the DO levels affected the population of the bacterial community in bioreactors (Park and Noguera 2004). Between 2013 and 2014, regardless of the summer treatment seasons, the bacterial population size in the Pond Inlet WSP was higher in 2014 than in 2013. The possible reason for this difference was that the WSP environment was warmer in 2014 than in 2013, as indicated by the degree days above 5 °C in pond surface water temperature profile (Ragush et al. 2015). Therefore, the pond temperature affects the size of bacterial community in arctic WSPs.

Wastewater bacterial community studies have focused on studying the core bacterial community present in activated sludge in wastewater treatment bioreactors or plants located in various geographical locations (Xia et al. 2010; Ding et al. 2013; Ju et al. 2014; Ju and Zhang 2015). At the present time, there is limited knowledge about whether the raw municipal wastewater in different geographical locations shares any commonalities in terms of the bacterial communities. In this study, it was found that a core set of bacteria existed in raw wastewater samples from both Pond Inlet and Clyde River, showing that the composition and diversity of bacterial communities in raw wastewater from two geographically separated arctic communities were similar, even though the raw wastewater quality, for example, the $CBOD_5$ levels, was significantly different between the communities (Ragush et al. 2015). The core bacteria presented at the phylum level (Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria) were also found in activated sludge samples from five biological wastewater treatment reactors in China and the United States in microarray experiments (Xia et al. 2010). Also, the proportion of the

four phyla distribution between this study and their study showed similar trends, with Proteobacteria being the largest phylum and Bacteroidetes, Firmicutes and Actinobacteria present as the subdominant phyla. The dominance of Proteobacteria and Bacteroidetes in this study was also found in the treated wastewater in the recent tropical WSP study using the PCR-DGGE technique (Belila et al. 2013) and activated sludge samples from 14 WWTPs in China using 454 pyrosequencing (Ding et al. 2012). Within Proteobacterial community in raw wastewater, Gammaproteobacteria was the predominant group and Betaproteobacteria was the minor group. In treated wastewater from the tropical WSP, Betaproteobacteria was the largest group and Gammaproteobacteria was the second largest group. In the shared bacterial genera group in raw wastewater, most of the identified genera except *Comamonas* were not found in the tropical WSP study (Belila et al. 2013) and other activated sludge studies (Xia et al. 2010; Ding et al. 2012; Zhang et al. 2012; Zhang et al. 2014; Zhang and Ju 2015, Ibarbalz et al. 2013). *Comamonas,* which belongs to denitrifier genera group, was more abundant in mainland China's activated sludge in comparison with other activated sludge samples collected in Hong Kong, Singapore, the United States and Canada (Zhang et al. 2012). When comparing the core bacterial groups between this study and other studies mentioned above, bacterial community compositions are highly similar at the phylum level, suggesting that similarities in the distribution of bacterial phyla among different municipal wastewater treatment systems located at various geographical locations, under different operations, and different climates reflect the coherence of microbial ecology at high taxonomic levels. However, when the taxonomic division is investigated at a deeper level, there is less commonality for the bacterial genera detected in the present study and other studies. It is

possible that the wastewater in Pond Inlet and Clyde River, which consists of domestic wastewater with no influence of industrial or agricultural activities, led to differences in bacterial community structure compared to other wastewater studies. Also, the technical limitations and differences in sequencing methods, primers and depths, and PCR amplification bias between this study and other studies may affect the comparison of bacterial communities, making biologically meaningful comparisons difficult.

The WSP treatment processes, sampling time during the summer treatment season and sampling years effectively influenced both the alpha and beta diversities of the bacterial communities in Pond Inlet and Clyde River. For the bacterial richness measured by the observed numbers of OTUs and Chao1 in the alpha-diversity analysis, the treatment in the one-cell Pond Inlet WSP resulted in the reduction of bacterial richness from the raw wastewater to the treated wastewater and finally the effluent. The two-cell Clyde River WSP treatment also caused the reduction of bacterial richness from the raw wastewater to the treated wastewater in the primary and secondary ponds. In Pond Inlet, the bacterial richness in sludge samples increased from the beginning to end treatment season and coincided with the growth of bacterial population size as well. A possible reason for this increasing trend is the accumulation of solids settling from the water column to the sludge layer. The passive wastewater treatment caused a decrease in the Shannon diversity in the bacterial community along the WSP process in Pond Inlet and Clyde River. A possible reason for this decreasing trend was the low temperature and anaerobic environment in the pond. The Simpson evenness measure showed the evenness of bacterial communities had no significant differences among all samples regardless of treatment processes and summer treatment season effects. The values of the evenness

