Microbial diversity in the lake Elliðavatn and its rivers in the capital city of Iceland

Örveruflóra Elliðavatns og Elliðaáa

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Yfirlýsing höfundar

Hér með lýsi ég því yfir að ritgerð þessi er samin af mér og að hún hefur hvorki að hluta né í heild verið lögð fram áður til hærri prófgráðu.

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60 ects thesis as a part of *Magister Scientiarum* in Environmental Science and Natural Resources

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Abstract

The uniqueness of Elliðaár rivers, situated in Reykjavík is reflected by their origin from the lake Elliðavatn to their estuary, both being within the city limit. Few studies have been carried out on the rivers and lake microbial diversity. The objective of this study was to screen the microbial diversity and presence of faecal indicators in the rivers and lake. Samples were taken from three sampling sites in the lake and six sampling sites in the rivers four times over a nine-month period. Total viable count was estimated using flow cytometry and traditional culturing methods following incubation at 4°C and 30°C. The indicator species *Escherichia coli, Enterococcus* spp. and norovirus were used to estimate faecal pollution. Diversity of uncultured bacteria was analysed using the 16S rRNA gene sequencing method. The lake and rivers were categorised according to provisions in regulation no. 796/1999 on water pollution. The results indicate that the nearby urban area considerably influences the water quality of the lake and rivers. High faecal pollution caused by *E. coli* was measured in the lake and some parts of the rivers, and noroviruses were detected on three other sites, indicating faecal pollution. The lake was categorised as type D, "very polluted", and the rivers type B, "little pollution", on average. The microbial diversity depicted using the 16S rRNA gene sequencing method was quite diverse and differed between sampling sites. The method identified bacteria that are prevailing in natural freshwater and two genera were detected in all samples; *Flavobacterium* spp. and *Limnohabitans* spp.

Útdráttur

Elliðaárnar í Reykjavík eru einstakt vatnakerfi þar sem upptök þeirra í Elliðavatni allt til árósa í Elliðavogi er innan borgarmarka. Örveruflóra vatnakerfisins hefur lítið verið rannsökuð en markmið þessarar rannsóknar var að skoða náttúrulega örveruflóru vatnasviðsins og saurmengun frá stöðuvatni niður að ósum. Sýni voru tekin á þremur stöðum í vatninu og sex stöðum í ánum fjórum sinnum yfir níu mánaða tímabil. Heildarbakteríufjöldi var talinn með örverugreini og með ræktunum við 4°C og 30°C. Skimað var fyrir *Escherichia coli, Enterococcus* spp. og nóróveirum til að meta saurmengun. Fjölbreytileiki óræktaðra baktería var skoðaður og greint til tegunda með 16S rRNA gena raðgreiningu. Vatnið og árnar voru einnig flokkaðar m.t.t. reglugerðar nr. 796/1999 um varnir gegn mengun vatns. Niðurstöður rannsóknarinnar benda til þess að bæði árnar og vatnið séu undir talsverðum áhrifum byggðar. Mikil saurmengun fannst í vatninu og ákveðnum stöðum í ánum m.t.t. saurkólí baktería auk þess sem nóróveirur greindust á þremur öðrum stöðum sem bendir einnig til saurmengunar. Elliðavatn var flokkað í D flokk eða „verulega snortið vatn“ og árnar í B flokk eða „lítið snortið vatn“, að meðaltali. Örverufjölbreytileikinn sem greindist með 16S rRNA gena raðgreiningunni var nokkuð fjölbreytilegur og töluvert breytilegur milli sýnatökustaða. Aðferðin greindi þær bakteríur sem eru yfirgnæfandi í náttúrulegri örveruflóru ferskvatns en tvær ættkvíslir greindust í öllum sýnum, *Flavobacterium* spp. og *Limnohabitans* spp.

For my parents

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Abbreviations

BLAST	basic local alignment search tool
BOD	biochemical oxygen demand
bp	base pair
cfu	colony forming units
cm	centimetre
COD	chemical oxygen demand
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E. coli	*Escherichia coli*
EU	European Union
FACS	fluorescence-activated cell sorter
FB	phosphate buffer
FISH	fluorescence *in situ* hybridisation
kb	kilo base
h	hour
H_2S	hydrogen sulphide
HCl	hydrogen chloride
M	molar
mg/l	milligram per litre
min	minute
ml	millilitre
MPN	most probable number
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
NH_3	ammonia
NH_4-	ammonium
NH_4-N	total ammonium-nitrogen concentration
NO_3-N	nitrate nitrogen
O_2	oxygen
PCR	polymerase chain reaction
pH	acidity level
PO_4-P	phosphate
QPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	revolution per minute
s	second
SO_4^{2-}	sulphate
TGGE	temperature gradient gel electrophoresis
TOC	total organic carbon
Tot-N	total nitrogen
Tot-P	total phosphorus
UK	United Kingdom

µg/l	microgram per litre
µl	microlitre
USA	United States of America
y.m.	year missing
°C	degree Celsius

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1. Introduction

Iceland has the highest renewable freshwater availability per capita in Europe. Considering the average 2000 mm rainfall each year and the fact that Iceland is the most sparsely populated country in Europe, the country provides abundant water per capita and the majority of the population has access to plentiful freshwater supplies (Rist 1990). This has great benefits; generally there is good access to good quality drinking water, especially in areas where lava has run in recent times and the bedrock is very permeable. There, the precipitation seeps through the ground creating large groundwater reservoirs and springfed rivers. Springfed rivers have stable runoff of clear water throughout the year. These waters provide habitats for spectacular plant and animal communities that are very different from North American, Greenland and Scandinavian lakes on similar altitudes that drain old continental shield rocks. These waters are a valuable contribution to North European biodiversity (Jónasson 1992). Furthermore, the European Union's water framework directive classifies Iceland's freshwaters as unique eco-regions (Umhverfisstofnun 2012).

Productive rivers and lakes are important for recreational activities and some are great for fisheries. Salmon and trout recreational fisheries are popular in Iceland and have been of high economic value since their initiation. The economic importance depends on region, but contributes as much as 50% of the total income for residents in productive salmon areas (Hagfræðistofnun Háskóla Íslands 2004; Umhverfisstofnun 2010). Freshwater is also of importance and high economic value for energy production (hydro- and geothermal power). Geothermal water has been used for centuries in Iceland for hygiene and health and in more recent years for space and domestic heating. A large proportion of electricity in Iceland is produced by hydropower and some is obtained from geothermal sources (Kristmannsdóttir & Halldórsdóttir 2008). Although abundant, these resources are not limitless and not evenly distributed across the country. There is a growing public awareness and demand for nature conservation and to implement sustainable management in the utilisation of hydro- and geothermal energy sources (Umhverfisstofnun 2010).

1.1. Aquatic environments

Of all the water found on Earth, 97% is marine, and only 3% is fresh, a great part of which is bound in glaciers. Marine and freshwater environments create unique niches for many specialised microorganisms needing habitats with a continuous water phase. Aquatic environments have varied surface areas and volumes, and are found in rivers, lakes, oceans and even in soils. These environments can range from alkaline to extremely acidic. In aquatic environments, microorganisms can function in the temperature range of -5 to -15°C at the lower range to at least 121°C in geothermal areas. The dominant factors that control microbial communities in freshwater and marine environments are the mixing and movements of nutrients, oxygen (O_2), and waste products (Prescott *et al.* 2005).

1.1.1. Freshwater environments

Most of the freshwater on Earth that is not locked up in ice sheets, glaciers, or groundwater is found in rivers and lakes. Lakes and rivers provide microbial environments that are different from the larger oceanic systems in many important ways. For example, mixing and water exchange can be limited in lakes, which creates vertical gradients over short distances. In rivers, changes occur over distance and/or time as water flows through river channels (Prescott *et al.* 2005).

Lakes

Lakes vary in nutrient status. Some lakes are oligotrophic or nutrient-poor, while others are eutrophic or nutrient-rich. Nutrient-poor lakes remain aerobic throughout the year, and seasonal temperature changes do not result in distinct oxygen stratification. Nutrient-rich lakes, however, usually have bottom sediments that contain organic matter. In eutrophic lakes there is often an aerobic, warm, upper layer (epilimnion) and an anaerobic, deeper, colder bottom layer (hypolimnion) that are separated by a zone called the thermocline. There is little mixing of water between the two layers, except in the spring and fall when the two layers turn over because of differences in temperature and specific gravity. After such mixing, motile bacteria and algae can migrate within the water column to find their most suitable environment again. When nitrogen and phosphorus are added to the water in sufficient amounts, eutrophication (nutrient enrichment) takes place and stimulates the growth of bacteria, algae and plants. This may occur over many centuries or very rapidly, depending on the body of water and the rate of nutrient addition. If phosphorus is added to oligotrophic freshwater, cyanobacteria play the major role in nutrient accumulation, even if there is no extra nitrogen (Prescott *et al.* 2005). Cyanobacteria function more efficiently in higher pH conditions (8,5-9,5) and higher temperatures (30-35°C) (Sushchik *et al.* 2001; Prescott *et al.* 2005; Agha *et al.* 2012), while algae generally prefer a more neutral pH and have lower optimum temperatures (Sushchik *et al.* 2001; Prescott *et al.* 2005). Cyanobacteria and algae often compete for dominance, but cyanobacteria have more competitive advantages (Prescott *et al.* 2005).

Streams and rivers

In streams and rivers there is sufficient horizontal water movement to minimise vertical stratification, in contrast to most lake environments. In addition most of the functional microbial biomass is attached to surfaces. Only in the largest rivers will a greater portion of the microbial biomass be suspended in the water. The source of nutrients may vary depending on the size of the stream or river. Nutrients may come from photosynthetic organisms that produce nutrients in-stream, or they can originate from outside the stream, including runoff sediment from the edge of the river, or leaves and other organic matter falling directly into the water. Chemoorganotrophic microorganisms in the water metabolise the available organic material and provide an energy base for the ecosystem. Under most conditions, the amount of organic matter added to the streams and rivers will not exceed the system's oxidative capacity, which results in the maintenance of productive streams and rivers. Streams and rivers have a limited capacity to process excessive amounts of organic matter. If too much organic matter is added, the water may become anaerobic. This is especially the case with

streams and rivers located adjacent to urban and agricultural areas. Inadequately treated municipal wastes and other materials released from a specific location along a river, can cause point source pollution. Such point source additions of organic matter can produce distinct and predictable changes in the microbial community and available oxygen, creating an oxygen sag curve. Runoff from fields and feedlots that flows into the river at a non-specific location is called a non-point source pollution (Prescott *et al.* 2005). Non-point source pollution is the main cause of impact on water quality in urban areas (Winter & Duthie 1998; Faulkner *et al.* 2000; Paul & Meyer 2001). Non-point source pollutants that are most detrimental to water quality include nutrients, particularly phosphorus (Osborne & Wiley 1988; Walsh *et al.* 2001), heavy metals and other toxic substances (Legret *et al.* 1994; Watts & Smith 1994), oil and gasoline runoff from roadways (Ourso & Frenzel 2003), microbial and organic pollution (Faulkner *et al.* 2000; Casper 1994). In addition, urbanisation can change the hydrologic characteristics of a stream and thereby affect the water quality (Mancini *et al.* 2005).

River chemistry in Iceland

River chemistry has been monitored in Iceland since 1998. Based on available data (Stefánsson & Ólafsson 1991; Ólafsdóttir 2006) riverine transport has been estimated. The total N runoff with Icelandic rivers is estimated to be 4360 tonnes per year. The quantity of both NO_3-N and PO_4-P is higher in East Iceland than in the South. In the period from 1997-2001 the nutrient status of 59 lakes in Iceland was measured (Malmquist *et al.* 2000). Forty-nine lakes were observed to have nitrate nitrogen (NO_3-N) concentrations lower than 0,005 mg/l, and all the lakes had less than 0,05 mg/l. For total nitrogen (Tot-N) 52 lakes had less than 0,3 mg/l, five had 0,3-0,75 mg/l and two lakes had concentrations 0,75-1,5 mg/l. For total phosphorus (Tot-P) 48 lakes had Tot-P less than 0,025 mg/l, and 11 lakes had Tot-P 0,025-0,125 mg/l. Many Icelandic lakes seem to have rather high natural concentrations of phosphorus, possibly due to high weathering rate of volcanic bedrock (Umhverfisstofnun 2010).

1.2. Microorganisms in freshwater

Microorganisms along with other life forms thrive very well in water. Aquatic microbiology has advanced greatly in recent years concomitantly with the development of new methods and technology (Hurst *et al.* 1997). Microbial populations of freshwater bodies tend to be affected mainly by the availability of oxygen and light. In many ways, light is the more important resource because photosynthetic algae are the main source of organic matter, and hence the energy, for the lake. These organisms are the primary producers of a lake that supports a population of bacteria, protozoa, fish and other aquatic life (Tortora *et al.* 2002).

Large numbers of microorganisms in a body of water generally indicate high nutrient levels in the water. In water with low nutrient levels, microorganisms tend to grow on stationary surfaces and on particulate matter. By this way the microbes tend to be in better contact with the nutrients than if they were randomly suspended in the body of water, floating freely with the current. Many bacteria whose main habitat is water often have appendages and holdfasts that attach to various surfaces, such as *Caulobacter* spp. (Tortora *et al.* 2002). In the top layer of freshwater where there is

sufficient oxygen concentration pseudomonads and species of *Cytophaga, Caulobacter* and *Hyphomicrobium* spp. are common. In deeper waters where the oxygen concentration is low and there is less light purple and green sulphur bacteria are more common. These bacteria are anaerobic photosynthetic organisms that metabolise hydrogen sulphide (H_2S) to sulphur and sulphate in the bottom sediments. Sediments often include bacteria such as *Desulfovibrio* spp. that use sulphate (SO_4^{2-}) as an electron acceptor and reduce it to H_2S. Methane-producing bacteria are also part of these anaerobic benthic populations. *Clostridium* spp. are common in bottom sediments and may include botulism organism (Tortora *et al.* 2002).

The world-renowned microbiologist Sergius Winogradski designed the Winogradski column to study the relationship between different types of microorganisms in mixed communities in the aquatic environment. The column demonstrates in a simple way how different microorganisms perform their interdependent roles. These columns are complete, self-contained recycling systems, driven only by energy from light. Initially all the organisms are presented in low numbers, but when the tube has been incubated for 2-3 months the different types of microorganisms proliferate and occupy distinct zones where the environmental conditions favour their specific activities. In time different zones emerge in the column; in the bottom there is an anaerobic sediment where *Clostridium* and *Desulfovibrio* spp. are dominant. Between the anaerobic sediment and anaerobic water purple S bacteria and green S bacteria are dominant where H_2S and SO_4 exchange. In the anaerobic water purple non-S bacteria are dominant with cyanobacteria and sheathed bacteria in the topmost aerobic water (Deacon y.m.)

1.2.1. Freshwater bacteria

Freshwater bacteria are at the centre of biochemical cycles and control water quality in lakes. Despite this, little is known about major lake bacteria, about their identity and ecology. For this reason, Newman *et al.* (2011) presented new freshwater phylogeny constructed from all published 16S rRNA gene sequences from lake epilimnia. Examination of the database revealed that 21 phyla have been recovered from lake epilimnia, with 5 phyla being common; Proteobacteria, *(*especially Beta-proteobacteria), Actinobacteria, Bacteroidetes, Cyanobacteria and Verrucomicrobia. This is in correlation with previous studies (Zwart *et al.* 2002) and in general agreement with FISH-based studies. The remaining 16 phyla make up only ~2,6% of the total sequences collected (Newman *et al.* 2011).

Phylum Actinobacteria

The phylum Actinobacteria constitutes of Gram-positive bacteria with a high mol% G+C DNA composition. Freshwater Actinobacteria are small (<0,1μm^3) with a rod, coccus, or selenoid shape and are pigmented. They are free-living, open-water defence specialists, with possible photo- and heterotrophic energy generation life-styles. Actinobacteria are common and often a numerically important component in a variety of freshwater habitats. Actinobacteria are also found in soil and marine environments. They can be found in a variety of limnic systems, such as rivers, brackish seas, bays and glacial ice. Actinobacteria in freshwater lakes actively synthesise DNA and proteins and have provided evidence that the phylum is an

indigenous resident of freshwater. They are ubiquitous and abundant in freshwater lakes. In fact, Actinobacteria are often the numerically dominant phylum in lakes where they can contribute over 50% of the bacteria in the surface water (epilimnion). Actinobacteria are also present in the bottom waters (hypolimnion) of lake, but their abundance often decreases with decreasing oxygen concentrations. Actinobacteria are spread across the globe and have been found in lakes in North America, Europe, Africa, Asia, Australia, South America and Antarctica. Species include *Candidatus Planktophila limnetica* (Newman *et al.* 2011) also found in this study.

Phylum Bacteroidetes

Bacteroidetes are Gram-negative, bacillus bacteria. The phylum was formerly known as the Cytophaga-Flavobacterium-Bacteroides phylum. The phylum exhibits enormous phenotypic and metabolic diversity. The members of this phylum are found in soil, aquatic environments, or as symbionts of plants, animals, and humans. Most described isolates of the Bacteroidetes are chemoorganotrophs. Within the Bacteroidetes there are three distinct classes: Bacteroidales, Flavobacteriales, and Sphingobacteriales (Newman *et al.* 2011), all found in this study. In lake epilimnia, the Bacteroidetes may comprise a large proportion of particle-associated bacteria and seem to play a particularly important role in the degradation of complex biopolymers. The freshwater Bacteroidetes are often found in high abundance during periods following cyanobacterial blooms, and have been measured to contribute more than 40% of the total bacterial biomass in a lake, as measured by fluorescence *in situ* hybridisation (FISH) probes (Newman *et al.* 2011).

Phylum Cyanobacteria

The ecological importance of the phylum Cyanobacteria has been recognised for a long time. Similar to eukaryotic phytoplankton, the freshwater Cyanobacteria perform oxygenic photosynthesis but rely only on chlorophyll *a* and an assortment of phycobilins for photosynthesis. Some aquatic Cyanobacteria contain heterocysts, which are cells dedicated solely to nitrogen fixation. These Cyanobacteria along with many others that do not contain heterocysts, but fix nitrogen, play a key role in nutrient cycling in lakes. Cyanobacteria species are often considered nuisance organisms, as some species form large floating mats and may release toxins into lake waters. Common freshwater lake genera include *Microcystis, Anabaena, Aphanizomenon, Oscillatoria, Planktothrix, Synechococcus,* and *Cyanothece* (Newton *et al.* 2011). Cyanobacteria were found in this study using flow cytometry.

Phylum Proteobacteria

The phylum Proteobacteria is a group of Gram-negative bacteria encompassing the majority of recognised industrially, medically, and agriculturally relevant bacteria. Six classes of Proteobacteria are currently recognised: the Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, Gammaproteo-bacteria, and Zetaproteobacteria (Newton *et al.* 2011).

Class Alphaproteobacteria
The Alphaproteobacteria are found in all imaginable habitats and display enormous plasticity in their genomes and in their lifestyles. The Alphaproteobacteria are at the centre of the global nitrogen cycle, because symbiotic members of the phylum often facilitate atmospheric nitrogen fixation by plants. The Alphaproteobacteria are numerically dominant in many marine ecosystems, but are less numerous in freshwater lakes. Alphaproteobacteria are resistant to predation (grazing), they are competitive under conditions of low nutrient/substrate availability but are also capable of degrading complex organic compounds, and are widely distributed in lakes all over the globe. Members of this class are generally not very abundant, suggesting that unknown mechanisms are keeping the abundance of the Alphaproteobacteria low in freshwater lakes. Species include *Sphingopyxis* and *Brevundimonas* (Newton *et al.* 2011), both of which were found in this study.

Class Betaproteobacteria
Betaproteobacteria are recognised for their morphological and physiological diversity. Betaproteobacteria are often numerically abundant in freshwater lakes but are in relatively low abundance in the ocean. They are abundant in many different freshwater lake habitats, sometimes constituting up to 60 to 70% of the total number of cells, measured using FISH probes. Members of the Betaproteobacteria are often cocultured with algae, associated with Cyanobacteria and are often particle associated. Freshwater Betaproteobacteria are fast growing, nutrient-loving and have a size-dependent vulnerability to grazing pressure. Species include *Limnohabitans* (Newton *et al.* 2011), found in this study.

Class Gammaproteobacteria
The class Gammaproteobacteria contains the most studied of all bacterial organisms, the Enterobacteriales. Many enteric organisms, such as *Escherichia coli*, can be found in freshwater lakes, however, they are generally considered transient members originating from anthropogenic or zoonotic sources and are generally not detected with the 16S rRNA method. This low recovery suggests that they may be transient members of lake communities brought in from the surrounding environment, or they could be common lake members existing at low abundances. The Gamma-proteobacteria are generally more abundant in saltwater environments such as the ocean or saline lakes than in freshwater. Gammaproteobacteria are generally found in the upper water in lakes (Newton *et al.* 2011). *E. coli* was found in this study using traditional culture.

Phylum Verrucomicrobia
Verrucomicrobia species have been identified in lakes, soil, oceans, and human faeces and even as ecto- and endosymbionts of eukaryotes. Members of the Verrucomicrobia do not seem particularly abundant in lakes, ranging between <1% and 6% based on clone recovery. However, application of FISH probes has yielded up to 20% of the total bacterial community. Methane oxidising members of the phylum have been isolated from extremely low pH, thermophilic environments. Members of the Verrucomicrobia have also been observed in both surface and hypolimnetic waters, suggesting a variety of metabolic strategies within the group. Some members of the

phylum seem to be associated with high-nutrient environments or algal blooms (Newton *et al.* 2011).

Three bacterial genera were the most commonly found in the rivers Elliðaár and lake Elliðavatn by 16S rRNA gene sequencing; *Flavobacterium, Limnohabitans* and *Verrucomicrobium* spp. These genera are commonly found in freshwater bodies and sediment.

Flavobacterium

Flavobacterium is a genus of Gram-negative, non-motile and motile, rod-shaped bacteria that consists of ten recognised species (Bergeys manual). Flavobacteria are found in soil and freshwater in a variety of environments. Several species are known to cause disease in freshwater fish (Bernardet *et al.* 1996). The genus *Flavobacterium* was established in 1923 to accommodate Gram-negative, non-spore-forming, yellow-pigmented rods that produce acid from carbohydrates weakly. Because of this limited definition the genus rapidly acquired many poorly defined species and consequently became very heterogeneous. However, through successive emendations, the genus *Flavobacterium* was restricted to non-motile and non-gliding species and thus achieved what could be considered reasonable homogeneity in *Bergey's Manual of Systematic Bacteriology. Flavobacterium aquatile* is the type species of this genus (Bernardet *et al.* 1996).

