



**Microbes and the groundwater  
amphipod *Crangonyx islandicus* in  
spring sources in Iceland**

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**Faculty of Life and Environmental Sciences  
University of Iceland  
2020**



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Dissertation submitted in partial fulfillment of a  
*Philosophiae Doctor* degree in Biology

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# Abstract

*Crangonyx islandicus* is a groundwater amphipod endemic to Iceland. Genetic analysis suggests that the species has been diverging in Iceland for at least 4.8 Myrs indicating it has survived in a subglacial refugia as Iceland was repeatedly covered by glaciers during that time period. The species has probably been inhabiting Iceland since before the island was formed, when the land bridge to Greenland collapsed into the ocean approximately 15 Mys ago. Currently, their habitat is in the subsurface of spring sources within the lava fields along the tectonic plate boundary. These spring sources act as a window into the groundwater, but they are also a complex ecotone where groundwater mixes with surface water and the terrestrial ecosystem. In this thesis, the microbial community composition associated with the amphipods and their habitat was examined both to inspect if more taxa could be found in this unique habitat and to elucidate which processes are likely to shape the community composition of microbial species in the habitat. The results showed that the amphipods are accompanied by a few ciliate and bacteria taxa that are unique to these amphipods but can only be marginally detected in the spring source. Both stochastic and deterministic processes were found to shape the bacteria and ciliate communities in the spring source. Variables such as pH, temperature, presence of fish and geographical location were found to shape the bacterial community while temperature and dispersal was shaping the ciliate communities. The bacterial community in the water from spring sources and in the biofilms harbored chemolithoautotrophic taxa, indicating primary production in the groundwater system, thus, providing a possible explanation for the subglacial survival of the amphipods during Ice age.

# Útdráttur

Íslandsmarflóin, *Crangonyx islandicus*, er grunnvatnsmarflóartegund sem finnst eingöngu á Íslandi. Erfðarannsóknir hafa sýnt að hún hefur aðgreinst hér á landi í um 4,8 milljónir ára sem bendir til þess að hún hafi lifað af undir ísaldarjökli en á þessu tímabili hefur Ísland endurtekið verið þakið jöklum. Líklega hefur tegundin verið á Íslandi allt frá því að forveri Íslands varð að eyju, þegar landbrúin til Grænlands rofnaði fyrir um 15 milljónum ára. Núverandi þekkt útbreiðsla tegundarinnar er í grunnvatni hraunalinda á flekaskilum landsins. Þessar lindir eru eins konar náttúrulegur gluggi inn í grunnvatnskerfið en þar má finna nokkuð flókið vistkerfi á mótum grunnvatns, yfirborðsvatns og yfirliggjandi jarðvegi. Í þessari ritgerð er örverusamfélag þessa svæðis og marflónna skoðað, bæði til að kanna frekara líf í þessu lítt þekktu kerfi og til að skoða þá ferla sem hafa mótað samfélögin. Niðurstöðurnar benda til þess að á marflónum finnast nokkrar tegundir af bifdýrum og bakteríum sem ekki finnast í uppsprettunum nema að litlu leyti. Bæði slembiferlar og umhverfisþættir reyndust stýra tegundasamsetningunni fyrir örveruhópana í uppsprettunum. Þættir eins og sýrustig, hitastig, hvort fiskur fannst í sama búsvæði og landfræðileg staðsetning mótuðu bakteríusamfélögin á meðan hitastig og far réðu mestu um samsetningu bifdýrasamfélaganna. Í bakteríusamfélaginu var að finna ýmsa efnatillíffandi hópa sem bendir til þess að einhver frumframleiðni á sér stað í grunnvatnskerfinu. Það gæti því mögulega útskýrt hvernig marflærnar gátu lifað af jökulskeið í grunnvatni landsins.

*I dedicate this work to Olgeir for  
his unconditional love and support*





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# List of Original Papers

This thesis is based on the following papers, of which all have been published. Hereafter they will be referred to by their numbers as follows:

- Paper I:** Guðmundsdóttir R, Kornobis E, Kristjánsson BK, Pálsson S (2018) Genetic analysis of ciliates living on the groundwater amphipod *Crangonyx islandicus* (Amphipoda: Crangonyctidae). *Acta Zoologica* 99:188-198
- Paper II:** Guðmundsdóttir R, Kreiling AK, Kristjánsson BK, Marteinsonn V, Pálsson S (2020) Ciliate diversity in cold water spring sources in Iceland. *Aquatic Microbial Ecology*. 84:191-203.
- Paper III:** Guðmundsdóttir R, Kreiling AK, Kristjánsson BK, Marteinsonn V, Pálsson S (2019) Bacterial diversity in Icelandic cold spring sources and in relation to the groundwater amphipod *Crangonyx islandicus*. *PLoS One* 14:e0222527
- Paper IV:** Guðmundsdóttir R, Björnsdóttir SH, Marteinsonn V, Pálsson S (2020) Comparison of the gut microbiota in the groundwater amphipod *Crangonyx islandicus* to biofilms in its spring-source habitat. *Journal of Crustacean Biology*. [Doi: 10.1093/jcbiol/ruaa065]

## Other papers published during this study:

Pálsson S, Guðmundsdóttir R, Kristjánsson B. K. (2020) Líf í grunnvatni í hraunalindum Íslands. *Náttúrufræðingurinn* 90(1):16-22.

# List of Published Nucleotide Sequences

The nucleotide sequences that were produced during the course of this study were made publicly available at the NCBI GenBank, SRA and ENA under the following numbers:

DDBJ/ENA/GenBank accession numbers MF188925 - MF188934 for partial 18S rRNA sequences for ciliates associated with amphipods from Iceland, France, United Kingdom, Slovenia and the United States, referred to in **Paper I**.

DDBJ/ENA/GenBank accession numbers MF188935 – MF188966 for partial ITS sequences for ciliate populations associated with amphipods from Iceland, referred to in **Paper I**.

BioProject accession number PRJNA623026 containing NGS 18S rRNA gene amplicon sequences for ciliate communities in spring sources, referred to in **Paper II**.

BioProject accession number PRJEB32879 containing NGS 16S rRNA gene amplicon sequences for bacteria communities in spring sources, referred to in **Paper III**.

BioProject accession number PRJNA632885 containing NGS 16S rRNA gene amplicon sequences for bacteria in amphipod guts and from biofilms in the amphipod habitat with the BioSample accession numbers: SAMN14925544 - SAMN14925552, referred to in **Paper IV**.

DDBJ/ENA/GenBank accession numbers MT471353-MT471371 and MT472106-MT472125 containing partial 16S rRNA gene sequences for bacteria cultured from amphipod guts, referred to in **Paper IV**.

# Abbreviations

aLRT – Approximate Likelihood Ratio Test

ASV – Amplicon sequence variant

DADA2 – Divisive Amplicon Denoising Algorithm, version 2

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic Acid

GTR – General Time Reversible substitution model

ITS – Internal transcribed spacer region

HTS – High throughput sequencing

mtDNA – Mitochondrial DNA

NGS – Next Generation Sequencing

NMDS – Nonmetric Multi-Dimensional Scaling

OTU – Operational taxonomic unit

PCoA – Principal Coordinate Analysis

PCR – Polymerase Chain Reaction

rRNA – Ribosomal Ribonucleic Acid

SRA – Sequence Read Archive





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# 1 Introduction

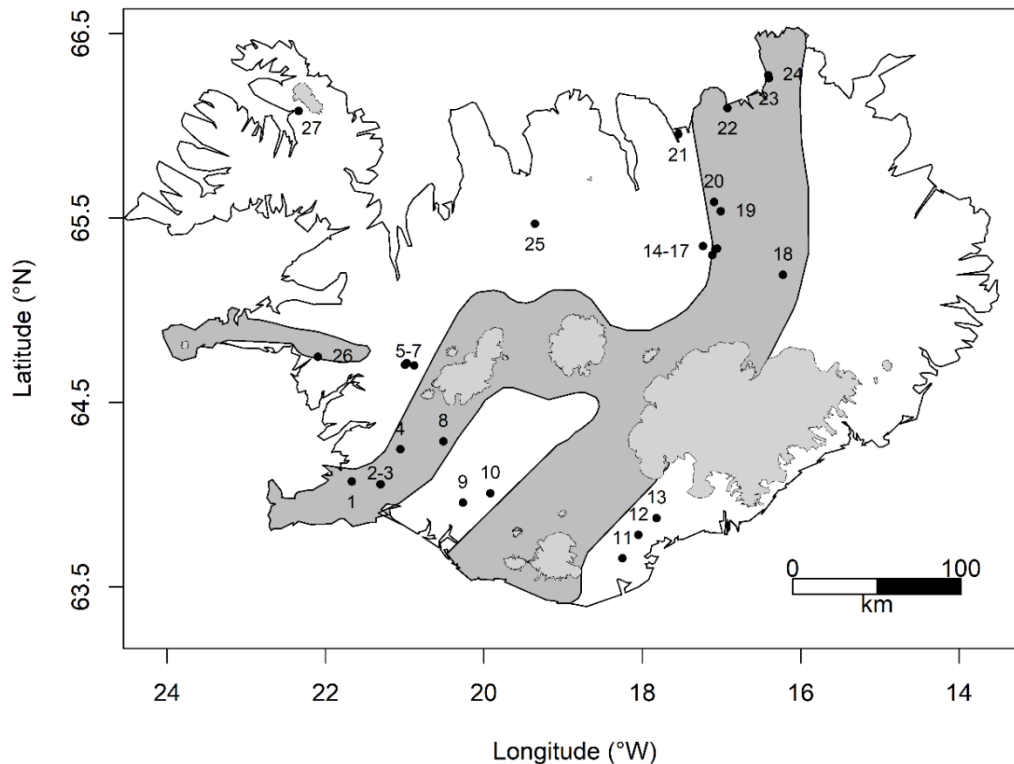
## 1.1 Groundwater amphipods

Stygofauna is a term used for those members of the animal kingdom that have some degree of affiliation with aquatic subterranean life. Stygoxens and stygophiles actively exploit resources from the groundwater realm but are not obligated subterranean biota while stygobites are restricted to the subterranean habitat (Gibert et al. 1994). In Iceland two stygobites have been described. These are the endemic groundwater amphipods *Crymostygius thingvallensis* Kristjánsson and Svavarson, 2004 and *Crangonyx islandicus* Svavarsson and Kristjánsson, 2006. *Crymostygius thingvallensis* has been found alive in a spring at Lake Þingvallavatn, Southwest Iceland and in Arctic charr stomachs in a spring in South Iceland (Skarðslækur) and in a spring in Northeast Iceland (Herðubreiðalindir).



