Microbial Biogeography of the Arctic Cryosphere

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Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Microbial Biogeography of the Arctic Cryosphere

Ph.D. thesis submitted 13th of January 2017 to the Ph.D. school of

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To me, it is a lovely sensation to have confirmed that even the tiniest creatures in our land have an important place in the large living community. It is these little creatures that in the middle of March make the ice of the Disko Bay dirty on the underside. After a week or so, the hunters who set their yarns in the Uummannaq Fjord find that the there has grown dirt under the ice and under the icebergs that are frozen into the ice. In the coming days you hear, that the same has been seen by Upernavik and finally all the way up in Thule, and by then everyone will know that the winter is ending and spring is on its way.

H. C. Petersen, The Cunning Raven and other tales of the living in my country

Preface

I have had the great privilege of coming from a background in microbial ecology into a world of bioinformatics. The overwhelming options for analysis and data to obtain for any study initially put some insecurity in me. Through the time spent working on this thesis and the three projects that comprise it I have gained experience with some of these methods and data types and eventually I am learning how to make choices in bioinformatics for microbial ecology.

In part I as an Introduction I would like to make the argument that in the world of Big Data less can be more. Knowing what you are interested in figuring out, making hypotheses and selecting the right kind and amount of data to assess these hypotheses makes for clearer results. Clear results are of better use to the colleagues in our fields of research, in this case microbial biogeography of the Arctic cryosphere.

The three studies that comprise this thesis presented in part II utilize a range of sequencing technologies and bioinformatical tools. From an explorative study of a remote environment, to a study that assesses a clear hypothesis and lastly to a study which enabled the generation of novel hypotheses.

The first study explores the microbial diversity and community composition of snow on North Pole ice floes (Paper I). In this study, I was responsible for conducting all the bioinformatical analyses, the initial writing of the manuscript and the submission and revision of the manuscript in the *Extremophiles* journal.

The second study investigates the link between glaciers and the ocean through a freshwater network on the West coast of Greenland (Paper II). I had the pleasure of partaking in some of the sampling. I was responsible for all of the bioinformatical analyses as well as the statistical analyses. I wrote the initial manuscript and was responsible for submitting and revising the manuscript through an extensive and fruitful reviewing process in *Frontiers in Microbiology*.

The final study deals with microbial communities on the Greenland ice sheet. In this study I was co-responsible for getting the samples sequenced. Besides primary quality check of binned genomes I did all the bioinformatical analyses, as well as the statistical

analyses and wrote the initial manuscript. I submitted the manuscript, which is now undergoing revision.

In part III as a conclusion on this thesis I would like to tie the methodological review and discussion in part I to the results presented in part II.
Finally, as a perspective it is important for me as a researcher and a Greenlander to give perspectives on how the research field of this thesis has importance not just to us as researchers but also to the inhabitants of the Arctic.

Copenhagen, Denmark, January 2017

Aviaja Lyberth Hauptmann

CONTENTS

Abstract

Microbial biogeography has become a recognized field of research within the science of microbial ecology. Technological advances such as the high throughput sequencing of genetic information with next-generation sequencing (NGS) technologies have made us able to "see" the diversity of microbial communities. This has considerably improved our understanding that even harsh and seemingly barren environments such as the cryosphere, the frozen parts of our planet, is inhabited by diverse life.

This thesis presents three studies in microbial biogeography of the Arctic cryosphere utilizing a range of NGS approaches.

The first study of this thesis explores microbial diversity and community composition in snow on North Pole ice floes. It was the first example of 16S rRNA gene amplicon sequencing of North Pole snow. The results of this study showed that snow in different sites on the North Pole can harbor different microbial communities, but these communities are more similar to each other than they are to the surrounding ice and the ocean. The second study confirmed the hypothesis that freshwater networks connected to the cryosphere are inoculated with cryosphere-specific microbial communities. It showed also, that these communities represented about a quarter of the diversity of the microbial community in the estuary. Lastly, this study illustrates the advantages that amplicon sequencing can have over shotgun metagenomics in certain well-defined studies. The final study included in this thesis utilizes the full potential of shotgun metagenomics, which enabled the binning of microbial genomes from metagenomes. Putative genomes showed signs of adaptation to and origin from contaminated habitats. This lead to the hypothesis that the Greenland ice sheet might be a contaminated habitat to a previously unacknowledged degree.

The overall aim of this thesis is to illustrate the advantages that NGS has given in the field of microbial biogeography with the Arctic cryosphere as an example. The most important point in the following is that in order to utilize these advantages to their full potential, we need to put emphasis on hypothesis-driven research and acknowledge the caveats that come with NGS in microbial ecology. If we can do this, cryosphere microbial biogeography can be of value not only to us as microbial ecology researchers but also to researchers in other fields and finally to the inhabitants of the Arctic.

Sammenfatning

Mikrobiel biogeografi er blevet et anerkendt forskningsfelt indenfor mikrobiel økologi. Teknologiske fremskridt som eksempelvis moderne høj-kapacitets metoder til fremskaffelsen af genetisk information har gjort os i stand til at "se" diversiteten af mikrobielle samfund. Dette har ledt til anerkendelsen af, at selv barske og tilsyneladende golde miljøer såsom de frosne dele af vores planet, *kryosfæren*, er beboet af forskelligartet liv. Denne afhandling præsenterer tre studier indenfor mikrobiel biogeografi i den Arktiske kryosfære. Studierne benytter et spektrum af moderne metoder til fremskaffelse af genetiske informationer. Det første studie udforsker den mikrobielle diversitet og sammensætning i sne fra Nordpolen. Dette studie var det første eksempel på benyttelsen af metoden amplicon-sekventering af genet for 16S rRNA i Nordpol sne. Resultaterne fra studiet viste, at sne fra forskellige steder på Nordpolen har forskellige mikrobielle samfund. Samtidig viste studiet, at disse samfund ligner hinanden mere end de ligner de mikrobielle samfund i de underliggende miljøer af is og hav. Det andet studie i denne afhandling bekræfter hypotesen, at en elv, der er i forbindelse med kryosfæren, får tilført mikrobielle samfund, som kommer fra de frosne omgivelser. Studiet viste også, at disse mikrobielle samfund fra elven udgør op mod en fjerdedel af det mikrobielle samfund i fjorden udfor elvens udløb. Endelig illustrerede dette studie, at metoden amplicon-sekventering kan have fordele i forhold til metoden shotgun metagenomics i specifikke vel-definerede studier. Det sidste studie i denne afhandling benytter det fulde potentiale, som metoden shotgun metagenomics har. Brugen af denne data-intensive metode gjorde det muligt at isolere mikrobielle genomer fra metagenomer. Dette resulterede i konklusionen, at indlandsisen på Grønland er et forurenet miljø i forhold til det mikrobielle samfund i en hidtil ukendt grad.

Det overordnede formål med denne afhandling er at illustrere fordele ved brugen af moderne metoder til opnåelsen af genetiske informationer indenfor forskningsfeltet mikrobiel biogeografi med Arktisk mikrobiologi som eksempel. Den vigtigste pointe i det følgende er, at for at kunne udnytte disse fordele må vi lægge fokus på hypotese-drevet forskning og anerkende de forbehold man må have, når man benytter disse metoder til mikrobiel økologi. Hvis vi er i stand til det, så vil mikrobiel biogeografi kunne have værdi ikke bare for os som forskere men også for befolkningen i Arktis.

Eqikkaaneq

Bakterissat sumiittarneri massakkut ilisimatusuunut soqutiginarsinikuuvoq. Teknologiit nutaat atorlugit sorlu annertusuumik aamma sukkassumik DNA-minngaaniik paasissutissat angusinnaangornikuullugit takusinnaangulerparput qanoq bakterissat assigiinngitsigisut. Taamatut paasivarput avatangiisiup qerisortai toqusimarpasittigalutik aamma uumasoqartartut assigiinngitsunik.

Una ilisimatuutut allaatigisaq ilisimasassarsiornerit pingasut saqqummerpai tamaasa nunat issittut avannaarliiniit. Ilisimasassiaq siulleq mississorpaa bakterissat suut nordpoliup aputaaniittut. Taanna ilisimasassiaavoq siullerpaaq teknologii amplicon sekventering atornikuusaa nordpoliup aputaanut. Taassumingaanniik inernerit takutippaat aput assigiinngitsumeertut bakterissat assigiinngitsut. Kisianni aamma takutippaa bakterissat apummiittut assigiinngitsumingaaneersut imminnut assiginnerusut siku-minngaaniik aamma imarminngaaniik. Ilisimasassiaq aappaa takutippaa Qeqertarsuarmi Kuussuup imaaniittut bakterissat sermerminngaaneertut. Imarmi Kuussuup ataani bakterissat sisamararterutaat kuumminngaaneerput. Taanna ilisimasassiaq aamma takutippaa teknologii amplicon sekventering ilaannikkut pitsaanerusinnaasoq teknologiit nutaanerningaanniik. Ilisimasassiaq kingulleq atorpaa teknologiit nutaanerpaat DNA-mingaaniik ilisimasat anguniarneraanni. Teknologiit nutaanerpaat atorlugit bakterissat genomii immikkortinneqarsinnaanngorput. Taamatut paasivarput Kalaallit Nunaanni sermersuaq bakterissaanut mingutsitsisoq. Imaassinnavoq siunissami bakterissat atorlugit paasilluarsinnaangussagipput pinngortitaq sumi mingutsinneqarnikuusoq taavalu taanna nalunngisaq atorlugu Kalaallit Nunanni uumasut inuillu nerisassai mingutsinneqarnissai pinaveersaarsinnaagipput.

Una ilisimatuutut allaatigisami anguniagaq pinngaarnerpaavoq takutillugu qanoq teknologiit nutaat DNA-minngaaniik ilisimasat angunissaanut pitsaasumik atorneqarsinnaasut paasiniaraanni bakterissat suut sumiittartut nunat issittut avannaarliinni. Taavalu aamma pikkunarpoq takutillugu teknologiit nutaat atussagaanni isummiussaagallartut paaseqqaartarlugit ilisimatuutut misissuissasugut. Iluamik suut paaseerusunnerlugit nalunngikkutsigut taava teknologiit nutaat atorluarlugit bakterissat sumiittarneri inunnut tamanut atorneqarsinnangorsinnaavoq.