Limnohabitans

The genus *Limnohabitans* (Comamonadaceae, Betaproteobacteria) has been recently described and established by Hahn *et al.* (2010a). The genus currently contains three described species. Some *Limnohabitans* spp. were classified as *Rhodoferax* spp. before. The type strains of all three *Limnohabitans* species described so far were all isolated from the water columns of stagnant freshwater systems located in Central Europe. A novel strain from a pond located in subtropical South America has recently been described. This novel strain is closely related to previously described *Limnohabitans* spp. (Hahn *et al.* 2010b). The bacteria are free-living and have a planktonic lifestyle (Simek *et al.* 2001). They are a significant contribution to total cell numbers of freshwater bacterioplankton (up to 30%; Simek *et al.,* 2010). The bacteria have a broad habitat range including, for instance, acidic and alkaline systems (Simek *et al.* 2010); and the potential for rapid growth under *in situ* conditions (Simek *et al.* 2006; Hahn 2010b).

Verrucomicrobium

Verrucomicrobia is a recently described phylum of bacteria that only contains a few described species. The species that have been identified were isolated from fresh water, marine sediments, hot springs, soil and human faeces. Various members of Verrucomicrobia are estimated to constitute up to 10% of all bacteria in soil, but very few have been grown in culture, and little is understood about their ecological role in the environment. Recent phylogenetic analyses of 16S rRNA sequences suggest that Verrucomicrobia form a clade with *Planctomycetes, Clamydiae, Lentisphaerae* and Poribacteria. (Hou *et al.* 2008). Several Verrucomicrobia live in association with eukaryotes, some as obligate endosymbionts in ectoparasitic nematodes of the genus

Xiphinema, while others live as ectosymbionts on hypotrich ciliates of the genus *Euplotidium* and defend their host against predation in a unique way by being ejected. Recent and future studies can be expected to considerably extend and deepen the knowledge of the phylum Verrucomicrobia, and to shed more light onto one of the most enigmatic branches of the bacterial tree of life (Wagner & Horn 2006).

1.2.2. Faecal pollution in freshwater

Water has been recognised as a potential carrier of disease causing agents for a long time. Many important human pathogens can survive in water and infect humans. When waters are used for recreation or as a source of food that is consumed uncooked, the possibility for disease transmission certainly exists (Prescott *et al.* 2005).

Faecal bacteria originate from the faeces of humans and warm-blooded animals. Their presence and quantity is used to measure faecal pollution in water. Water in the environment is however not an optimal habitat for faecal bacteria and their number declines rapidly after excretion. The quantity of these bacteria will therefore be greatly reduced after some time in the environment. Sunlight, saltiness/salt content, temperature and grazing are the main factors that lead to the death of faecal bacteria in water. Death rate is higher during summer because of higher temperatures and light (Þórðarson 2004; Prescott *et al.* 2005). Wild mammals are few in Iceland and therefore it is unlikely that faecal bacteria in water originate from them. Birds are more common and some species live at or near water. Therefore faecal bacteria from avian origin are more likely to be found in lakes that are unaffected by human activity (Þórðarson 2004). However, it takes a large number of birds or an unusually small and still lake for faecal bacteria of avian origin to be measurable in any quantity. Faecal pollution in freshwater is therefore most often caused by humans; either by the humans themselves, their pets or livestock (Þórðarson 2004; Prescott *et al.* 2005).

1.3. Methods to detect microbes in freshwater

The most common traditional method of determining bacterial community structure involves culturing the organisms from the habitat in question and identifying the cultures by standard techniques. However, very few bacteria can be cultured using traditional methods (Amann *et al.* 1995; Hurst *et al.* 1997). Therefore molecular methods will give much better information on the microbial diversity. Molecular methods only need the bacteria cells from which DNA can be extracted without cultivation. Such methods include the DNA-DNA hybridisation and 16S rRNA gene sequencing methods. The latter method has proved very useful in community structure analysis (Hurst *et al.* 1997).

1.3.1. Microbes as indicators of faecal pollution in freshwater

A wide range of viral, bacterial, and protozoan diseases results from the pollution of water with human faecal wastes. Although many of these pathogens can be detected directly, indicator organisms are generally used as an index of possible water pollution by human pathogens (Prescott *et al.* 2005). Indicator organisms are used

globally as a warning of possible pollution and as an index of water quality deterioration. The presence of enteric pathogens in drinking and recreational waters is of great concern. As a result of the danger to public health due to the presence of pathogens, it is extremely important to determine the microbiological safety of these waters. This practice is not perfect and there is considerable variety in the ways that different indicator microorganisms are applied in various geographical areas and situations, however, public health concerns are generally well served (Toranzoz & McFeters 1997). Commonly used indicator species are *E. coli, Enterococcus* spp. and norovirus, and are used as indicator species in this study.

Escherichia coli

E. coli is a Gram-negative, rod shaped bacterium commonly found in animal intestinal tracts. Most *E. coli* strains are harmless but some serotypes are important pathogens (Tortora *et al.* 2002). *E. coli* is the type species of the genus but several other species have been described. Strains of *E. coli* are usually motile and some, especially those from extra-intestinal infections produce a polysaccharide capsule. *E. coli* grows well on non-selective media, over a wide range of temperature (15-45°C). Some strains are more heat-resistant than others and can survive temperatures up to 60°C for 15 min or 55°C for 60 min. Most strains ferment lactose with the production of acid and gas within 24-48 h, but some do so only after extended incubation or are non-lactose fermenters. *E. coli* is widespread and present wherever there is faecal pollution. *E. coli* appears not to lead an independent existence outside the animal body, and is for this reason used as an indicator of faecal pollution of water sources, drinking water and food (Lewis 1997). *E. coli* satisfies most of the criteria of the total and faecal coliforms and has additional characteristics that make it a useful microbiological indicator of water quality. In particular, *E. coli* has been demonstrated to be a more specific indicator for the presence of faecal pollution than the faecal coliforms (Toranzos & McFeters 1997).

***Enterococcus* spp.**

Enterococcus is a genus of lactic acid bacteria of the phylum Firmicutes. Enterococci are Gram-positive cocci that often occur in pairs (diplococci) or short chains. Enterococci are often difficult to distinguish from streptococci on physical characteristics alone, but the genus used to be classified as Group D *Streptococcus* (Gilmore *et al.* 2002). Enterococci are facultative anaerobic organisms, i.e. they are capable of cellular respiration in both oxygen-rich and oxygen-poor environments (Fischetti *et al.* 2000). Enterococci cannot form spores but are tolerant of a wide range of environmental conditions; they can tolerate extreme temperature (5-65°C), pH (4,5-10,0) and high sodium chloride concentrations. This enables them to colonise a wide range of niches (Fisher & Phillips 2009). An important feature of the genus is its high level of intrinsic antibiotic resistance (Ryan & Ray 2004). Faecal enterococci are useful as indicators of microbiological water quality since they are common inhabitants of the intestinal tracts of humans and lower animals. Like coliforms (*E. coli*), some of these organisms have persistence patterns that are similar to those of a range of potential waterborne pathogenic bacteria (Toranzos & McFeters 1997).

Norovirus

Human noroviruses are a major cause of acute non-bacterial gastroenteritis in people of all age groups worldwide. Noroviruses are highly heterogeneous and are classified in the genus *Norovirus* that is in the family Caliciviridae, and include five genogroups, GI to GV. Noroviruses of GI, GII and GIV infect humans (Haramoto *et al.* 2009). Noroviruses are non-enveloped viruses, 27-35 nm in diameter, and possess a single-stranded RNA genome of 7.5-7.7 kb (Gregory *et al.* 2011). Norovirus are highly infectious. The faecal-oral route of transmission via polluted water or food is a predominant mode of transmission. Human noroviruses are found in high concentration in the faeces of an infected person. Recent studies have demonstrated the prevalence of human noroviruses in aquatic environments, such as raw sewage, treated sewage, river water, seawater and even tap water by using polymerase chain reaction (PCR) (Haramoto *et al.* 2009). Norovirus is commonly associated with human sewage and is responsible for numerous cases of waterborne and food borne gastroenteritis every year (Gregory *et al.* 2011). Due to a lack of suitable cell culture systems, noroviruses are detected by molecular methods (Gregory *et al.* 2011). Norovirus outbreaks due to polluted drinking water occur worldwide. These outbreaks often happen where sewage pollutes groundwater. In the last 28 years six norovirus outbreaks have occurred in Iceland. Viruses are highly persistent in water, and even more so in cold water, which makes groundwater supplies in cold climate such as Iceland especially vulnerable (Gunnarsdóttir 2012).

1.3.2. Microbial diversity analysis by 16S rRNA gene sequencing

Advances in the field of genetics have greatly improved the quality and accuracy of microbiological diversity. The use of the 16S rRNA gene sequencing method to study phylogeny and taxonomy of bacteria has become the most popular used method for many reasons; the 16S gene is present in all bacteria, the function of the gene has not changed over time which suggests that random sequence changes are a more accurate measure of evolution, and the 16S rRNA gene (1.500 bp) is large enough to store necessary information (Patel 2001; Janda & Abbott 2007). The 16S rRNA gene sequencing method is used to assess community structure in this study.

Use of the 16S rRNA gene sequencing method has caused an explosion in recognised species names. In 1980, 1.791 valid species names were recognised in the *Approved Lists*, today over 14.686 names are listed (Euzéby 2012). This explosion in number of recognised taxa can be attributed to the 16S rRNA method and how easy it is to use compared to more cumbersome methods, such as DNA-DNA hybridisation investigations. The DNA-DNA hybridisation method is the standard method used for proposed new species and for the definitive assignment of a strain with ambiguous properties to the correct taxonomic unit (Janda & Abbott 2007).

One of the best advantage of the 16S rRNA gene sequence method is that it can provide genus and species identification for isolates that do not fit any recognised biochemical profiles; for strains that only generate “low likelihood” or “acceptable” identification according to other methods (Janda & Abbott 2007); and that it is not necessary to culture the bacteria, instead the method is based on 16S rRNA screening from a DNA mass collected. It is generally believed that only 0,1-1% of bacteria can be cultivated using traditional methods, but with DNA isolation and 16S rRNA

screening the other 99% can be reached (Amann *et al.* 1995; Marteinsson *et al.* 2004). Studies suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65-83%), with 1-14% of the isolates remaining unidentified after testing (Drancourt *et al.* 2000; Mignard & Flandrois 2006; Woo *et al.* 2003; Janda & Abbot 2007).

By using culture-independent molecular techniques, a large number of new lineages in the domains Bacteria and Archea have been identified from the environment. Only a small fraction of existing microorganisms have been grown in pure cultures and characterised. Therefore, the ecophysiological role of the majority of microorganisms is not known. For that reason, molecular methods such as 16S rRNA sequencing are important to increase our understanding and knowledge of microorganisms (Okabe *et al.* 2010). Many studies have used the 16S rRNA gene sequencing method to identify genus or species of bacteria found in a specific freshwater lake (Pearce *et al.* 2005; Hahn *et al.* 2009) or stream/river (Belt *et al.* 2007; Beier *et al.* 2008; Lee *et al.* 2010; Porat *et al.* 2010; Souza *et al.* 2006; Hahn *et al.* 2009; Conradle *et al.* 2008). The 16S rRNA sequencing method has been used to show species diversity (Pearce *et al.* 2005; Souza *et al.* 2006; Belt *et al.* 2007; Beier *et al.* 2008; Porat *et al.* 2010). A few studies have used 16S rRNA gene sequencing to describe new species (Hahn *et al.* 2009; Strahan *et al.* 2011). Others have used the 16S rRNA gene sequencing method to re-identify known species (Conradle *et al.* 2008).

Although the 16S rRNA sequencing method plays an important role in identification of bacteria, the method is not foolproof and applicable in every situation. The disadvantage of the 16S rRNA method is that it recognises best the species in most abundance with other species often left undiscovered (Marteinsson *et al.* 2004); it has difficulties recognising novel taxa; too few sequences are deposited in nucleotide databases; species that share similar or identical 16S rRNA sequences; and nomenclature problems that arise from singe species or complexes being assigned the same genomovars (DNA groups). The method also has low phylogenetic power at the species level and poor discriminatory power for some genera. However, the 16S rRNA gene sequencing is the method that shows best natural diversity in the ecosystem excluding 16S tag pyro-sequencing (Marteinsson *et al.* 2004) and is a widely used method in environmental microbiology for which a large database exists.

1.4. Regulations concerning water quality in Iceland

Water quality in Iceland is generally good. However, like for everything else legislations are required to help keep it that way and prevent pollution. The legislation on water quality is divided in five regulations; regulation on the prevention of water pollution no. 796/1999, regulation on groundwater no. 797/1999, regulation on sewage and wastewater no. 798/1999, regulation on nitrates originating from agriculture no. 804/1999, and regulation no. 536/2001 on drinking water. These regulations are based on directives from the European Union (EU). Furthermore, Iceland has implemented the water framework directive (WFD) no. 2000/60/EC from the EU, with act number 36/2011 on the management of water and regulation no. 535/2011 on classification, characterisation, pressure analysis and monitoring of water bodies. The WFD is a framework for community action in the field of water policy. It commits European Union member states to achieve good qualitative and

quantitative status of all water bodies (including marine waters up to one nautical mile outside the base line for the territorial water). Present regulation no. 796/1999 is in effect until environmental quality standards based on ecological classification regarding regulation no. 535/2011 is completed before January 1st 2015. The new classification system is currently under development. Until then, Icelandic waters are regulated by regulation no. 796/1999.

Regulation no. 796/1999 on the prevention of water pollution is the leading regulation on surface water pollution in Iceland. Its purpose is to reduce and prevent water pollution from human impact. It also aims to limit the consequences of current water pollution (pollution that has already occurred) and that water classification obeys to certain rules and regulations. The regulation classifies water into five categories, A-E, as seen in Table 1.4.1.

Table 1.4.1. Classification of water according to regulation no. 796/1999

Class	Condition/state
A	Natural water (unpolluted)
B	Little pollution
C	Some pollution
D	Very polluted
E	Unsatisfactory water quality

Faecal bacterial pollution

Faecal bacterial pollution in surface water is classified into five categories. Environmental indicators should fit into each category in 90% of samples, unless otherwise instructed.

Environmental marks are:
I: Very little or no faecal pollution
II: Little faecal pollution
III: Some faecal pollution
IV: High faecal pollution
V: Unsatisfactory water quality/mixing zone

Table 1.4.2. Faecal bacteria threshold values according to regulation no. 796/1999

Environmental mark	I	II	III	IV	V
***E. coli* or Enterococcus spp. (cfu/100ml)**	<14*	14-100	100-200	200-1000	>1000

*Faecal pollution can reach 43 colony forming units (cfu)/100 ml in 10% of samples.

In regulation no. 798/1999 it further states that environmental quality standards for special pollutants such as coliforms/*Enterococcus* spp. should not exceed 1000 cfu/100 ml in 90% of at least 10 samples in less sensitive areas outside mixing zones.

In sensitive areas, coliforms should not exceed 100 cfu/100 ml in 90% of at least 10 samples outside mixing zones.

Nutrients
Threshold values for nutrients and organic compounds in water are classified into five categories, the same as for faecal bacteria.

Table 1.4.3. Nutrient status according to regulation no. 796/1999

Environmental mark	Nutrients in lakes and rivers
I	Oligotrophy
II	Oligo-/mesotrophy
III	Meso-/eutrophy
IV	Eutrophy
V	Hypertrophy

Table 1.4.4. Threshold values for nutrients in lakes according to regulation no. 796/1999

Environmental mark indicator compound	I	II	III	IV	V
Tot-P (mg P/l)					
Shallow water	<0.02	0.02-0.04	0.04-0.09	0.09-0.15	>0.15
Deep water	<0.01	0.01-0.03	0.03-0.05	0.05-0.10	>0.10
Tot-N (mg N/l)	<0.30	0.30-0.75	0.75-1.50	1.50-2.50	>2.50

Table 1.4.5. Threshold values for pollution indictors in rivers according to regulation no. 796/1999

Environmental mark indicator compound	I	II	III	IV	V
BOD	<1.5	1.5-3.0	3-6	6-10	>10
COD	<3.0	3-10	10-20	20-30	>30
TOC (mg O_2/l)	<1.5	1.5-3	3-6	6-10	>10
Ammonia NH_3 (mg/l)	<0.01	<0.025	<0.10	<0.25	>0.25
PO4-P (mg/l)	<0.01	<0.02	<0.05	<0.10	>0.10
Tot-P (mg P/l)	<0.02	<0.04	<0.09	<0.15	>0.15
Tot-N (mg N/l)	<0.30	0.30-0.75	0.75-1.50	>1.50	>2.50

These are the environmental quality standards the results in this study will be compared to, since the new threshold values are not yet published, and will not take effect until 2015.

1.5. Lakes and rivers in urban areas

Much of the surface in urban and suburban areas is covered by buildings and pavement that do not allow rain and snowmelt to soak into the ground. Instead the runoff from roofs and paved areas is carried to nearby waterways by storm drains. The storm water runoff carries pollutants such as oil, chemicals, dirt, and lawn fertilisers directly into streams and rivers, where they can seriously affect water quality. When large amounts of storm water flow into streams, its excessive volume and power can cause damage to the streambanks, damaging streamside vegetation and the aquatic habitat. These increased storm flows can carry sediments from construction sites and other denuded surfaces and eroded streambanks. They often carry higher water temperatures from rooftops, streets and parking lots, which can be harmful to the health and reproduction of aquatic life. Urbanisation can also lower stream flow during dry weather and cause profound groundwater charges. Urbanisation increases the variety and amount of pollutants carried into streams, rivers, and lakes. These pollutants include; oil, grease, and toxic chemicals from motor vehicles; sediment; pesticides and nutrients from lawns and gardens; bacteria, viruses, and nutrients from pet waste and failing septic systems; heavy metals from roof shingles, motor vehicles, and other sources; road salts; and thermal pollution from dark impervious surfaces such as streets and rooftops. These pollutants can harm fish and wildlife populations, kill native vegetation, foul drinking water supplies, and make recreational areas unsafe and unpleasant (EPA 2003). Major sources of pollution in lakes and rivers in Reykjavík are thought to be wrong sewage connections, unsatisfactory septic tanks, manure, fertiliser dispersion, agriculture, aquaculture, street and drain water (Reykjavík Public Health Authority).

1.5.1. The Elliðaár river catchment area

The Elliðaár river catchment area reaches North far into Mosfellsheiði, to the East to Hengill and to the South of Bláfjöll (Rist 1969; Þórðarson 2004). The entire catchment area is thought to be 286 km^2 (Línuhönnun verkfræðistofa 2002; Þórðarson 2004). The catchment area is situated on a very permeable bedrock, so in most of the area, surface water cannot be found. Approximately 75% of precipitation that falls in the area becomes groundwater (Þórðarson 2004). A large part of the Elliðaár catchment area is a protected water area. The Reykjavík Energy Company harness water from the area but it is not considered to have any effect on the water flow to lake Elliðavatn (Þórðarson 2004). The catchment area is on the Icelandic Nature Conservation Register (Umhverfisstofnun 2012).

The Elliðaár rivers catchment area, Reykjavík, Iceland, is a unique water system. The rivers whole catchment area, from their origin in lake Elliðavatn to the estuary in Elliðavogur is within the Reykjavík city limit. The Eastern river of the Elliðaár rivers is also considered a good fishing river, which adds to the uniqueness of the river.

Salmon is the dominating breed found in the river but river trout and sea trout are also common in the water system (Þórðarson 2004).

Most lakes and rivers in Reykjavík were classified during the period 2001-2007. In 2002 lake Elliðavatn was classified as a type A-B lake, and in 2003 the rivers Elliðaár were classified as type A-C rivers according to provisions in regulation no. 796/1999 on water pollution (Reykjavík Public Health Authority).

1.5.2. Impact of urbanisation

The Elliðaár catchment area is still mostly untouched and uninhabited, but there is an urban area close to the lake Elliðavatn and around Elliðaár valley. In Elliðaár valley is a large stable area. At the North part of the catchment area are many bridle paths commonly used. At lake Elliðavatn there are also many old summerhouses, a sheep farm and a chicken farm. Around Hólmsá there are old summerhouses, whole year houses, stables and gravel mine. Along Suðurá are also a few summerhouses. At the Eastern part of the catchment area there is a gliding flight airport, gravel mine, kindergarten and a diner. At the East and Southeast border are the largest ski-areas of the greater Reykjavík area (Þórðarson 2004).

Wastewater from residential settlements around lake Elliðavatn and Elliðaár valley is discharged to the ocean with a double wastewater treatment system. However, street and drain water flows into the rivers through a collecting system but direct input of sewage to the rivers is not known (Þórðarson 2004). Since the early 2000ds the residential area around Elliðavatn has grown considerably and the area continues to develop, resulting in increased human impact on the lake and the rivers. Recent studies have shown this, like the Reykjavík Public Health Authority study in 2009.

1.6. Freshwater studies in urban lakes and rivers

Many studies have been carried out on freshwater quality, using microbial indicator species as a measurement. Monitoring and detection of indicator and disease-causing microorganisms are a major part of sanitary microbiology. Monitoring freshwater quality in urban areas is very important, as these areas are often a place for recreational activities and pathogen transmission from water to humans can easily occur (Prescott *et al.* 2005).

Faecal bacteria have been used as an indicator for freshwater quality in urban areas, in streams/rivers (Mancini *et al.* 2004; Servais *et al.* 2007; Carvalho *et al.* 2011; Thorn *et al.* 2011; Vavias *et al.* 2011; Viau *et al.* 2011), lakes (Haugland *et al.* 2005; Chandran *et al.* 2008) and coastal waters (Schiff *et al.* 2003), in Rome, Italy (Mancini *et al.* 2004), UK (Thorn *et al.* 2011), France (Servais *et al.* 2007), Greece (Vavias *et al.* 2011), Portugal (Carvalho *et al.* 2011), USA (Schiff *et al.* 2003; Haugland *et al.* 2005), O'ahu, Hawaii (Viau *et al.* 2011), New Zealand (Till *et al.* 2008) and India (Chandran *et al.* 2008). Further, specific indicators have been related to urban water quality; *E. coli* (Mancini *et al.* 2004; Chandran *et al.* 2008; Servais *et al.* 2007; Till *et al.* 2008; Thorn *et al.* 2011; Viau *et al.* 2011), total coliforms (Mancini *et al.* 2004), faecal coliforms (Mancini *et al.* 2004; Servais *et al.* 2007), faecal streptococci (Mancini *et al.* 2004), enterococci (Viau *et al.* 2011; Haugland *et al.* 2005),

Salmonella (Till *et al.* 2008; Chandran *et al.* 2008; Viau *et al.* 2011), *Campylobacter* (Till *et al.* 2008; Viau *et al.* 2011), *Vibrio cholerae* (Chandran *et al.* 2008), *Vibrio parahaemolyticus* (Chandran *et al.* 2008; Viau *et al.* 2011), *Vibrio vulnificus* (Viau *et al.* 2011) and *Staphylococcus aureus* (Viau *et al.* 2011) have been used as indicator bacteria for urban water quality. Norovirus has also been used as an indicator of water quality since it is commonly associated with human sewage and is responsible for many cases of water- and foodborne gastroenteritis every year (Gregory *et al.* 2011). Most of the studies reported poor water quality (Mancini *et al.* 2004; Chandran *et al.* 2008; Viau *et al.* 2011; Vavias *et al.* 2011; Carvalho *et al.* 2011; Servais *et al.* 2007; Schiff *et al.* 2003) and none of the studies reported good water quality. Many studies linked human influence to the poor water quality (Schiff *et al.* 2003; Mancini *et al.* 2004; Servais *et al.* 2007; Chandran *et al.* 2008; Carvalho *et al.* 2011; Thorn *et al.* 2011; Vavias *et al.* 2011; Viau *et al.* 2011).