stayed in the range from 0.02 to 0.06 in both Pond Inlet and Clyde River. The range of the Simpson evenness index is between 0 to 1 (Mulder et al. 2004). The low value (0.02 to 0.06) of the evenness index indicated that the bacterial community was not evenly distributed in both WSPs. It is not unexpected as Proteobacteria was the most abundant bacterial phylum at the 70-90% relative abundance in all wastewater samples regardless of treatment types, summer treatment seasons, and sampling years. This bacterial phylum is known to inhabit in eutrophic ponds and is responsible for removal of the organic waste in municipal wastewater (Wagner et al. 2002).

Using the weighted UniFrac metric in the beta diversity analysis, the diversity of wastewater bacterial communities was significantly ($p<0.05$) different along the passive treatment processes from the raw wastewater to the effluent in Pond Inlet WSP and from the raw wastewater to the treated wastewater in the secondary pond in Clyde River WSP, respectively. Within the predominant Proteobacterial phylum, there was a shift in the dominant Proteobacterial classes from the raw wastewater to the treated wastewater. In Pond Inlet WSP, Gammaproteobacteria was the predominant class in the raw wastewater, however, Betaproteobacteria became the predominant class in the pond during the later part of the treatment season. In Clyde River WSP, Gammaproteobacteria was the predominant class in the raw wastewater, Epsilonproteobacteria turned out to be the predominant class in the primary pond and Betaproteobacteria was the most prevalent and abundant group in the secondary pond, respectively, during the later part of the treatment season. In other municipal wastewater treatment systems, Gammaproteobacteria seemed to dominate in the activated sludge in an conventional activated sludge system, a membrane bioreactor, and an anaerobic/aerobic-membrane

bioreactor located at different geographic places (Xia et al. 2010), and the Betaproteobacteria and Gammaproteobacteria classes dominated in the tropical WSP consisting of four connected cells in series (an anaerobic, a facultative, and two maturation cells) (Belila et al. 2013). By looking at the genus profile, the *Rhodoferax* and *Geobacter* genera were the minor group in the raw wastewater in Clyde River, however, they gradually became the dominant genera from the primary to the secondary pond. The *Rhodoferax* genus is most often found in activated sludge in sewage treatment plants (Zhang et al. 2012). The species in the *Geobacter* genus are often found in the anaerobic conditions from soil and aquatic environment (Lovley et al. 1987), indicating the presence of this non-fecal genus in the pond was more likely coming from the surrounding environments.

The alpha and beta diversities, distribution of bacterial community differed during the course of the summer treatment season and sampling years in both WSPs. For the alpha diversity analysis, as the measured pond wastewater temperature increased mid-season, the richness and Shannon diversity also increased in the WSPs, showing that the mid-season provided a favorable environment to enhance the richness and diversity of the bacterial community in the WSPs. This coincided with previous reports of the mid-season improved removal of fecal indicator and pathogenic bacteria and nutrients (Huang et al. 2017; Ragush et al. 2015), suggesting that disinfection and nutrient removal performances are related to bacterial richness and diversity in WSPs. In Pond Inlet, the WSP had higher levels of bacterial richness and diversity indexes in 2014 than in 2013, especially during the middle of the treatment season. Pond Inlet experienced a warmer summer in 2014 than in 2013, especially during the first half of the treatment season

(Huang et al. 2017). It may be that the warmer environment affected this difference in alpha diversity in pond samples.