Acknowledgements

First and foremost I want to thank my initial supervisor Nikolaj Blom for having the courage to hire me for this Ph.D. project. Your courage creates opportunities all around you and for me it created a unique chance to change my setting from my training in microbial ecology to a world of bioinformatics. Because of that I was able to learn more in these past years than I could have ever hoped for. Second to Nikolaj but primary as a role of a supervisor I want to thank Jacob Bælum. Thank you Jacob for your time, your patience, your great advice and for knowing to teach me things in all fields of science whether that be how to log in to the CBS servers or how to review a paper and write a grant application. I could not have asked for a better supervisor. When my dream-team of supervisors decided to leave the Technical University of Denmark I was fortunate that professor Thomas Sicheritz-Pontén was willing to take on my project. Thomas, you were able to fill in for Nikolaj and Jacob and enable me to continue my project as efficiently as before. I am very grateful for the effort you have put into the project despite the circumstances and I have been very pleased with our synergy as a supervisor-student team.

I want to thank all current and previous members of the Metagenomics group, particularly TNP, Simon, Josef, Damian, Bjørn, Henrik, Ida, Asli, Agnieszka, Bent and Kosai. You have all been amazing colleagues and given me help many many many times. You are all greatly appreciated.

Also thank you to all of DTU Bioinformatics and especially Peter Wad Sackett, who taught me the one skill that I appreciate the highest among the ones acquired at DTU, namely Perl. Thank you also to Dorthe Kjærsgaard, the Computerome team, Marlene Dalgaard and to John Damm Sørensen, for his ability to be so skilled and at the same time able to kindly and thoughtfully help someone who was far from that level of skill and who probably asked too many stupid questions.

While wonderful colleagues, lots of help and great lessons were gained from DTU Bioinformatics my project would not have existed was it not for the Center for Permafrost (CENPERM) at the University of Copenhagen and particularly Professor Carsten Suhr Jacobsen and Marek Stibal. Carsten, I cannot thank you enough for all the opportunities that you have given me, which eventually resulted in this thesis. I am very

grateful for all the things that you have included me in and I will do my best to live up to the responsibilities that come with these amazing opportunities. Marek, thank you for all the time you have put into our collaborative work, for your honest advice, for your friendship and for everything that you have taught me about microbial ecology and about writing a scientific paper. I have been very lucky to have the opportunity to learn from you. A great thanks to Bo Elberling for allowing me to become an integrated part of the fantastic group at CENPERM. Thank you Karen Bjerre for making me feel at home at CENPERM and to everyone else at CENPERM, you truly are fantastic colleagues and I have valued my space at CENPERM very highly.

The most exciting time of the project was enabled by Professor Janet Jansson. Thank you Janet for welcoming me first to the Lawrence Berkeley National Laboratory and then to the Pacific Northwest National Laboratory and for giving me the opportunity to work with your group and the exciting data that you create. I have gained invaluable experience from working with permafrost data and I have enjoyed every minute of my time with you at PNNL. Thank you also to Lee Ann McCue and Neslihan Tas Baas for supervising me during my external stay in the USA. Thank you Monica Moffett for your helpfulness, kindness, your friendship and for showing me some fantastic American culture. In this context I also want to thank the funding, which enabled this thesis and the travels. Thanks in particular to the Novo Nordisk Center for Biosustainability. Thanks also to Otto Mønsted Fonden, to the Oticon Foundation, to Dudo and Povl Brandt's Foundation, to the Niels Bohr Foundation, to Jordbrugsakademikerne, to Lektor Bent Lantows Mindelegat and to Jens Peter Jensens Mindelegat.

Finally, and very importantly, thank you Nils for being a fantastic partner and the most amazing father any mother could wish for her son. You have made it possible for me to dedicate myself to this thesis.

Research included in this thesis

Paper I: Bacterial diversity in snow on North Pole ice floes

Hauptmann, Aviaja L.; Stibal, Marek; Bælum, Jacob; Sicheritz-Pontén, Thomas; Brunak, Søren; Bowman, Jeff S.; Hansen, Lars H.; Jacobsen, Carsten S.; and Blom, Nikolaj (2014). Bacterial diversity in snow on North Pole ice floes.
Extremophiles 18(6): 945-951 doi: 10.1007/s00792-014-0660-y

Paper II: Upstream Freshwater and Terrestrial Sources Are Differentially Reflected in the Bacterial Community Structure along a Small Arctic River and Its Estuary

Hauptmann, Aviaja L.; Markussen, Thor N.; Stibal, Marek; Olsen, Nikoline S.; Elberling, Bo; Bælum, Jacob; Sicheritz-Pontén, Thomas; and Jacobsen, Carsten S. (2016) Upstream Freshwater and Terrestrial Sources Are Differentially Reflected in the Bacterial Community Structure along a Small Arctic River and Its Estuary.
Front. Microbiol. 7:1474 doi: 10.3389/fmicb.2016.01474

Paper III: Contamination of the Arctic reflected in microbial metagenomes from the Greenland ice sheet

Hauptmann, Aviaja L.; Sicheritz-Pontén, Thomas; Cameron, Karen A.; Bælum, Jacob; Plichta, Damian R.; Dalgaard, Marlene; and Stibal, Marek (2017). Contamination of the Arctic reflected in microbial metagenomes from the Greenland ice sheet.
Submitted

List of Abbreviations

16S rRNA	16S ribosomal RNA. S is the unit Svedberg for sedimentation rate describing particle size
ATP	Adenosine tri phosphate
dNTP	Dideoxy nucleoside tri phosphate
EPS	Extracellular polymeric substance
NGS	Next-generation sequencing
OTU	Operational taxonomic unit
PE	Paired-end
PCR	Polymerase chain reaction
WRT	Water residence time

List of Terms

Ablation	Snow or ice removal from a glacier or iceberg due to melting or evaporation
Allochthonous	Originating from outside of the present position
Amplicon	A nucleotide string resulting from amplification of a specific nucleotide target
Annotation	The assignment of a gene name and/or function to a nucleotide strand
Autochthonous	Indigenous to the present position
Binning	Clustering, in this case of genomic elements from the same genome
Bioinformatics	Computational methodology for handling and analyzing biological information, in this thesis DNA information
Contig	Short for contiguous, a set of overlapping DNA segments that make up a consensus DNA region
Cosmopolitan	World citizen, Globally distributed

Cryoconite	Microbe-mineral aggregates on ice surfaces
Cryosphere	The parts of Earth composed of frozen water
Endemic	Restricted to a certain location
Homotherm	With a constant temperature
in situ	On site, studies performed in the natural setting
omics	Suffix of data-intensive research fields such as metagenomics, i.e. data intensive genomics including all organisms of a given environment
Shotgun metagenomics	High-throughput sequencing of genetic information utilizing universal primers with no specific target
Supraglacial	At the surface of a glacier

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Part I

1 Introduction

Polar research in its earliest form was only available to the bravest. The brave, who wanted to make their name in the world through proving themselves capable of surviving some of the harshest environments on the planet. Today polar research covers the broader Arctic including not only the harsh polar environments such as the cryosphere but also the impact of these environments on their inhabitants and their surroundings. It has become a field of research with relevance to the common public and in particular those that inhabit the Arctic.

Microbial ecology plays a central role in modern Arctic research as microbial ecosystems and dynamics are determinants in the exchange of nutrients and gases and therefore comprise a key element of climate change - the centerpiece of today's Arctic research.

Our understanding of microbial ecology has undergone dramatic expansion in the current wave of next-generation sequencing (NGS) of genetic information. This is owed to the development of stronger computational tools as well as efficient and low-cost nucleotide sequencing. The large amounts of nucleotide data available from NGS have enabled the exploration of extreme environments and the development of novel hypotheses on microbial ecology and biogeography.

This thesis encompasses research in the field of microbial ecology and more specifically cryosphere microbial biogeography using bioinformatical methodology.
The following sections comprise a very short introduction to the cryosphere and to microbial biogeography followed by a critical review of the use of bioinformatics and NGS technologies in microbial ecology with biogeography of the cryosphere as an example. Technologies and methods relevant to this thesis will be highlighted in method-boxes throughout part I.

1.1 The Cryosphere

The cryosphere, from Latin *kryos*, ice, and *sphaera*, globe, is the part of our planet consisting of frozen water. As cold and inhospitable as it may seem it is the home of microbes, fungi, birds, mammals and people. The cryosphere covers a remarkable 11% of Earth's surface (Benn and Evans 2010).

The cryosphere was once believed to be only a depository of microbes not able to sustain its own ecosystem (Lutz et al. 2016; Boetius et al. 2015). Through the advancement of molecular techniques the cryosphere has now been fully recognized as a biome (Boetius et al. 2015; Anesio and Laybourn-Parry 2012).

1.2 Microbial Biogeography

Modern microbial biogeography is a synthesis of the tradition-bound scientific discipline of ecology and the newly developed field of bioinformatics, which has become a field of research in its own right.

Biogeographers deal with the distribution of the diversity of organisms over time and space (Ramette and Tiedje 2007; Martiny et al. 2006). While biogeography of macroorganisms has been a well-established discipline for centuries, it was not recognized until recently that microorganisms also show biogeographic patterns (Ramette and Tiedje 2007; Martiny et al. 2006; Staley and Gosink 1999). At a time when microorganisms were characterized by culturing there was a general belief that this domain of life was not as diverse as macroorganisms (Martiny et al. 2006; Staley and Gosink 1999). An early study that utilized nucleotide composition for describing microbial diversity in an environmental sample was performed by Vigdis Torsvik and colleagues published in 1990 (Torsvik, Goksyr, and Daae 1990). Based on thermal denaturation and reassociation of DNA measured with spectrophotometry the results of this study suggested that a gram of soil contain more than 4000 species (Torsvik, Goksyr, and Daae 1990). The study argued that soil is so diverse that the biodiversity can only be approximated with difficulty and that most of the diversity in soil cannot be cultured (Torsvik, Goksyr, and Daae 1990). One particular development in microbiology changed the paradigm of bacterial diversity. Carl Woese and colleagues pioneered the use of

genetic signatures in assessing phylogeny and taxonomy of microorganisms (Woese 1987). This made it possible to compare prokaryotes at a much greater resolution than previously. Since then nucleotide-based methods and particularly the 16S rRNA gene have been widely used in microbial biogeography for estimating diversity (Nemergut et al. 2013; Ramette and Tiedje 2007; Staley and Gosink 1999). Today it is acknowledged that "*microbial diversity is beyond practical calculation*" (Wilson 1999; Fierer and Lennon 2011).

In microbial biogeography there is one seminal hypothesis that dominates. The Baas-Becking hypothesis proposed by Lourens G. M. Baas-Becking in 1934 states that *Everything is everywhere, but the environment selects* (Baas-Becking 1934). This idea still dominates hypotheses in microbial biogeography and has yet to be finally accepted or rejected (Fierer and Lennon 2011; Martiny et al. 2006; Staley and Gosink 1999).
The Baas-Becking hypothesis suggests that microbes are cosmopolitans, that are globally distributed, and local differences can be owed to selection in a specific habitat (Staley and Gosink 1999).