1.7. Previous studies in the rivers Elliðaár and lake Elliðavatn

Few studies have been done on the microbial conditions in Elliðaár and Elliðavatn by cultivation methods. The Reykjavík Public Heath Authority has carried out most of the studies as a part of monitoring rivers and water quality in the Greater Reykjavík area.

In 2001-2002 a research was carried out on the environmental quality of lake Elliðavatn for the Environment and Health Department of Reykjavík (Þórðarson 2003). In 2003 a research was carried out to evaluate the human impact on the rivers Hólmsá, Suðurá and Elliðaár that are all a part of the Elliðaár catchment area, for the Environment and Health Department of Reykjavík. Samples were collected in four sites along the rivers (Þórðarson 2004). In 2009, lake Elliðavatn (one sampling site) and the rivers Elliðaár (six sampling sites) were included in a large study in the greater Reykjavík area along with other lakes and rivers in the area (Reykjavík Public Health Authority). In these studies the lake and rivers were categorised according to provisions in the regulation on the prevention of water pollution no. 796/1999. Faecal pollution (*E. coli*, *Enterococcus* spp.), chlorophyll, indicator compounds (NH_4-N, Tot-N, Tot-P, TOC) and metals (Cu, Zn, Cd, Pb, Cr, Ni, As) were measured (Þórðarson 2003,2004; Reykjavík Public Health Authority).

In the 2001-2002 study lake Elliðavatn was categorised as a trophic lake, and no obvious signs of pollution or recent degradation of water quality were seen. A high TOC concentration of was observed and not fully explained, but considered mostly natural. The same results were obtained for ammonium. Because of the high flushing rate and a high proportion of springwater in the throughflow, Elliðavatn was not considered very sensitive to pollution loading. Elliðavatn was given the pollution status A – unpolluted. A proposal was made for the continued monitoring of the lake. A yearly monitoring of faecal bacteria was suggested despite good results, because of continued development in the catchment area. A continued monitoring of the nutrients was suggested because of high concentrations of TOC, NH_4-N and Tot-P. Little metal pollution was found in the lake, but it was expected to increase with continued development so monitoring was advised two years later. Future water quality goal was set as pollution status A (Þórðarson 2003).

In 2003, the overall outcome for the Elliðaár rivers was good, but least for the West channel of the rivers, which was estimated to be clearly influenced by human contamination. In that study a proposal was made for a condition of natural state as the long-term water quality goal. Proposals were also made for the monitoring of the rivers. It was proposed to monitor one sampling site two years later and three sampling sites four years later. The proposal took into account that human impact was considerable in the rivers catchment area and would increase in the years and decades to come. At the same time urbanisation of the catchment area was expected to expand. Since nutrients, organic carbon and faecal bacteria often have the same source it was considered wise to monitor these simultaneously. According to the study the rivers were categorised as type A-C rivers (Þórðarson 2004).

In 2009, the overall outcome for the Elliðaár rivers was good. The rivers were categorised as A-E rivers, on average type B rivers for faecal bacteria and nutrients. The lake was categorised as an A-C lake for faecal bacteria and an A-B lake for nutrients, being oligo-/mesotrophic lake (Reykjavík Public Health Authority). Pollution had increased in lake Elliðavatn and the rivers Elliðaár since the 2001-2003 studies.

The Reykjavík Public Health Authority has conducted studies on coastal waters by cultivation techniques in Reykjavík, including the Elliðavogur inlet, since 2003. In the last two to three decades great improvements have been made in domestic wastewater treatment in the Reykjavík city area. This has resulted in reduced faecal pollution with coastal waters around Reykjavík of generally good condition (Ólafsdóttir & Steinarsdóttir 2010; Ólafsdóttir *et al.* 2011).

1.7.1. Fish pathogens in Elliðaár rivers

Aeromonas salmonicida subsp. *salmonicida* infection was diagnosed in salmon in Elliðaár rivers in the summer of 1995. This was the first time this infection was diagnosed in Iceland. During that summer and fall 200 salmons were infected and killed by the disease. This represented approx. 7% of the fish stock in the river that year, and approx. 20% of the catch. The outbreak was localised in Elliðaár rivers with only a few exceptions. The disease has not been diagnosed since May 1996. *Aeromonas salmonicida* ssp. *achromogenes* was a problem in the entire country for a while but due to constant vigilance of fishermen and constant improvements in cleanliness the problem has been mostly eradiated (Jónsson 1995,1996,1997; Sandholt and Jónsson 1995). Outbreaks like this one are a reminder how important it is to monitor the rivers regularly and to have knowledge of their water quality status.

1.8. Research objective, questions and limitations

The research objective was to study the natural environmental microbial flora in the rivers Elliðaár catchment area and to screen for faecal pollution. Conditions in the river and lake were evaluated four times over three seasons and nine months for faecal pollution and once for the environmental microbial flora. The project conducted a practical examination of the lake and river water with traditional cultures and genetic/ molecular methods.

Research questions:

1. What is the total number of bacteria in the lake and at different sites along the rivers in different seasons?
2. Is the microbial diversity similar in the lake and rivers and what are the dominant taxa?
3. Is there a seasonal faecal pollution in the lake and rivers?
4. To which category can we classify the rivers and lake according to water quality standards?

Limitations:
It was clear from the beginning that the number of samples and analysis would be limited. One sample was collected from each segment of the river and lake during three seasons; summer, fall and winter. Only one season could be selected for analysis by 16S rRNA sequencing and a limited number of clones were sequenced from each sampling site.

2. Materials and methods

2.1. Study sites and sampling

2.1.1. Lake Elliðavatn

Lake Elliðavatn is located at the border of Reykjavík and Kópavogur and is located at 64° 5,298'N, 21° 47,017'W (ja.is 2012) and is 76.5 m above sea level (Þórðarson 2004). Elliðavatn was originally two lakes, Vatnsendavatn in Kópavogur and Vatnsvatn in Reykjavík. The two lakes were separated by a cape called Þingnes and connected by a narrow channel (see Figure 2.1.1). In 1924-1928 a dam was built at Elliðavatnsengi and the lake area grew 40% to its current size. Lake Elliðavatn is now 2 km^2 and its average depth is 1 m (Kópavogsbær 1998).

Figure 2.1.1. Pre-dam Elliðavatn (Morgunblaðið 2000)

Lake Elliðavatn is a springfed lake. Most of the water in the lake flows into it through the ground as groundwater. Two streams flow into the lake, Hólmsá and Suðurá. One river flows out of the lake, Elliðaá, that later splits into two rivers (Kópavogsbær y.m.). Water stays in the lake for a relatively short time compared to other lakes, or only 4.6-4.9 days (Þórðarson 2003). The lake was used as a reservoir for the hydropower station in the river (Þórðarson 2004).

Samples were taken at three locations in the lake. One sample was taken where the river Elliðaá flows from the lake, close to the residential area (A), one was taken close to the residential area (B) and one sample was taken further away from residential area (C), see Figures 2.1.2 – 2.1.5.

Figure 2.1.2. Sampling sites in lake Elliðavatn. Arrows show location of sampling sites. Sampling sites are labelled A-C. A is where the river flows from the lake close to the residential area, B is close to the residential area and C is farthest away from the residential area. Note that this picture is from 2009, and the residential area is now bigger than shown here.

Figure 2.1.3. Sampling site A (close to the origin of the river and residental area) in lake Elliðavatn, September 2011

Figure 2.1.4. Sampling site B (close to the residental area) in lake Elliðavatn, September 2011

Figure 2.1.5. Sampling site C (farthest away from residential area) in the lake Elliðavatn, September 2011

2.1.2. The rivers Elliðaár

The rivers Elliðaár flow from the lake Elliðavatn through the Elliðaár valley in Reykjavík city, a popular outdoor recreational area. The estuary of the river is in Elliðavogur. The river branches out in the middle of the valley and flows to the ocean in two segments (see Figure 2.1.6.) (Reykjavíkurborg y.m.). Early in the 20th century the river was harnessed for power and a dam was built (Antonsson & Árnason 2011). Above the Árbær dam, waters are calm and the rivers runs along scrublands and down some rocky steppes. Below the dam, the current increases somewhat until reaching the generation station. There, small flats take over that end at the cascades of Sjávarfoss, the first holding spot for salmon seeking the freshwaters of the Elliðavogur inlet (SVFR 2010). Through the years the riverbed of the river has been changed by human impact (Antonsson & Árnason 2011).

The length of the rivers Elliðaár to lake Elliðavatn is 6 km and from there to the origin are 23 km (Rist 1969; Þórðarson 2004). The average river flow over a 65 year period at Elliðaárstöð is 5.03 m^3/s (Gagnabanki vatnsmælinga 1996; Þórðarson 2004). New information from the Icelandic Meteorology Office indicates 4.56 m^3/s flow at Heyvað that is at the top of the river (Þórðarson 2004).

Samples were taken from 6 locations along the river. The first sample (1) was taken midway from the origin of the river to the place where it branches out into two separate rivers. Two samples (2 & 3) were taken from each river before they merge in a dam, Árbær dam. Two samples (4 & 5) were taken midway in each river that runs from the dam to the rivers estuary and one sample (6) was taken at the estuary, see Figures 2.1.6 – 2.1.12.

Figure 2.1.6. Map of rivers Elliðaár and vicinity. Sampling sites are labelled 1-6. A sample was taken at each segment of the rivers. Note the estuary is at the top of the picture. Salmon fishing is allowed at the top of the river to where the river branches out (sampling site 1) and in the East river (sampling sites 3 and 5).

Figure 2.1.7. Sampling site 1 (top of the river) in the Elliðaár rivers, September 2011

Figure 2.1.8. Sampling site 2 (West river, above the Árbær dam) in Elliðaár rivers, September 2011

Figure 2.1.9. Sampling site 3 (East , river above the Árbær dam) in Elliðaár rivers, September 2011

Figure 2.1.10. Sampling site 4 (West river, in Elliðaár Valley) in Elliðaár rivers September 2011

Figure 2.1.11. Sampling site 5 (East river, in Elliðaár valley) in rivers, September 2011

Figure 2.1.12. Sampling site 6 (the rivers estuary) in Elliðaár rivers, September Elliðaár 2011

2.1.3. Sampling conditions

Samples of river water were collected from three sampling sites in lake Elliðavatn and six sampling sites along Elliðaár rivers during June/July and September 2011 and January and February 2012 (see Figures 2.1.2 and 2.1.6). The water samples were collected in 5 L sterile plastic bottles for diversity analysis by culturing methods and DNA extraction for 16S rRNA gene analysis. The water samples were taken from the surface of the water using a long scoop. During sampling, water temperature was measured with a handheld thermometer, pH was evaluated with pH strips and pictures were taken at each location. All samples were taken to the laboratory at MATÍS for immediate analyses, except the samples taken in January and February, they were collected on Sunday, stored in a refrigerator at 4°C and processed early Monday morning. Samples for 16S rRNA gene analysis (after filtration) were stored in -80°C until processing. See further description in chapters 2.3 – 2.5.5.

When the first samples (June 16th) were collected the air temperature was around 10-11°C. It was mostly cloudy with sunshine in between and very low wind. Weather had been similar the days prior to sampling day. Samples from sampling site 6 were collected on low tide. On July 13th and 27th the air temperature was around 12-13°C with heavy wind and rain. The days leading up to sampling day were dryer with less wind, but similar temperature. When the fall samples were collected (September 20th)

the air temperature was around 9°C with a blend of sunshine and rain mixed. There was a storm in the last few days prior to sampling day. Samples at site 6 were collected on high tide. In January (15th) air temperature was around 1°C with short showers and clouds and low wind. Snow and ice had covered everything since the end of November with frost most days (except for a little break in early January) but ice melting had started. Sampling site 2 was overflowing and had ice covering most of it. Sampling site 3 was frozen so samples were collected beneath the dam instead. Sampling site A had a lot of ice, but enough water to collect samples, but sampling sites B and C were frozen to the bottom of the lake, so no samples could be collected. All other sampling sites were easily reached. Samples at site 6 were collected on high tide. In February (19th) the air temperature was about 1-3°C and raining. There was frost and snow in the days prior to sampling day. A lot of ice and snow had already melted. Lake and river were overflowing. Ice was broken to reach samples at sites A, B, C, 2 and 3. Samples at site 6 were collected on high tide.

2.2. Media and cultural conditions

All water samples were filtered through a sterile gridded 0.22 μm cellulose membrane filter (Millipore Corporation, MA, USA) to capture microbial cells. The filter was placed directly on media plates for cultivation. For media recipes see Appendix A.

2.2.1. Total viable count

To evaluate total viable count 0.1, 1, 10 and 100 ml samples were filtered through a sterile 0.22 μm cellulose membrane filter and placed onto Reasoner's 2A (R2A) agar (Difco, Kansas, USA) and incubated in 30°C for 4-5 days for evaluation of total viable count. Samples taken in July (Elliðavatn only) and September were additionally cultured at 4°C and 22°C. The 4°C samples needed a few extra days in incubation to observe growth. Samples taken in January and February were cultured at 4°C and 30°C. For better results, 20 ml of sterile phosphate buffer (FB) was used with the 0.1 and 1 ml samples to increase the volume filtered, allowing better dispersion of cells to be grown on the filter paper. In some cases colonies could not be counted due to large numbers or overgrowth. In those cases colonies were counted in 5-10 squares on the filter paper and the average multiplied by 107 (according to Gæðahandbók Matís).

2.2.2. Total count of faecal bacteria

Water samples were filtered through a sterile 0.22 μm cellulose membrane filter to cultivate *E. coli* and *Enterococcus* spp. Ten and 100 ml were filtered and the filter placed on m FC basal medium (Difco) (*E. coli*) and Bile Aesculin (BA) agar (Oxoid, Hampshire, UK) (*Enterococcus* spp.) for total faecal count. The m FC agar was incubated at 44°C for 24 h and the BA agar was cultivated at 37°C for 48 h. *E. coli* bacteria were counted and confirmed using liquid lactose broth (Oxoid), incubated in a 44°C water bath for 24 h. *E. coli* ferment lactose which is a distinctive feature and used to confirm *E. coli.* For confirmation, 10 colonies were picked from each sample. *Enterococcus* spp. count was obtained and confirmed by using Slanets & Bartley (S&B) agar (Oxoid). The filter was moved from the BA agar onto S&B agar and

incubated at 44°C for 2 h. During that time the *Enterococcus* spp. colonies formed a ferric precipitation, a distinct feature that can be used to accurately count the *Enterococcus* spp. colonies.

2.2.3. Strain isolation and purification

Representatives of most colony types growing on R2A in samples taken in June and July were picked and transferred to a fresh R2A agar to obtain pure cultures. The colonies were picked and re-cultured three times to isolate pure strains. The strains were incubated at the temperature from which they originated, 4°C, 22°C or 30°C. Colonies were compared and at the end, 101 possible pure strains were collected and frozen in 87% glycerol at -80°C for future analysis.

2.3. Cell count with flow cytometer

For cell count, 5 ml of water sample from each sample were fixed in 0.12% gluteraldehyde and then frozen at -80°C for cell count. About 300 µl of sample were mixed with 3 µl of 1/100 diluted SYBR Green and incubated at room temperature for 20 min. About 25 µl of counting beads (CountBright™ absolute counting beads, Invitrogen) was put into the sample before they were placed in flow cytometer BD FACSAria™ II. Samples flew through the cytometer for 30 s (elapsed time) and then events were recorded and counted for 1 min. Results were viewed using BD FACSDiva Software. Cells were then calculated using the formula:

A/B * C/D = concentration of sample as cells/µl

Where:
A = number of cell events
B = number of bead events
C = assigned bead count of the lot (beads/50µl)
D = volume of sample (µl)

In this case 25 µL of beads were used, i.e. 24750 beads/25 µl (C) (CountBright™ 2005).

2.4. Chemical analysis

Water samples were analysed for nutrient contents of nitrate/nitrite, phosphate and ammonium. Nitrate/nitrite was analysed by method 353.2, Determination of Nitrate/nitrite Nitrogen by Automated Colorimetry from the Environmental Monitoring Systems Laboratory, Office of Research and Development, U.S. Environmental Protection Agency (O'Dell 1993a). Phosphate was analysed by method 365.1, Determination of Phosphorus by Semi-automated Colorimetry from Environmental Monitoring Systems Laboratory, Office of Research and Development, U.S. Environmental Protection Agency (O'Dell 1993b). Ammonium was analysed by using a fluorometric method that gives a precise measurement of

ammonium, originally developed by Holmes *et al.* (1999), and improved by Taylor *et al.* (2007).

2.5. Diversity analysis with molecular methods

2.5.1. DNA extraction and PCR amplification

For the extraction of total DNA from river water, 1 l of sample was filtered through a sterile gridded 0.45 μm pore size cellulose membrane filter (Millipore Corporation) for microbial cell capture, followed by DNA extraction performed by using the KingFisher method (Reynisson *et al.* 2008).

Extracted DNA was PCR-amplified in a DNA engine Terad®2 Peltier Thermal Cycler from Bio Rad using the 20 μM primer pair 9F (GAGTTTGATCCTGGCTCAG) and 805R (GACTACCAGGGTATCTAATCC). One μl of each primer was used, 0.8 μl dNTP, 4.0 μl 10x buffer, 0.8 μl Teg polymerase and 0.8 μl betaine. Thirty-eight μl mix and 2 μl sample was used. The polymerase chain reaction (PCR) reaction was performed as follows: after 5 min of denaturation at 95°C, 35 thermal cycles were performed as follows: 40 s at 95°C, 40 s at 52°C and 1 min at 72°C, followed by a final extension step at 72°C for 7 min. The PCR product was then incubated at 4°C until collected.

2.5.2. GFX procedure

PCR amplification products were visualised on 1% agarose gel containing ethidium bromide and run at 100V or 150V depending on gel size. DNA was cut from the gel under UV-light and cleaned using a gel band purification kit (GE Healthcare, illustra™, GFX™) according to manufacturers instructions with slight modifications. Then 600 μl capture buffer was placed on the gel containing the DNA, incubated at 60°C with shaking for 15-20 min or until the gel had dissolved. This was placed in a column for 1 min and centrifuged for 30 s at 13.000 rpm. Then 600 μl wash buffer was added, centrifuged for 30 s and the supernatant was discarded again, centrifuged again for 15 s and remaining supernatant was discarded again. Columns were carefully placed in a new eppendorf tube and 20 μl of H_2O were added. The tubes were stored at -20°C until further analysis.

2.5.3. 16S rRNA gene amplification and cloning

Adenine (A) was added to the 3' end of the GFX product by using 10 μl GFX product, 0.5 μl dNTP, 1,5 μl 10x buffer, 0.1 μl Teg polymerase (Matís) and 2.9 μl H_2O (15 μl total). PCR reaction program AD 72 was performed as follows: incubation at 72°C for 10 min followed by incubation at 10°C until collected.

The PCR products from the biomass were cloned directly by the TA cloning method by using a TOPO TA cloning kit according to the instructions of the manufacturer (Invitrogen). Cloning was done as follows: 1 μl salt solution, 4 μl GFX/PCR product

and 1 µl topo vector mixed carefully together, followed by incubation at room temperature for 20 min. Then 3.5 µl of the solution were carefully mixed with top 10 cells, incubated on ice for 30 min, placed in 42°C water bath for 30 s and back on ice. 250 µl SOC solution (stored at 20°C) was added to the solution and shaked (950 rpm) at 37°C for 60 min. The solution was placed on two lysogeny broth plates containing antibiotic ampicillin (LB/AMP) and cultured overnight at 37°C in a loosely closed plastic bag. Clones that grew overnight were picked and placed in a 96-well plate with liquid LB/AMP medium and incubated at 37°C overnight. Twenty-four clones were re-cultured from each sample.

PCR reaction was performed from the clones using 0.4 µl dNTP, 3.0 µl 10x buffer, 0.18 µl 100µM M13F primer, 0.18 µl 100 µM M13R primer, 0.2 µl Teg polymerase and 26.0 µl H_2O; 30 µl total. PCR reaction was performed as follows: denaturation at 94°C for 2 min, 30 thermal cycles of incubation at 94°C for 40 s, 50°C for 40 s and 72°C for 1 min 30 s, followed by a final extension step at 72°C for 7 min and then incubated at 4°C until collected. The PCR product was then placed on agarose gel to make sure the clones contained the amplified product (partial gene) and the rest frozen at -20°C.

2.5.4. 16S rRNA gene sequencing

Plasmid DNA from single colonies was isolated and sequenced. Four µl Exo/Sap mix (0.25 µl EXO/SAP and 3.75 µl H_2O) was added to 2.5 µl of the clone/PCR product and placed in PCR for a cleaning step; incubation at 37°C for 25 min and 80°C for 15 min followed by incubation at 4°C until collected.

The next step was thermal cycling. A mix of 1.0 µl 3.5 µM R805 primer, 0.5 µl Big Dye, 1.5 µl 5x buffer and 2.0 µl H_2O was added to the Exo/Sap product and placed in PCR where 25 thermal cycles were conducted at 99°C for 30 s, 50°C for 15 s 60°C for 4 min, followed by incubation at 4°C until collected.

The sequencing was performed by Alexandra María Klonowski and Steinunn Ásbjörg Magnúsdóttir (Matís) on an ABI 3777 DNA sequencer by using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according to Marteinsson *et al.* (2001). Results were viewed using the program Sequencer 4.8.

2.5.5. Sequences analysis

Sequences were viewed and edited by using the program Sequencer 4.8 from ABI. Bacteria phylogenetic trees were constructed using the results from sequencing obtained by the NCBI Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=WGS&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch). To construct a tree, sequences were exported from Sequencer 4.8 to the program Bio Edit a Sequence Alignment Editor and MEGA 5.0 used for tree construction.

2.5.6. Norovirus detection and PCR amplification

One litre of pre-filtered water was used for viral isolation. The water pH was adjusted to ca. 6.5-7.0 using 1 molar (M) hydrogen chloride (HCl). Water was filtrated using Zeatpor, an electropositive filter with pore size of 45 μm. Filter was placed on petridish with 3 ml of 50 μM glycine sodium hydroxide (NaOH) (containing 1% beef extract, pH 9.5) buffer. The filter and solution were shaken for 20 min and then 600 μl 0,1M HCl were added. The sample and buffer were placed into an Amicon Ultra Spin column following centrifugation at 2500 rpm for 5-10 min. The sample was reduced to a volume of ca. 140 μl. If the volume was greater, centrifugation was repeated. Sample was transferred to a 1.5 ml eppendorf tube and is ready for RNA isolation. Stored at -20°C.