**Figure 1** The groundwater amphipod *Crangonyx islandicus*. Scale: 1 mm

*Crangonyx islandicus* (Fig 1) has been found more widely in springs at the edge of lava fields throughout the volcanic active zone of Iceland, from southwest to northeast (Fig 2). *Crymostygius thingvallensis* belongs to a new family, Crymostygiidae, whereas *C. islandicus* is a member of a known freshwater subterranean amphipod family, Crangonyctidae. Both families are members of the superfamily Crangonyctoidae that has a worldwide distribution and is mainly subterranean (Väinölä et al. 2007). This discovery is quite remarkable and one of the larger biological discoveries in Iceland in the recent years. In Iceland endemism is low, and most of the biota are thought to have colonised Iceland after the last glaciation that ended 10-15 thousand years ago (Geirsdóttir et al. 2007).



**Figure 2** Location of spring sources in Iceland included in this dissertation. The volcanic active zone is represented in dark grey and glaciers in light grey. Samples outside the volcanic zone, except nr 25 and 27, are found in lava fields which originate in the volcanic zone. Further information on these sampling locations and the studies in which they were included, is listed in Table 2.

Studies on mitochondrial DNA variation in *C. islandicus* have shown that there are five distinct lineages within Iceland that have survived in separate subglacial refugia along the tectonic boundary. The first split between those groups occurred 4.8 Myr ago (Kornobis et al. 2010). As Iceland was repeatedly covered with ice sheets during glacial periods of the Ice Age, a viable subterranean ecosystem must have thrived under these conditions that would have been based on the primary production of chemosynthetic bacteria and archaea. Evidence of such microbial activity has been shown in subglacial waters in Iceland (Gaidos et al. 2004, Gaidos et al. 2009, Marteinsson et al. 2013) but this has not been studied in the habitat where *C. islandicus* is found.

### 1.1.1 Groundwater amphipods and resources

The subsurface has historically been considered as a food-scarce place that is dependent on allochthonous material originated in surface systems (Poulson & Lavoie 2000). Recent studies have, however, shown the existence of subterranean systems independent of sunlight, but based on primary production based on chemosynthesis which support complex food chains (Por 2007, Dattagupta et al. 2009, Flot et al. 2014). In these cases, the main bedrock type is limestone, leaving the groundwater rich in sulphur that can be utilized by chemoautotrophic bacteria. The studies of the *Niphargus* amphipods in the Frassassi cave system in Italy and the Movile cave system in Romania indicate that the relationship between the amphipods and the *Thiothrix* sulphur oxidizing ectosymbiotic bacteria might be widespread in Europe (Flot et al. 2014). These systems are found within karst caves,

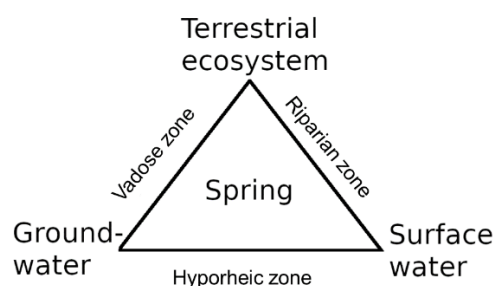
deep in the earth and isolated from the surface; they are considerably different from the geological settings in Iceland and other volcanic islands.

Currently, there is a considerable knowledge gap in the role of chemolithoautotrophic activity in the food webs in subsurface habitats. Chemolithoautotrophic organic matter is considered to have increased available resources for groundwater biota and possibly provided an evolutionary refugia for number of taxa at the Edwards Aquifer in Texas, USA. There, stable isotopes from stygobionts and microbial mats from springs, caves and wells in the aquifer have indicated that autochthonous organic material was responsible for sustaining higher trophic levels in the food web (Hutchins et al. 2016).

### 1.1.2 The habitat of *C. islandicus*

Groundwater amphipods in Iceland have been found in spring sources at the edges of lava fields where the groundwater enters the surface. Iceland has numerous groundwater resources due to high precipitation rate and little evaporation (Árnason 1976, Koreimann et al. 1996) and large glaciers (Sveinbjörnsdóttir & Johnsen 1992).

Springs are groundwater dependent habitats that are at the intersection between the groundwater and surface waters (Fig 3). Spring sources are where the groundwater emerges from the ground and they act as a window into the groundwater but they are also a mixture between three systems, namely the groundwater, surface water and the overlying terrestrial system (Barquín & Scarsbrook 2008). For this reason, they are referred to as ecotones where two or more ecosystems meet or as the transition zone between different systems (van der Maarel 1990). The spring sources can be further categorized into the surface and the subsurface. At the surface, there is a sun exposure and free flow of water, although under constrains while the subsurface of the springs is the below ground counterpart of the surface and is only accessible through the source. In the subsurface there is a permanent darkness and the water flow is controlled by the geological settings, water pressure e.g. by adjacent glaciers, topology of the bedrock, lava and sediments. This subsurface part of the spring is the habitat of *C. islandicus*.



**Figure 3 Three-dimensional aspect of the spring source. The spring is an ecotone, or zone of interaction, between the groundwater, surface water and the terrestrial surrounding and overlaying ecosystem. Hyporheic zone: streambed interstices, riparian zone: stream banks and vadose zone: unsaturated soils. Redrawn with permission from Scarsbrook et al. (2007).**

Spring sources are stable in terms of chemical and physical properties such as temperature and pH (van der Kamp 1995, Szczucińska & Wasielewski 2013) within a site but variable

across sites. The cold groundwater springs within the volcanic zone of Iceland are high in pH and they follow the mean annual air temperature of the region (Einarsson 1994).

When the topology of the area surrounding a spring is steep, a stream forming spring may result. This is also referred to as rheocrene spring. Another common type of springs in Iceland are pool forming springs, or limnocrene spring. Both rheocrene and limnocrene springs have a distinct opening where the groundwater emerges from the ground, so the source is well-defined. A third type of spring, also common in Iceland, is a marsh forming spring or helocrene spring. Helocrene springs are not considered in this thesis as the emerging point of groundwater is not a distinct point but rather groundwater diffusing the surface, forming a marsh or a bog (Glazier 2009), so the source is less well-defined than for the rheocrene and limnocrene springs. Furthermore, it has not been reported that the groundwater amphipods have been found in this type of springs. Spring type is one of the main factors shaping the invertebrate communities in springs in Iceland (Govoni et al. 2018, Kreiling et al. Submitted). This classification of springs, above, is based on the habitat type they provide (Glazier 2009) but springs are also classified in various other ways based on geology and bedrock type (Pérez 1996), hydrology and magnitude of discharge (Meinzer 1927), water chemistry and total dissolved solids (Danks & Williams 1991) and water temperature (Tuxen 1944, Kreiling et al. 2018)

Cold spring sources are common, especially in the volcanic active zone of Iceland (Fig 2) where the bedrock type is basaltic lava that is highly permeable (Sigurdsson & Stefansson 2002). The volcanic active zone in Iceland extends from the southwest corner of the island to the northeast corner and follows the tectonic plate boundaries. This is the youngest rock formations with late Pleistocene basalt lavas and hyaloclastites and Holocene lava grounds. On both sides, older rock formations are found that are less permeable with tertiary basalts and early Pleiocene basalt lava. Springs that are located in these older areas of Iceland are a result of surface runoff and can often be found in the unconsolidated strata (Einarsson 1994). The young basaltic lava fields are rich in minerals and they weather easily, resulting in waters rich in minerals. Basalt is known to be easily soluble and rich in minerals that becomes available for bacteria and archaea through chemical weathering processes (Gislason et al. 1996) whereas iron (Fe) and manganese (Mn) are known to be a potential energy source for bacterial growth (Kelly et al. 2010, Cockell et al. 2011) making the groups of iron and manganese utilizing bacteria of interest for our system as potential primary producers and a source of energy for the amphipods. The Icelandic cold groundwater spring sources within the volcanic active zone are categorized as Fennoscandian mineral rich springs (category C2.111, European Environmental Agency) according to the European Nature Information System (EUNIS) classification system, and they have a high conservation value according to the Bern convention.

## 1.2 Microbes

Microbes play a critical role in aquifers and groundwater habitats in the recycling of nutrients through the microbial loop (Gibert et al. 1994, Goldscheider et al. 2006), not only bacteria and archaea (Griebler & Lueders 2009) but also protists (Novarino et al. 1997) and fungi (Livermore & Mattes 2013). In this study the focus was put on ciliates, a monophyletic and unicellular protist group (**Paper I** and **II**) and bacteria (**Paper III** and **IV**). An attempt was made at amplifying archaea and the results for that are reported in **Paper III**.

### **1.2.1 Ciliates**

Ciliates are an important link in food webs where they primarily prey on bacteria and smaller protists (Sherr & Sherr 2002). The prokaryotes are mainly found in biofilms attached to sediments and substrates (Griebler et al. 2002) that again attract protozoan grazers like ciliates among others (Kota et al. 1999). This microbial eukaryote community is a food source for meiofauna (Hancock et al. 2005), e.g., copepods and chironomid larvae (Stoecker & Capuzzo 1990, Mieczan et al. 2015) and even fish larvae (de Figueiredo et al. 2005). Therefore, it is an essential link for energy transfer and nutrient recycling in many ecosystems (Beaver & Crisman 1989, Carlough & Meyer 1991).