Proving or disproving the Baas-Becking hypothesis has shown to be a difficult task (Ramette and Tiedje 2007; Martiny et al. 2006; Staley and Gosink 1999). One of the major reasons for this is the dynamic and promiscuous character of microbial genomes, which results in genetic changes over relatively short periods of time (Martiny et al. 2006; Staley and Gosink 1999). A second reason is the difficulty in agreeing on a definition of a microbial species (Martiny et al. 2006; Staley and Gosink 1999). Based on 16S rRNA gene sequences, or parts thereof, a number of studies have concluded in support of the Baas-Becking Hypothesis (Lutz et al. 2016; Abell and Bowman 2005; Cho and Tiedje 2000; Oda et al. 2003). Other methods that assess diversity at a higher resolution such as rep-PCR fingerprinting have revealed unique genotypes isolated to specific niches in support of endemic organisms (Cho and Tiedje 2000). Specifically, it has been shown that a set of nine genes is required in order to reach a resolution that indicates endemic clades (Whitaker, Grogan, and Taylor 2003).

Trying to discern whether the Baas-Becking hypothesis holds for all prokaryotes might not even be possible. For one thing, to prove that a microorganism is absent from a habitat is nearly impossible (Martiny et al. 2006; Staley and Gosink 1999). Furthermore, different microbes may very well have different dispersal patterns, such as suggested for spore-forming bacteria, that may more easily survive long-distance transportation (Staley and Gosink 1999).

The cryosphere has been proposed as the ideal habitat for the studying of microbial biogeography (Staley and Gosink 1999). The clear bi-polar distribution of cold habitats on Earth makes a good case for studying microbial distribution (Margesin and Miteva 2011). Despite the potential of cold habitats as model habitats for the studying of microbial biogeography, these habitats have not given any final answers on cosmopolitan versus endemic distribution of microorganisms (Margesin and Miteva 2011).

Box 1: Indicator Species

Dufrene and Legendre developed the Indicator Species concept, which calculates an Indicator Value (Equations 1-3) based on the abundance of a species within a subgroup (cluster) of samples (i.e. replicates) compared to the abundance across all samples (Equations 1-3) (Dufrene and Legendre 1997). High Indicator Values result from species that are present only within a subgroup of samples and in none of the other samples while a species will not be an Indicator if it is found in approximately equal abundance across all samples. This Indicator Species measure was used in Paper II, where Indicator Species were denoted Indicator OTUs, defined as OTUs having a higher abundance at one site compared to other sites with Indicator Values $d \geq 0.3$ at a significance level of $p \leq 0.05$.

$$f_{i,c} = \frac{\sum_{j \epsilon c} p_{i,j}}{n_c} \quad \text{(Equation 1)}$$

where $p_{i,j}$ = pres/abs of species i in sample j ; n_c = number of samples in cluster c

$$a_{i,c} = \frac{(\sum_{j \epsilon c} x_{i,j})/n_c}{\sum_{k=1}^{K}((\sum_{j \epsilon k} x_{i,j}/n_k)} \quad \text{(Equation 2)}$$

where $x_{i,j}$ = abundance of species i in sample j

$$d_{i,c} = f_{i,c} \times a_{i,c} \quad \text{(Equation 3)}$$

Trying to describe a general pattern that holds true for all microbes seems a formidable task at a point where we have acknowledged the incredible diversity of the microbial world and technologies have developed that enable us to study the diversity at a high resolution. Studying the biogeography of a defined group of organisms in a defined ecosystem type and with regard to a defined hypothesis seems a better use of an investigators time rather than trying again to reject or confirm the Baas-Becking hypothesis on a general level (Ramette and Tiedje 2007). Identifying the scale of underlying biogeographic principles and identification of *indicator taxa* has been suggested for future research (Ramette and Tiedje 2007) (Box 1: Indicator Species). Additionally, it has been suggested that future biogeography research should sample systematically to better distinguish between contemporary and historical factors (Martiny et al. 2006). As stated therein:

"*If they do not, the field of microbial biogeography will probably become mired in phenomenological description, instead of tackling the mechanisms that generate the patterns.*" (Martiny et al. 2006)

Box 2: Genomes from Metagenomes

A missing piece in metagenomics has long been the linking of taxonomy and function. Within recent years a variety of tools have been developed for clustering or binning contigs or genes from a metagenomic sample into genomes, which represent organisms from the sample. In this way, also genomes of uncultured organisms may be obtained. Different strategies have been used to detect DNA fragments that belong to the same genome. Some methods use either marker genes or signature oligos that utilize the notion that each organism has a certain signature in its nucleotide composition (Kang et al. 2015; Laczny et al. 2015; Y. Wu et al. 2014; Alneberg et al. 2014; Sunagawa et al. 2013; Dick et al. 2009). Other methods are based on underlying statistics in the distribution of reads sequenced at different depth (Albertsen et al. 2013; Nielsen et al. 2014). The Metagenomic-species (MGS) method used for Paper III is such a tool based on the logic that DNA fragments from a single entity will be sequenced to the same depth (Nielsen et al. 2014). Across many samples, the entities, which are always present to the same depth across all the samples will belong to the same entity, whether this be a genome, a plasmid, a virus or other DNA entities.

1.3 Cryosphere Biogeography through Next-generation Sequencing

Next-generation sequencing (NGS) technologies have been defining in the transition from the paradigm of the uninhabitable cryosphere to the now acknowledged paradigm of the cryosphere as one of Earth's biomes (Boetius et al. 2015; Anesio and Laybourn-Parry 2012). Just as it has been seminal for our acknowledgement of the incredible diversity of the prokaryotic domains of Life and the development of the field of microbial biogeography (Ramette and Tiedje 2007; Staley and Gosink 1999). NGS has made it possible for us to "see" the diversity and functions of life in the microbially dominated cryosphere, which was not visible to us before.

As illustrated by the difficulty in discerning the validity of the long proposed Baas-Becking hypothesis in microbial biogeography, the large amounts of data made available with NGS technologies also poses some challenges. This has lead to ongoing debates about the integrity of old-fashioned research with new methods such as the debate of data-driven versus hypothesis-driven research in microbial ecology (Jansson and Prosser 2013).

There has been a natural development in the type of research deemed to be interesting based upon the novelty of the method in use. In the beginning of genome sequencing the publication of a microbial genome, however descriptive, was a sensation on its own. Today, sequenced genomes are not published as peer reviewed papers (Hauptmann et al. 2013). The same development has been seen for metagenomic sequencing where 16S rRNA gene amplicon sequencing studies of descriptive character had an interest in the early examples of using this technique, but today both in shotgun and amplicon metagenomic studies there is a requirement for studying some question of interest rather than announcing the first use of the method in a certain environment. Lately, the novelty of shotgun metagenomics has been the possibility to link taxonomy and function of uncultured organisms through computational binning of genomes from metagenomes (Box 2: Genomes from Metagenomes).

In the following sections three themes will be reviewed with relation to the use of NGS technologies in microbial biogeography of the cryosphere. First, the discussion of data-driven versus hypothesis-driven research will be quantitatively assessed. Next, the less

data-intensive amplicon metagenomic sequencing and the more data-intensive shotgun metagenomic sequencing technologies will be compared and discussed. Finally, the impact of the usage of NGS technology in microbial ecology on measures of biodiversity, the key measure of microbial biogeography, will be presented and discussed before part II of this thesis.

1.3.1 Is Data-intensive research less Hypothesis-driven?

There are two dominant and opposing opinions on the use of NGS technologies in microbial ecology. One opinion is that NGS and the data intensive *omics* are at an opposite of hypothesis-driven research and leads to "*an overemphasis on descriptive approaches*" and that "*It is easier to describe and compare data than to construct and test hypotheses*." (Jansson and Prosser 2013). Another opinion is that *omics* and hypothesis-driven research go hand in hand and that "*The data will themselves be hypothesis-generating*." (Jansson and Prosser 2013; Schloss 2008).

To quantitatively test these statements the combined research articles within the fields of research in part II of this thesis (n=49) were assessed for selected words that relate to either ecology (hypothesis-focused) or technology (data-focused) (List of words Appendix 1). We might see less use of hypothesis-focused words when we are overwhelmed by technological advances, which then take the place of the theoretically and ecologically important content.

Through text-processing scripts written in Perl language the list of words were counted in each of the papers and within each year (Perl script Appendix 2). Within the two categories the counts of the words were summed for each year and divided by the number of total research articles included for that year (Figure 1). A similar calculation was made including only words related to the term "hypothesis" (Figure 2).

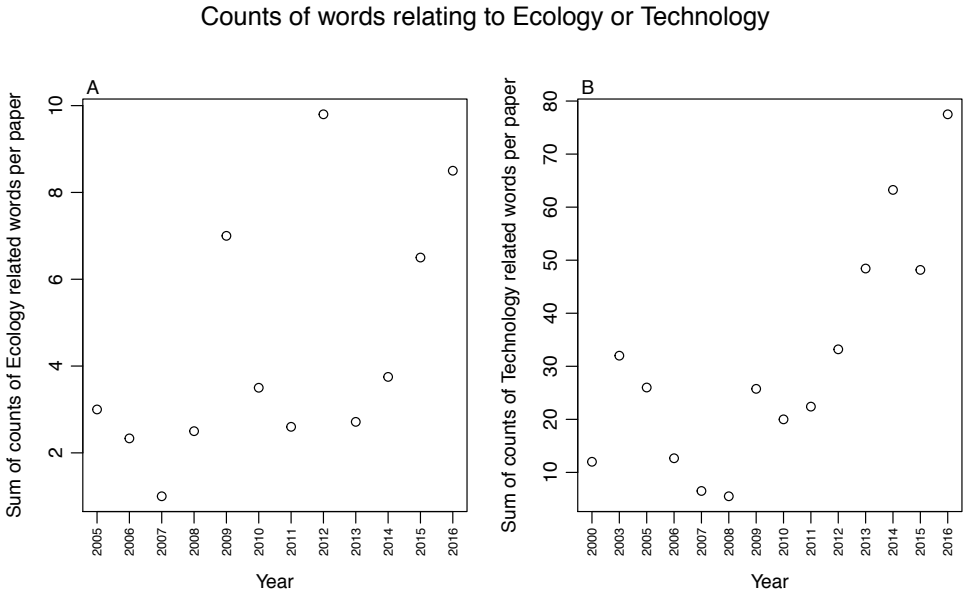

Figure 1: Development in ecology and technology related words in cryosphere biogeography. Within the two categories, the counts of the words were summed for each year and divided by the number of total research articles included for that year. Words are listed in Appendix 1.