For viral RNA purification, a kit (QiaAmp Viral RNA, Quiagen, Maryland, USA) was used. The product was then placed in Real Time PCR using a mix (12.5 μl Master Mix, 0.5 μl 20 μM Primer F9, 0.5 μl 20 μM Primer R805, 0.5 μl Probe, 1.0 μl RT Enzyme and 5 μl H_2O). Real Time PCR program was performed as follows: the denaturation step was conducted at 50°C for 30 min and 95°C for 10 min after which 40 thermal cycles were undertaken (95°C for 30 s, 50°C for 30 s and 72°C for 30 s), followed by a final extension step at 72°C for 7 min. Both undiluted and 10x diluted samples were PCR amplified along with a positive control.

3. Results

3.1. Samples characteristics

Samples were collected from 6 sampling sites in the rivers Elliðaár and 3 sampling sites in lake Elliðavatn four times in June/July, September 2011, January and February 2012. Additional samples were collected in the lake in July 2011 for faecal bacterial cultures. Results from the environmental measurements are presented in chapters 3.1.1 – 3.1.3 and Figures 3.1.1 – 3.1.3.

3.1.1. Water temperature

Water temperature was measured on each sampling site in the rivers and lake using a handheld thermometer. Results are given in Table 3.1.1 and Figure 3.1.1.

Table 3.1.1. Water temperature (°C) in the rivers and lake

Sampling time/ site	June	July	July (extra)*	September	January	February
1	12.1	-	-	8.6	0.3	0.1
2	12.3	-	-	8.5	0.1	0.2
3	12.4	-	-	8.4	0.3	0.0
4	12.7	-	-	8.3	0.6	0.9
5	12.8	-	-	8.2	0.4	0.4
6	12.3	-	-	8.1	0.5	0.5
A	-	12.9	12.3	8.9	0.5	0.2
B	-	13.3	11.8	8.8	-	0.2
C	-	12.2	11.7	7.6	-	0.3

- Samples not collected
*Note that samples taken in July (extra) were extra samples only for faecal bacteria culture.

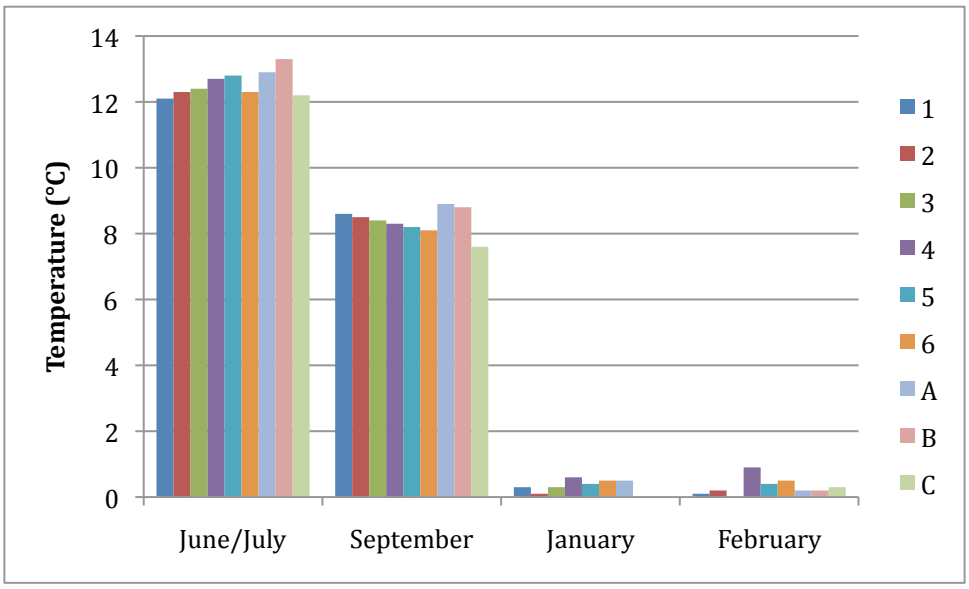

Figure 3.1.1. Water temperature in the rivers and in the lake

3.1.2. Conductivity

Conductivity was measured for each sampling site in the rivers and the lake in all samples collected. Results are given in table 3.1.2 and figure 3.1.3. One sample (sampling site 6 in September) measured much higher than other samples, see table 3.2.1.

Table 3.1.2. Conductivity (µS/cm) in the rivers Elliðaá and lake Elliðavatn

Sampling time/ site	June/July	September	January	February
1	90.3	93.8	104.0	92.9
2	91.6	94.2	113.5	95.8
3	95.0	94.1	110.8	96.0
4	109.9	106.4	144.9	233.0
5	94.9	95.1	109.2	106.7
6	4.48	2650.0*	124.0	110.2
A	97.3	95.0	101.0	94.6
B	94.5	91.8	-	80.9
C	101.7	99.4	-	83.6

- Samples not collected

* Note high conductivity in sampling site 6 in September.

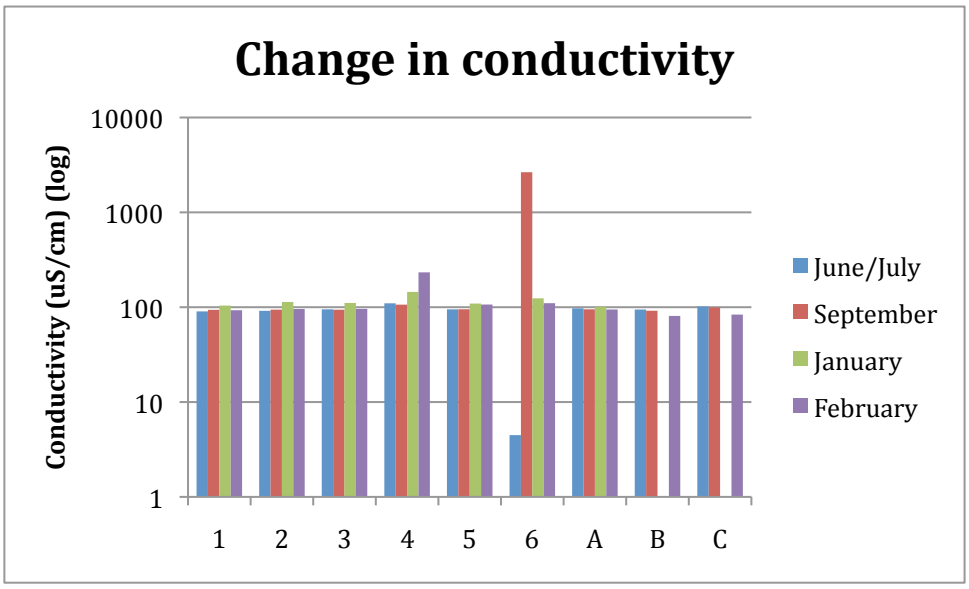

Figure 3.1.2. Change in conductivity in the rivers and lake on a log scale

3.1.3. pH

The pH was evaluated at each sampling site of the rivers and lake in all samples collected. The pH was measured using pH strips to get approximate pH value of the water. Most samples had a pH value of 7, while lower values were observed in

January samples 5 and A (pH 6.5) and higher pH in all February samples (pH 7.5) and two June samples (site 1= pH 8 and site 2= pH 7.5).

3.2 Bacterial load of the lake and rivers

3.2.1. Total bacterial count

Total count of bacteria in the rivers and lake samples was evaluated using two methods; by culture on the non-selective R2A medium and with culture-independent quantification by flow cytometry. Results are given in Tables 3.2.1 – 3.2.4 and Figures 3.2.1 – 3.2.6.

Viable plate count method

Water from all samples was filtered and cultured onto R2A agar for 5 days at 30°C and 4°C for all samples except in June 2011. Results are presented as colony forming units (cfu) per 100 ml in Tables 3.2.1 – 3.2.2 and Figures 3.2.1-3.2.2.

Table 3.2.1. Total viable counts (10^3 cfu/100ml) of water samples obtained by cultivation (30°C)

Sampling time/ site	June/July	September	January	February
1	3	58	86	80
2	6	67	96	84
3	6	158	138	59
4	10	83	116	141
5	5	76	147	41
6	31	642	1498	81
A	205	428	963	37
B	237	149	-	70
C	117	151	-	8

- Samples not collected

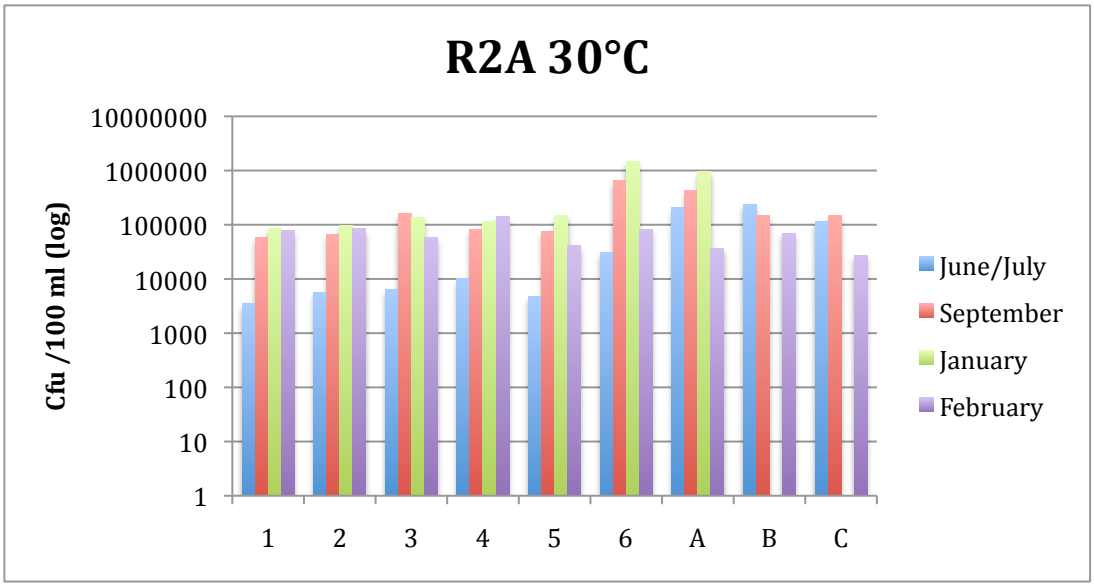

Figure 3.2.1. Total viable counts (cfu/100 ml) of water samples obtained by cultivation (30°C)

Table 3.2.2. Total viable counts (10^3 cfu/100 ml) of water samples obtained by cultivation (4°C). June samples were not cultivated in 4°C

Sampling time/ site	July	September	January	February
1	-	49	1391	33
2	-	42	96	25
3	-	185	104	28
4	-	66	176	86
5	-	61	62	10
6	-	200	1017	31
A	94	84	107	21
B	161	78	-	22
C	123	428	-	7

- Samples not collected

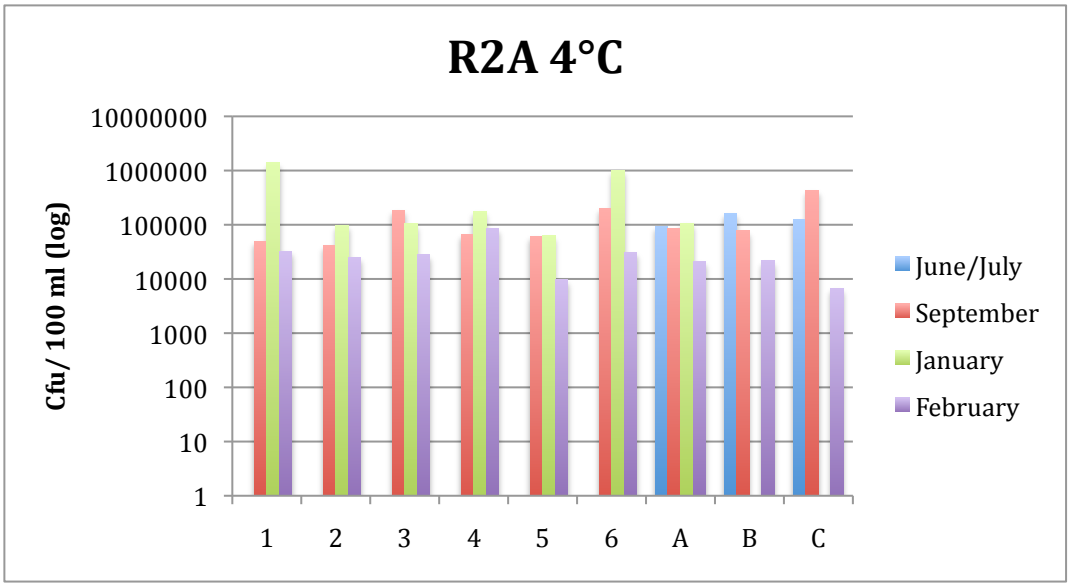

Figure 3.2.2. Total viable counts (cfu/100ml) of water samples obtained by cultivation (4°C)

Cell count by flow cytometry

Cell count was estimated using a flow cytometer for all samples collected. The cytometry counts bacterial and cyanobacterial cells, and cell count per ml was calculated using the formula in chapter 2.3. Results in 100 ml are presented in Tables 3.2.3 – 3.2.4 and Figures 3.2.3 – 3.2.6.

Table 3.2.3. Bacterial cell count (10^6 cells/100ml) of water samples obtained by flow cytometry

Sampling time/ site	Bacterial cell count (cells/100 ml)			
	June/July	September	January	February
1	65	100	31	55
2	54	104	37	97
3	92	97	28	39
4	36	86	34	51
5	47	99	36	47
6	91	216	41	187
A	118	112	76	74
B	115	112	-	70
C	123	79	-	45

- Samples not collected

Table 3.2.4. Cyanobacterial cell count (10^6 cells/100 ml) of water samples obtained by flow cytometry

Sampling time/ site	Cyanobacterial count (cells/100ml)			
	June/July	September	January	February
1	23	30	16	19
2	21	27	17	29
3	27	30	17	16
4	16	25	16	20
5	20	27	18	18
6	21	23	18	38
A	58	31	21	24
B	42	30	-	27
C	34	23	-	20

- Samples not collected

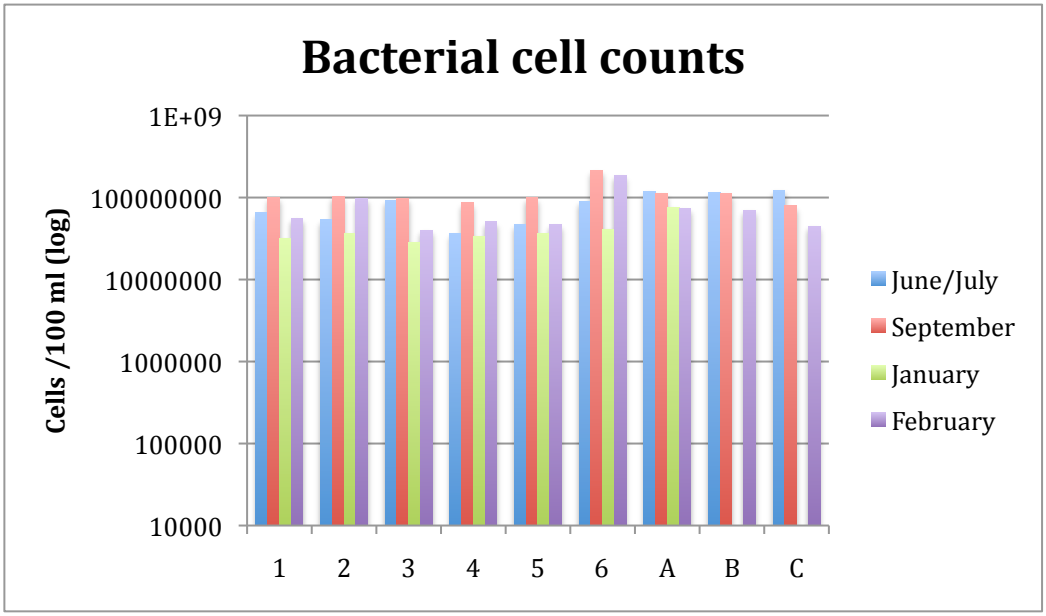

Figure 3.2.3. Bacterial cell count (cells/100 ml) of water samples obtained by flow cytometry. Bacterial and background could not be separated, introducing some inaccuracy in counts. However, comparison among samples can be made.

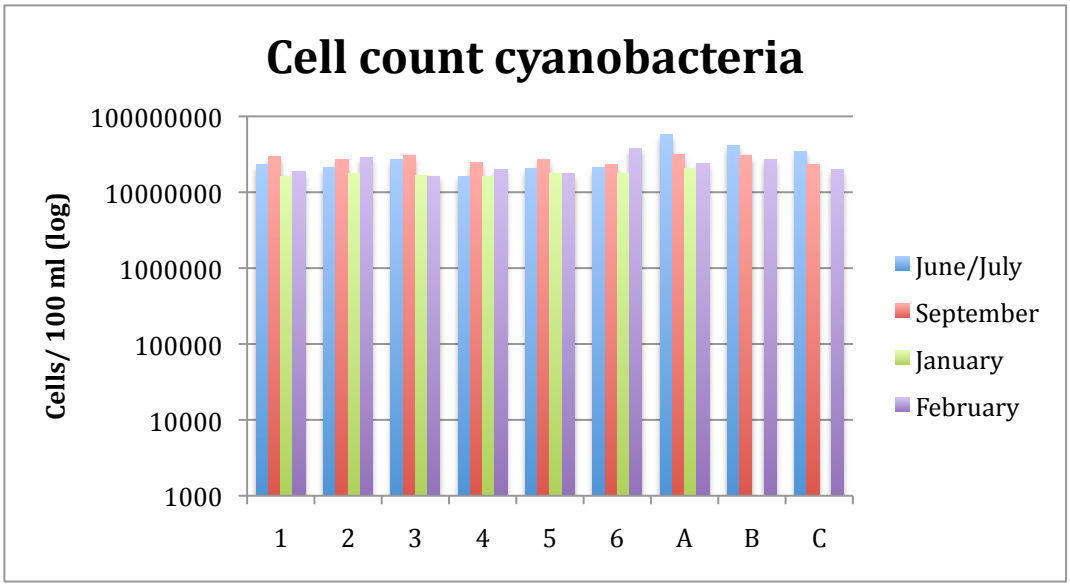

Figure 3.2.4. Cyanobacterial count (cells/100 ml) of water samples obtained by flow cytometry

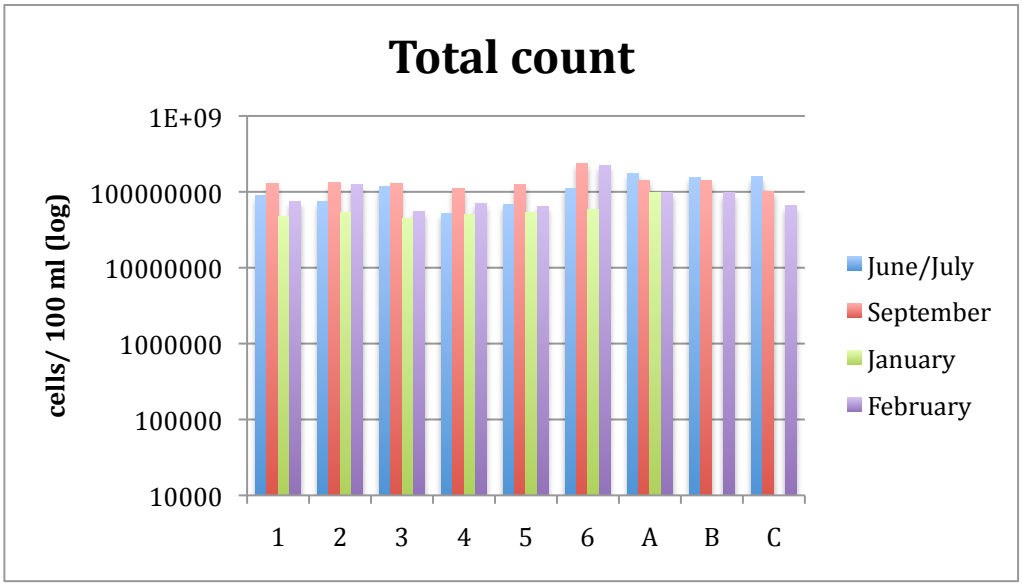

Figure 3.2.5. Total counts (bacterial and cyanobacterial cells/100 ml) of water samples obtained by flow cytometry

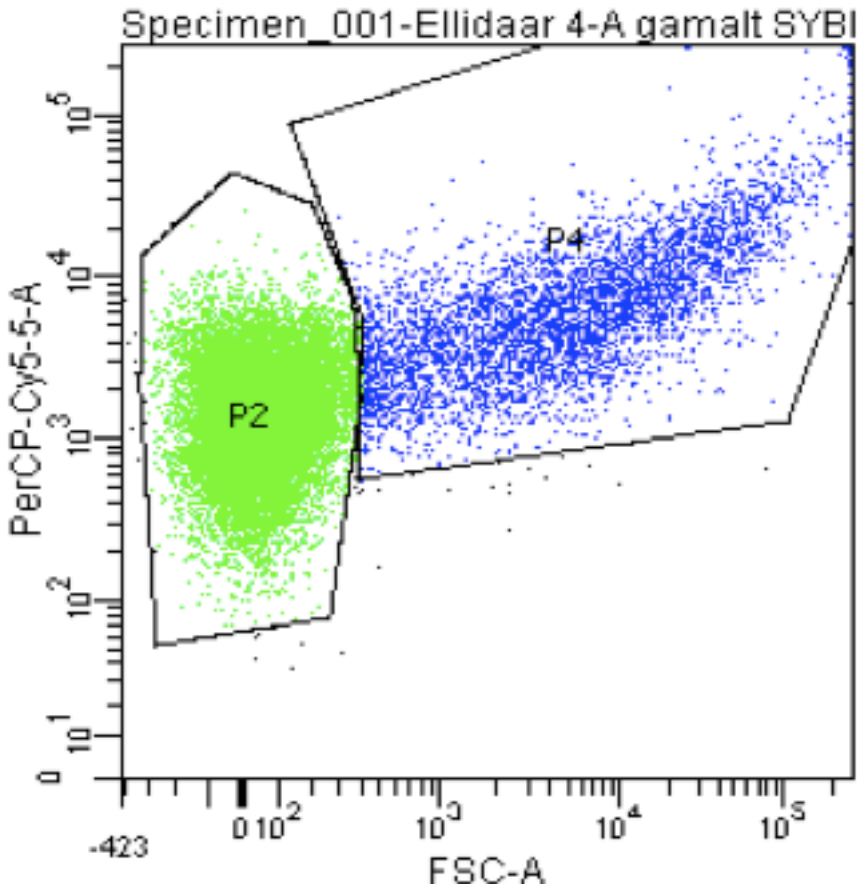

Figure 3.2.6. Example of cell count in flow cytometry. Sample from site A, February 2012. P2 (green) is bacteria (and background) and P4 (blue) is cyanobacteria.

3.2.2. Total count of faecal bacteria

Total count of faecal bacteria (*E. coli* and *Enterococcus* spp.) was evaluated by cultivation in all samples collected from the rivers and the lake. Results are given in Tables 3.2.5 – 3.2.7 and Figures 3.2.7 – 3.2.8.