### **1.2.2 Bacteria**

The energy within subterranean ecosystems has been considered to originate at the surface and from there penetrate into the system (Poulson & Lavoie 2000, Foulquier et al. 2010). However, there is recent evidence that indicates that chemolithoautotrophic microbial activity (Table 1 for overview of energy sources and nutritional demands of microorganisms) is also important as the base of food webs in aquifer and groundwater systems (Nowak et al. 2017, Schwab et al. 2017) and that endemic groundwater microbiota might exist (Mehrshad et al. 2020), but this has been under debate (Danielopol & Griebler 2008, Griebler & Lueders 2009).

**Table 1 Main nutritional types of microorganisms. Based on Prescott et al. (2002).**

	Energy	Carbon source	Example of taxa
Photoautotroph	Light	CO <sup>2</sup>	Algae, cyanobacteria
Photoheterotroph	Light	Organic C and CO <sup>2</sup>	Purple non-sulfur bacteria Green non-sulfur bacteria
Chemolithoautotroph	Inorganic chemical energy (S, H, N, Fe)	CO <sup>2</sup>	Sulfur oxidizing bacteria, Hydrogen bacteria, Nitrifying bacteria, Iron oxidizing bacteria
Chemolithoheterotroph	Inorganic chemical energy (S, H, N, Fe)	Organic C	<i>Shewanella</i> , <i>Geobacter</i>
Chemoorganoheterotroph	Organic chemical energy	Organic C	Protozoa, fungi, most non-photosynthetic bacteria
Mixotroph	Organic and inorganic energy	Inorganic and organic C	<i>Thiomonas</i>

Prokaryotes in groundwater are attached to sediment particles and surfaces such as rocks where they form biofilms (Griebler et al. 2002) and there are indications that traits such as biofilm formations might be selected for in groundwater and aquifers (Mehrshad et al. 2020). A study on hyporheic biofilms in a subsurface streambed showed that biofilms formed by bacteria and fungi in that particular habitat was a potential food source for stream invertebrates in the hyporheic zone (Barlocher & Murdoch 1989).

### 1.2.3 Biodiversity, microbes and spring sources

Biodiversity, the variety of life on earth, is a central topic in ecology and evolutionary biology and various fundamental patterns have been detected such as latitudinal-diversity patterns (Hillebrand 2004, Fuhrman et al. 2008), species-abundance distributions (Marquet et al. 2014, Matthews & Whittaker 2014, Locey & Lennon 2016), species-area relationships (Zhou et al. 2008), distance-decay relationships (Soininen et al. 2007, Astorga et al. 2012, Power et al. 2018) and species-time relations (Zhou et al. 2012). The processes behind generating and maintaining these patterns of diversity have likewise gained considerable attention in evolutionary and ecological research: contrasting neutral and deterministic processes, which have been referred to as neutral and selectionist debate in



evolutionary biology and with the neutral vs. niche debate as a central theme in community ecology. According to the neutral theory, species that have equal fitness and stochastic processes such as dispersal, colonization, extinction and speciation will be mainly responsible for shaping the communities (Hubbell 2005). In contrast, the assumption of the niche theory is that species differ in their abilities to compete for resources, whether biotic (competition, predation, mutualism etc.) or abiotic (physical and chemical) and this will result in differences in occupancy of the niches (Hutchinson 1959), thus the community structure will be shaped by environmental filtering and biological interactions among species.

Microorganisms are ubiquitous. They are small in size, have a fast generation time and conduct a horizontal gene transfer. As a result they were thought to have cosmopolitan distribution patterns and Baas-Becking (1934) referred to his hypothesis on this as “everything is everywhere but the environment selects”. The central theme of this hypothesis is that the microorganisms have a global metapopulation and huge population size resulting in worldwide distribution biodiversity patterns. This view is now under debate: Fenchel et al. (2019) argued that protists such as ciliates do have large metapopulations and ubiquitous distribution if they are smaller than 1 mm, while the model of “moderate endemicity” (Foissner 2008) states that microorganisms have similar limitation to geographical distribution as macro-organisms. The role of dispersal has, therefore, gained considerable attention in shaping diversity patterns for microorganisms (Wilkinson et al. 2012, Van Eaton et al. 2013, Chu et al. 2017). Along with dispersal other processes such as stochastic drift, environmental selection and diversification are important in shaping the microbial community composition (Hanson et al. 2012, Nemergut et al. 2013). Thus, there is evidence for both niche and neutrally driven communities and the two are not mutually exclusive.

Spring sources are interesting study sites in ecology due to the stability in chemical and physical properties and the fact that the spring sources form island-like structures in the landscape. For this reason, they have been used for testing ecological hypothesis about community assembly theory and how ecological gradients affect the community structure, including microbial organisms such as bacteria (Power et al. 2018) and diatoms (Teittinen & Soininen 2015).

#### **1.2.4 Microbial diversity analysis**

The assessment of the microbial communities has been advanced with the introduction of the next generation sequencing (NGS) technique, also referred to as high throughput sequencing, that allows for cultivation-free approach to analyze bacteria and archaea. This has led to a revolution in the field of environmental microbiology since prior to the NGS technique, only a fraction of the microbial taxa could be revealed by the cultivation methods needed before identification. This has been referred to as the “great plate count anomaly” (Staley & Konopka 1985).

Identification of organisms is possible using DNA identification utilizing the methods described above, targeting marker genes of interest. For diversity analysis of bacteria, the 16S rRNA gene has mainly been used as it is a well conserved gene encoding for protein synthesis. Amplicon sequencing based on part of the 16S rRNA gene has provided valuable insight into diverse communities such as in soil (Baldrian et al. 2012, Bartram et al. 2014), drinking water (Bruno et al. 2017), seeps (Woycheese et al. 2015) and springs

(Headd & Engel 2014, Karwautz et al. 2017, Power et al. 2018) to name few, but it also has shortcomings like all methods. Although the 16S rRNA gene allows insight into the phylogenetic relations of the community members and taxonomic assignment, it does not provide much information about the functional capabilities for the individual taxa identified or the community as whole (e.g. Fuhrman 2009). Another shortcoming is that not all taxa are equally amplified with the same primer pairs so some taxa might be missing altogether. This can be inspected beforehand using *in silico* PCR for instance to inspect how universal the primers are (Ficetola et al. 2010). The third problem of note is that not all taxa have equal copy numbers of the 16S rRNA gene, therefore, some taxa might be over- or underrepresented in the dataset (Vetrovsky & Baldrian 2013). So called *r* strategists, or taxa that grow exponentially when resources are plentiful usually have more copies of the 16S rRNA gene than taxa that grow more slowly, so called *K* strategists (Klappenbach et al. 2000), which may better utilize the environment at high density (Gill 1974). In an oligotrophic habitat such as the cold groundwater spring sources it can be expected that slow growing taxa are common (Griebler & Lueders 2009) with low copy numbers of the 16S rRNA gene.

## Environmental DNA and metabarcoding

Environmental DNA or eDNA, *sensu lato* is the genetic material, DNA, that can be found within environmental samples such as soil, water, ice or air (Taberlet et al. 2012). This genetic material can be of intracellular or extracellular origin. Intracellular DNA is still bound within the cellular membrane. These cells can either be living unicellular organisms such as prokaryotes and unicellular eukaryotes or they can be from multicellular organisms that have been shed from their origin. This intracellular DNA is usually in good condition, especially when coming from living unicellular organism. The extracellular DNA is prone to degradation as it is not protected by the cellular membrane from the sunlight, microbes, temperature and other elements that can destroy the DNA molecule (Barnes & Turner 2016).

Originally, the research using eDNA came from the field of microbiology where DNA molecules were extracted directly from sediment samples (Ogram et al. 1987). The research field of eDNA has extended to broader usage and is now widely applied in fields ranging from microbes to metazoans for taxa identification and diversity assessments (Klymus et al. 2017, Power et al. 2018).

Metabarcoding, often referred to as amplicon-based metagenomics or amplicon sequencing especially in microbiology research, is a type of NGS where only one gene, or a part of one gene is targeted. The gene part of interest is amplified with polymerase chain reaction (PCR) and then sequenced with NGS. The first study to conduct a metabarcoding, although not using this terminology, was Giovannoni et al. (1990) to assess diversity of bacterioplankton in the Sargasso Sea using cloning as this was before the NGS era. The metabarcodes are usually rather short segments of DNA but taxonomically informative with conserved sequences at each side to serve as primer anchors. For bacteria, the nuclear 16S rRNA gene with its nine variable regions is the standard approach for microbial diversity assessments (Tringe & Hugenholtz 2008). Different length of fragments will yield a different taxonomical resolution: fragments covering the whole 16S rRNA gene and all nine variable regions will give a good resolution down to the species level while shorter fragments covering only one variable region will hold the potential of discriminating between taxa at the family or genus level (Kim et al. 2011).

Environmental DNA (eDNA) together with metabarcoding provide a powerful tool to assess microbial diversity. It is now possible to extract all DNA from an environmental sample, targeting both intracellular and extracellular DNA via metabarcoding and thus generating a list of taxa living within the environment of interest (Taberlet et al. 2012, Ji et al. 2013). However, the list of taxa always relies on the databases available (Nilsson et al. 2006, Edgar 2018). These methods enable us to examine the biodiversity of inaccessible systems such as by sampling water from the groundwater springs where amphipods are known to live.