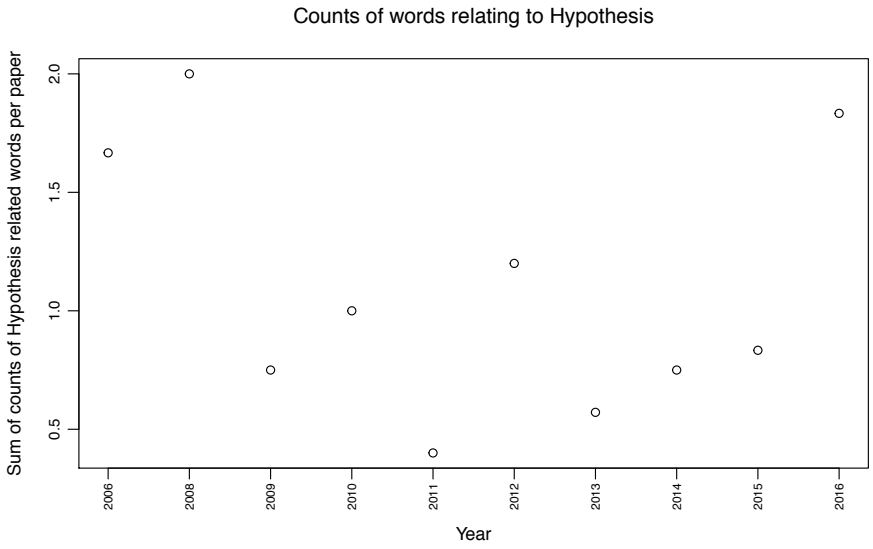

Figure 2: Development in hypothesis-related words in cryosphere biogeography. Counts of words related to "hypothesis" were summed for each year and divided by the number of total research articles included for that year. Words are listed in Appendix 1.

If data-intensive research such as research utilizing amplicon sequencing and shotgun metagenomics are at an opposite of hypothesis-driven research, it could be expected that the use of words related to microbial ecology theory would decline as the use of these data-intensive technologies increase. As expected, the use of words relating to technology such as *amplicon, sequencing, metagenomic,* and *bioinformatics* increase in recent years (Figure 1B). Particularly from 2013 where the first NGS-based studies in cryosphere biogeography were published.
The same tendency is evident when looking at words related to ecology, where there is a particularly high count per paper in 2012 (Figure 1A). For words only relating to "hypothesis" the use seems to be lower after 2008, with a small local optimum at 2012, but is at a high again in 2016 after an increasing tendency from 2013 (Figure 2).

This limited quantitative assessment of data-driven versus hypothesis-driven research does not indicate that there is a decreasing focus on microbial ecology theory in research that utilizes NGS technologies. These results however suggest that data-intensive research results in an increased usage of words related to microbial ecology, and therefore seem to support the notion that data-driven research helps generate hypotheses in microbial ecology.

The gradually increasing pattern in ecology related words after around 2012-2013 (Figure 1A) also indicates that while data-intensive research is not at an opposite to hypothesis-driven research, in the initial phase of newly applied technologies there will be a period of descriptive studies before novel hypotheses can be developed.

The counts of hypothesis-related words shown above might include counts of words that are not stated hypotheses of the research article in question but rather previously established hypotheses referred to in the article. An assessment of the clear statement of a hypothesis was conducted. In 14 out of 27 research articles in cryosphere biogeography using NGS technologies there was no clearly stated hypothesis (Lopatina, Medvedeva, and Shmakov 2016; Ruiz-González, Niño-Garcia, and del Giorgio 2015; Cameron et al. 2015; Lutz et al. 2016; Musilova et al. 2015; Choudhari, Lohia, and Grigoriev 2014; Michaud et al. 2014; Møller et al. 2013; Lopatina, Krylenkov, and Severinov 2013;

Larose et al. 2013; Liu et al. 2011; Edwards et al. 2013; Edwards et al. 2014; Larose, Dommergue, and Vogel 2013), in 11 articles the hypothesis of the paper was clearly stated up-front (Gokul et al. 2016; Franzetti et al. 2016; Cameron et al. 2016; Niño-García, Ruiz-González, and del Giorgio 2016; Peter and Sommaruga 2016; Stibal et al. 2015; Maccario, Vogel, and Larose 2014; Hell et al. 2013; Fortunato et al. 2013; Fortunato et al. 2012; Larouche et al. 2012) while in two articles the hypothesis was vaguely indicated as either an "idea" (Crump, Amaral-Zettler, and Kling 2012) or later in the discussion section of the article (Hisakawa et al. 2015).

It is not new to scientific method that one needs to observe and describe prior to stating a hypothesis (Popper 1935). What might seem problematic in the era of NGS in microbial ecology is that one might generate or alter ones hypothesis *after* designing and conducting ones study, which does not comply with traditional scientific practice. In an enormous amount of data there will most likely be something that pops out as significant that one might then state a hypothesis about. Moreover, it becomes increasingly difficult to falsify research results with increasing amounts of data. The worry about data-driven research might also be, that we are at risk of being able to justify unclear and hard-to-test results that do not necessarily advance our understanding of microbial ecology.

Advancing our understanding of microbial ecology and biogeography at this point in time, does not only require greater amounts of data. Maybe more importantly it requires the right type of sampling, hence the concept of *Think before you sequence* (Jansson and Prosser 2013). A lot of data from the wrong samples does not advance our understanding of cryosphere biogeography.

Indeed, when looking into the literature of microbial biogeography of the cryosphere, conclusions commonly refer to the need for additional sampling over spatial and temporal ranges (Maccario, Vogel, and Larose 2014; Crump, Amaral-Zettler, and Kling 2012; Fortunato et al. 2013; Ruiz-González, Niño-Garcia, and del Giorgio 2015; Niño-García, Ruiz-González, and del Giorgio 2016; Cameron et al. 2016; Edwards et al. 2013) while studies have not been found to include sequencing depth as part of the future needs of cryosphere microbial biogeography.

Box 3: The 454 Pyrosequencing Platform

Pyrosequencing with the 454 Life Sciences GS FLX platform was used for amplicon sequencing of 16S rRNA genes from snow on North Pole ice floes as described in Paper I. Pyrosequencing employs the sequencing-by-synthesis principle, which allows the detection of the incorporated nucleotide during DNA synthesis (Ronaghi, Uhlén, and Nyrén 1998). In short, the 454 platform reads light emission from a PicoTiterPlate device consisting of wells that contain one bead each, which under optimal conditions is coated with one clone of a DNA fragment. The clonal DNA fragments on the beads are synthesized by adding one type of dNTP at a time, which in turn releases pyrophosphate. The pyrophosphate is then converted to ATP by ATP sulfurases and lastly the ATP is used to activate the process of converting luciferin to oxyluciferin under the emission of light. In this way light is emitted only if the added type of dNTP is incorporated, which allows the base calling. If a clone contains more than one of the added nucleotide the incorporation of the nucleotides will result in the emission of light proportional to the number of nucleotides incorporated. This specific feature of the pyrosequencing platform results in a higher error rate in DNA regions of homopolymer sequences as the proportionality gets decreasingly accurate as the length of the homopolymer increases (Ronaghi, Uhlén, and Nyrén 1998). 454 Pyrosequencing can yield reads with length up to 500 bases.

1.3.2 Amplicon Sequencing versus Shotgun Metagenomics

There seems to be a general belief that shotgun metagenomics is an advancement over the first established amplicon sequencing. This can be seen for one thing in the declining use of the 454 pyrosequencing platform, which generates longer reads appropriate for amplicons, replaced by the Illumina platforms (Heather and Chain 2016) (Box 3 and 4: NGS Platforms).

Box 4: The Illumina Platform

Illumina MiSeq 250bp paired-end sequencing was used for amplicon sequencing of 16S rRNA genes from the Red River freshwater network and its estuary as described in Paper II and Illumina HiSeq 100bp paired-end sequencing was used for shotgun sequencing of cryoconite shotgun metagenomes as described in Paper III.

The Illumina platform employs sequencing-by-synthesis but differs from 454 pyrosequencing in that all four nucleotides are added at once and the detection of the nucleotide incorporated is done through the detection of the different colors emitted when the differentially fluorescently labeled nucleotides are excited by a light source (Heather and Chain 2016). In summary, a flow cell is coated with clonal clusters of a DNA fragment as a result of bridge amplification of singular DNA fragments on the flow cell. Fluorescently labeled dNTPs are added and the complementary dNTP to the DNA strand is incorporated. Only one dNTP can be incorporated at each cycle. After each step the clusters are excited with a light source resulting in a fluorescent signal corresponding to the nucleotide incorporated. This procedure is first done with one strand of the DNA fragments but may be complemented by also sequencing the reverse strand afterwards resulting in paired-end (PE) data. The MiSeq platform can yield reads with length up to 300 bases, while HiSeq has a limit of 150 bases.

There are a number of obvious limitations to the most common usage of amplicon sequencing in microbial biogeography, which is the sequencing of the 16S rRNA gene. First and foremost the 16S rRNA gene does not allow the interpretation of the functional capabilities of the microbial community (Nemergut et al. 2013; Staley and Gosink 1999). Furthermore, 16S rRNA genes are often sequenced only in part comprising one to three variable regions and therefore do not allow the detection of taxonomy at species level (Staley and Gosink 1999). Commonly, we also assume that one 16S rRNA gene amplicon equals one organism, while one organism may in fact have several 16S rRNA genes in its genome (Nemergut et al. 2013). This could potentially lead to an over-estimation of diversity, which is also a bias introduced at several other steps of the amplicon sequencing and analysis process (Fierer and Lennon 2011) (More details in section on Biodiversity in the era of Next-Generation Sequencing and Box 5: Alpha Diversity).

What 16S rRNA gene amplicon sequencing is ideal for is the assessment of biodiversity, a principal measure in microbial ecology (Box 5: Alpha Diversity). Diversity measures have the advantage that they can be compared between studies and between habitats.

At this point in time shotgun metagenomics does not give a direct measure of diversity, as we have not yet been able to sequence samples exhaustively (Nemergut et al. 2013; Fierer and Lennon 2011; Staley and Gosink 1999). Any functional gene is not per se suitable for assigning taxonomy both due to the potential for horizontal gene transfer but also due to our lack of knowledge of the functions of specific gene sequences in the environment resulting in very limited base of knowledge to compare to. Another reason is that despite a very high level of sequencing efforts, targeting all genes in a pool of organisms will increase the level of undersampling, so that rare species will be even harder to detect (Nemergut et al. 2013).