E. coli

E. coli were cultivated and counted as cfu in 100 ml and classified with provisions according to regulation no. 796/1999 (see chapter 1.4). Results are presented in Table 3.2.5 and Figure 3.2.7. Higher *E. coli* counts were counted in the lake than in the rivers in all months except February. Sites 3, 4, A and B measured with the highest counts and were all over the regulation limit. Three largest counts were in samples 3 (September), A (September) and B (July). Extremely high concentration was measured at sampling site A in September.

Table 3.2.5. Number of *E. coli* cfu in 100 ml and classification according to regulation no. 796/1999

Sampling time/ Site	June	July	July (extra)	September	January	February
1	1^{a}	-	-	31^{b}	35^{b}	0^{a}
2	3^{a}	-	-	14^{b}	19^{b}	0^{a}
3	28^{b}	-	-	470^{d}	36^{b}	1^{a}
4	22^{b}	-	-	48^{b}	56^{b}	56^{b}
5	4^{a}	-	-	31^{b}	32^{b}	0^{a}
6	4^{a}	-	-	60^{b}	36^{b}	2^{a}
A	-	150^{c}	240^{d}	7490^{e}	240^{d}	5^{a}
B	-	780^{d}	41^{b}	25^{b}	-	0^{a}
C	-	10^{b}	49^{b}	6^{a}	-	0^{a}

- Samples not collected
a, unpolluted; b, little pollution; c, some pollution; d, very polluted; e, unsatisfactory water quality

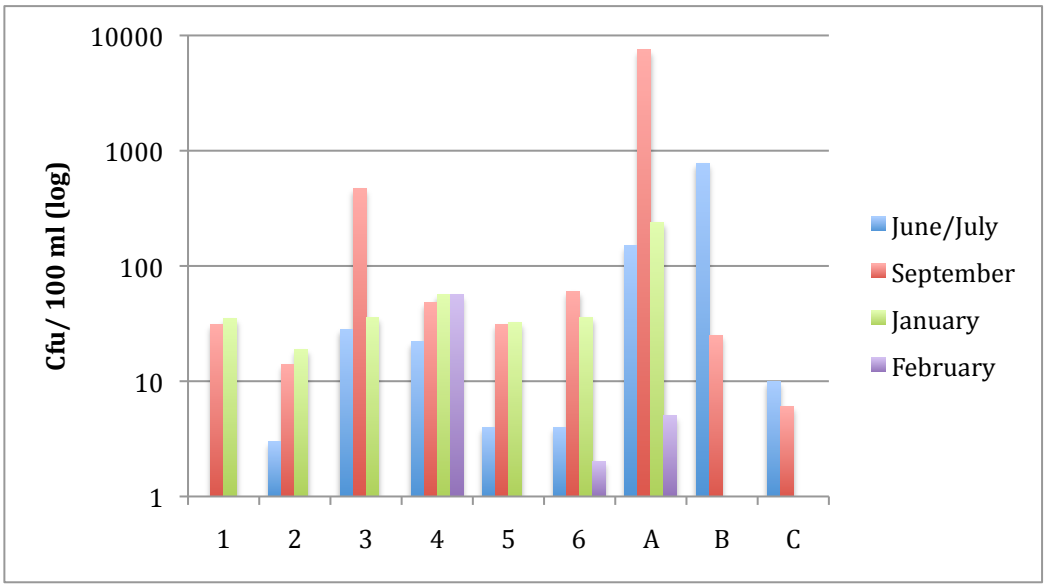

Figure 3.2.7. *E. coli* (cfu/100 ml) on a log scale excluding extra samples in July

Enterococcus spp.

Enterococcus spp. were cultivated and counted as cfu in 100 ml from all samples collected and classified with provisions according to regulation no. 796/1999 (see chapter 1.4). Results can be seen in Table 3.2.6 and Figure 3.2.8. High counts were generally measured at site A. September values are notably high in general followed by January samples.

Table 3.2.6. Number of *Enterococcus* spp. (cfu/100 ml) and classification according to regulation no. 796/1999

Sampling time/ site	June	July	July (extra)	September	January	February
1	1[a]			5[a]	12[a]	1[a]
2	1[a]			6a	11[a]	3[a]
3	11[a]			70[b]	6[a]	0[a]
4	3[a]			7[a]	17[b]	6[a]
5	3[a]			40[b]	14[b]	4[a]
6	4[a]			10[a]	18	2[a]
A		51[b]	127[c]	17[b]	210[d]	4[a]
B		12[a]	13[a]	20[b]		0[a]
C		8[a]	8[a]	2[a]		0[a]

- samples not collected
a, unpolluted; b, little pollution; c, some pollution; d, very polluted; e, unsatisfactory water quality

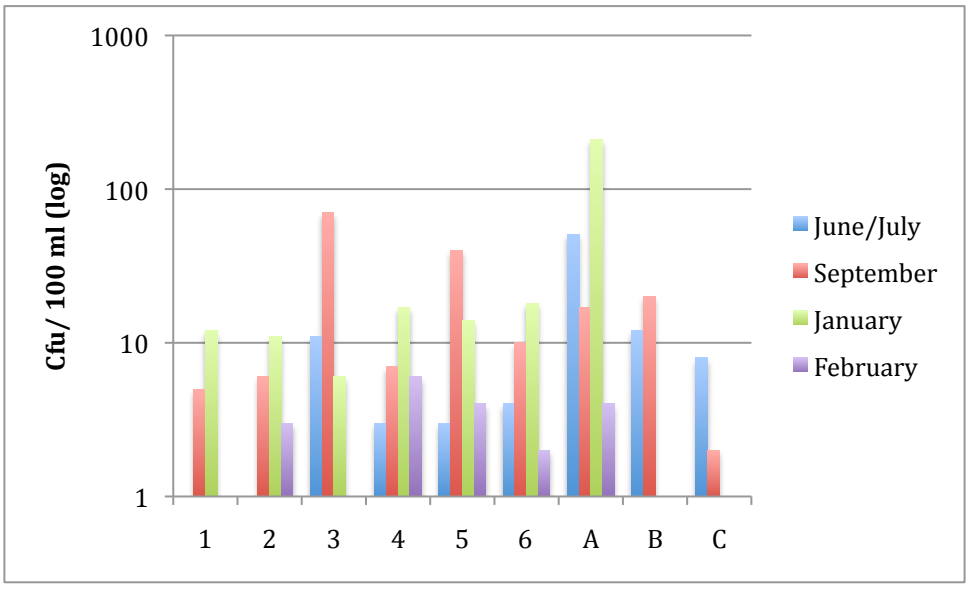

Figure 3.2.8. *Enterococcus* spp. (cfu/100 ml) on a log scale excluding extra samples in July

3.3. Analysis of nitrite, nitrate, phosphate and ammonium concentration

Nitrite, nitrate, phosphate and ammonium concentrations were measured in summer samples collected in July 2012. Results are presented in Table 3.3.1. All samples were observed to be lower than the detection limit.

Table 3.3.1. Results of nitrite, nitrate, phosphate and ammonium analysis

Compound/ site	NO_3-N [mg/l]	NO_2-N [mg/l]	PO_4-P [mg/l]	NH_4-N [mg/l]
1	<0.01	<0.05	<0.1	<0.05
2	<0.01	<0.05	<0.1	<0.05
3	<0.01	<0.05	<0.1	<0.05
4	<0.01	<0.05	<0.1	<0.05
5	<0.01	<0.05	<0.1	<0.05
6	<0.01	<0.05	<0.1	<0.05
A	<0.01	<0.05	<0.1	<0.05
B	<0.01	<0.05	<0.1	<0.05
C	<0.01	<0.05	<0.1	<0.05
Detection limit	0.01 mg/l	0.05 mg/l	0.1 mg/l	0.05 mg/l

3.4 Diversity analysis by 16S rRNA gene sequencing

3.4.1. Analysis of 16S rRNA gene sequences and species diversity

Summer samples (June/July) were chosen for diversity analysis to get an overview of the dominant taxa in the natural bacterial flora of the rivers and lake. DNA was isolated from the concentrated cell mass and 16S rRNA genes were sequenced after successful PCR amplification with universal bacterial primers and cloning. About 500-700 bp from each gene were obtained. Genes from 24 clones from each sample were analysed. In most samples 1-2 clones could not be sequenced, giving a total of 204 clones being sequenced. The clones were among the phyla Actinobacteria, Bacteroidetes, Verrucomicrobia and Proteobacteria with different class; alphaproteobacteria, betaproteobacteria, gammaproteobacteria, deltaproteobacteria. Most of the bacteria analysed were related to species that have previously been detected in freshwater and soil habitats. No pathogens were detected with this method. Most of the clones had a known relative with over 95% homology. A few clones showed homology with less than 95%, and it is likely that those represent new species. The closest relatives to the clones are presented in Table 3.4.1 and for each sample a phylogenetic tree is shown in Figures 3.4.1 – 3.4.10.

Four bacterial taxa are common in most samples; *Flavobacterium* spp. were present in all samples, *Limnohabitans* spp. were identified in all samples except A and B, Actinomycetales were detected in all samples except 6 and A, and Actinobacteria were found in all samples except 2, 3 and 4. Other bacteria were unique to a sample or detected in few samples. Sample 6 (the estuary) counted several unique taxa, except it shared five common taxa; *Flavobacterium* spp., *Limnohabitans* spp., *Cytophaga* sp., *Flectobacillus* sp. and Actinobacteria. Lake samples A and B differed in taxa composition; their most dominant single taxon was *Verrucomicrobium* spp. which was not found in other samples. *Limnohabitans* spp. a common taxon in all other samples, was not found in samples A and B. Lake sample C however resembled the river samples and was identical in taxa composition to sample 1. Taxa diversity changed down the river, see Table 3.4.1 and Figure 4.4.1.

Table 3.4.1. BLAST results. Taxa found at each sampling site.

Site / BLAST identification	1	2	3	4	5	6	A	B	C
Flavobacterium spp.	x	x	x	x	x	x	x	x	x
Limnohabitans spp.	x	x	x	x	x	x			x
Pseudorhodobacter sp.	x								x
Actinomycetales bacterium	x	x	x	x	x			x	x
Actinobacteria	x				x	x	x	x	x
Micrococcineae bacterium	x	x					x		x
Burkholderiales bacterium		x							
Rhodoferax sp.		x	x						
Uncultured bacterium		x							
Sphingopyxis sp.		x	x	x					
Sandarakinorhabdus limnophila		x							
Spingobacterium sp.		x	x				x	x	
Cellulophaga sp.		x			x				
Comamonadaceae bacterium			x		x				
Unc. Alpha Proteobacterium			x						
Aricella sp.			x	x	x				
Canditus Planktophila limnetica			x				x		
Fluviicola sp.				x					
Cytophaga sp.				x		x	x		
Flexibacter sp.				x	x		x		
Rhodobacter sp.				x					
Flectobacillus sp.					x	x			
Hyphomicrobiaceae bacterium					x				
Ideonella sp.					x				
Polaribacter sp.						x			
Flaviramulus basaltis						x			
Bacteroidetes bacterium						x			
Fimicutes bacterium						x			
Methylobacter sp.						x			
Roseovarius aestuarii						x			
Desulfuromusa succinoxidans						x			
Unc. Delta Proteobacterium						x			
Desulfobacterium zeppelinii						x			
Desulfobacula sp.						x			
Sulfurimonas sp.						x			
Verrucomicrobium sp.							x	x	
Prostetobacter vanneervenii							x		
Brevundimonas sp.							x		
Micavibrio sp.								x	
Xanthomonadaceae bacterium								x	
Roseomonas sp.								x	
Leptothrix sp.								x	
Thaurea sp.								x	
Methylophilaceae bacteria								x	
Number of taxa per site	**6**	**11**	**10**	**9**	**11**	**16**	**10**	**11**	**6**

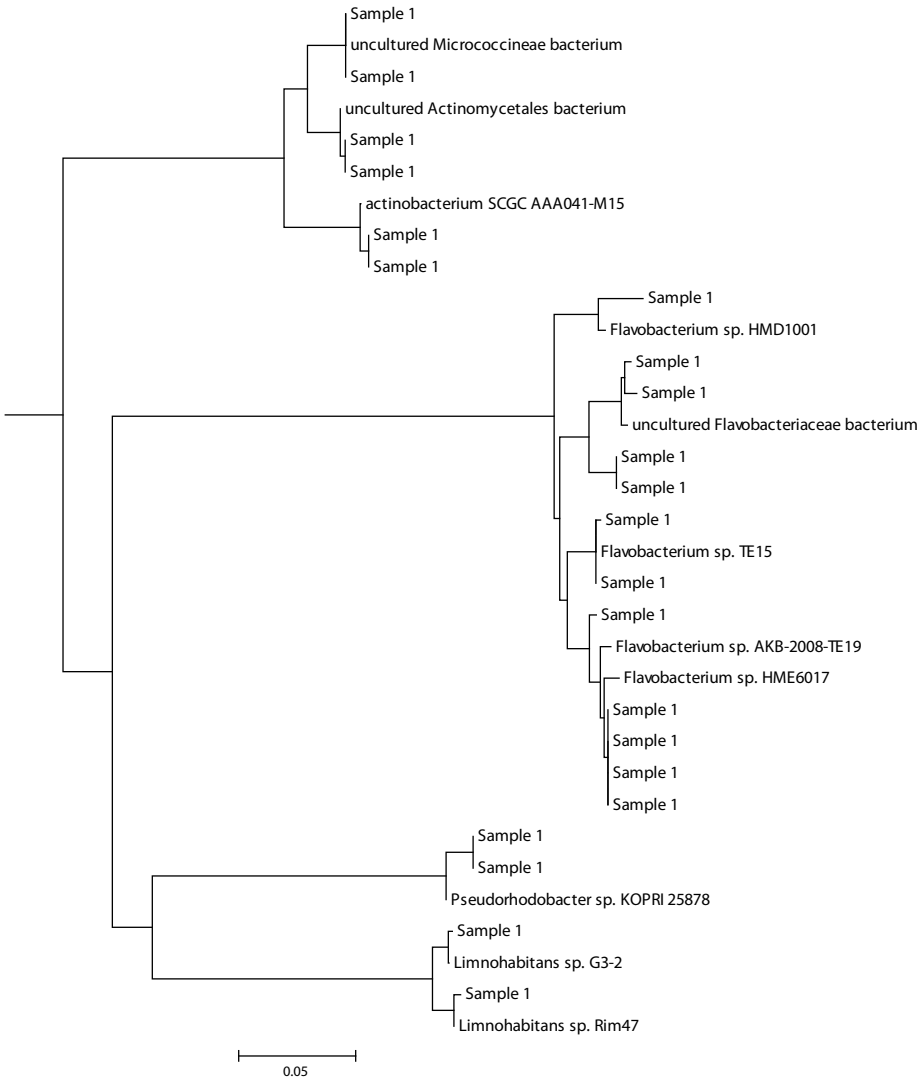

Figure 3.4.1. Neighbour-joining tree of sequences from river sample 1. The 16S rRNA clone libraries shows phylogenetic relationships with four major taxa, Flavobacteria (12 clones, 96-99% sequence similarity), Actinobacteria (6 clones, 99-100% sequence similarity), *Limnohabitans* spp. (2 clones, >99% sequence similarity) and *Pseudorhodobacter* sp. (2 clones, >99% sequence similarity). The scale bar represents the expected % of substitutions per nucleotide position and *Thermus thermophilus* was used as an outgroup.

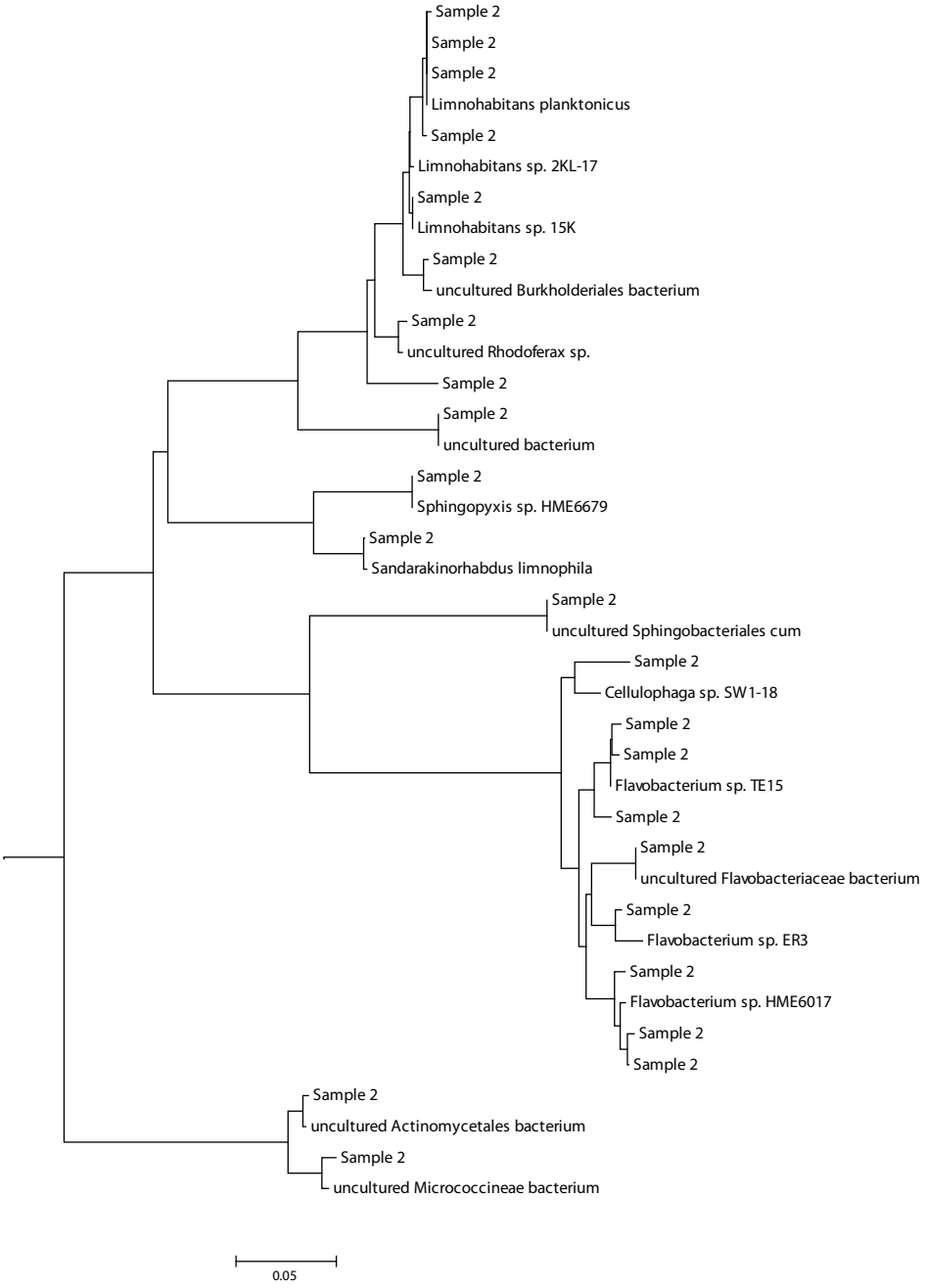

Figure 3.4.2. Neighbour-joining tree of sequences from river sample 2. The 16S rRNA clone libraries shows phylogenetic relationships with three major taxa, Flavobacteria (8 clones, 98-100% sequence similarity), Actinobacteria (2 clones, >99% sequence similarity), *Limnohabitans* spp. (5 clones, 99-100% sequence similarity). The scale bar represents the expected % of substitutions per nucleotide position and *T. thermophilus* was used as an outgroup.

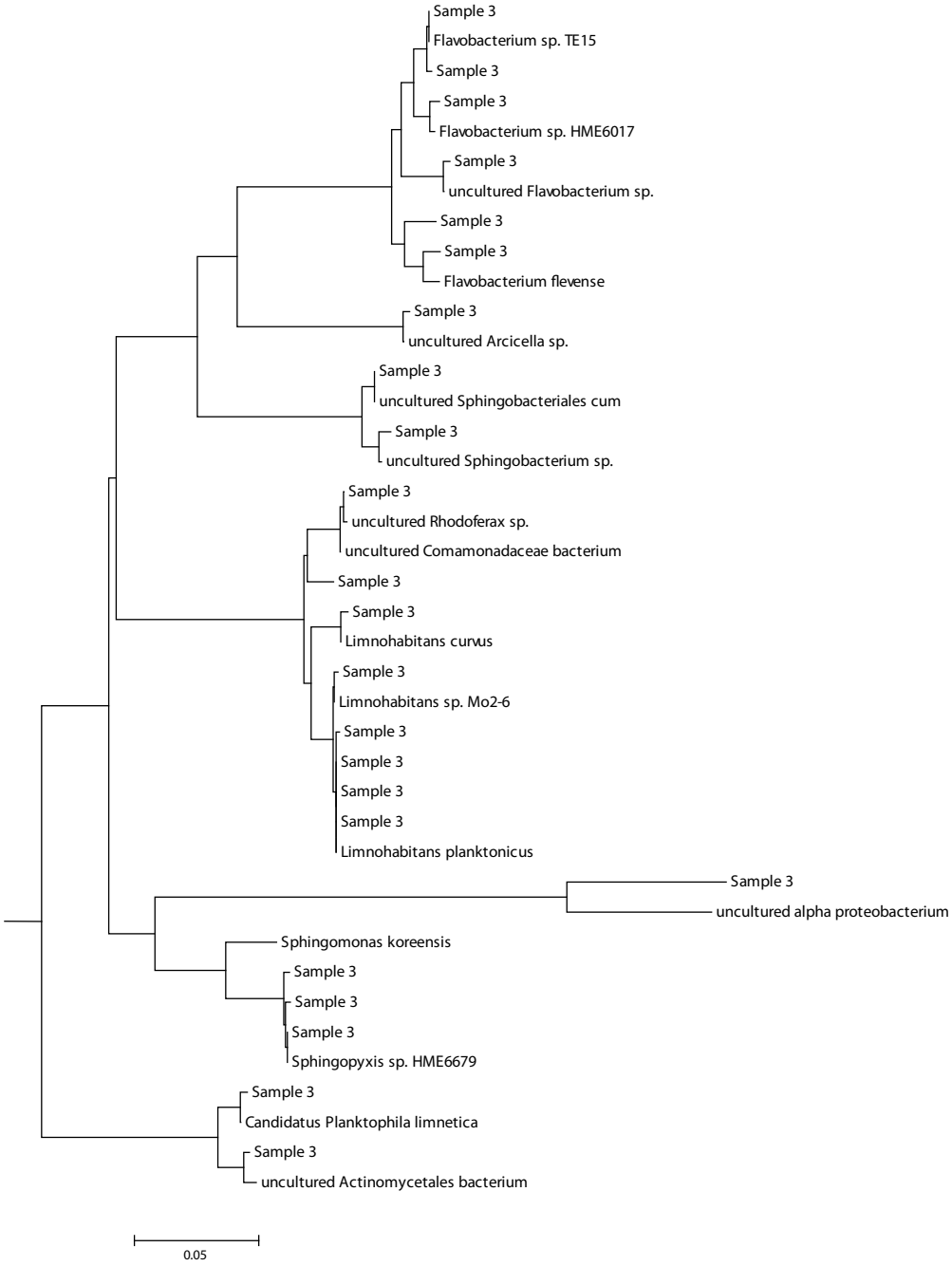

Figure 3.4.3. Neighbour-joining tree of sequences from river sample 3. The 16S rRNA clone libraries show phylogenetic relationships with two major taxa, *Flavobacterium* spp. (6 clones, 97-100% sequence similarity), and *Limnohabitans* spp. (6 clones, 99-100% sequence similarity). The scale bar represents the expected % of substitutions per nucleotide position and *T. thermophilus* was used as an outgroup.