The beta diversity of bacterial communities in both WSPs was affected by the sampling time during the summer treatment season and the sampling year. Among the three measured parameters in the pond environment (temperature, pH and DO), only temperature is significantly ($p<0.05$) consistent with the beta diversity of wastewater bacterial communities as determined by the nonparametric statistical test through permutations (ANOSIM). Between the two sampling years (2013 and 2014), the bacterial diversity differed, probably due to the warmer pond environment in 2014 than in 2013. Temperature has also been found to be one of the most important factors to influence the bacterial community diversity in past wastewater bacterial communities studies (Ding et al. 2013; Wells et al. 2009). In Pond Inlet WSP during the middle treatment season as the pond temperature increased, Betaproteobacteria became the most prevalent and abundant group in the pond wastewater community. Betaproteobacteria were also found to be the predominant bacteria in the tropical WSP study (Belila et al. 2013). The results showed that the Betaproteobacterial class tend to grow in the warm environment in WSPs. In the Clyde River WSP from the beginning to the end of the summer treatment seasons in both years, the relative abundance of *Dechloromonas* kept increasing. Species belonging to *Dechloromonas* genus are identified as phosphate accumulating bacteria in enhanced biological phosphorus removal reactors (Liu et al. 2005). The information about the presence and roles of other genera in WSPs are limited.

The functional content of wastewater bacterial communities tended to have higher carbohydrate and energy metabolisms during the middle of the treatment season than

other treatment seasons in the one-cell Pond Inlet WSP. A previous study had indicated that the middle treatment season was the best season to reduce nutrient contents or $CBOD_5$ levels (Ragush et al. 2015). The higher temperature measured in the pond and the higher carbohydrate and energy metabolisms that bacterial communities had during the middle treatment seasons were more likely to support the previous findings. In the two-cell WSP in Clyde River, the higher carbohydrate and energy metabolisms in the secondary pond bacterial communities may cause the further reduction of $CBOD_5$ level in comparison with the treatment in the primary pond as observed in the previous study (Ragush et al. 2015). Overall, the results from the functional profiling of wastewater bacterial communities supported the summer treatment season and the treatment effects in the removing of biodegradable wastes as observed in the previous study (Ragush et al. 2015).

Lastly, using the weighted UniFrac beta-diversity metric and PCoA tool, the diversity of bacterial communities in WSPs differed between the two geographic arctic communities, showing that the different climatic conditions and treatment systems between the two communities are likely to affect the bacterial diversity in wastewater ponds that completely exposed to the arctic environment. Ragush and other researchers (2015) found that the WSP in Pond Inlet (for example, degree days above 5 ºC was 313 days in 2013) generally had warmer water temperature environment than in Clyde River (degree days above 5 ºC was 243 days in the primary pond and was 246 days in the secondary pond, respectively, in 2013) in the 2012-2014 studies during the summer treatment seasons.

4.6 Conclusions

The study investigated the population size, composition, distribution, diversity and functional content of bacterial community in arctic WSPs treating municipal wastewater in Pond Inlet and Clyde River, Nunavut, Canada. The outcome of this study can be divided into five findings. 1) The bacterial population size was highly dependent on the treatment process and the sampling time during the summer treatment season. 2) The composition and diversity of the microbiome in raw wastewater did not differ between two arctic communities. 3) The bacterial alpha and beta diversity, and the composition and distribution of bacterial communities was significantly influenced by the treatment process, the sampling time during the summer treatment season, the sampling year and different geographic locations. 4) The predicted gene functions (KEGG pathway study) confirmed that the middle of the treatment season was the optimal time for removal of nutrients, represented by $CBOD_5$ values, observed in Pond Inlet, and the treatment in the secondary pond in Clyde River constituted a better treatment for $CBOD_5$ removal than the primary pond. 5) The KEGG ortholog prediction results supported the observation in both Pond Inlet and Clyde River WSPs there was an absence of bacterial ammonia removal (oxidation) in the anaerobic pond environments present during the 2012-2014 study period. Beside the significant temperature effect in the pond environment to affect the bacterial community variances, there are a lot of unpredictable or uncontrollable factors probably involved in the treatment processes taking place in passive WSPs operated under the extreme climate conditions. Further studies, probably bench-scale settings with much simpler and more controllable conditions, are needed to clarify the effect and significance of each variable (e.g., temperature, DO, pH and nutrients) on the WSP bacterial community population size, composition, diversity and potential function.