### 1.3 Aims and objectives

The aim of this study was to explore the diversity associated with *C. islandicus* and the spring sources within its habitat, to gain more knowledge of this unique and understudied habitat. The previously unidentified epibiotic features on the surface of *C. islandicus* were identified in **Paper I**, using phylogenetic methods. The population structure of the most abundant form of epibionts was examined to see if it reflected the same divergence patterns as its host. The few observed living things associated with the amphipods turned out to be ciliates and this was taken further in **Paper II** where the ciliate community in the spring sources was examined. In this study the questions asked were i) if the epibionts previously found on *C. islandicus* could be detected in the spring water ciliate community, ii) could more taxa be detected accompanying *C. islandicus* using metabarcoding, iii) which, if any, environmental and geographical factors affect the community composition of ciliate communities in the spring sources especially focusing on geographical distribution patterns.

In **Paper III**, the focus was shifted towards the bacteria with the aim of exploring the bacterial diversity and community composition associated with *C. islandicus* and its habitat. For this, the water at the spring sources was used. The questions asked were i) which, if any, environmental and geographical factors affect the community composition of bacteria in this habitat, ii) are there chemolithoautotrophic bacteria in the spring source community or associated with *C. islandicus* as this could be related to the subglacial survival of the amphipods.

In **Paper IV**, the aim was to inspect the biofilms that were found within the habitat and investigate the community structure. The bacterial diversity within the gut of *C. islandicus* was also analyzed. The question asked were i) is there an overlap between the gut content of *C. islandicus* and the naturally occurring biofilms in its habitat and ii) are there chemolithoautotrophs in the naturally occurring biofilms as this could shed light onto possible resources for *C. islandicus* and be related to the subglacial survival of the amphipods. The second aim of this study was to predict the possible habitat of viable heterotrophic bacteria in the gut of *C. islandicus* and put it into the context of the spring source habitat as an ecotone between groundwater, surface water and the terrestrial ecosystem. The specific questions asked were i) do these cultured bacteria belong to groundwater, surface water or soil or are they animal associates and ii) how common are they in the gut and in the naturally occurring biofilms.



## 2 Methods

### 2.1 Sample collection

Water samples were obtained from 26 spring sources across the volcanic active zone of Iceland, glass beads were incubated at eight sites, amphipods were obtained from five springs and a subsurface biofilm sample was obtained from one location (Table 2, Fig 2). The focus was set on cold rheocrene and limnocrene springs. The temperature range was 3.36-7.45°C and the pH was 5.26-9.43, although within these ranges most springs tended towards colder and alkaline. Two warmer springs were also included to get an indication of the effect of warmer temperatures. These were in Hengill (11.5°C and pH 7.4) and Steinsstaðir (42°C and pH 8.48), Sites 2 and 25 respectively (Fig 2).

**Table 2** Sampling sites at spring sources in Iceland included in this dissertation. Site numbers are the same as in Fig 2. Global position is displayed in decimal degrees.

Nr	Site	LAT °N	LON °W	Paper	Samples <sup>1</sup>	Spring <sup>2</sup>	Temp <sup>3</sup>	pH
1	Lækjarbotnar Rvk	64.07	-21.67	<b>II</b>	W	R	5.32	8.69
2	Hengill 6a	64.06	-21.30	<b>III</b>	W	R	11.5	7.4
3	Hengill 7	64.06	-21.31	<b>II</b>	W	R	5.77	7.64
4	Vatnsvik	64.25	-21.06	<b>I, II, III, IV</b>	A, B, C, G, W	L	4.34	8.99
5	Hrauná	64.70	-21.00	<b>II</b>	W	R	5.58	8.45
6	Kiðárbotnar	64.70	-20.88	<b>II</b>	W	L	3.39	9.43
7	Sílatjörn	64.71	-20.98	<b>II</b>	W	R	3.99	7.60
8	Miðhúsaskógur	64.29	-20.51	<b>II, III</b>	C, G, W	L	3.36	9.38
9	Lækjarbotnar Hol	63.96	-20.26	<b>I, II, III</b>	A, W	R	5.47	7.90
10	Galtalækur	64.01	-19.92	<b>II, III</b>	C, G, W	R	5.00	7.99
11	Botn	63.65	-18.25	<b>II</b>	W	L	7.45	7.92
12	Kirkjubæjar- klaustur	63.78	-18.05	<b>II, III</b>	C, G, W	R	5.5	

13	Þverá	63.87	-17.82	<b>II</b>	W	L	5.11	7.45
14	Krakárbotnar	65.33	-17.08	<b>II</b>	W	R	5.91	8.67
15	Mótunga	65.30	-17.12	<b>II</b>	W	R	4.5	8.96
16	Svartárvatn	65.35	-17.23	<b>I, II, III</b>	A, C, G, W	L	3.66	8.96
17	Hagalækur	65.33	-17.06	<b>II</b>	W	R	7.27	8.6
18	Herðubreiðar- lindir	65.19	-16.23	<b>II</b>	W	L	5.6	6.8
19	Grænavatn Suður	65.54	-17.01	<b>II</b>	W	L	4.23	8.82
20	Síkid	65.59	-17.09	<b>II</b>	W	L	5.05	7.7
21	Sandur	65.95	-17.55	<b>I, II, III</b>	A, C, G, W	L	3.77	7.4
22	Lón	66.10	-16.93	<b>II</b>	W	L	4.92	8.02
23	Presthólar	66.26	-16.40	<b>II, III</b>	W	R	4.11	7.95
24	Klapparós	66.27	-16.41	<b>I, II, III</b>	A, C, G, W	L	4.5	7.6
25	Steinsstaðir	65.47	-19.36	<b>II, III</b>	W	R	42.62	8.48
26	Staðarhraun	64.75	-22.10	<b>II</b>	W	R	4.57	5.26
27	Kaldalón	66.08	-22.34	<b>II</b>	G		6.6	

1: Samples legends: A: Amphipods, B: Biofilms, C: Cell counts, G: Glass beads, W: Water.

2: Spring: Spring type, L: Limnocrene, R: Rheocrene.

3: Temp: Temperature °C.

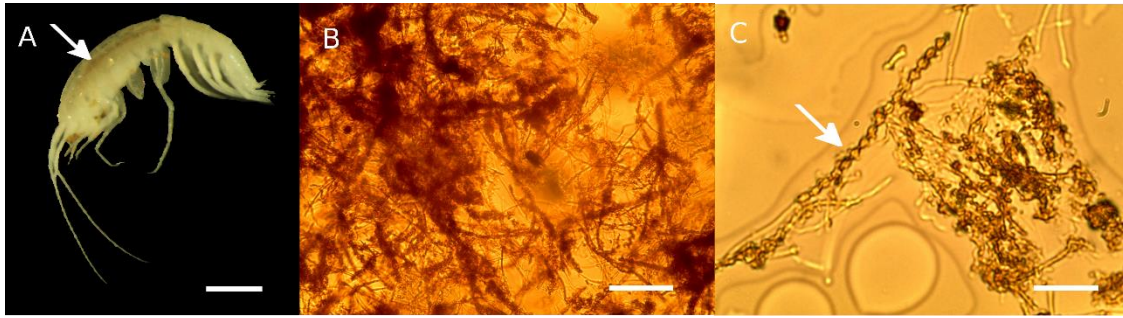
### 2.1.1 Biological samples

#### Amphipods

The amphipods were collected with electrofishing gear at the spring sources and caught by net when they floated out of the sources, paralyzed by the electrical shock that was given. All amphipods were placed in 96% ethanol for further storage except a sample which was used for a cut cultivation experiment (**Paper IV**); they were brought alive to the lab where they were dissected.

In **Paper I**, amphipods that had been collected in the spring and summer 2012 and 2013 were inspected for epibionts. Five specimens from each location (Sandur, Klapparós and Thingvallavatn), were examined in a stereoscope (Leica MZ 95) with 80x magnification.

In **Papers II** and **III**, DNA extracts from whole amphipods, that had been collected in the summers of 2012 and 2013, were used and for **Paper IV**, DNA extracts from the amphipod guts were used (Fig 4a). The last mentioned were collected in the summer of 2018.



**Figure 4** A *Crangonyx islandicus* with red gut content (arrow), scale bar 1 mm. B Red biofilm from the groundwater spring source, 400x magnification. Filaments are visible, scale bar 55  $\mu\text{m}$ . C Chain-like structure from the biofilm, resembling *Gallionella* sp., 1000x magnification, scale bar 135  $\mu\text{m}$ .

## Water samples

Water samples (5 litres) were collected in the summers of 2014 and 2015 at the spring sources (example of spring source Fig 5) into plastic bottles that had been washed with hydrochloric acid (10% v/v HCl) and autoclaved prior to use. The water samples were stored in a cooler and then filtered through Sterivex<sup>TM</sup> filters within 24 h of sampling. Filters were stored frozen at  $-25^{\circ}\text{C}$  until the DNA was extracted. These filtered water samples were used in **Papers II** and **III**.

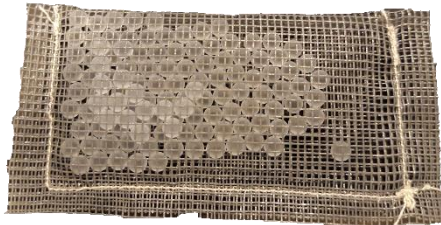


**Figure 5** Spring source in Klapparós, Site 24, north east Iceland.

## Glass beads

Glass beads ( $4 \pm 0.3$  mm in diameter, Carl Roth GmbH, Karlsruhe, Germany) were incubated at the spring sources as an artificial support for collection of biofilms for eight to ten weeks in the summer of 2014. The initial idea was to place them within the subsurface of the spring source but due to the structure of the sources at the lava edges this was not possible. The glass beads were thus placed as far as possible at each source, but this was around 2 to 10 cm into the sources. As the roughness of the surface of an artificial support can affect the formation of the biofilm e.g. (Voisin et al. 2016) two surface treatment types of glass beads were placed at each sampling site: Treatment 1) the surface of the glass beads was treated with a strong base, Armor Etch® (sodium difluoride) for 10 minutes as recommended by the manufacture and treatment 2) where the glass beads were sandblasted for 20 minutes with pressurised air and sand. The first treatment resulted in an evenly

uneven surface on the glass beads while the sandblasting resulted in scratches across the surface of the glass beads. Each treatment type was put into a net envelope (120 glass beads in each net with a surface area of 60.3 cm<sup>2</sup> and weight of 10 ± 0.1 g) (Fig 6) and both treatments were placed at each spring source. This sample type was used in **Papers II and III**.