As long as undersampling is an unresolved issue, a targeted approach to estimating diversity will most likely give more accurate results. At this point databases of 16S rRNA genes and curation of these are at a higher level than for the general gene databases and

therefore supports a more accurate basis for comparison. While amplicon sequencing has several identified steps in which diversity measures may become inflated, calculating diversity based on shotgun metagenomics certainly will also lead to an overestimation of diversity (Ranjan et al. 2016). In a comparison between 16S rRNA gene amplicon sequencing and shotgun metagenomics in human microbiome studies the conclusion was in favor of the shotgun sequencing of whole genomes (Ranjan et al. 2016). The authors were stuck by the result that 16S rRNA gene amplicon sequencing results in a lower diversity when compared to shotgun metagenomics, which had taxonomy assigned through the MG-RAST pipeline. MG-RAST assigns taxonomy based on the taxonomy of the organism of the gene, which the query sequence matches best (Meyer et al. 2008). While 16S rRNA genes do not allow detection at species level, this gene was selected based on the fact that it was the best candidate for sorting out phylogeny (Woese 1987). Any functional gene from a shotgun metagenome does not live up to the same characteristics as the 16S rRNA gene and cannot predict taxonomy to the same accuracy and will therefore result in a highly inflated diversity of a sample.

Several tools are being developed that estimate diversity and taxonomy based on shotgun metagenomic data (Meyer et al. 2008; Huson et al. 2011; M. Wu and Eisen 2008) (Box 6: MGmapper). These tools aim at not just detecting species but advancing into the detection of organisms at strain level. This is an advancement from the current use of 16S rRNA genes for species assignment.

In time, when shotgun metagenomics allows the exhaustive sequencing of a sample and databases have been updated and curated based on the new knowledge that shotgun metagenomics allows, then this technology will approach the potential for the equal detection of diversity at the highest possible level and the simultaneous detection of functional potential of a microbial community.

Box 5: Alpha Diversity

Alpha diversity is the measure of diversity and species evenness within a microbial community. There are a number of used metrics for alpha diversity in microbial ecology. The ones used in Papers I-II are observed richness, the diversity metric Shannon index and Chao1 richness. Metrics in the included papers were chosen based on the metrics used in comparable studies. Chao1 is a non-parametric estimator of true species richness based on the notion that the larger fraction of species that are found at least twice, the closer the sample is at fully representing true diversity (Chao 1984) (Equation 4). In Paper I and II Chao1 richness was calculated using the Qiime pipeline (Caporaso et al. 2010). It is worth noting that this metric depends on the fraction of singletons in a sample. The global singletons, sequences that are only found once across all samples, are conventionally removed prior to sequence analysis, which might impact the Chao1 index. Accordingly, the threshold for minimum number of sequences for an OTU in Paper I and II have been set to 2, in hopes of minimizing the influence on the calculated Chao1 richness.

$$chao1 = S_{obs} + \frac{F_1^2}{2F_2} \qquad \text{(Equation 4)}$$

Where F_1 and F_2 are the count of singletons and doubletons respectively

The Shannon Index was conceived in 1948 originally proposed for text analysis in communication (Shannon 1948). This measure quantifies the uncertainty of predicting an entity based on the proportion of entities belonging to different groups in a sample, in microbial ecology the proportion of reads belonging to a certain OTU. In Paper I and II the Shannon Index was calculated using the Qiime pipeline (Caporaso et al. 2010), which calculates the Shannon Index with default logarithm base 2 (Equation 5).

$$H = -\sum_{i=1}^{s}(p_i log 2 p_i) \qquad \text{(Equation 5)}$$

Where s is the number of OTUs and p_i is the proportion of the community represented by OTU i.

There seems to be a consensus in cryosphere biogeography that there is no need to argue for the choice of methods when it comes to sequencing. The reasoning behind this might be the existence of the notion that newer methods are inherently better and choosing sequencing is based only on what the investigators can afford. Since sequencing is the foundation for these studies the different methods and their applicability should be rigorously tested and debated in the scientific community (Mizrahi-Man, Davenport, and Gilad 2013). Before spending additional resources by choosing shotgun metagenomics over amplicon sequencing, we need to ask; does more data give clearer results or is there a tendency to sequence the whole haystack, when one could have targeted the needle?

For microbial ecology the knowledge base is low compared to e.g. human microbiome-studies and the diversity potentially higher, therefore one needs to consider carefully what kind of data answers ecological research questions best. Cryosphere biogeography is one end of an extreme with the other end being clinical biogeography, for which we have a much deeper understanding and detailed knowledgebase. We are able to track exactly how human pathogens are distributed because we know what we are looking for from decades of detailed studies of isolated cultures that are now often fully sequenced and annotated. In cryosphere biogeography we are at a much earlier stage and the great amounts of data provided by shotgun metagenomics might not always be the optimal choice of advancing our understanding.

We know that it is nearly impossible to disprove the major microbial biogeography hypotheses, such as *Everything is everywhere* (Martiny et al. 2006). This is still true today despite the much larger amount of data available. One reason is that we do not yet sequence exhaustively, another is that we are still not able to accurately assign organisms to their precise taxonomy, which would require complete whole genome sequences. Developments in sequencing technologies and computational tools such as binning (Box 2: Genomes from Metagenomes) are bringing us closer to be able to do this. In the future, the extraction of the entire set of complete genomes from metagenomes holds promise of finally testing the Baas-Becking hypothesis.

For the massive amounts of data generated by NGS technologies to make sense we need to have solidly curated databases for comparison. Therefore, advancements in

NGS go hand in hand with developments in culturing and *in situ* detection through e.g. FISH (Nemergut et al. 2013; Staley and Gosink 1999).

Until shotgun metagenomics allows us to fully sequence a sample we need to consider our hypothesis before choosing a sequencing strategy. This is particularly important in cryosphere biogeography, where our base for comparison is limited and more data does not necessarily equal more knowledge. 16S rRNA amplicon sequencing has merit in usage for answering research question that involves measures of diversity. 16S rRNA gene amplicons are also useful for tracing dispersal in the environment, where the DNA sequence serves as a tracer of microbial communities, rather than a detection of a specific strain (Hauptmann et al. 2016).

1.3.3 What Next-generation Sequencing Taught Us about Microbial Biogeography of the Cryosphere

Potentially the most important outcome of the application of NGS technologies in microbial biogeography of the cryosphere is the acknowledgement of the cryosphere as a biome (Boetius et al. 2015; Anesio and Laybourn-Parry 2012). The cryosphere is a microbially dominated habitat and thus the life in this habitat was not fully visible to us prior to the advancement of molecular techniques for the assessment of the diversity and functions of microbial life. Through NGS technologies the cryosphere has been shown to harbor a distinct and diverse microbial community.

In this final section of part I the caveats of utilizing NGS technologies for estimating diversity and community composition will be discussed and examples from cryosphere microbial biogeography will be presented.

1.3.3.1 Biodiversity in the era of Next-generation Sequencing

Snow was once believed to harbor only an allochthonous microbial community, and was not considered a habitat sustaining its own adapted community (Boetius et al. 2015). The first studies of snow using 16S rRNA gene amplicon sequencing emerged in 2013 (Møller et al. 2013; Hell et al. 2013). With these studies the diversity of snow increased from an estimated species richness of below 100 (Lopatina, Krylenkov, and Severinov 2013; Chuvochina et al. 2011; Harding et al. 2011) to several hundred (Hell et al. 2013)

and even up to almost 10.000 OTUs per sample (Møller et al. 2013). With this notable increase in estimated diversity of a seeming uninhabitable environment, snow no longer seemed to be only a depository of inactive microbes. Furthermore, the results indicated that the samples had not yet been sequenced exhaustively (Møller et al. 2013).

The measurement of diversity using NGS technologies and bioinformatical tools has been reviewed thoroughly and there are several important caveats to consider when measuring diversity using NGS data (Ranjan et al. 2016; Sinclair et al. 2015; Mizrahi-Man, Davenport, and Gilad 2013). For the two first NGS studies of snow both of them employed the 454 pyrosequencing platform (Box 3: The 454 Pyrosequencing Platform). One study targeted 16S rRNA gene variable regions V1-V3 (Hell et al. 2013) the other targeted variable regions V3 and V4 (Møller et al. 2013). Due to the limited length of amplicons available with commonly used sequencing platforms (Box 3 and 4: NGS Platforms) it is conventional to sequence only selected variable regions of the ca. 1500bp length of the 16S rRNA gene. This ultimately means that studies are often not comparing the same signature of diversity. Also, within a study sequences are conventionally clustered into operational taxonomic units (OTUs), roughly equivalent to species, at 97% identity (Nguyen et al. 2016). This threshold was based on results from the whole length of the 16S rRNA gene (Konstantinidis and Tiedje 2005) and is in fact not appropriate when using only selected variable regions (Nguyen et al. 2016). A recent study showed that paired-end sequencing of variable regions 3 or 4 gives the most effective study design but the study also emphasized the lack of consensus in the literature reviewing sequencing and data analysis strategies for 16S rRNA gene amplicons (Mizrahi-Man, Davenport, and Gilad 2013).

454 pyrosequencing has been used extensively for amplicon sequencing of the 16S rRNA gene due to its ability to produce longer reads. However, its higher error rate in regions of homopolymers results in an artificially high number of operational taxonomic units (OTUs), which then inflates the resulting diversity (Box 3: The 454 Pyrosequencing Platform). Therefore, it has become mandatory to denoise pyrosequencing amplicon data to reduce the noise from artificial OTUs (Reeder and Knight 2011). Today Illumina sequencing, in particular MiSeq, is widely used for amplicon sequencing (Sinclair et al. 2015) (Box 4: The Illumina Platform). Illumina

MiSeq yields shorter reads than 454 pyrosequencing but has the advantage of allowing paired-end sequencing, which has shown to give a better balance between precision and coverage in the results (Mizrahi-Man, Davenport, and Gilad 2013).

Before amplicon data analysis the data is quality checked. In addition to denoising of the data, removal of chimeric sequences is also important to avoid inflation of the detected diversity (Edgar et al. 2011). Chimeras are artifacts of PCR amplification, which occur when short fragments from incomplete amplification anneal to target sequences during subsequent PCR cycles and thereby act as primers. This results in chimeric sequences between the incomplete fragment and the target fragment to which it annealed. Commonly used software for removing chimeras is the UCHIME algorithm from the USEARCH package (Edgar et al. 2011).