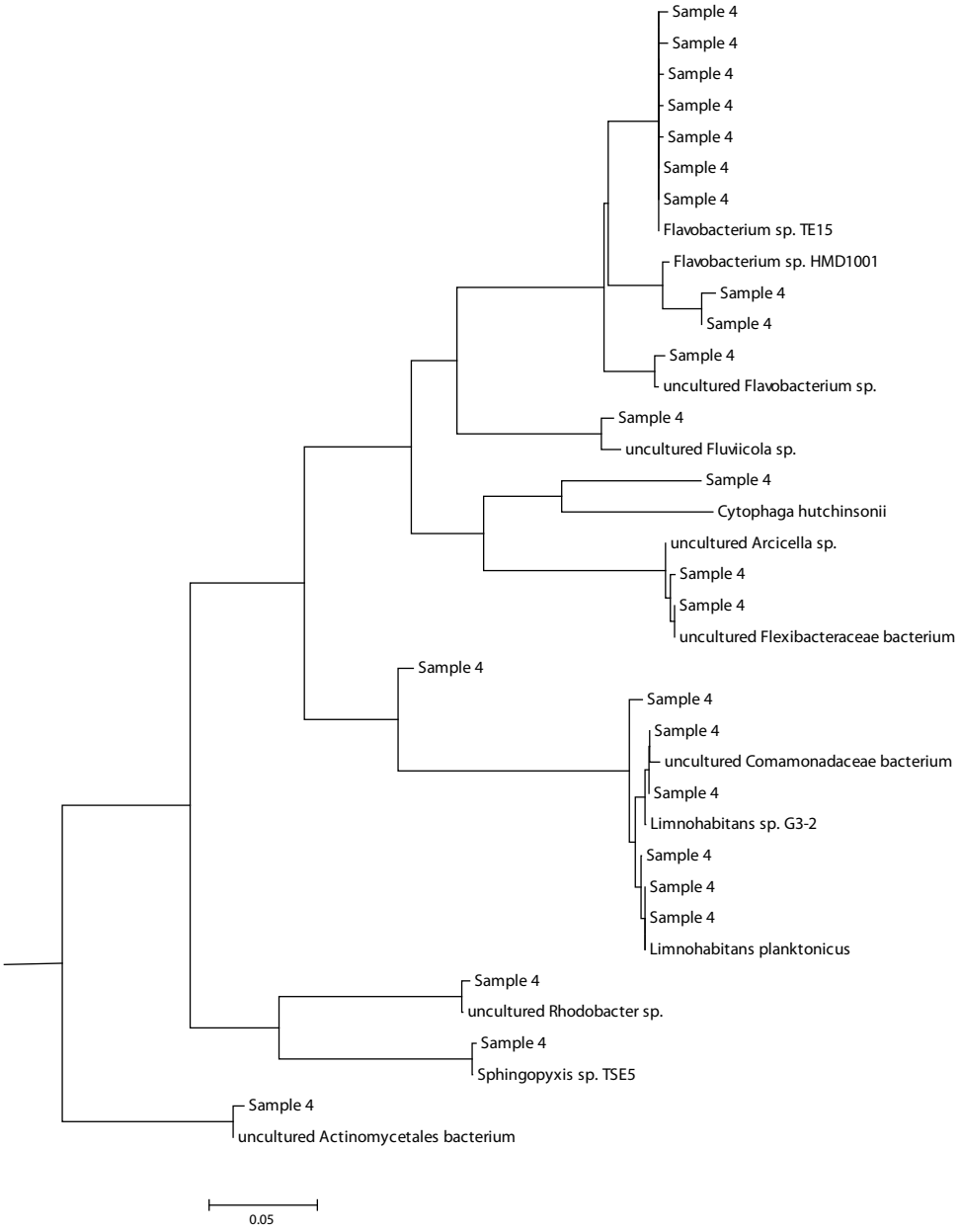

Figure 3.4.4. Neighbour-joining tree of sequences from river sample 4. The 16S rRNA clone libraries show phylogenetic relationships with two major taxa, *Flavobacterium* spp. (10 clones, 95-100% sequence similarity), and *Limnohabitans* spp. (6 clones, 95-100% sequence similarity). The scale bar represents the expected % of substitutions per nucleotide position and *T. thermophilus* was used as an outgroup.

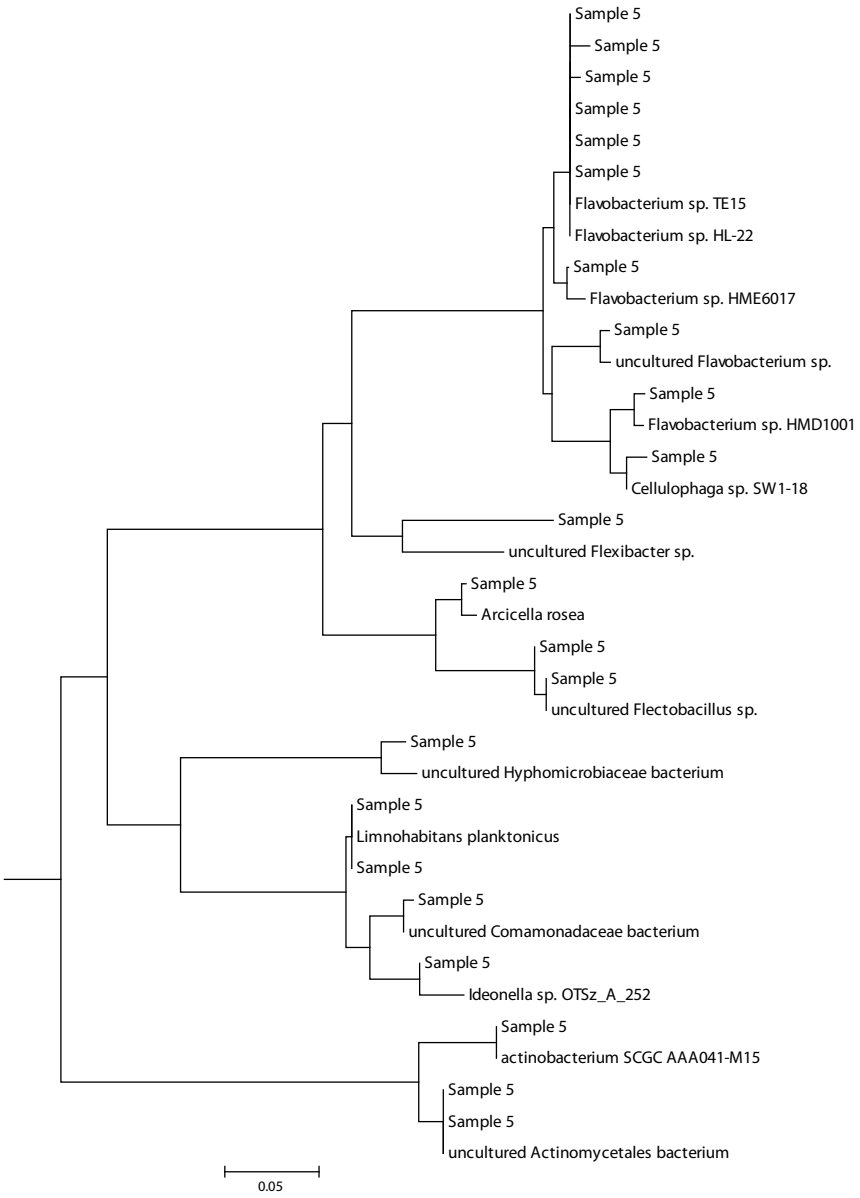

Figure 3.4.5. Neighbour-joining tree of sequences from river sample 5. The 16S rRNA clone libraries show phylogenetic relationships with three major taxa, *Flavobacterium* spp. (10 clones, 96-100% sequence similarity), Actinobacteria (3 clones, 99-100% sequence similarity) and *Limnohabitans* spp. (2 clones, 100% sequence similarity). The scale bar represents the expected % of substitutions per nucleotide position and *T. thermophilus* was used as an outgroup.

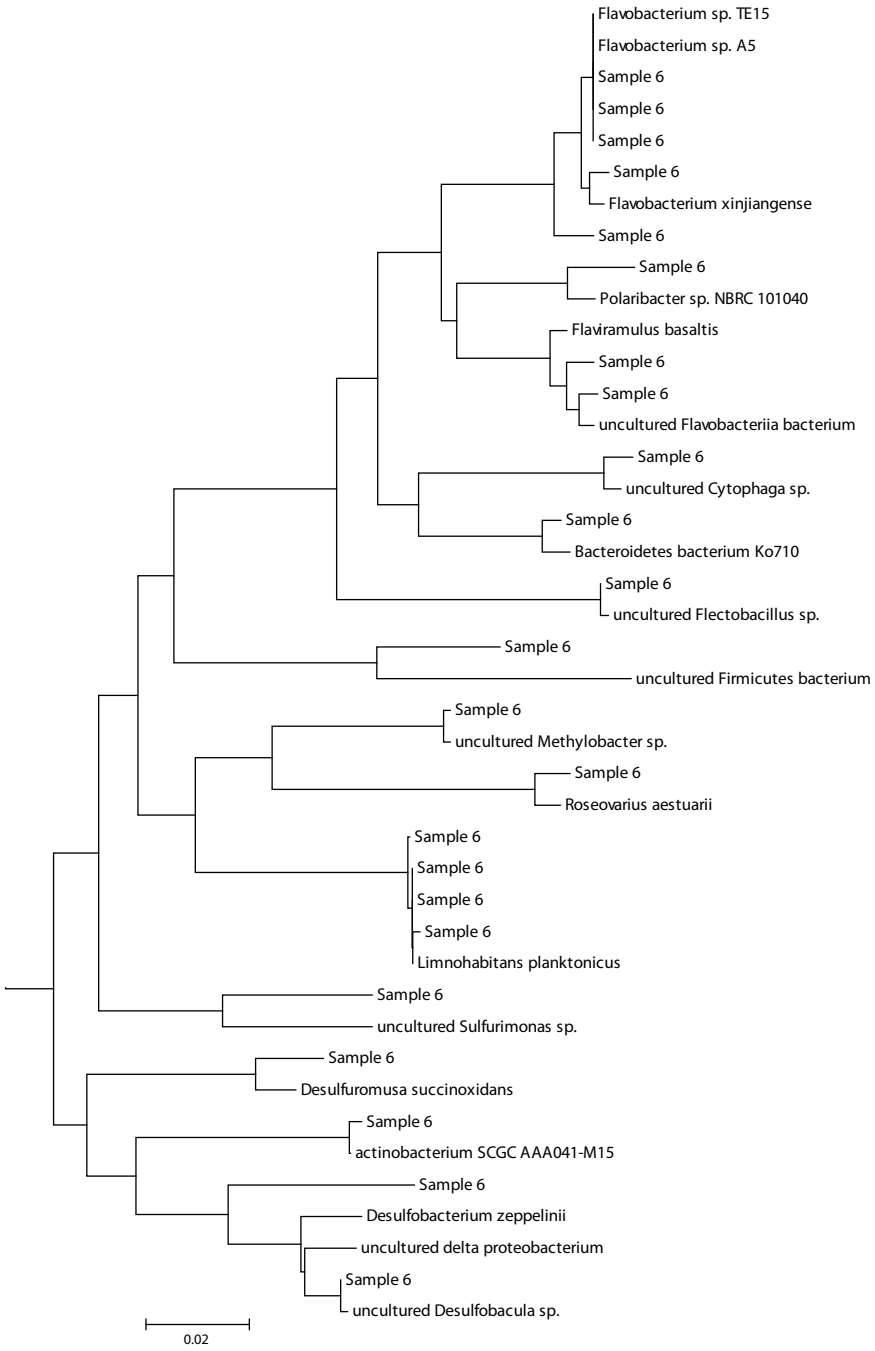

Figure 3.4.6. Neighbour-joining tree of sequences from river sample 6. The 16S rRNA clone libraries shows phylogenetic relationships with two major taxa, Flavobacteria (5 clones, 96-100% sequence similarity), and *Limnohabitans planktonicus* (4 clones, >99% sequence similarity). The scale bar represents the expected % of substitutions per nucleotide position and *T. thermophilus* was used as an outgroup.

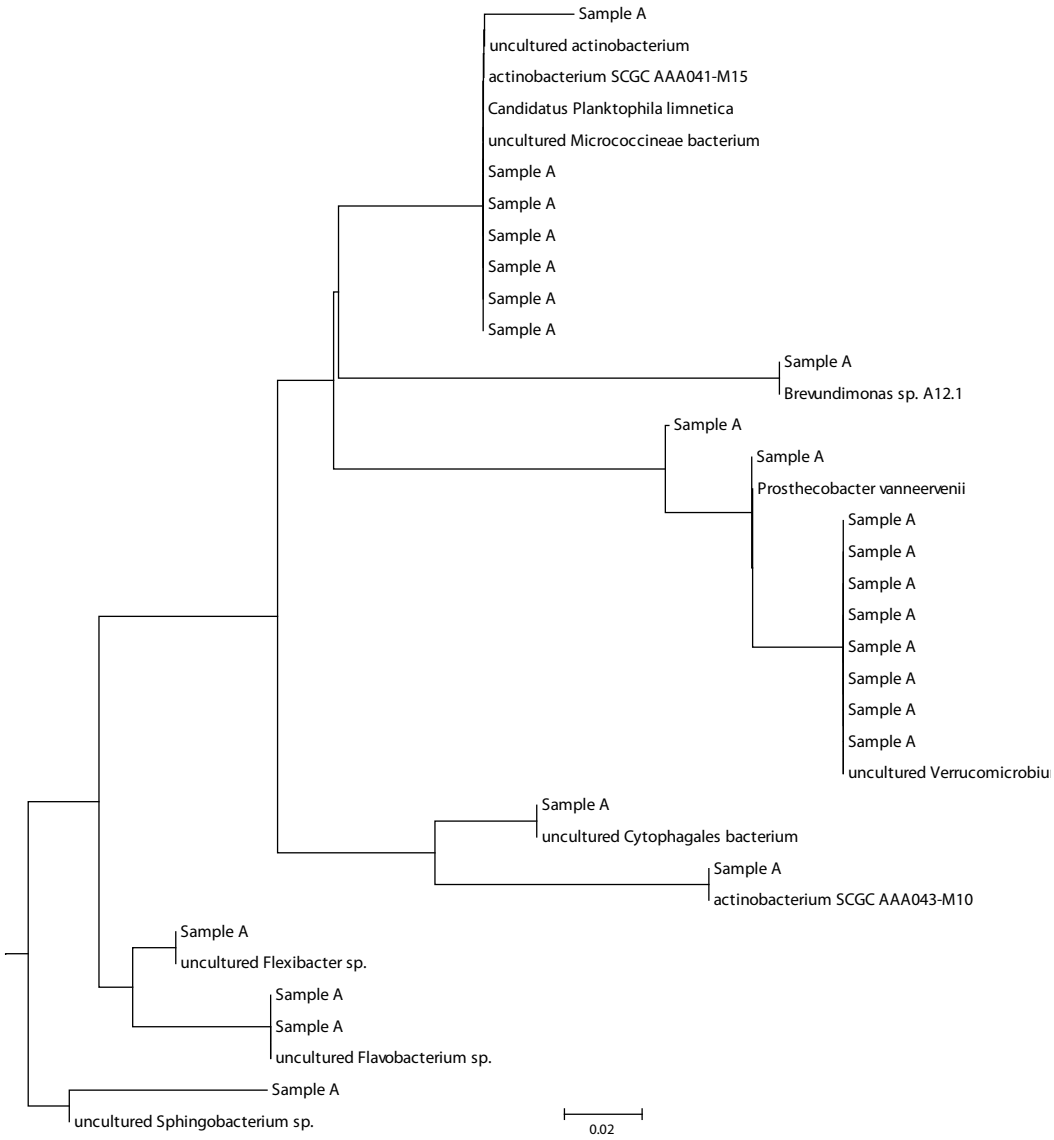

Figure 3.4.7. Neighbour-joining tree of sequences from lake sample A. The 16S rRNA clone libraries show phylogenetic relationships with three major taxa, *Flavobacterium* sp. (2 clones, >99% sequence similarity), Actinobacteria (7 clones, 99-100% sequence similarity) and Verrucomicrobia. (8 clones, 98-99% sequence similarity). The scale bar represents the expected % of substitutions per nucleotide position and *T. thermophilus* was used as an outgroup.

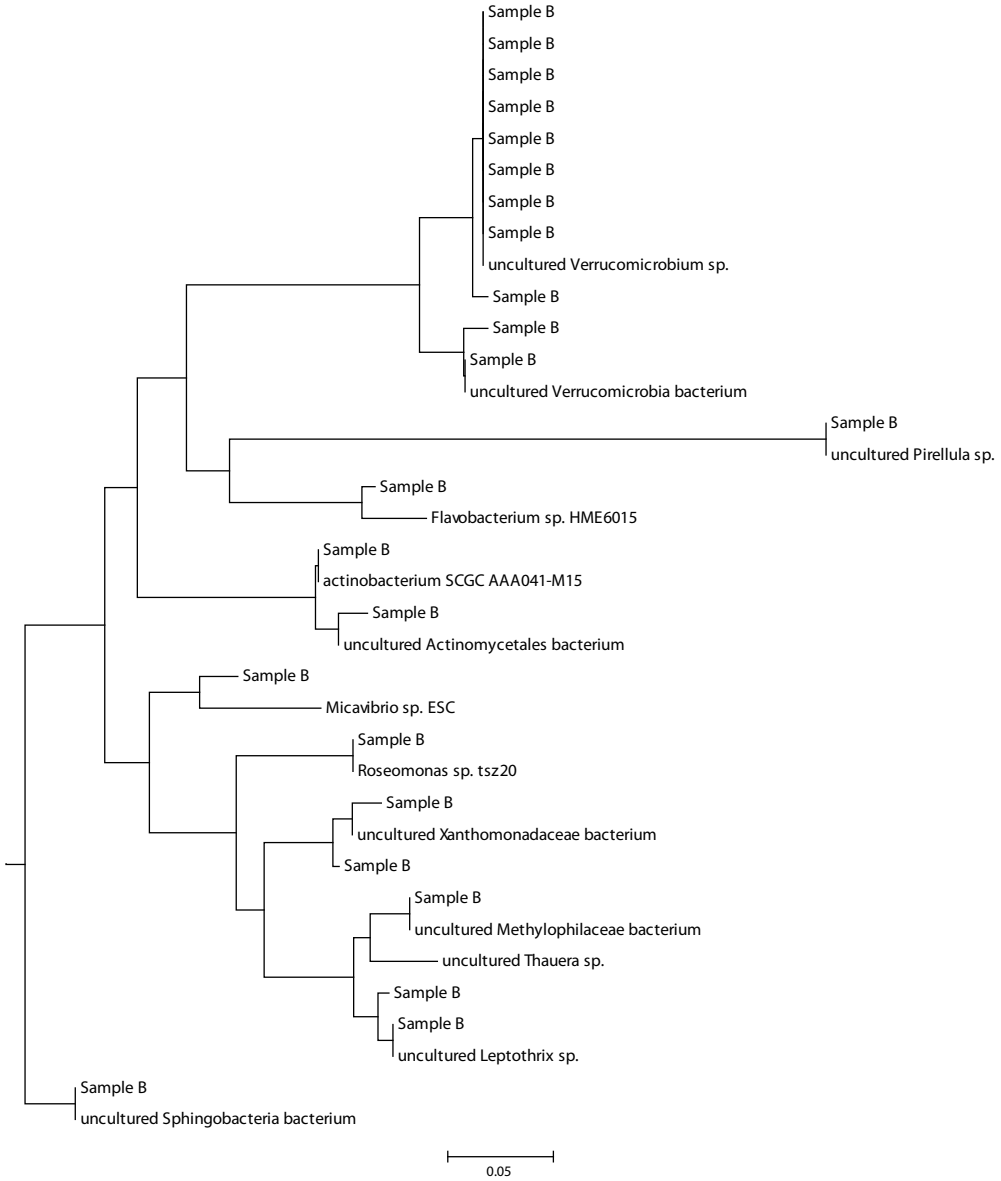

Figure 3.4.8. Neighbour-joining tree of sequences from lake sample B. The 16S rRNA clone libraries show phylogenetic relationships with two major taxa, Actinobacteria (2 clones, >99% sequence similarity) and Verrucomicrobia. (11 clones, 98-100% sequence similarity). The scale bar represents the expected % of substitutions per nucleotide position and *T. thermophilus* was used as an outgroup.

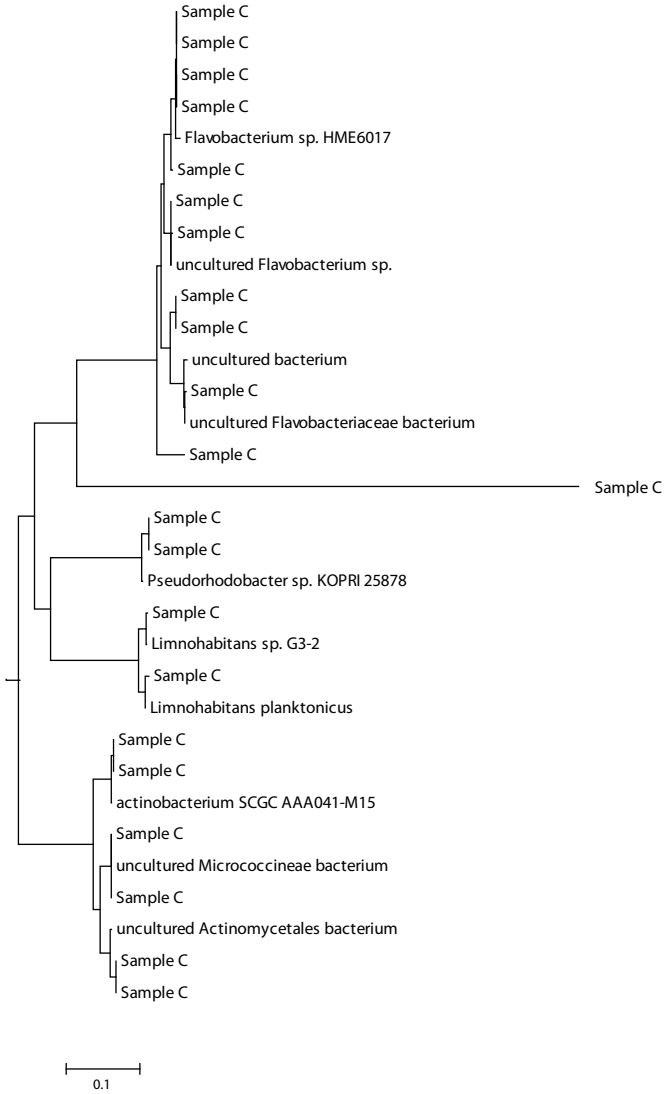

Figure 3.4.9. Neighbour-joining tree of sequences from lake sample C. The 16S rRNA clone libraries show phylogenetic relationships with three major taxa, Flavobacteria (11 clones, 96-99% sequence similarity), Actinobacteria (6 clones, >99% sequence similarity) and *Limnohabitans* spp. (2 clones, >99% sequence similarity). The scale bar represents the expected % of substitutions per nucleotide position and *T. thermophilus* was used as an outgroup.