**Figure 6** Glass beads in an envelope used as artificial support for collecting biofilms in the spring sources. Size of envelope: 8×4 cm.

Analysis showed that there was no difference between the two treatment types regarding community composition or counts of unicellular biomass, so they were treated together in the down stream analysis.

## Biofilms

Naturally occurring biofilms were collected in the spring 2018 from the subsurface of the spring sources in Vatnsvik, Lake Thingvallavatn with a thin wire that was put up to 30 cm into the spring sources and shaken back and forth. This resulted in pieces of biofilms floating out of the spring source that was collected with a dip net. Examples of microscope pictures of biofilms can be seen in Fig 4 b and c. The biofilms were preserved at -20°C until the DNA was extracted. Biofilm samples was used in **Paper IV**.

### 2.1.2 Environmental and geographical variables

Environmental variables were measured at the same time as the collection of the water and glass bead samples. In 2014, temperature was recorded, and samples collected for chemical composition (including nitrate, nitrite, phosphate, sulphur, iron and manganese) of the springs (see **Paper III** for description). In 2015, temperature, pH, oxygen saturation and water conductivity were measured.

Due to the stability of the spring source environment (van der Kamp 1995, Glazier 2009, Eiríksdóttir & Gíslason 2013, Szczucińska & Wasielewski 2013), the pH measurements recorded in 2015 were used to infer the pH of samples collected in 2014. The presence of fish was also reported.

The geographical variables used in these studies were geographical location (longitude and latitude), elevation above sea level, spring type (limnocene and rheocene springs). The samples were also either collected at the source (source samples) and approximately two meters downstream from the source (referred to as “surface” samples here after). Geographical area within Iceland was also used for the analysis. This was based on different groundwater basins that are also reflected in distinct mitochondrial DNA (mtDNA) lineages of *C. islandicus*.



## 2.2 Microbial diversity analysis

### 2.2.1 Ciliates

In **Paper I** and **II**, the ciliate diversity associated with the amphipods and in the spring water was assessed, respectively. In **Paper I**, ciliate DNA was amplified from whole amphipods using both a conserved phylogenetic marker (18S rRNA) and a more variable marker (internal transcribed spacer, ITS) for population structure (Table 3 for primer information). The variation in the ITS sequences was summarized by the number of haplotypes and the haplotype and nucleotide diversity using the program Arlequin 3.5 (Excoffier & Lischer 2010) and the partition of the pairwise sequence difference between the different samples were summarized with  $\Phi_{ST}$  and tested by permuting the individual sequences among samples 1000 times. Each part of the amphipod body was carefully studied, and the position of ciliates was recorded for each body part. The sequencing was performed on an ABI 3500xL Genetic Analyzer at the University of Iceland.

In **Paper II**, the ciliate diversity was examined in the spring water, from the biofilms collected by the glass beads and in the amphipods using hypervariable V2-V3 region of the 18S rRNA gene and sequences with NGS (Table 3). The community structure was modelled against environmental and geographical factors.

**Table 3 Overview of taxa targeted in each study, the marker gene targeted, and the variable region covered.**

Taxa	Marker	Primers	Region	Sample type	Sequencing	Paper	Ref
Ciliates, phylogenetic	18S rRNA	CS322F, 1147R	V2-V5	Amphipods	Sanger	<b>I</b>	Puitika et al. (2007), Dopheide et al. (2008)
Ciliates, populations	ITS	ITS1F, 28SR		Amphipods	Sanger	<b>I</b>	Chu et al. (2001), Kornobis and Pálsson (2013)
Ciliates community	18S rRNA	CiliF, CiliR	V2-V3	Amphipods, water, glass beads	MiSeq	<b>II</b>	Tapio et al. (2016)
Bacteria community	16S rRNA	341F, 785R	V3-V4	Amphipods, water, glass beads	MiSeq	<b>III</b>	(Klindworth et al. 2013)
Bacteria community	16S rRNA	341F, 785R	V3-V4	Amphipod gut, biofilms	MiSeq	<b>IV</b>	Klindworth et al. (2013)
Bacteria isolates	16S rRNA	9F, 805R	V1-V4	Amphipod gut	Sanger	<b>IV</b>	Skirnisdottir et al. (2000)

### 2.2.2 Bacteria

In **Papers III** and **IV** the bacterial diversity in the spring water, both water samples and from biofilms from artificial support (glass beads), in the amphipods (both whole animal and the gut) and in the biofilms from the subsurface part of the spring was targeted using the 16S rRNA gene (hypervariable region V3-V4, Table 3) and sequenced with NGS.

In **Paper III**, the community structure was modelled against environmental and geographical factors to infer whether deterministic or stochastic were dominant in shaping the spring community.

#### Flow cytometry

In **Paper III**, the number of cells in the spring source water and on glass bead samples were assessed with flow cytometry to estimate the unicellular biomass for each sample type. This was then correlated with the numbers of reads obtained for the corresponding sample types.

## Cultivation of bacteria in the gut

In **Paper IV**, bacteria were cultivated from the gut of amphipods. This was done to predict the possible habitat of bacteria in the gut as this might be a potential food source of the amphipods. The guts were extracted and streaked out on R2A media (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, US) and the bacteria cultivated at three different temperatures (4°C, 10°C and 20°C). The R2A media is a low nutrient media specially designed for drinking water microbes and other microbes that are potentially slow growing (Reasoner & Geldreich 1985). Colonies were picked up and re-streaked on fresh media to isolate pure strains. DNA was then extracted from the cultures and put into Sanger sequencing that was done at Microsynth AG in Switzerland (<https://www.microsynth.ch/home-ch.html>).

## Sequencing data pipeline for the NGS data

The raw sequence data was processed in OBITools (Boyer et al. 2016). The initial data handling steps involved pairing the forward and reverse reads (R1 and R2), quality filtering and dereplication while the binning into operational taxonomic units (OTUs) and the classification step was done using the SILVA database (Quast et al. 2013). This was done for the datasets in **Paper II** and **III**. In **Paper IV**, QIIME2 (Bolyen et al. 2018) and DADA2 package (Callahan et al. 2016) in R (R Core Team 2019) was used for the initial data handling, quality filtering and making of amplicon sequence variants (ASVs) and SILVA database was used for the classification. For convenience the ASVs were binned together under the taxonomic name assigned to each sequence variant and, therefore, forming OTUs. The binning of sequences into ASVs, also termed Exact Sequence Variants (ESVs) is emerging and has been recommended over the use of the conventional OTUs for better taxonomic resolution and facilitating of comparing datasets (Callahan et al. 2017). It has however been debated whether this approach yields more accurate estimates for the microbial community assessments and picking up ecological signals (Glassman & Martiny 2018). Therefore, for the interpretation of the results obtained in the studies included in this dissertation, it does not make a difference whether ASVs or OTUs are used.

## 2.3 Statistical methods

In **Papers I-IV**, all statistical analysis were done in R (R Core Team 2019). For the NGS datasets (**Papers II-IV**), samples were standardized using rarefaction, to account for uneven sample sizes, which could arise due to different densities but could also be an artifact of sequencing. The command used for rarefying was `rrarefy` in the `vegan` package (Oksanen et al. 2017) which produces a randomly rarefied community with a given sample size, usually to the level of fewest reads per sample. This is an important step to account for uneven library sizes, but this approach has also been criticized since it could result in discarding rare sequence variants or OTUs and thus underestimate diversity (McMurdie & Holmes 2014). Nonetheless, Weiss et al. (2017) recommend including the rarefying to reduce the potential noise of uneven library sizes. Therefore, this step was included as a first step prior any analysis.

### 2.3.1 Alpha diversity

Diversity within sites, also known as alpha diversity indices, was included in the analysis and used for model building. This was done in **Papers II-III**. The indices that were explored for the datasets were richness, Shannon entropy and diversity and Pielou's and Shannon evenness as these may reflect different aspect of the data. Richness, the simplest of the alpha diversity indices, is the count of taxonomic units in each sample and it does not consider the abundance of the taxa involved. Shannon entropy, often referred to as Shannon index or Shannon-Wiener index, is a robust index for diversity assessments (Haegeman et al. 2013). It has been suggested that the use of the Shannon diversity index (calculated as the exponent of the Shannon entropy) could also be used as it might be more easily interpreted than the Shannon entropy as it directly refers to number of taxa rather than an abstract index number (Hill et al. 2003). A commonly used evenness index is the Pielou's evenness index that scales the Shannon entropy with the number of taxa (Borcard et al. 2018). Another frequently used evenness index is the Shannon evenness, which like the Shannon diversity index, represents the number of taxa that are needed to produce a given value of the diversity index and is perhaps more easily interpreted than the Pielou's index (Borcard et al. 2018).

In **Papers II-III**, linear models were built with the alpha diversity indices as response variables and the environmental and geographical variables as predictors or independent variables. The model predictors were tested for collinearity and excluded if  $|r| > 0.7$  (Dormann et al. 2013). The model building was done with a stepwise model building in R, using the step command in R that selects the best model based on the Akaike criterion (AIC) and model diagnostics evaluating the Cook's distance (Cook 1977). The normality of the model residuals were tested using a Shapiro-Wilk test of normality (Shapiro & Wilk 1965) and transformed if needed.