A bioinformatical pipeline for 16S rRNA gene processing is employed after quality checking of sequencing data, which allows the classification and clustering of operational taxonomic units (OTUs). The difference in clustering methods should be taken into close consideration when comparing results from one study to another. Using default settings of a commonly used bioinformatics pipeline for amplicon data Qiime (Caporaso et al. 2010) has shown to inflate the resultant diversity measures over ten fold (Sinclair et al. 2015). Reviews however also show that general trends in alpha and beta diversity were conserved and corresponded well regardless of the clustering algorithm (Sinclair et al. 2015). Encouragingly, comparisons within a single study where all samples are processed alike can be expected to be comparable and general trends among different studies can also be compared when the known discrepancies are kept in mind and absolute richness estimates are not compared directly (Sinclair et al. 2015).

The first study of microbe-mineral aggregates on ice, termed cryoconite, using shotgun metagenomics was published prior to the use of 16S rRNA gene amplicon sequencing in this habitat (Edwards et al. 2013). This study did not give any measures of diversity but estimated taxonomy through the commonly used tool MG-RAST (Meyer et al. 2008).

A number of bioinformatics software packages are available for estimating taxonomic composition of shotgun metagenomic samples (Meyer et al. 2008; Huson et al. 2011; M. Wu and Eisen 2008) (Box 6: MGmapper). A caveat when estimating diversity

and taxonomy from shotgun metagenomic samples is the *genome length bias*, which results in more reads from longer genomes (Kerepesi and Grolmusz 2016). This bias makes it problematic to make quantitative comparisons among different taxa within samples. Some tools estimate taxonomic fractions based on single copy marker genes and are able to circumvent the genome length bias (M. Wu and Eisen 2008). Other tools, including MG-RAST, have been shown to wrongfully estimate quantities of taxa (Kerepesi and Grolmusz 2016).

In addition to bioinformatical tools there are a number of other potential biases when employing NGS technologies to microbial communities such as sample collection, storage, DNA/RNA extraction, Reverse Transcriptase, PCR, sequencing and statistical analyses (Sinha et al. 2015; Sinclair et al. 2015). The magnitude of these biases and the reproducibility of NGS-based studies have not yet been thoroughly tested (Sinha et al. 2015). At the frontier of NGS-based studies are human microbiome projects, where initiatives such as The microbiome quality control project have just started assessing the potential biases and reproducibility of NGS-based studies of microbial communities (Sinha et al. 2015).

As we rightfully marvel at the technological advances that will lead to great scientific discoveries such as the recent in-depth sequencing of 10,000 human genomes (Telenti et al. 2016) we need to take special care in rigorously and critically reviewing our methodologies in the light of our specific field of research.
In well-defined fields of research such as cryosphere microbial biogeography we need to let the technologies be aids in developing our understanding of our field rather than the focus of our research. In the end of the day we need to put emphasis on hypothesis-driven research in microbial ecology, use our hypothesis for choosing our methods, as this is the prerequisite for obtaining truthful and useful knowledge about microbial ecology through bioinformatics.

Box 6: MGmapper

MGmapper is a reference-based tool for assigning taxonomy to raw shotgun metagenomic sequences (unpublished data, personal communication). Reliable annotations down to strain level are enabled through post-processing analysis. MGmapper maps NGS sequencing reads to designated databases for e.g. Bacteria, Vira, Fungi, Human gut microbiomes, Vertebrates, Plants etc. Per default MGmapper evaluates taxonomy assignments to be true if they are properly paired reads with alignment score >30 and minimum coverage 80%. Additionally, a minimum of 10 reads must map to a strain for it to be evaluated as true. These settings were used for taxonomy assignment of shotgun metagenomic samples in Paper III.

Part II

2 Cryosphere Microbial Biogeography

The current thesis comprises three studies of microbial biogeography in three different habitats of the cryosphere. The three studies are examples of different next-generation sequencing approaches. Three chapters in this part will be opened with a short introduction to each of the habitats of the cryosphere followed by an overview of the results and utilization of NGS methodology in this habitat. Each chapter will be concluded with the paper concerning the specific cryosphere habitat.

Chapter 2.1 is an exploratory study of snow on North Pole ice floes using 16S rRNA gene amplicon sequencing (Paper I). This chapter illustrates the first use of NGS methodology in one extreme and remote habitat.

Chapter 2.2 addresses the potential link between the cryosphere, specifically a glacier on the Disko Island of Greenland, and the ocean through a freshwater network also utilizing 16S rRNA gene amplicon sequencing (Paper II). This chapter illustrates how the cryosphere interacts with the downstream ecosystems.

Finally, chapter 2.3 describes the use of shotgun metagenomics in assessing the microbial communities around the Greenland ice sheet. In this chapter it is discussed how the cryosphere potentially takes part in global interactions, receiving input from anthropogenic activities and potentially influences ecosystems and food chains of the Arctic (Paper III).

The three chapters together illustrate a range of the possibilities and impact that NGS technologies enables in cryosphere microbial biogeography. From a purely explorative study, through a study illustrating the interactions of the cryosphere at a local scale and lastly to the interaction of the cryosphere at a global scale.

2.1 Snow

Snow is the largest frozen habitat on Earth covering up to 35% of the land surface area (Margesin and Miteva 2011). There has been a long interest in microbes on snow due to the visibility of these microbes resulting in phenomena such as red snow (Hardy and Curl 1968). The current interest in these microbes lies in their potential for lowering albedo of snow surfaces resulting in an accelerated melting of snow (Lutz et al. 2014; Lutz et al. 2016).

Despite the long history of interest in snow microbes our understanding of the dynamics of snow microbial communities is still limited. It has been hypothesized that snow is inoculated from proximate terrestrial habitats, as snow microbial communities showed closest resemblance to soil microbial communities and surprisingly less similarity to communities from close-by cryosphere habitats (Cameron et al. 2015). A similar result was obtained also in a study of the different environments on the Greenland ice sheet (Musilova et al. 2015). In here, snow and dust microbial communities showed resemblance but did not seem to establish themselves in the more long term communities of cryoconite holes (Musilova et al. 2015). In a recent study of red pigmented snow algae, results showed no apparent distinct communities at different sites in favor of a cosmopolitan distribution on red snow algae (Lutz et al. 2016).

While snow microbial communities have shown to be important from a global climate change perspective (Lutz et al. 2014), our understanding of the microbial biogeography and adaptation of these communities to the cryosphere is still in an early phase.

PAPER I: Bacterial diversity in snow on North Pole ice floes

Paper I was one of the first applications of NGS technologies in snow. The relevance of the paper was in its example of an extremely isolated location, the North Pole, with minimal influence from terrestrial and anthropogenic sources.
The study was a purely explorative study, which was linked to previous results on ice from the same locations (Bowman et al. 2012).

Results from pyrosequencing of the 16S rRNA gene and qPCR showed a less diverse and less abundant community than snow communities on land. This study acts as a negative control to support the idea that a fraction of land-based snow communities from other NGS studies originate from terrestrial environments.

The microbial community sampled from the three different sites on the North Pole had one major difference between them in that one sample site (C) had notably few Cyanobacteria when compared to the two other sites. This Cyanobacteria discrepancy was also observed in Paper III, where it is discussed further.
Despite the notable difference among the snow samples the samples were still similar enough that they clustered clearly when compared to the nearby ice and ocean environment as shown by principal component analysis. This aligns with more recent results that have shown a different community in snow when compared to ice (Cameron et al. 2015; Musilova et al. 2015).

Finally, the results from Paper I support the idea that snow microbial communities are globally distributed at the resolution enabled by 16S rRNA amplicon sequencing. It is still unknown to which degree the snow environment selects and if an autochthonous community inhabits snow or if it is simply too transient to support its own distinct microbial community. As described in chapter 2, the residence time in a habitat can be determining for the microbial community composition and the degree to which it is adapted to its environment. Whether this is true for snow remains to be answered.

Extremophiles (2014) 18:945–951
DOI 10.1007/s00792-014-0660-y

ORIGINAL PAPER

Bacterial diversity in snow on North Pole ice floes

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Received: 6 March 2014 / Accepted: 18 May 2014 / Published online: 21 June 2014

Abstract The microbial abundance and diversity in snow on ice floes at three sites near the North Pole was assessed using quantitative PCR and 454 pyrosequencing. Abundance of 16S rRNA genes in the samples ranged between 43 and 248 gene copies per millilitre of melted snow. A total of 291,331 sequences were obtained through 454 pyrosequencing of 16S rRNA genes, resulting in 984 OTUs at 97 % identity. Two sites were dominated by *Cyanobacteria* (72 and 61 %, respectively), including chloroplasts. The third site differed by consisting of 95 % *Proteobacteria*. Principal component analysis showed that the three sites clustered together when compared to the underlying environments of sea ice and ocean water. The Shannon indices ranged from 2.226 to 3.758, and the Chao1 indices showed species richness between 293 and 353 for the three samples. The relatively low abundances and diversity found in the samples indicate a lower rate of microbial input to this snow habitat compared to snow in the proximity of terrestrial and anthropogenic sources of microorganisms. The differences in species composition and diversity between the sites show that apparently similar snow habitats contain a large variation in biodiversity, although the differences were smaller than the differences to the underlying environment. The results support the idea that a globally distributed community exists in snow and that the global snow community can in part be attributed to microbial input from the atmosphere.

Keywords Polar microbiology · Arctic · Bacterial diversity · Pyrosequencing · Snow

Introduction

The extent of sea ice in the Arctic is diminishing due to climate change (Perovich and Richter-Menge 2009), and

Communicated by A. Oren.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-014-0660-y) contains supplementary material, which is available to authorized users.

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it is expected that an ice-free Arctic Ocean will become a reality in coming summers (Wang and Overland 2009). Snow on North Pole ice floes is an example of a virtually unknown microbe-dominated ecosystem undergoing profound change. While the snow microbial community in the high Arctic, Antarctica and Asia has been receiving increasing attention with regard to community structure and biogeographical dispersal of microorganisms (Amato et al. 2007; Liu et al. 2009; Bowers et al. 2009; Larose et al. 2010; Harding et al. 2011; Møller et al. 2013; Hell et al. 2013; Lopatina et al. 2013), there is little knowledge of the diversity of microbes in snow with no direct influence by terrestrial and/or anthropogenic sources. The abundance of bacteria in snow has been found to range from 0.02×10^3 (Harding et al. 2011) to 720×10^3 (Liu et al. 2009) cells per millilitre of melted snow. Recently, it has been shown that microbial abundance in snow may be influenced by anthropogenic sources/activity resulting in higher abundance related to higher input of microorganisms closer to human activity (Lopatina et al. 2013). The North Pole serves as an example of a remote microbial habitat with little impact from human activity.