Chapter 5 Conclusions

The arctic climate, especially the temperature variations over the summer treatment season and between sampling years, impacted the microbial WSP treatment processes in Pond Inlet and Clyde River, that is the disinfection and removal of human bacterial pathogens as well as the bacterial community structure including population size, distribution, diversity and potential functionalities. During the three-year study period (2012-2014), the ponds stayed close to anaerobic with no discernable algal blooms and pH values remained constantly between 7.5 and 7.8.

WSP treatment of municipal wastewater in both communities successfully reduced the content of generic *E. coli* to levels that are in compliance with the Nunavut Water Board (2014) regulatory limits (4-6 Log MPN/100 mL). Further treatment would be necessary if the stricter regulations were to be implemented. The single-cell WSP in Pond Inlet was able to impart a marked removal of *Salmonella* spp. (0.7-0.9 log) and pathogenic *eae*-positive *E. coli* including O157:H7 (~1.0 log) but not *L. monocytogenes* from the wastewater. The two-cell Clyde River WSP provided better treatment in regards to disinfection and removal of bacterial pathogens with reductions of 1.0-1.5 log, provided the primary pond was used as the only recipient of raw wastewater which then after a settling period was transferred to the secondary pond for further treatment. The variation of pond temperatures during the course of the summer treatment season appeared to influence the treatment efficiency. The best removal of fecal indicator bacteria and pathogens was achieved mid-season, likely due to the warmer water temperatures.

Through investigations of the microbial ecology during treatment of municipal wastewater in the communities, it was observed that the bacterial population size and

diversities were highly influenced by the treatment train and the time of sampling during the summer treatment season. The bacterial diversities in raw wastewater were not different ($p>0.05$) between the two arctic communities. However, the bacterial diversities in wastewater treated in the WSPs differed significantly ($p<0.05$) between the two locations. The predicted gene functions from PICRUSt confirmed that the middle of the summer treatment season in Pond Inlet WSP was the optimal time for microbial activities, which coincided with enhanced nutrient removal, as shown by $CBOD_5$ values. Similarly, the predicted functionality and community size pointed to the processes in the Clyde River secondary pond constituting a better treatment for $CBOD_5$ removal than the primary pond. The predicted gene content from PICRUSt supported the observation of the absence of bacterial ammonia removal (oxidation) in the anaerobic pond environments during the 2012-2014 study period in Pond Inlet and Clyde River.

In summary, this is the first study to look at the microbiology of the WSPs systems in Arctic Canada in the past 30-50 years. The results from Chapter 3 showed that even though arctic WSPs achieved significant removal of fecal and pathogenic bacteria from municipal raw sewage, partially treated wastewater still containing low levels of bacterial pathogens (2-4 Log CFU/100 mL) is released into the receiving environment during the annual decant. Since Arctic communities rely on their local surroundings to harvest food, collect drinking water and recreational activities, from a public health perspective, it may be prudent to assess whether the wastewater effluents pose a potential risk to human health and the receiving environment. Assessment of the potential risks would help the authorities decide whether it is necessary to change the current wastewater effluent standards in the Territory. To understand more about wastewater microbial ecology and

improve the biological wastewater treatment in arctic WSPs under the extreme and unpredictable environmental conditions, it is recommended that further bench-scale studies using controlled conditions should be performed to clarify the effect and importance of environmental (e.g., temperature) and operational parameters (e.g., DO, pH, and nutrients) on the WSP bacterial community population size, distribution, diversity and functionality.

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Appendix A Supplementary Material for Disinfection and Removal of Human Pathogenic Bacteria in Arctic Waste Stabilization Ponds

Table S1. Primers and qPCR protocols for detection of bacterial pathogens.