### 2.3.2 Beta diversity

In **Papers II-III**, the community composition among springs was estimated using Bray-Curtis (BC) dissimilarity index (Bray & Curtis 1957) accounting for abundances. The BC was calculated using the function `vegdist` in the `vegan` package (Oksanen et al. 2017) in R. The principal coordinate scores (PCoA) were visualized using R. For calculating the difference in community composition among sites the impact of environmental and geographical variables on the community variability was evaluated with the permutational analysis of variance (PERMANOVA) using the `adonis` function in the `vegan` package (Oksanen et al. 2017) in R.

## 3 Results and discussion

### 3.1 Microbes associated with *C. islandicus*

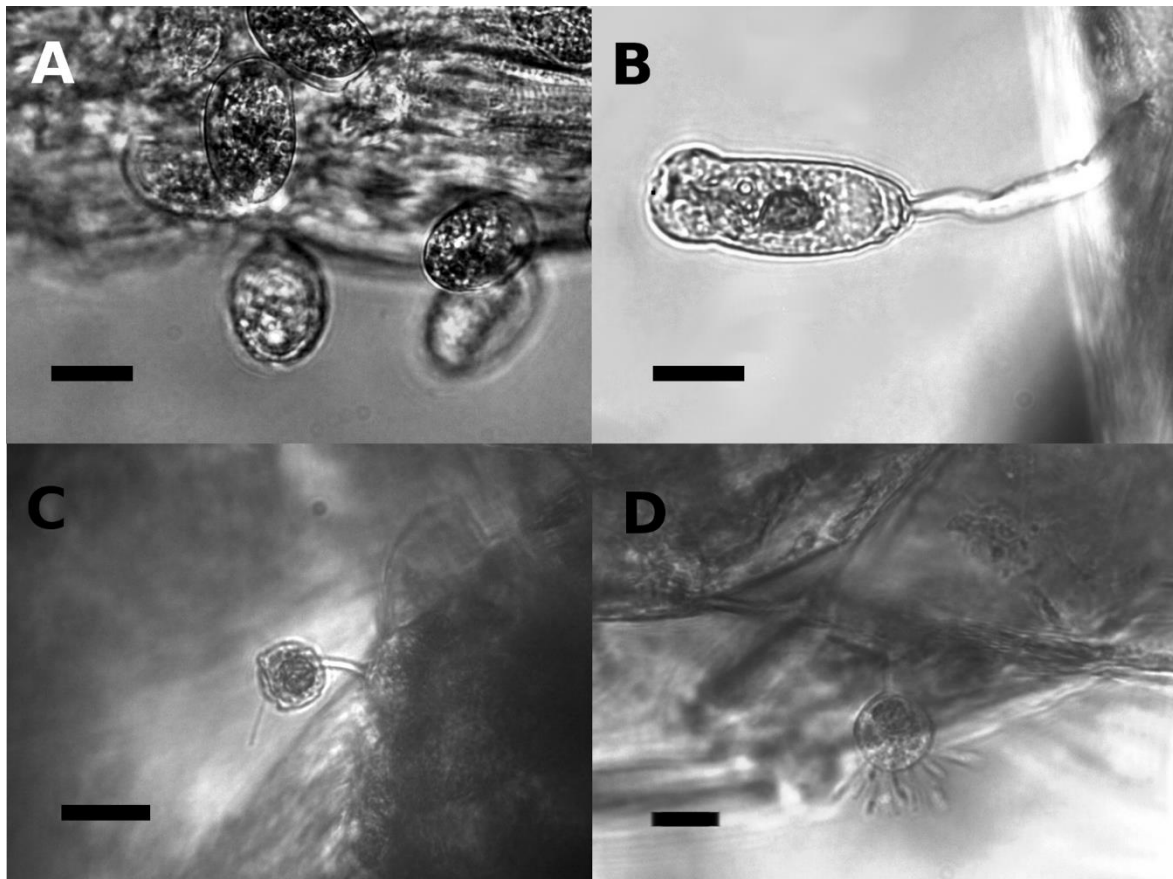
*Crangonyx islandicus* was found to be accompanied by both ciliate and bacterial taxa unique to the amphipods, and in much higher frequencies, in comparison with the environmental samples collected. These taxa range from being epibiotic symbionts or parasites (**Paper I-III**) to a potential food source as for the bacteria found in the gut samples (**Paper IV**). The amphipods are harboring specific microbiome comprised of few taxa of ciliates and bacteria.

#### 3.1.1 Ciliates

Apostome ciliates (**Paper I**) were found to accompany the amphipod *C. islandicus* both under the microscope and with molecular methods using a fragment of the 18S rRNA gene. They appear as egg shaped attachments (Fig 7 A) on the amphipods and were identified as phoront forming exuviotrophic ciliate in the order Apostomatida. These are specialized crustacean epibionts with life cycle adapted to the molting process of their crustacean hosts.

Very few additional taxa were observed in this part of the study. Philasterida ciliates were detected with 18S rRNA gene sequencing in Lake Thingvallavatn and in Sandur. three specimens of non-identified taxa were observed under the microscope that indicated peritrichs or suctorians. This study showed that few taxa are found to be associated with the *C. islandicus*, a sharp contrast to more vivid systems in the ocean and freshwater lakes where complex ciliate communities have been described for their hosts (Threlkeld & Willey 1993, Ólafsdóttir & Svavarsson 2002, Fernandez-Leborans & Von Rintelen 2007) pointing to an oligotrophic and isolated habitat.

When NGS was applied to the amphipods (**Paper II**), four other parasitic taxa were detected: Apostomatida, *Trichodina*, *Paranophrys* and *Hypocoma* along with two free living: *Paramecium* and *Euplotes*. The latter two might be a possible food source and just in low abundance in the environment and, therefore, not detected in the environmental samples. The low numbers of ciliates accompanying the amphipods in this part of the study is in line with the results from **Paper I** where few epibionts were observed on the amphipods, therefore, reflecting a oligotrophic habitat. However, the samples size for the amphipods in this study was low (n=3) so more work is needed to shed better light on the ciliate diversity accompanying the amphipods.



**Figure 7** A Oval-shaped epibionts located on pleopod from *Crangonyx islandicus* in 400× magnification. B Epibiont Type 1 found as a single specimen, located on pleopod from *C. islandicus* in 400× magnification. Size was recorded to be 55.7 μm for the length of the head and the width was 20.4 μm. The length of the stalk was 61.0 μm. C Epibiont Type 2 found as a single specimen, placed on pleopod from *C. islandicus* in 400× magnification. Size is recorded to be 12.8 μm for the length of the head and the width is 14.2 μm. The length of the stalk is 9.4 μm. D Epibiont Type 3 found on *C. islandicus* from Sandur. Found as a single specimen. Diameter of head is 11.4 μm. Scale bar: a, b, c = 20 μm. d: 10 μm. From Gudmundsdottir et al 2017.

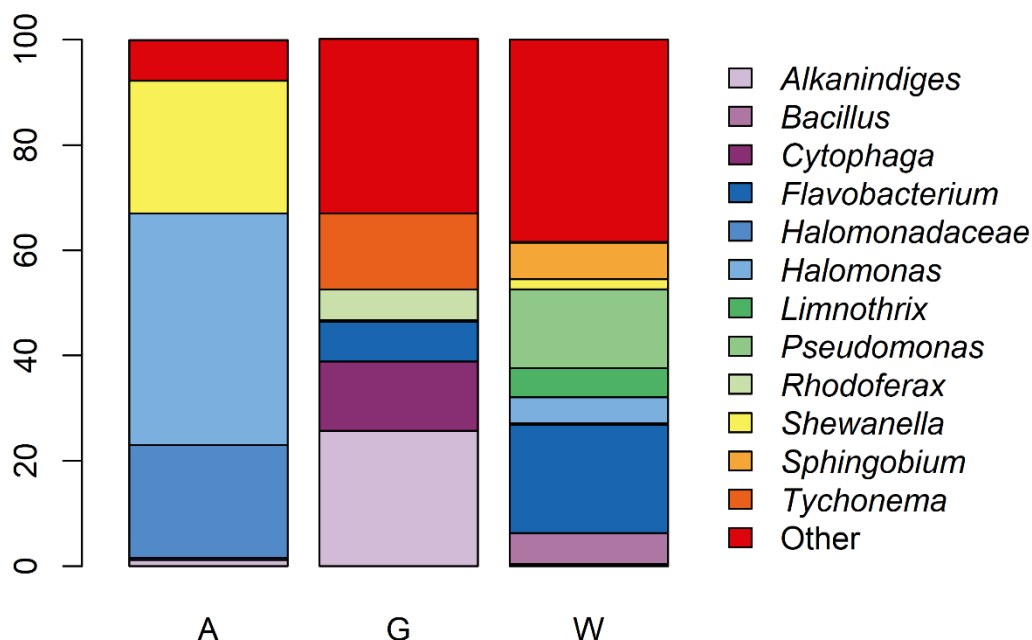
### Population structure of the ciliate epibionts

The same species of Apostomes were found to accompany the amphipods in all studied locations with the furthest spring sources being approximately 300 km apart (**Paper I**). Eleven different haplotypes of the variable ITS sequences were observed in three populations groups, one in south Iceland (Thingvallavatn) and two in north Iceland (South Thingeyjasýsla, Sandur and Svartárvatn, and North Thingeyjasýsla, Klapparós). We did not date the split of the ciliate populations but the pairwise  $\Phi_{ST}$  for the different populations but the ranking followed the same pattern as for their host, *C. islandicus*, showing the largest divergence between Klapparós in North Thingeyjasýsla from the other samples ( $\Phi_{ST} = 0.33$ ,  $p < 0.001$  for North Thingeyjasýsla and Thingvallavatn and  $\Phi_{ST} = 0.17$ ,  $p = 0.049$  for North and South Thingeyjasýsla).