Whether the bacterial community in snow is globally distributed or if local sources of microorganisms determine the bacterial composition remains unclear (Harding et al. 2011; Larose et al. 2013). Studies from remote environments may help clarify whether snow is merely a reservoir of local sources of organisms or if it hosts a community specific to snow as a habitat, and they may also serve as a substantial contribution to the discussion of the range of dispersal of microorganisms.

Bacteria found in snow may represent recent deposition events, as suggested by studies showing great spatial and temporal variability in the bacterial community composition (Hell et al. 2013; Lopatina et al. 2013) and relation to local environments (Liu et al. 2009; Harding et al. 2011). There are indications, however, that the microbial community in snow is dominated by certain bacteria, including *Betaproteobacteria* that seem to be dominant in most snow habitats followed by other *Proteobacteria, Cyanobacteria, Actinobacteria* and *Bacteroidetes* (Larose et al. 2010; Møller et al. 2013; Hell et al. 2013; Lopatina et al. 2013).

In this paper, we examine the abundance and community composition of microorganisms in snow samples collected in the vicinity of the geographical North Pole during the LOMROG II expedition in 2009, using quantitative PCR and 454 pyrosequencing. We aim to contribute to further insight into the biodiversity of this extreme and remote environment and the possible dispersal mechanisms of microorganisms to snow.

Materials and methods

Field sites and sample collection

Samples of snow were collected during the LOMROG II expedition at three different sites in the vicinity of the North Pole (Supplementary Figure S1). Sample A was collected at 85°15′N, 18°18′E on 5 August 2009, sample B was collected on 11 August at 88°55′N, 99°45′E, and sample C on 30 August 2009 at 87°27′N, 16°13′E. All the sites were accessed by helicopter. 120 l of snow was collected from the upper layer of loose snow (max. 20 cm) using a flame-sterilized snow shovel at each site. The snow was placed in 10-l plastic buckets, pre-cleaned with sterile deionized water, and later transferred to sterile 30-l polypropylene bags. The samples were thawed for 36 h in a heated lab on board the ship and, prefiltered through 2.0-µm polypropylene filters and filtered through Steripak GP 0.22-µm polyethersulfone filters (Millipore, Billerica, MA, USA). 30-ml sucrose buffer (0.75 M sucrose) was added to the filters to lyse the DNA. The filters were then capped at both ends with sterile caps, frozen to −20 °C and transported frozen to Copenhagen.

DNA extraction

Prior to extraction, the Steripak filters were left to thaw in a laminar flow cabinet at room temperature, and the thawed sucrose buffer was refiltered through the filters into sterile 15-ml polypropylene tubes. DNA was then extracted from the filters using the manufacturer's extraction protocol for the PowerWater Sterivex DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), modified for the larger volume of Steripak filters. The volumes of solutions ST1B and ST2 added to the Steripak filters were increased ninefold to 13.5 ml, the resulting lysate ('new lysate') was evacuated from the filters using sterile 50-ml syringes and split evenly into nine glass bead tubes from the PowerWater DNA isolation kit. DNA contained in the original sucrose buffer filtrate was precipitated using the following protocol. The filtrate was mixed with 2-M NaCl and cold ethanol (1:0.1:2 v/v/v filtrate:NaCl:ethanol) in a 50-ml polypropylene tube and incubated at −20 °C for 1 h. The tubes were then centrifuged at 15,000*g* for 15 min, the supernatant was decanted and 1 ml of 70 % ethanol was added. The centrifugation step was then repeated, supernatant decanted, and the pellet was air-dried for 10 min, resuspended in 0.5 ml of DNA-free water, and then added to the glass bead tubes containing the new lysate. In the last elution step of the extraction protocol DNA from 3 tubes was pooled into one, thus resulting in 3 tubes containing 100 µl of DNA extract for each sample. These three subsamples were treated as replicates. This method gave

the highest DNA yield compared to a method using the Sterivex protocol only without adding the precipitated DNA from the filtrate and with a method based on proteinase K lysis, as tested on filtered tap water samples (data not shown).

Quantitative PCR

The abundance of 16S rRNA genes in the extracts was determined using quantitative PCR (qPCR) performed with the PRBA338f and P518r primers (Muyzer et al. 1993) and standards with known quantities of the gene extracted from 1.8 ml of a culture of *E. coli* using an UltraClean microbial DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. The qPCR was set up under DNA-free conditions in a clean, UV sterilized (3 h on a daily basis) lab with an HEPA-filtered air inlet. The setup was as follows: 20-µl reactions containing 10 µl of SYBR Premix Ex Taq II (TaKaRa, Kyoto, Japan), 0.8 µl of the primers (final concentration 0.4 µM) and 1 µl of template DNA. The reaction was then carried out on a CFX96 Touch qPCR system (Bio-Rad, Hercules, CA, USA). The cycle program was 95 °C for 1 min followed by 50 cycles of 95 °C for 30 s, 30 s at 60 °C and 72 °C for 30 s. The reaction was completed by a final 72 °C elongation step for 6 min. All qPCR reactions were performed in triplicate, and confirmatory melting curve analysis was performed on all qPCR products. Negative controls containing no template were used to assess the contamination potential.

Pyrosequencing

The diversity of 16S rRNA genes in the snow samples was determined by pyrosequencing. Amplicons of 466-bp flanking the V3 and V4 regions of the 16S rRNA gene were amplified using the primers 341F (5′-CCTAYGGGRBG CASCAG-3′) and 806R (5′-GGACTACNNGGGTATC TAAT-3′) followed by a second PCR where primers with adapters and 10-bp tags were used (Hansen et al. 2012). PCR amplification was performed using 1 × AccuPrime buffer II which contained 0.2 mM dNTP's, 0.75 U AccuPrime Taq DNA Polymerase High fidelity (Invitrogen, Carlsbad, CA, USA), 0.5 µM of each of the primers, and 1 µl of DNA extract to a total of 25 µl per reaction. PCR was performed with a DNA Engine Dyad Peltier Thermal Cycler (MJ Research, Massachusetts, USA). The cycle conditions were 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 20 s, annealing at 56 °C for 30 s and elongation at 68 °C for 40 s, and a final elongation step at 72 °C for 5 min. The conditions of the second PCR were as the first PCR, except that the number of cycles was reduced to 15 cycles. The PCR products were run on a gel and the appropriate fragments were cut and purified using the Montage DNA Gel Extraction kit (Millipore, Bedford, Massachusetts, USA). The amplified fragments with adapters and tags were quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) and mixed in approximately equal concentrations (1×10^7 copies μl^{-1}) to ensure equal representation of each sample. Samples were run on a GS FLX Titanium Pico TiterPlate using a GS FLX Titanium Sequencing Kit XLR70 according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA).

Bioinformatics

The 454 pyrosequencing data were analyzed using the software package QIIME version 1.7.0 (Caporaso et al. 2010). The data were denoised to remove characteristic sequencing errors for 454 pyrosequencing, specifically faulty additional operational taxonomic units (OTUs) caused by long homopolymers (Reeder and Knight 2010). Chimeras were removed with the USEARCH toolbox version 7.0.1001 UCHIME reference-based chimera detection using the Greengenes database gg_13_5 release downloaded on 13 February 2014 as reference (DeSantis et al. 2006; Edgar et al. 2011). Samples were assigned to multiplex reads with the following default parameters: min. quality score of 25, min. length 200, max. length 1000 and no ambiguous bases and mismatches allowed in primer sequences. De novo OTU picking in QIIME was done by assigning similar sequences to a single OTU at a 97 % similarity threshold. Taxonomy of the OTUs was assigned through QIIME with BLAST (Altschul et al. 1990), the RDP Classifier (Wang et al. 2007) and the QIIME RTAX classifier. Rarefaction curves (Hughes et al. 2001), Shannon indices (Magurran 1988) and richness indices (Chao 1984) were obtained through QIIME. Further analysis of sequences matching cyanobacterial OTUs was done through manually blasting with the NCBI Standard Nucleotide BLAST against the nr/nt database using Megablast (Altschul et al. 1990).

Statistical analysis

The multivariate data analysis software Canoco 5 (ter Braak and Šmilauer 2012) was used to conduct principal component analysis (PCA) of the relative abundance data to analyze the total variation within the snow dataset, as well as between snow samples and ice and ocean samples. PCA is an unconstrained ordination method that describes the axes of maximum variability in the data and helps discern patterns within datasets. The data were $\log(x + 1)$ transformed prior to analysis.

Results and discussion

A total of 291,331 16S small subunit ribosomal RNA gene (variable regions 3 and 4) sequences were obtained from the snow samples collected at three sites in the vicinity of the North Pole (Table 1). The average number of sequences per sample was 29,994 and the minimum number was 13,737, which is a hundred- to thousand-fold higher than the numbers obtained from previous snow diversity studies using cloning and Sanger sequencing or bacterial isolation (Carpenter et al. 2000; Amato et al. 2007; Larose et al. 2010). Denoising of the raw sequencing data corrected 182,913 sequences, leaving 269,944 sequences that were subsequently assigned to 984 OTUs. The rarefaction curves (Fig. 1) showing the numbers of observed species as a function of sequences per sample do not reach a plateau, showing that the diversity is still undersampled despite the relatively high number of sequences obtained.

Table 1 Overview of the 16S rRNA gene sequence dataset

Total number of sequences	291331
Number of sequences after denoising and chimera removal	269944
Average sequences per sample	29994
Minimum sequences per sample	13737
Maximum sequences per sample	72931
Total number of OTUs	984

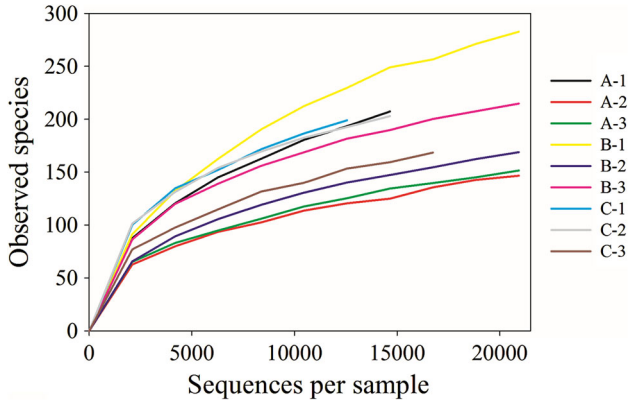

Fig. 1 Rarefaction curves for replicates of samples A, B and C showing observed number of species (OTUs at 97 % identity) as a function of sequences per sample

Abundances ranged between 43 and 248 16S rRNA genes per ml of melted snow (Table 2), falling in the lower end of what has been shown in other studies with abundances ranging from 20 (Harding et al. 2011) to 720,000 (Liu et al. 2009) cells ml^{-1}. A recent study on snow around Russian Antarctic stations showed microbial abundances of 1×10^3 to 46×10^3 cells ml^{-1} and a ten- to hundred-fold increase in DNA content in snow sampled in the proximity of human activity (Lopatina et al. 2013). The low abundances of 16S rRNA genes found in this study indicate that snow from North Pole ice floes is a habitat with low rates of microbial input. Sample A showed the lowest 16S rRNA gene abundance as well as the lowest diversity; however, the highest abundance was determined in sample B and the highest diversity in sample C. This shows that the diversity did not depend on the absolute amount of DNA, or microbial abundance, in the samples alone.