3.4.2. Norovirus detection

All samples from the rivers and the lake were screened for norovirus. Norovirus type GII was detected in samples 2, 3 and C in February. Other samples were negative. Results are shown in Table 3.4.2 and Figure 3.4.11.

Table 3.4.2. Results for norovirus analysis

Sampling time/ site	June/July	September	January	February
1	-	-	-	-
2	-	-	-	+
3	-	-	-	+
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
A	-	-	-	-
B	-	-		-
C	-	-		+

(+) positive (-) negative

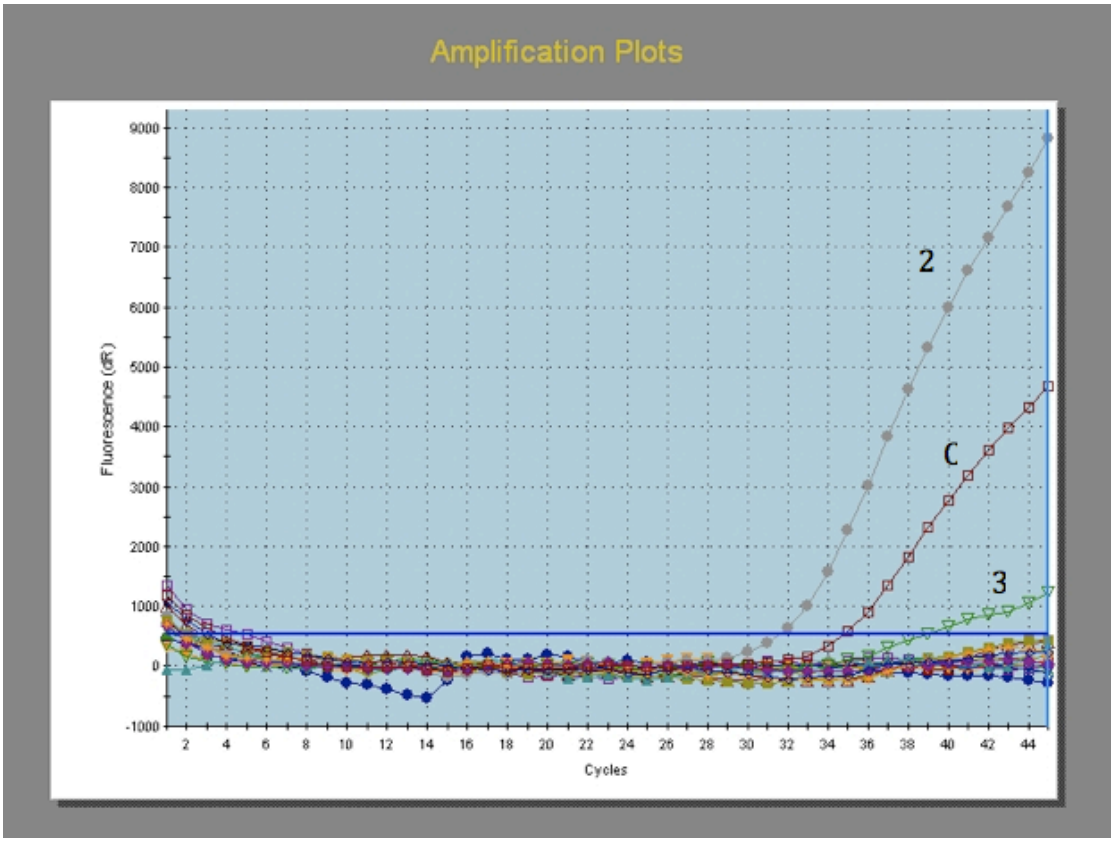

Figure 3.4.11. Real time PCR showing positive results for norovirus type GII from samples 2, 3 and C in February. Other samples were negative.

4. Discussion

4.1 Sample characteristics

4.1.1. Water temperature

Water temperature was measured at all sampling sites every time a sample was collected. Water temperature was similar in all sampling sites on each sampling day, only small variations were observed; in February in the rivers and July and September in the lake. The variation in the rivers in February can possibly be explained by the runoff water from the streets flowing into the river at sampling site 4 that was 0.4°C higher than the second warmest site, number 6. Street water is known to raise temperature in rivers in urban areas (EPA 2003) and this was possibly the case here, since it was raining and ice had started to melt after a long period of snow and frost. Difference in temperature in the lake in July (Δ1.1°C difference between sites B and C) and September (Δ1.3°C difference between sites A and C) is not as easily explained. Fluctuations in water temperature are natural and follow the temperature gradient of the environment. Temperature decreased with colder seasons. Water temperature was highest during the summer period (average 12.6°C) when the weather was good and lowest in January and February when there was a lot of snow and long periods of frost (average 0.4°C in January and 0.3°C in February). Difference in average water temperature reached 12.3°C between the warmest and coldest months. More difference in water temperature was measured between sampling sites in the lake than in the rivers in June/July (Δ1.1°C difference between sites B and C; Δ0.7°C difference between sites 1 and 5) and September (Δ1.3°C difference between sites A and C; Δ0.5°C difference between sites 1 and 6). The movement of the water might explain this; running water has a more uniform temperature than still water. Although a greater temperature difference was observed between sampling sites in the rivers than in the lake in February (Δ0.9°C difference between sites 3 and 4; Δ0.1°C difference between sites A and C) it could be explained by the amount of street water at sampling site 4 that raised the temperature. However, the ice covering most parts of the lake probably maintained a more uniform temperature beneath the ice.

4.1.2. Conductivity

Conductivity of a river is generally stable over time and can be used to assess input of chemicals into a river or water body (EPA 2012). The conductivity of the rivers and the lake was relatively stable over the study period, except for sampling site 4 in the rivers in September and February. In September there was heavy rain and in February snowmelt from the streets around the river was flowing into the river around site 4. Street water can carry road salts, chemicals, dirt, sediments etc. that can increase the conductivity of a river (EPA 2003). Sampling site 6 also showed a very high conductivity, especially in September. Those samples were collected from the estuary of the river at high tide which most likely caused mixing of river water and seawater, resulting in higher conductivity. The conductivity was very low at sampling site 6 in

June. That sample was collected at low tide, which might explain the difference. The natural conductivity of the rivers and lake is a little under 100 μS/cm, which is a normal state for this type of river in Iceland (Gíslason 2010).

4.1.3. pH

The pH measured in the rivers and the lake was around 7 in most cases, except a little higher in sampling site 1 in June and a little lower in sampling sites 5 and A in January. Average pH was slightly higher (7.5) in February than in other months. The pH strips are not a very accurate measurement of pH, but it gives a general picture of the water pH. The pH level in the rivers and lake was in general quite stable, which was anticipated, as pH is not shifted significantly unless there is high pollution.

4.2. Bacterial load of the lake and rivers

4.2.1. Total count

Total viable counts were different at 4°C compared to 30°C and the highest load varied between samples, though a trend for higher count in 30°C was seen. The highest counts at 4°C were observed in samples 1 and 6 collected in January (see Figure 3.2.2) and in samples 6 and A collected in September and January (see Figure 3.2.1) for 30°C.

Total cell count without cultivation estimated by flow cytometry was considerably much higher (1000-fold) than total viable cell count grown on R2A agar (see Figure 4.2.1). This suggests that a high portion of bacteria present in the water was unculturable with the growth technique used in this study. This is in correlation with theories that only 0,1-1% of bacteria can be cultivated using traditional methods (Amann *et al.* 1995; Marteinsson *et al.* 2004). Higher cell counts were obvious in sampling sites 6 (the estuary), A, B and C (the lake) than in the rivers (1-5). Lower cell counts were frequently measured in the colder months (January and February) and the highest cell counts were typically obtained in September. This trend of seasonal counts of bacteria is in agreement with other studies that show the highest cell counts in summer and early fall (Velimirov & Walenta-Simon 1992). However, a clear trend was not seen in all samples – the rivers, estuary and lake did not follow the same trend. Cyanobacterial counts were higher in summer and fall samples (June, July and September) than in winter samples (January and February). Cyanobacterial counts were also higher in the lake than in the rivers during these months. This is maybe not so surprising as the river has a rapid flow rate while the lake is much more untroubled and cyanobacteria can accumulate in a defined area. There seems to be no connection between the cell counts on R2A agar plates to those estimated by flow cytometry, see Figure 4.2.1. Flow cytometry samples 1-C are marked with an F, and cell count on R2A 1-C is marked with a C. Much higher cell counts were obtained by flow cytometry than by cultivation on R2A as evidenced in Figure 4.2.1, where the lines for the flow cytometry counts are all well above the total viable count lines.

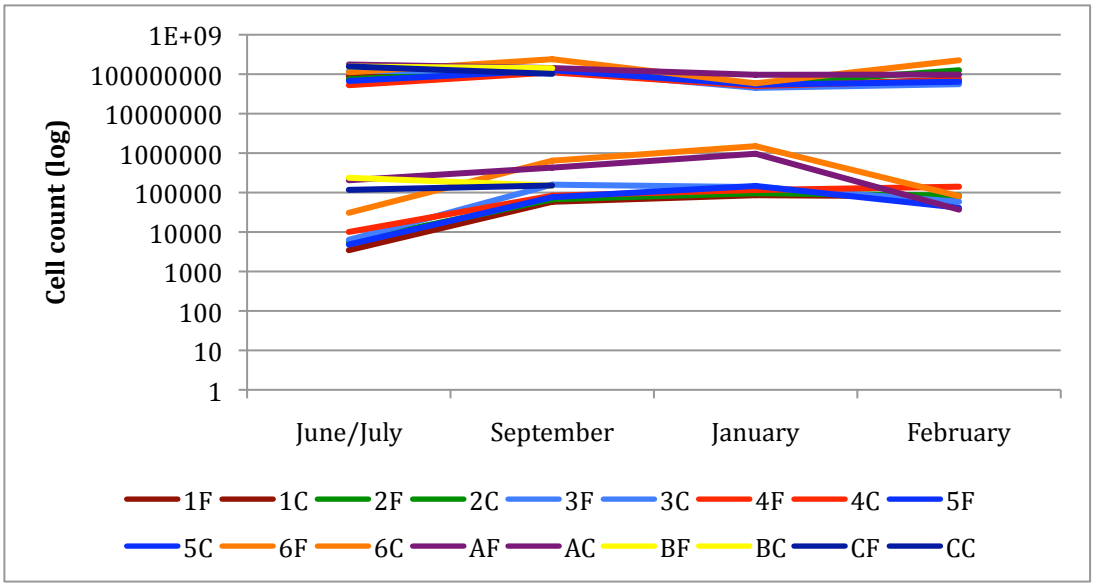

Figure 4.2.1. Comparison between cell counts using flow cytometry (1F-CF) to total viable counts obtained by cultivation on R2A agar (1C-CC) using a log scale. Flow cytometry samples 1-C are marked with an F, and cell count on R2A 1-C is marked with a C. Each sample is marked with the same colour in both flow cytometry and cell count.

4.2.2. Total count of faecal bacteria

E. coli and *Enterococcus* spp. were cultivated from water in the rivers and the lake to evaluate the faecal pollution of the water, and how much it was with regards to regulation no. 796/1999 on water pollution. The results showed that for *E. coli*, the lake was on average a type D lake (695 cfu/100 ml on average), the rivers type B on average (44.35 cfu/100 ml) and the estuary type B on average (25.5 cfu/100 ml). Eleven out of 37 samples (29.7%) taken in the rivers were over the regulation limit allowing 43 cfu/100 ml for *E. coli* in 10% of samples (see Table 4.2.1), which is nearly three times the amount allowed in the regulation. Twenty-five percent of the samples were over this limit in sampling sites 3, 6, B and C, and 75% in sampling site 4 and 80% in sampling site A, for *E. coli*. *Enterococcus* spp. were measured in much smaller quantities; 10,8% of the samples were over the regulation limit, 25% in sample site 3 and 60% in sampling site A. Considering total faecal pollution (*E. coli* and *Enterococcus* spp.), 15 of 74 samples collected were over the limit, or 20.3% which is twice the legal limit. Sampling site A was on average a very polluted site (see Figure 4.2.4), especially in September when the *E. coli* concentration reached nearly 7500 cfu in 100 ml. Most samples (89%) fell into pollution categories A and B (see Figure 4.2.5) for both *E. coli* and *Enterococcus* spp. Five samples in sampling sites 3, A and B fell into categories C and D, and one sample fell into category E for *E. coli* (site A) and two samples fall into categories C and D in sampling site A for *Enterococcus* spp.

Table 4.2.1. Classification of sampling sites according to regulation no.796/1999

Sampling site	Total number of samples	Number of samples over 43 cfu/100 ml (*E. coli*)	Proportion of samples over 43 cfu/100 ml (*E. coli*)	Number of samples over 43 cfu/100 ml (*Enterococcus* spp.)	Proportion of samples over 43 cfu/100 ml (*Enterococcus* spp.)
1	4	0	0	0	0
2	4	0	0	0	0
3	4	1	25%	1	25%
4	4	3	75%	0	0
5	4	0	0	0	0
6	4	1	25%	0	0
A	5	4	80%	3	60%
B	4	1	25%	0	0
C	4	1	25%	0	0
Total/ **Average**	37	11	**29.7%**	4	**10.8%**

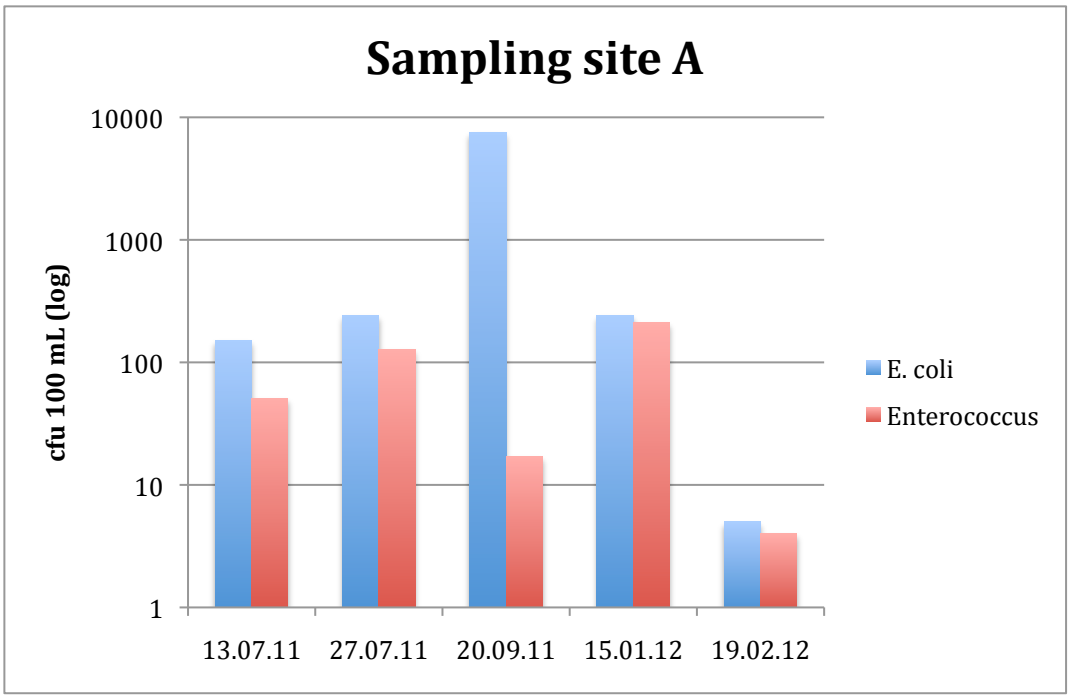

Figure 4.2.4. Levels of *E. coli* and *Enterococcus spp.* in sampling site A on a log scale

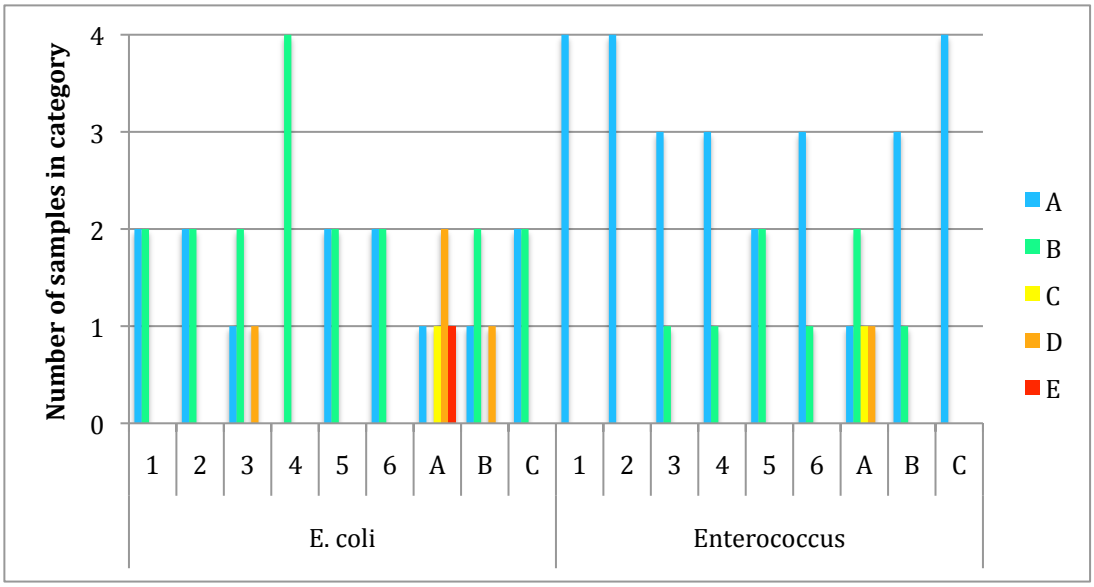

Figure 4.2.5. Number of samples in each pollution category (A-E) according to provisions in regulation no. 796/1999 for both *E. coli* and *Enterococcus* spp.

When compared to previous studies conducted in the rivers and lake it is clear that faecal pollution has increased considerably since the first study in 2001-2002 (see Table 4.2.2). At that time the lake was considered "not sensitive to pollution loading" and was given the pollution status "type A", or unpolluted, pristine water (Þórðarson 2003). However, recent development in the area may reflect the changes observed in water quality. In 2001-2002 when the first study was conducted, the lake Elliðavatn was not located in the middle of an urban area as today; it was rather in a rural area, surrounded by a dispersed residence. Since this original study was performed the urban area around the lake has grown considerably. In the first study the average *E. coli* level measured was 0.6 cfu/100 ml, and 0.4 cfu/100 ml for *Enterococcus* (Þórðarson 2003) compared to the average of 695 cfu/100 ml and 36.3 cfu/100 ml respectively in this study. In 2003 the average *E. coli* concentration in the rivers was 36.5 cfu/100 ml, and the average *Enterococcus* spp. concentration 15.5 cfu/100 ml compared to the average of 44.4 cfu/100mL for *E. coli* and 10,6 cfu/100 ml *Enterococcus* spp. in this study. In 2003 the rivers were considered type A-C rivers (Þórðarson 2004). In 2003 it was recognised that the urban area in the rivers catchment area would expand and with it the pressure on the rivers. When the lake and rivers were evaluated in 2009 the average concentration of *E. coli* were 42.6 cfu/100 ml and *Enterococcus* spp. 4.7 cfu/100 ml in the lake, reaching up to 170 cfu/100 ml for *E. coli.* The average *E. coli* concentration in the rivers was 285.4 cfu/100 ml, reaching over 2000 cfu/100 ml in six samples in two sampling sites. *Enterococcus* spp. was on average 25.5 cfu/100 ml, reaching over 2000 cfu/100 ml in two samples in two sampling sites. These pollution peaks were measured in late winter/early spring and in the fall (Reykjavík Public Health Authority).

Table 4.2.2. Comparison of faecal pollution (cfu/100 ml) in the lake and rivers

Indicators	2001-2002[i]	2003[ii]	2009[iii]	2011-2012[iv]	
E. coli	0.6[a]	-	42.6[b]	695[d]	**lake**
***Enterococcus* spp.**	0.4[a]	-	4.7[b]	36.3[b]	
E. coli	-	36.5[b]	285.4[c]	44.4[b]	**rivers**
***Enterococcus* spp.**	-	15.5[b]	25.5[b]	10.6[b]	

i – Þórðarson 2003; ii – Þórðarson 2004; iii – Reykjavík Public Health Authority 2009; iv – this study; a, unpolluted; b, little pollution; c, some pollution; d, very polluted

In 2003 it was speculated that there might be wrong sewage connections at some houses surrounding the lake Elliðavatn, causing the high faecal pollution measured. Drainage from individual septic tanks at some old houses was also thought to contribute to the pollution (Þórðarson 2004). The results in this study support that theory. The high faecal pollution peaks measured in sampling sites A and B in the lake, which are close to the residential area, and in sampling site 4 close to Reykjanesbraut highway, support the theory of point source pollution reaching the lake and rivers in these locations. Street water from Reykjanesbraut reaches the river close to where the samples at site 4 were taken and the street water may carry all kinds of pollution (EPA 2003). It is also possible that wrong sewage connections could cause high faecal pollution in sampling sites A and B. Additionally street water from the near neighbourhood may leach to sampling site A in the lake (see Figure 4.2.6).

Figure 4.2.6. Sampling site A in February where street water flows into the lake

Weather conditions can also play a role in the high faecal pollution measured in September. The weather some days before and at the sampling day was perceptible with strong winds and rain which could have increased the amount of faecal pollution to some extent. Birds residing around the lake and rivers might contribute to the faecal pollution to some extent, but it is unlikely they have a great effect.