### 3.1.2 Bacteria

The bacterial taxa associated with *C. islandicus* was first studied using whole amphipod DNA extracts (**Paper III**). In these samples the main groups of bacteria that got amplified were *Shewanella* sp. and *Halomonas* sp (Fig 8). These two taxa were found to be

dominating in all studied amphipod samples. They accounted for around 90% of taxa detected in the amphipod samples while only marginally detected in the environmental samples. These have been found to be probiotic for its hosts (Suantika 2013) and chitinolytic (Bowman et al. 1997) that might utilize the exoskeleton as energy source. *Shewanella* is chemolithoheterotrophic bacteria that can utilize sulphur and reduce iron and manganese (Newsome et al. 2018).



**Figure 8** Bacteria taxa with relative abundance more than 2% of all reads for each sample type. A = Amphipod samples (n = 6), G = Glass bead samples (n = 11), W= Water samples (n = 13). Other: Taxa with reads less than 2%.

### The amphipod gut samples

When just the amphipod gut was extracted (**Paper IV**) for the amphipods and sequenced with NGS, the most common taxa were *Thiomonas* (15.4 %) and *Streptococcus* (9.9 %). *Thiomonas* was common in the biofilm samples taken at the same location, so it becomes a question if it is a preferred food source of the amphipod or a transient, just passing through the gut. *Streptococcus* has been found in various gut samples from crustaceans (Kostanjsek et al. 2002, Tzeng et al. 2015) and insects (Allonsius et al. 2019). *Thermoanaerobacterium* (6.5%) that is a spore forming, thermophilic genus (Lee et al. 1993) and the methylotrophic *Methylocystis* (6.13%), (Bowman 2015) were also present in considerable amount. Other less abundant taxa were mostly heterotrophs. *Halomonas* was only briefly represented in the gut samples (0.25%) and *Shewanella* was not detected at all.

Based on the taxa that was cultivated from the amphipod guts both freshwater and terrestrial bacteria are present.

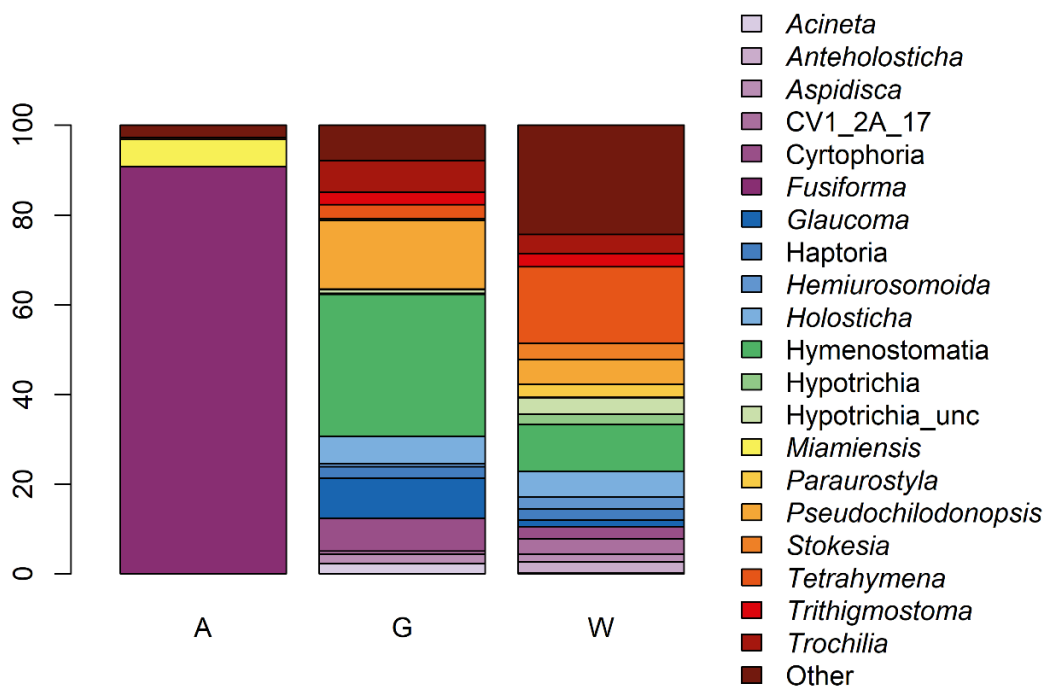
## 3.2 Microbial community in spring sources

The microbial community in the spring sources was examined in **Papers II and III**, focusing on ciliates and bacteria. The community was screened for the groups of ciliates and bacteria already found in the amphipod samples to determine if they were present in these environmental samples, however, they were not present to any great extent.

### 3.2.1 Ciliates

#### Water and glass beads

The most common ciliate taxa (Fig 9) found in the springs were free living, non-sessile ciliates that are known to feed on bacteria and small algae (Lynn 2008). The diversity of the ciliate communities increased with temperature in these cold groundwater springs even within the narrow temperature range of the springs under study. The community diversity was independent of other environmental variables tested but the evenness index indicated that limnocrene springs had fewer, more abundant taxa than the rheocrene springs so it might be that the pool forming springs offer a more stable habitat where the ciliate taxa manage to form populations than the stream forming where the taxa are in more even proportions, possibly just flowing by with the current. The richness of taxa was independent of other variables tested.



**Figure 9** Ciliate taxa with relative abundance more than 2% for all reads for each sample type. A = Amphipod samples, *Crangonyx islandicus* (n = 3), and spring sources, G = Glass bead samples (n = 13), W= Water samples (n = 42). Other: Taxa with reads less than 2%.

The diversity among springs was independent of both the environmental and geographical variables tested. This is interesting as the distance between the springs closest apart is about 1 km while for the ones furthest apart the distance is approximately 300 km.



### 3.2.2 Bacteria

#### Water and Glass beads

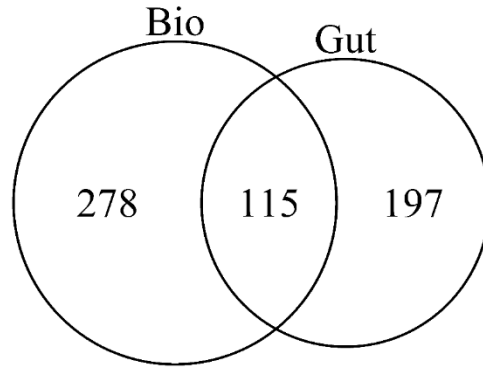
The bacterial community in the spring source water (Fig 8) was dominated by *Flavobacterium* (20.5%) and *Pseudomonas* (15.0%) but *Sphingobium* (6.9%), *Bacillus* (5.9%) and the cyanobacteria *Limnothrix* (5.5%) were also common (Table 3). The community that accumulated on the glass beads was significantly different in composition ( $F_{(1,12)} = 5.1, p = 0.001$ ). The main types that settled on the glass beads were *Alkanindiges* (25.7%), the cyanobacteria *Tychonema* (14.5%) and *Cytophaga* (13.2%).

The bacterial richness in water samples from the spring sources decreased with increased pH level ( $\log(b) = -5.6, p = 0.007$ ) but was independent of other variables tested. The diversity and evenness were independent of all tested variables. For the community composition among spring sources variation was found for different geographical areas ( $F_{5,12} = 1.8, p = 0.001$ ), presence of fish ( $F_{1,12} = 2.5, p = 0.004$ ) and temperature ( $F_{1,12} = 1.8, p = 0.002$ ).

The importance of pH for the within site richness has been reported for soil (Lauber et al. 2009, Bartram et al. 2014, Yun et al. 2016) and water (Power et al. 2018) and it has been hypothesized that pH influences microbial communities significantly as the availability of nutrients, cationic metal solubility and organic carbon characteristics are influenced by the pH of the environment (Lauber et al. 2009).