Assay type	Pathogenic bacteria	Primers (F and R) and probes (P)	Sequence 5' to 3'	qPCR protocols
TaqMan	*Listeria monocytogenes*	HlyQF	CATGGCACCACCAGCATCT	95 ℃ for 10 min; 40 cycles of 95 ℃ for 20 sec, 56 ℃ for 30 sec, 72 ℃ for 1 min
		HlyQR	ATCCGCGTGTTTCTTTTCGA	
		HlyQP	FAM-CGCCTGCAAGTCCTAAGACGCCA-TAMRA[a]	
	Eschericha coli (pathogenic EHEC/EPEC, intimin+)	EaeF	GTAAGTTACACTATAAAAGCACCGTCG	95 ℃ for 6 min; 40 cycles of 95 ℃ for 20 sec, 55 ℃ for 30 sec, 72 ℃ for 40 sec
		EaeR	TCTGTGTGGATGGTAATAAATTTTTG	
		EaeP	FAM-AAATGGACATAGCATCAGCATAATAGGCTTGCT-BHQ1[b]	

Assay type	Pathogenic bacteria	Primers (F and R) and probes (P)	Sequence 5' to 3'	qPCR protocols
TaqMan	*Campylobacter* spp.	CampF2	CACGTGCTACAATGGCATAT	95 ℃ for 6 min; 40 cycles of 95 ℃ for 15 sec, 60 ℃ for 1 min
		CampR2	GGCTTCATGCTCTCGAGTT	
		CampP2	FAM-CAGAGAACAATCCGAACTGGGACA-BHQ1	
	Salmonella enterica	InvAF	AACGTGTTTCCGTGCGTAAT	95 ℃ for 6 min; 40 cycles of 95 ℃ for 15 sec, 60 ℃ for 1 min
		InvAR	TCCATCAAATTAGCGGAGGC	
		InvAP	FAM-TGGAAGCGCTCGCATTGTGG-BHQ1	
SybrGreen	*Helicobacter pylori*	HPF	TTATCGGTAAAGACACCAGAAA	95 ℃ for 10 min; 40 cycles of 94 ℃ for 20 sec, 54 ℃ for 5 sec, 72 ℃ for 10 sec
		HPR	ATCACAGCGCATGTCTTC	

a. FAM – fluorescein

b. BHQ1- Black hole quencher

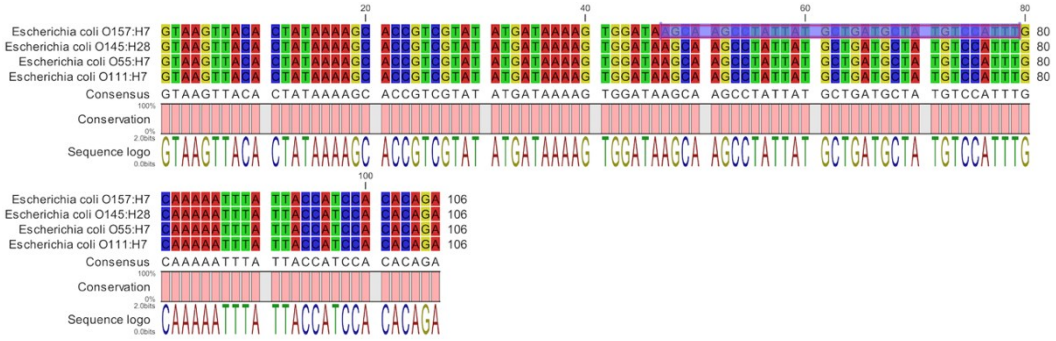

Figure S1. Alignment of the *eae* gene fragment amplified by the Ibekwe et al. (2002) primer pair from different pathogenic *E. coli* serotypes showing the binding site for the EaeP probe (CLC Genomics Workbench Version 9). Shown are enterohemorrhagic *E. coli* (EHEC) strains O157:H7 and O145:H28 and enteropathogenic *E. coli* (EPEC) strains O55:H7 and O111:H7. A search in the NCBI database (https://www.ncbi.nlm.nih.gov/) revealed that other EHEC and EPEC serotypes were also detected by this primer and probe set including EHEC strains O79:H7, O91:H14, O153:H2, O156:H25, and EPEC strains O55:K59 , O126:K71,O86:K61, O44:K74, O26:K60, O128:K67, O127:K63, O126:K71, O125:K70, O119:K69, O111:K59, O111:K58, O82:H11, O40:H19, O170:H49, O2:H40, O119:H25, O76:H7, O136:H21.

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