The results of this study clearly show that average faecal pollution in the rivers and lake is considerable, and some parts of the rivers and lake are clearly under some human pressure. The results show that according to regulation no. 796/1999 the rivers Elliðaár have shifted from type A-C to type A-D rivers and lake Elliðavatn has shifted from being pristine, type A to type A-E lake based on the indicator *E. coli*. If *Enterococcus* is used as indicator, the lake is not pristine or A-D and the rivers type A-B. For *E. coli*, the lake is on average a type D lake and the rivers type B, with the rivers estuary type B on average. The pollution status of the lake and rivers has deteriorated greatly in the last decade and will continue to do so unless some action will take place. If these are wrong sewage connections, they need to be found and corrected. The street water should also be discharged directly into the sea and not to the rivers or the lake.

4.3. Analysis of nitrite, nitrate, phosphate and ammonium concentrations

The nutrients nitrite, nitrate, phosphate and ammonium were measured from summer samples to get an overview of the nutrient status of the rivers and the lake. All measurements were below the detection limit. The nutrition status in the rivers and lake is low, making the water nutrient poor. This was anticipated, as there are no major farming or industrial activities around the rivers and the lake.

4.4. Diversity analysis by 16S rRNA gene sequencing of non-cultivated microbes in selected samples

The diversity of taxa was evaluated with cloning and sequencing of the 16S rRNA gene in samples taken in June/July from both of the rivers and the lake. Taxa diversity increases downstream of the catchment area, or along the river (see figure 4.4.1). Taxa diversity is lowest in the beginning and then increases and is relatively stable down the catchment area until its reaches the estuary with almost three times more taxa diversity compared to the top (upstream) of the rivers. The diversity was almost twofold lower at the top of the rivers (1) where it runs from the lake than at other sampling sites. This was also observed in the sample taken furthest away from the residential area in the lake (C). The other river samples (2, 3, 4 and 5) and lake samples A and B, contained similar numbers of taxa or 10.3 taxa on average. The taxa diversity was highest at the rivers estuary (sample 6) or 16 taxa. This high number of taxa could have been anticipated, as the water conditions are different at the estuary than in other parts in the river. At the estuary the river freshwater mixes with seawater and therefore with different environmental factors and nutrient conditions which could lead to high taxa diversity. The microbial composition was also quite different in the estuary than in the other sampling sites. The results also show higher taxa diversity at sampling sites with high faecal pollution. Both sampling sites A and B had high faecal pollution and are both richer in diversity than sampling site C that had lower faecal pollution. The same goes for the river; sample at site 1 had little faecal pollution and low taxa diversity.

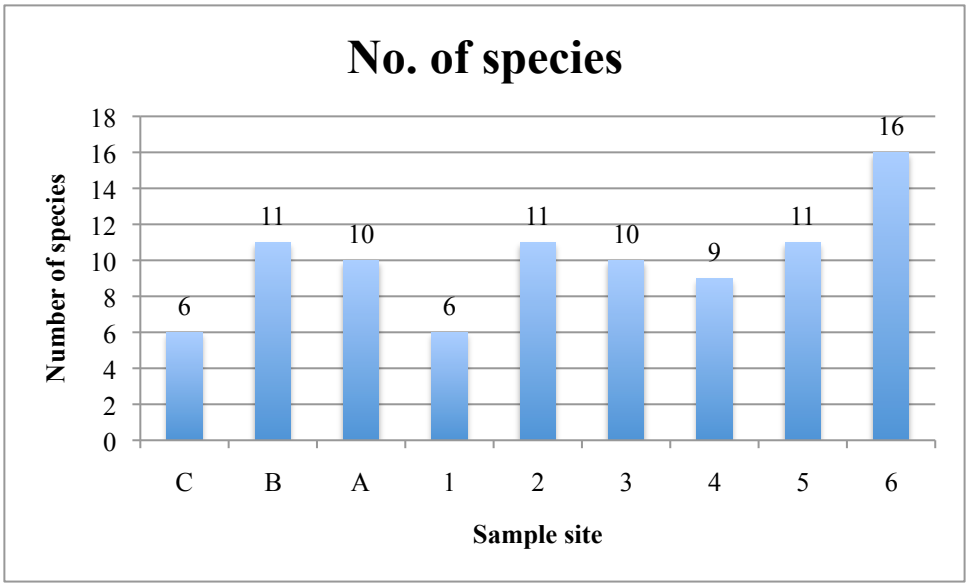

Figure 4.4.1. Numbers of species from the point where the rivers run from the lake (C) to the rivers end in the estuary (6)

Flavobacterium spp. and *Limnohabitans* spp. were the two bacterial genera in addition to Actinobacteria group that were prevailing in the rivers and the lake. These genera of bacteria are commonly found in freshwater and soil and are a part of the natural microbiota of freshwater. Some variation can be seen in interrelations within the dominant taxa indicating several different species (Figure 4.4.2). *Flavobacterium* spp. were recently found in the river Glerá, a river in North Eastern Iceland (Markúsdóttir *et al.* 2012). Many other naturally occurring genera were found in the water and some of them specific to their sampling location. This could suggest some variation in the microbial habitats of different parts of the rivers and lake. Sampling site 6 had the highest taxa diversity; apart from the three most dominating taxa and two additional ones, the taxa diversity was unique at this site and could be explained by the dynamic environment with influence from the tides. Taxa diversity in the rivers differs somewhat from the diversity in the lake; the same taxa were not often found in both places. Conditions in the lake are different from the rivers; there is much more movement of the water in the rivers than in the lake, which affects the habitat and therefore the taxa diversity; different species thrive in still water than in flowing water.

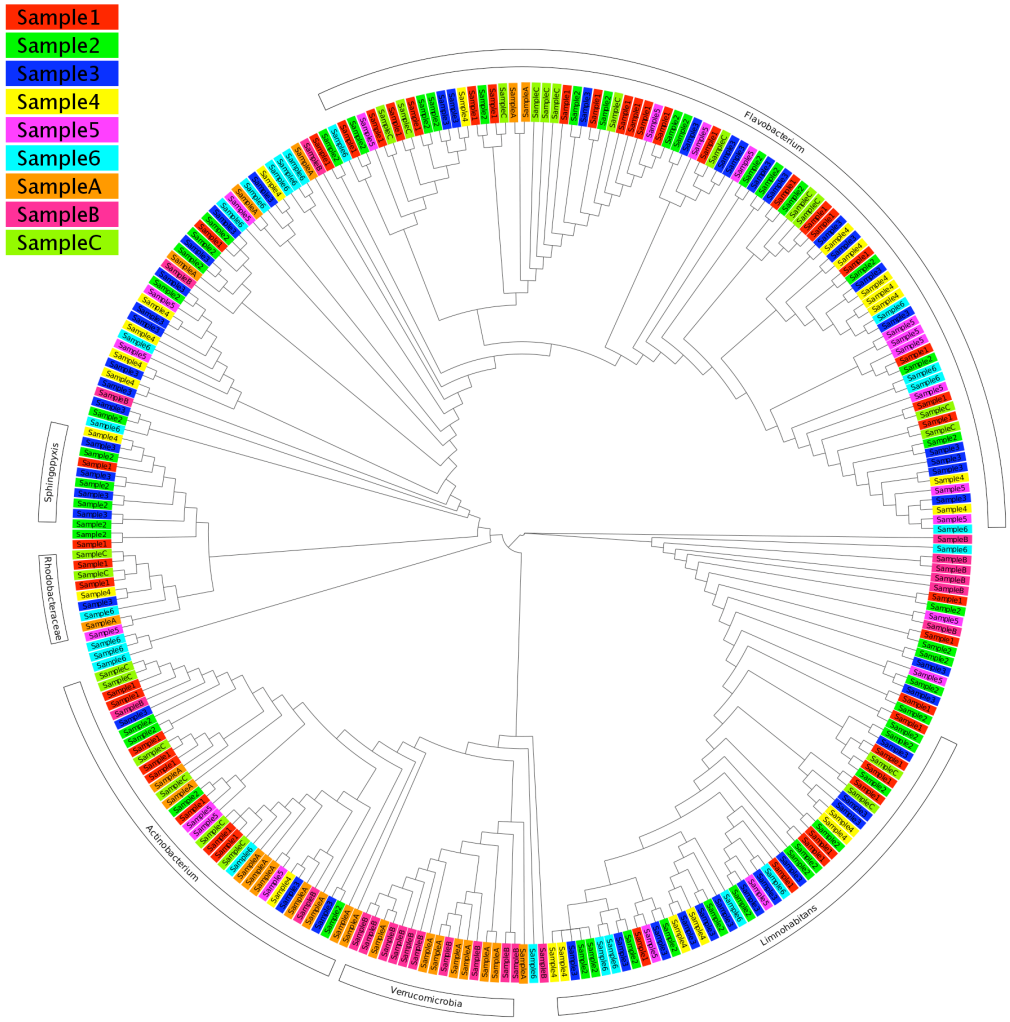

Figure 4.4.2. An overall phylogenetic tree showing interrelation of all clones sequenced. Sampling sites are colour-coded. The six largest taxa are *Flavobacterium* spp. (~31.6%), *Limnohabitans* spp. (~18.8%), Verrucomicrobia (~5.9%), Actinobacterium (~13.6%), Rhodobacteraceae (~3.3%) and *Sphingopyxis* spp. (4.4%).

4.4.1. Norovirus

Norovirus was found in three samples in February. It has been proposed that the presence of norovirus is a better indicator for faecal contamination of human origin than faecal coliforms (Gerba *et al.* 1979), e.g. shellfish that were not contaminated by indicator bacteria were infected with norovirus and caused an outbreak (Doré *et al.* 2010). The presence of noroviruses indicates a faecal pollution of human origin at sampling sites 2, 3 and C. Sampling sites 2 and 3 are both located above the Árbær dam and are surrounded by a large urban area. In February there was a lot of water in the rivers, so much that the two rivers had merged into a small lake above the dam. It is not certain how the pollution is reaching the river in these locations, but it is quite possible that sewage is somehow leaking into the rivers at these locations although

there is a low total count of faecal bacteria at these sites. Most likely there are incorrect sewage connections somewhere in the vicinity. Close to sampling site C there is one house that may be the cause of the faecal pollution detected, the septic tank from the house might have over run and polluted the lake. To find norovirus in surface water during winter season is in agreement with previous findings. Noroviruses have been detected under similar circumstances in other surface waters in the area of Reykjavík and elsewhere in 2011 (Sveinn Magnússon, personal communication).

5. Conclusion

Faecal pollution in lake Elliðavatn and the rivers Elliðaár is of concern. Faecal pollution has increased greatly in the last 10 years and the pollution status of the lake and rivers has decreased severely, from pollution status A (unpolluted) to B on average for the rivers (little pollution) and D for the lake (very polluted). The fact that noroviruses were detected in three samples adds to these concerns, since noroviruses are only found if the water is polluted by human sewage. Sewage is clearly reaching the lake and rivers, most likely through wrong or broken sewage connections that need to be corrected or replaced. Generally, higher microbial counts were in the lake than in the rivers, and in the summer and fall than in winter. A 1000-fold higher count was generally observed with flow cytometry than with the total viable count method. Bacteria from the five largest phyla of freshwater bacteria (Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria and Verrucomicrobia) were found in the lake and river water. Using the 16S rRNA sequencing method the six largest taxa found were *Flavobacterium* spp. (~31.6%), *Limnohabitans* spp. (~18.8%), Verrucomicrobia (~5.9%), Actinobacterium (~13.6%), Rhodobacteraceae (~3.3%) and *Sphingopyxis* spp. (4.4%). These taxa are all part of natural freshwater microbial flora. The microbial diversity was quite diverse and differed between sampling sites. In the rivers the taxa *Flavobacterium* spp. and *Limnohabitans* spp. were prevailing, but in the lake *Flavobacterium* spp. and Verrucomicrobia were the prevailing taxa.

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Figures

Appendix A

Medium recipes

Ætauppskriftir

BILE AESCULIN AGAR (BA) pH 7,1 ± 0,2
OXOID CM 888

Notkunarsvið: Notað til staðfestingar á *Enterokokkum* í vatni og sjó

44,5 g Bile Aesculin Agar
1 l eimað vatn

Leyst upp með suðu

Dauðhreinsað í gufusæfi í 15 mín við 121°C
Kælt í vatnsbaði niður í 45°C
Hellt á skálar

Geymsluskilyrði: Myrkur, 4°C
Geymsluþol: 2 vikur

Ætaeftirlit:
Jákvætt kontrol: Streptoc. Faecalis ATCC 29212 góður eink. vöxtur rauð/svört/útfell
Neikvætt kontrol: Escherichia coli ATCC 25922 hindraður vöxtur
Strikið kontrolstofni á ætið og ræktið við 37°C í 40-48 klst.

LACTOSE BROTH (LAKTÓSI) pH 6,9 ± 0,2
OXOID CM 137

Notkunarsvið: Notað til forræktunar á *kólígerlum* í lyfjum og til staðfestingar á *kóli- og saurkólígerlum* í vatni.

13 g Lactose broth
1 l eimað vatn

10 ml settir á túpuglös með durham

Dauðhreinsað í gufusæfi í 15 mín við 121°C

Geymsluskilyrði: Myrkur, stofuhiti
Geymsluþol: 2 vikur

***Æ*taeftirlit:**
Jákvætt kontrol: Escherichia coli ATCC 25922 góður eink. vöxtur og loftmyndun
Neikvætt kontrol: Streptoc. Faecalis ATCC 19433 vöxtur en ekki loftmyndun
Sáið kontrolstofni út í ætið og ræktið við 37°C í 18-48 klst

mFC AGAR pH 7,4 ± 0,2
DIFCO 267720

Notkunarsvið: Notað til ákvörðunar á *saurkólígerlum* við síun á vatni, sjó og árvatni.

26 g mFC agar
500 ml eimað vatn

Leyst upp með suðu

5 ml af 1% Rosolicsýru* settir út í 500 ml af æti
Hita í 1 mínútu eftir að rosolic sýran er komin út í

Hellt í dauðhreinsaða kolbu (vatn soðið í kolbu)
Kælt í vatnsbaði niður í 45°C
Hellt á skálar

Geymsluskilyrði: 4°C
Geymsluþol: 2 vikur

*1% Rosolicsýra
0,05 g rosolicsýra (difco 232281)
5 ml 0,2 N NaOH
(rosolicsýra útbúin samdægurs)

Ætaeftirlit:

Jákvætt kontrol:	E. coli	ATCC 25922	góður eink. vöxtur
Neikvætt kontrol:	Streptok. Faecalis	ATCC 29212	enginn vöxtur

Strikið kontrolstofni á ætið og ræktið við 37°C í 18-24 klst.

R_2A AGAR pH 7,0 ± 0,2

9,1 gr R_2A agar
500 ml eimað vatn

Leyst upp með suðu
Dauðhreinsað í gufusæfi í 15 mín við 121°C
Hellt á skálar

SLANETS & BARTLEY AGAR (S&B) pH 7,2 ± 0,2
OXOID CM 0377

Notkunarsvið: Notað til ákvörðunar á *Enterokokkum* við síun á vatni og sjó

42 g	S&B agar
1 l	eimað vatn

Leyst upp með suðu
Kælt í vatnsbaði niður í 45°C
Hellt á skálar

Geymsluskilyrði: Myrkur, 4°C
Geymsluþol: 2 vikur

***Æ*taeftirlit:**

Jákvætt kontrol:	Streptok. Faecalis	ATCC 29212	góður eink. vöxtur
Neikvætt kontrol:	E. coli	ATCC 25922	enginn vöxtur

Strikið kontrolstofni á ætið og ræktið við 37°C í 40-48 klst.

Appendix B

Pictures of total viable counts on R2A and faecal bacteria (*E. coli* and *Enterococcus* spp.), see figures A-D.

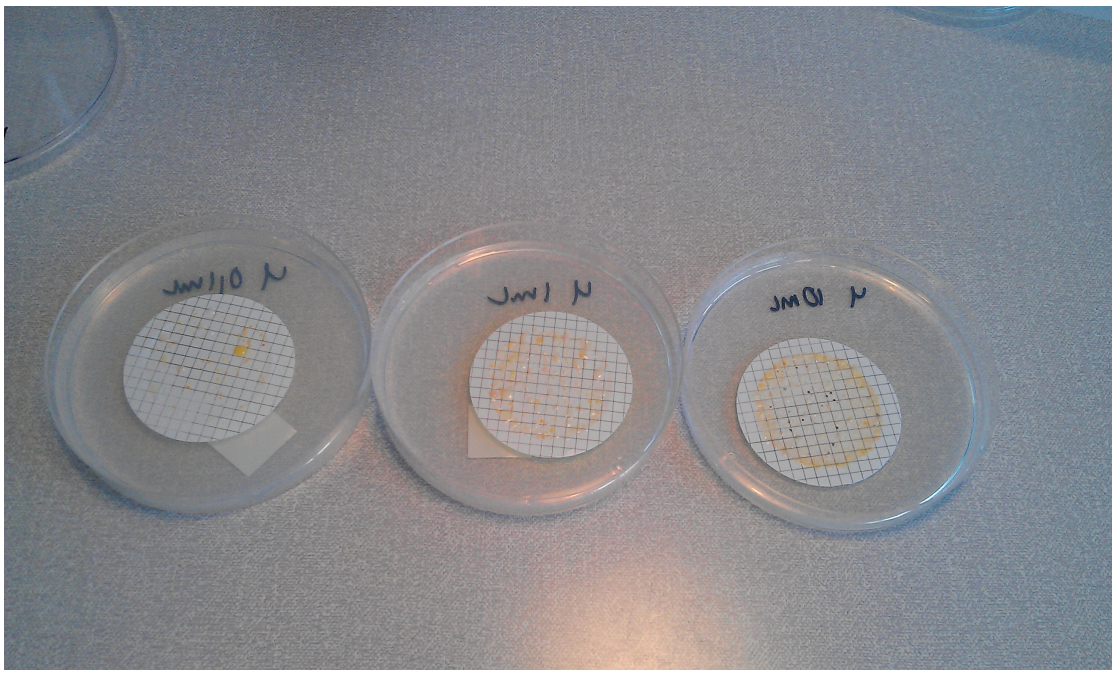

Figure A . 0.1, 1 and 10 ml filtered and cultured on R2A agar at 30°C from sample 4, February 2012

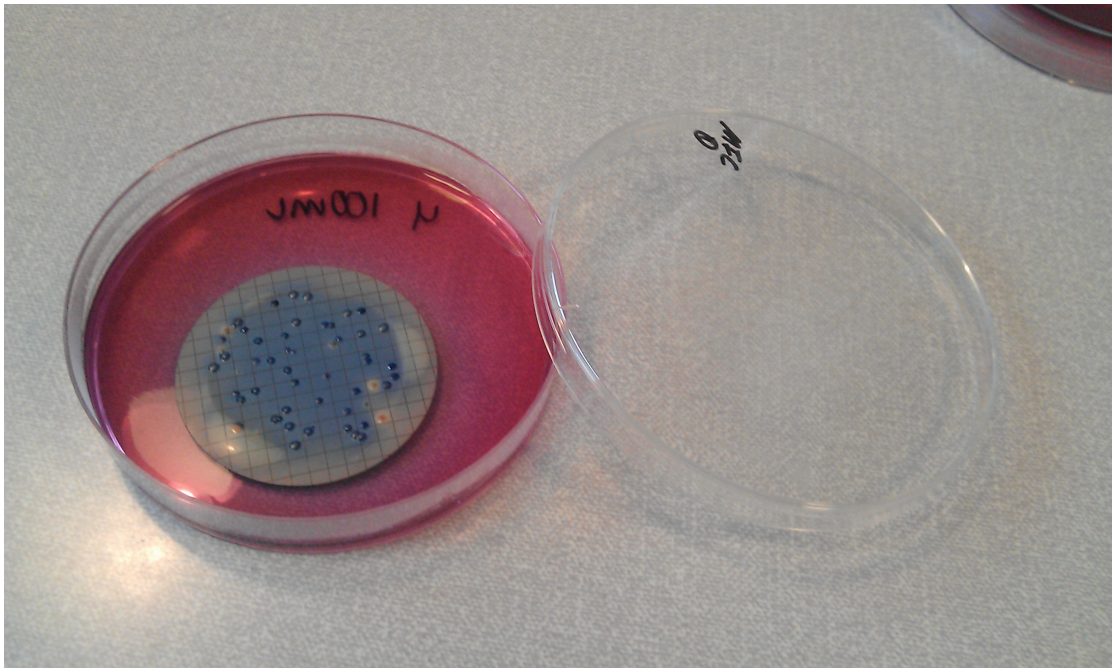

Figure B. 100 ml filtered and cultured on m FC agar for *E. coli* culture, from sample 4, February 2012

Figure C. *E. coli* colonies picked and re-cultured in liquid lactose medium for confirmation, February 2012.

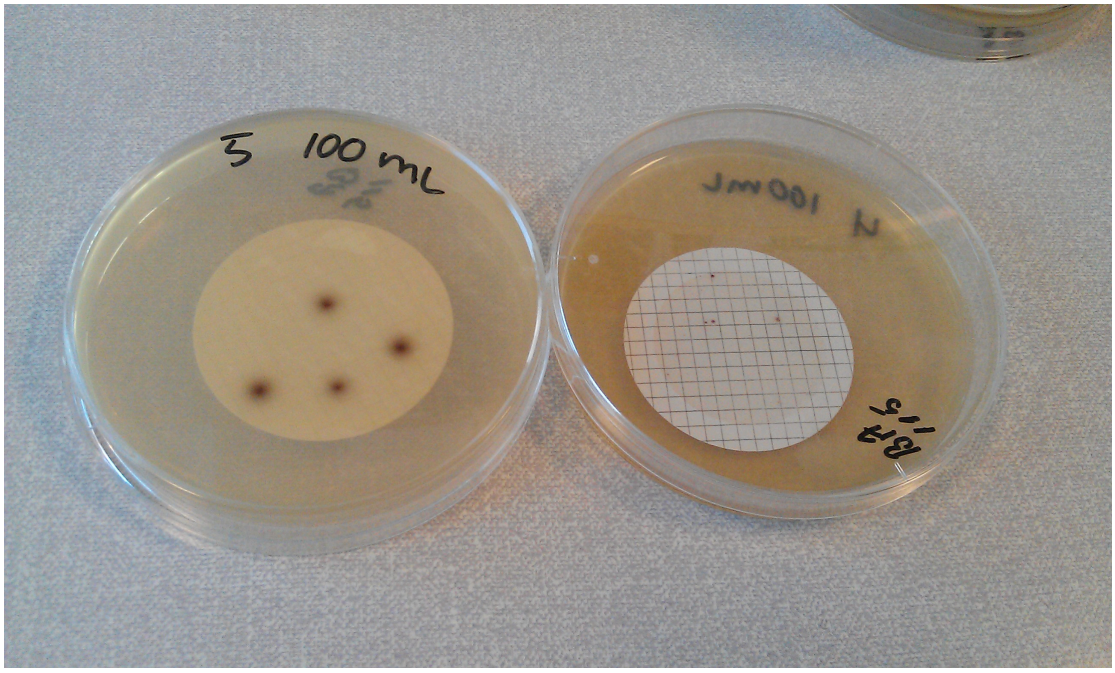

Figure D. *Enterococcus* spp. confirmed on SB agar where the colonies form a ferric precipitation (samples 4 and 5, February 2012)