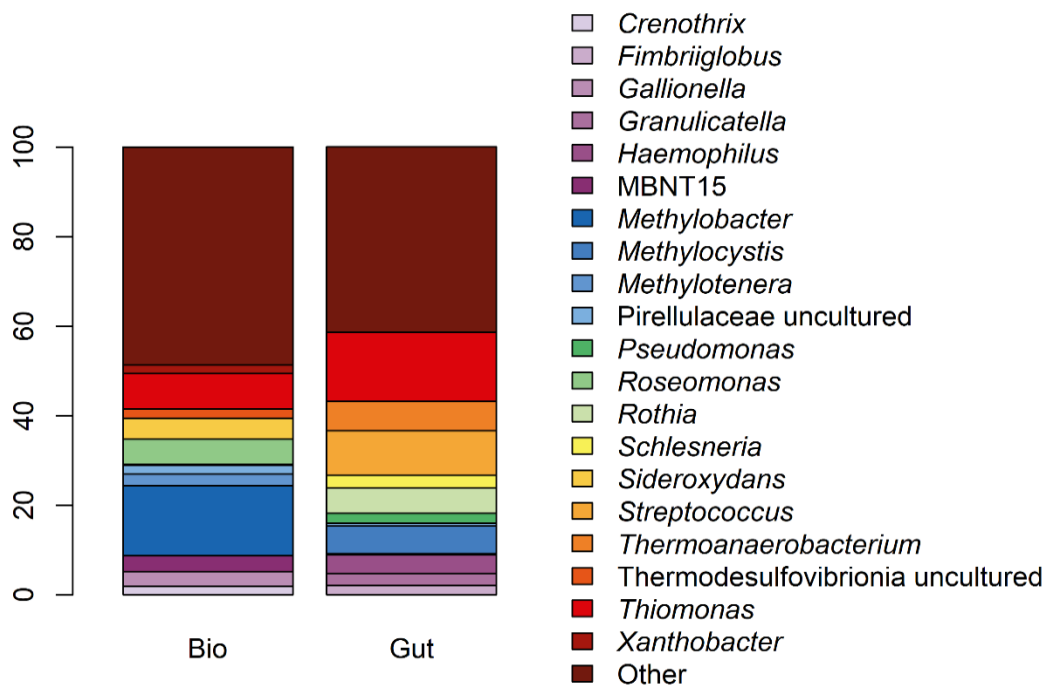
#### Biofilms

In the biofilms collected at the subsurface of the spring in Vatnsvik, Lake Þingvallavatn methane utilizing bacteria (methanotrophs) were noticeable with *Methylobacter* (15.6% of total reads for the biofilm samples), *Methylotenera* (2.7%) and *Crenothrix* (1.9%). *Crenothrix* has often been affiliated with iron oxidation (Emerson et al. 2010) as it is often common in iron rich springs (Cantonati et al. 2006) but studies have shown that it is rather a methane oxidizer (Stoecker et al. 2006, Oswald et al. 2017). Methanogens were detected but these are archaea, so they are not directly targeted by the primer pair used in the studies but show up none-the-less. Therefore, they are present in the samples but since they are not being specifically targeted, their relative abundance is perhaps skewed. *Thiomonas* was also common in the biofilm samples (7.9%). *Thiomonas* is a mixotrophic bacterium that grows best with organic carbon and reduced sulfur compounds and some can utilize ferrous iron (Kelly et al. 2007). It was also common in the gut of the amphipod so it might be an important food source. Approximately 30% of the taxa that was found in the biofilm was also present in the gut (Fig 10). For overview of which taxa was found in the biofilm and gut samples see Fig 11.



**Figure 10** Comparison of the number of OTUs detected in the biofilms and in the gut of *Crangonyx islandicus*. Bio: Biofilms (n=3), Gut: Amphipod guts (n=5).

Iron oxidizing bacteria were also common in the biofilms. This group consisted of *Sideroxydans* (4.7%) and *Gallionella* (3.3%) and the less common *Mariprofundus* (0.4%) and *Xanthobacter* (1.9%). *Sideroxydans* been reported to be chemolithoautotrophic (Weiss et al. 2007) but it is also metabolically flexible and can utilize sulphur compounds for lithotrophic growth and has a set of nitrogen fixation (Emerson et al. 2013). *Gallionella* has also the capacity of inorganic carbon fixation but is also capable of utilizing organic carbon and is, therefore, a mixotroph (Hallbeck & Pedersen 1991). *Gallionella* is a microaerophilic taxa and can be found where anoxic groundwater with ferrous iron reaches an oxygen rich environment (Hallbeck & Pedersen 2015). Along with the above-mentioned bacteria, heterotrophic bacteria were also common in the biofilm samples and methanogens and sulphur reducers were also present.



**Figure 11** Bacteria taxa relative abundance more than 2% from all reads for biofilm and gut samples. Bio: Biofilm (n=3) and Gut: Amphipod gut, *Crangonyx islandicus*. Other: Other taxa with 2% or less reads.

## 4 Conclusions and future perspective

With the discovery of *C. islandicus* in the groundwater it became apparent that in the groundwater in Iceland there is a vivid community of various living things. This thesis sheds light on a microbial community accompanying the amphipods as epibionts, in the spring source water and in a naturally occurring biofilms in the amphipod's habitat in the form of prokaryotes and single cellular eukaryotes. It also reveals some of the patterns that shape the community structures of these microbes inhabiting the spring sources.

Few taxa were found to accompany *C. islandicus* when looking at the surface of the amphipod (**Paper I**), all of which turned out to be ciliates. When DNA extracts from whole amphipods were sequenced (**Paper II**), a few more taxa were added. The few ciliate taxa associated with the amphipods might reflect that the subsurface system of the springs is oligotrophic and isolated or as the species-energy theory states that number of species go in line with the amount of energy available in the system (Wright 1983). Conversely, in resource rich surface water environments, both marine and freshwater, complex communities of epibionts and symbionts are found to accompany crustaceans (Threlkeld & Willey 1993, Ólafsdóttir & Svavarsson 2002, Fernandez-Leborans & Von Rintelen 2007).

In the alkaline cold mineral rich springs in Iceland, the diversity of ciliate communities (**Paper II**) are affected by temperature and the evenness was related to spring type where limnocene springs had fewer but more abundant taxa than rheocene springs. The diversity patterns, therefore, are influenced by environmental filtering. Likewise, the diversity among springs seems to be affected by neutral processes such as long-distance dispersal, possibly with wind, birds or flying insects and temporal fluctuations may dominate the possible association with environmental factors. If the dispersal is limited, we would expect some taxa to be abundant in some sites and lower in others but as there was no association with geographical distances, our findings suggest that the dispersal between sites is not limited for this group of organisms. Both deterministic and stochastic processes therefore seem to shape the community composition. Genetic marker with higher taxonomic resolution might reveal more fine-scaled pattern for the spring community. In **Paper I** we used the internal transcribed spacer region, ITS, a marker with a higher taxonomic resolution on the apostome ciliates associated with the amphipod and that revealed difference between north and south Iceland where the sampling locations are approximately 300 km apart. However, those ciliates are probably restricted to the subsurface part of the spring source along with the amphipods that have extremely limited dispersal capacities.

The bacterial communities in the spring sources (**Paper III**) vary with respect to geographical areas that are representing different groundwater basins, presence of fish in the habitat and environmental factors such as temperature and pH. They seem, therefore, to be shaped by environmental factors and be dispersal-limited where diversification and stochastic drift may play a role.

When considering the bacterial taxa from whole animal extracts (**Paper III**) few dominating taxa were revealed that probably belong to the surface and the exoskeleton of the amphipods, but when gut extracts were sequenced high proportion of unique taxa were revealed (**Paper IV**) that might indicate a specialized gut microbiome. It has been

suggested that the dependency of animals on symbiotic gut microbiome spans a continuum from high dependency, where the fitness of the host is tightly knitted to the presence and function of the gut microbiome to no dependency where the gut microbiome is nonexistent (Hammer et al. 2019). Amphipods potentially have a specific gut microbiome (Dittmer et al. 2012) and have been shown to harbor species specific gut microbiome that, at least partly, reflect the host's habitat and food choices (Abdelrhman et al. 2017). It has not been shown for amphipods if the microbiome has a role in the ability of colonize extreme habitats such as the subsurface although there has been evidence for difference in gut microbiome between populations inhabiting extreme habitats such as the hadal zone (Zhang et al. 2019). This has been tested for fish where no link was found between populations in the surface and in the subsurface (Ornelas-Garcia et al. 2018). It is a subject for future studies to investigate if the gut microbiome of the *C. islandicus* has played a role in the survival of the amphipods in the subglacial refugia during past Ice ages in Iceland.

Spring sources are groundwater dependent habitat and rely on the continuous flow of water from the subsurface. As it is an ecotone between the groundwater, surface water and the terrestrial system it can be expected that the microbial inhabitants come from these three realms. Cell counting (**Paper III**) indicated that the water harbored few cells and was in line with groundwater cell count (Pedersen et al. 2008) but was less than the numbers reported from surface water in lake Thingvallavatn (Guðmundsson 2014). The main microbial community in groundwater and aquifers are however found to be attached to surfaces and form biofilms and there is possibly a selection for this trait among the groundwater microbiome (Mehrshad et al. 2020). The resources within the groundwater system for metazoans such as *C. islandicus* therefore seems to be attached to the surface of the lava rather than being in the water current.

Chemoautolithotrophic iron oxidizers were common in the biofilm sample (**Paper IV**) and this raises questions regarding the subglacial survival of the amphipods. Iron holds little energy potential (Chapelle 2001, Emerson et al. 2010) but is abundant in the volcanic basalt lava, bound in different minerals (Stefansson 2001) that are also easily weathered (Gislason et al. 1996). It might, therefore, be able to sustain a viable population of metazoans during time periods when there is low nutrient input from the surface system such as during glacial periods of Ice age when Iceland was covered with glaciers. In the present-day system, it is likely that the complex community of the biofilms formed in the subsurface of the spring source, and probably extending into the aquifer, is the source of food for the amphipods. The presence of methanogens and methanotrophs indicate that decaying organic material is important and the occurrence of both iron oxidizing and iron reducing bacteria indicate that the biological iron cycle is present. Along with the chemoorganoheterotrophs the biofilm community along with the extracellular matrix make up a possible food source for the amphipods.

To reveal to what extent the food source of the *C. islandicus* is of subsurface origin it would be interesting to apply stable isotope analysis as was done in the Edwards Aquifer system in Texas, US (Hutchins et al. 2016). By using this method, it should be possible to disentangle if the food source is mainly of photosynthetic origin or coming from chemolithoautotrophic action.

Cold groundwater spring sources are a unique habitat type with their subsurface harboring endemic amphipod species relying on resources that might be partly generated within the subsurface by chemolithoautotrophic bacteria. The present study has demonstrated that taxa capable of chemolithotrophic primary production are present in considerable amount in the subsurface system in Iceland. This provides a basis for further research in this field

where the function of the system should be targeted, and the origin of the primary production traced and quantified. It is important to investigate the smallest components and organisms of an ecosystems as they are responsible for important ecosystem services such as nutrient cycling. Studying the processes behind the diversity patterns is crucial to understanding how diversity is generated and maintained, and it is fundamental to understanding the functioning of ecosystems.

This study highlights the presence of an interesting subsurface ecosystem in Iceland where the habitat of *C. islandicus* is and the results presented here form a good basis for further studies on the association between microbes and the amphipods, not only in the Icelandic subsurface springs but also in other systems. Future studies on this topic could for example focus on the role of microbes in the colonization of metazoans in extreme habitats such as the subterranean realm and if the interaction between the two played a role in making the subsurface an evolutionary refugia.



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# Paper I



## Paper II



## Paper III



## **Paper IV**