All samples showed a Chao1 index of less than double the number of the observed OTUs (richness) (Table 2), meaning that the observed number of species represents over 50 % of the estimated true number of species. Two other studies have assessed biodiversity in snow by means of 454 pyrosequencing. One study found 333 OTUs per sample in a High Arctic glacier snowpack (Hell et al. 2013) slightly higher than the mean richness of 193 OTUs per sample in our study. Another study showed a mean richness at Station Nord in Northeastern Greenland of 4620 OTUs per sample (Møller et al. 2013). The higher richness detected at Station Nord as well as a Chao1 index of 7841 could be partly attributed to the higher number of sequences obtained in the study. This supports the results of the rarefaction analysis (Fig. 1) showing that, with the number of sequences obtained in present study, the environment of Arctic ice floe snow was still undersampled. The diversity expressed as Shannon indices ranged between 2.23 and 3.76 (Table 2), which is also lower than what was found at Station Nord (5.06–5.60) (Møller et al. 2013). The lower richness and diversity found in snow on North Pole ice floes could be explained by a lower rate of microbial input to this remote site caused by selective elimination of microorganisms from the pool of microbial input from the atmosphere due to the extreme conditions that exist during atmospheric transportation. The higher bacterial diversity found in snow in the proximity of

Table 2 Diversity indices and 16S rRNA gene abundances in the North Pole snow samples

Site	Shannon index	Chao1 index	Richness	Dominance	16S rRNA gene abundance (gene copies per ml of melted snow)
A	2.226 ± 0.51	314 ± 54	168 ± 33	0.70 ± 0.17	$0.043 \pm 0.021 \times 10^3$
B	3.124 ± 0.22	353 ± 64	222 ± 57	0.31 ± 0.0003	$0.248 \pm 0.091 \times 10^3$
C	3.758 ± 0.35	293 ± 6,7	190 ± 19	0.27 ± 0.01	$0.118 \pm 0.016 \times 10^3$

Mean ± st.dev., $n = 3$

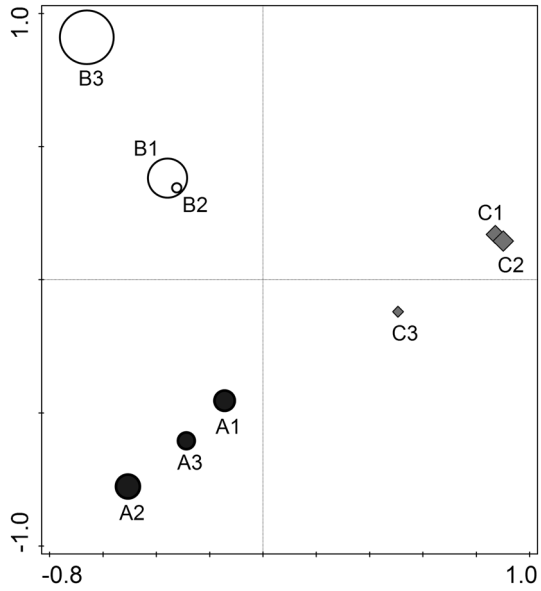

Fig. 2 Principal component analysis (PCA) ordination of the microbial diversity data in the snow from the three North Pole sites. The first axis explains 31.8 % of the variation in the data, and the second axis explains 25.9 % of the variation in the data. The size of the *circles* is determined by the richness of the samples

terrestrial and anthropogenic sources is likely to originate from the surrounding habitats, where microbial abundances are higher than compared to environments on the North Pole. This is supported by studies showing similarity of the snow microbial community to the community found in environments adjacent to the sampled snow (Liu et al. 2009; Harding et al. 2011).

The most abundant OTUs in sample A were chloroplasts (69.7 %), *Burkholderiaceae* (7 %) and *Sphingomonas* (4.5 %). Sample B was dominated by diatoms (*Bacillariophyta*, 30.9 %), (26.8 %), chloroplasts and *Shewanella* (11.6 %). The dominant OTUs in sample C were *Pseudoalteromonas* (26.8 %), *Herbaspirillum* (18.4 %) and *Sphingomonas* (7.9 %). The variation in diversity within samples (i.e., between replicates) was smaller than the variation between sites, which is visualised in a principal component analysis plot shown in Fig. 2, where replicates from the same site cluster together. This shows that errors potentially introduced during handling and analyses of the samples are smaller than the real differences between sites. At phylum level, the diversity of samples from site A and B was dominated by *Cyanobacteria*, including chloroplasts, (72 and 61 % respectively), *Proteobacteria* (23 and 35 %), *Firmicutes* (1.9 and 1.2 %) and *Bacteroidetes* (1.1 and 1.5 %) (Fig. 3). Sample C had similar abundances of *Bacteroidetes* (1.3 %) and *Firmicutes* (1.4 %) as in samples A and B, but was otherwise dominated by *Proteobacteria* (95 %) and lacked the high abundance of *Cyanobacteria* (1.4 %). Thus far most studies on snow have found *Proteobacteria* to be dominant, followed by *Bacteroidetes* and *Actinobacteria* (Bowers et al. 2009; Liu et al. 2009; Hell et al. 2013; Lopatina et al. 2013; Møller et al. 2013). *Firmicutes* have also shown to be common in snow (Bowers et al. 2009; Liu et al. 2009; Larose et al. 2010; Hell et al. 2013; Møller et al. 2013), as have *Cyanobacteria* (Bowers et al. 2009; Larose et al. 2010; Harding et al. 2011; Hell et al. 2013; Møller et al. 2013). The results of the present study are in line with previous findings that *Proteobacteria*, *Bacteroidetes, Firmicutes* and *Cyanobacteria* are the most abundant phyla in snow habitats. The similarity found between studies of snow from different locations supports the idea that a core community of bacteria might inhabit the snow habitat globally. Our study shows that the community composition at remote sites with minimum input from terrestrial and anthropogenic sources resembles that of sites in proximity to these sources and that the similarity in communities is, therefore, not a result of input from terrestrial ground, anthropogenic activities or as in our case the underlying environment of ice and ocean.

Samples A and B showed similarity also at the genus level confirming the results at the phylum level (Figure S2). Both samples had a high representation (>1 % of total OTUs) of *Cyanobacteria* and chloroplasts, *Sphingomonas* (*Alphaproteobacteria*), *Burkholderia* (*Betaproteobacteria*), *Pseudomonas* (*Gammaproteobacteria*) and various genera of the *Comamonadaceae* family of *Betaproteobacteria*. Sample C contained a greater variety of sequences related to bacteria known from marine environments than samples A and B, including 4.6 % of *Idiomarina* (Ivanova et al. 2000), 5 % of *Marinobacter* (Gauthier et al. 1992), 3.3 % of *Shewanella* (Hau and Gralnick 2007) and 1.2 % of *Alteromonas* (Baumann et al. 1972). *Shewanella* was the only predominantly marine genus found in samples A and B with over 1 %, representing 11.7 % of sequences in sample B. A higher number of marine-related bacteria suggest that the microbial community at site C had received greater input from the ocean compared to sites A and B at the time of sampling. The marine input may be caused by disruption of the ice and exposure of the snow to aerosols from the ocean, or by warmer or more saline ice, which could result in an easier migration of marine bacteria to the snow cover on the ice surface through brine channels (Ewert and Deming 2013). These factors may also partly account for the higher diversity found in this sample (Table 2). The differences in community composition at the three sites show that the bacterial community in snow is influenced by the surrounding environments, which potentially leads to an increase in biodiversity. It is highly unlikely that any of the three samples could have avoided receiving input from the underlying environment at some time point. The lack of the marine-related bacteria in samples A and B could be explained by these organisms

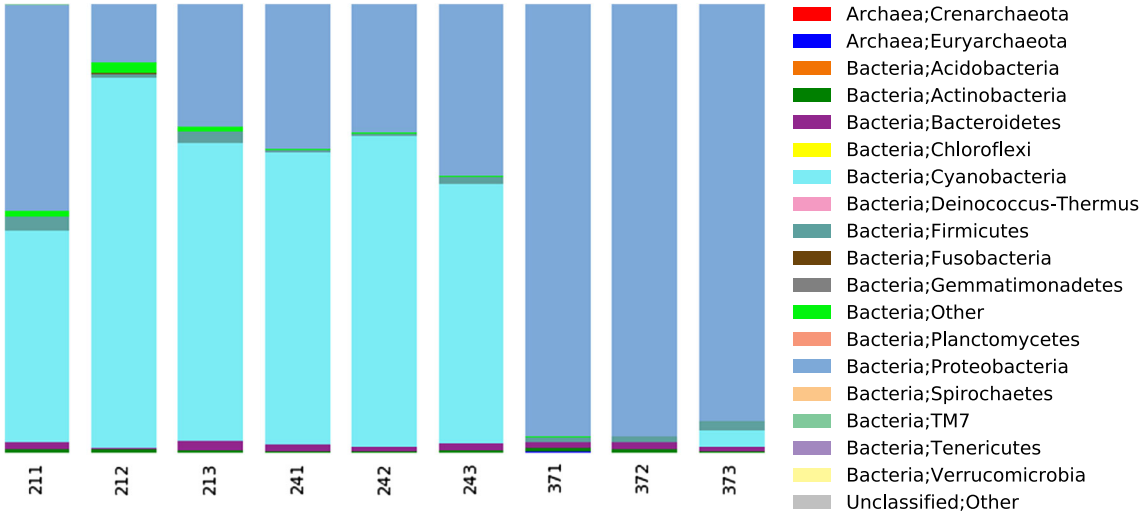

Fig. 3 Composition of bacteria in North Pole snow samples A–C at phylum level

not being able to sustain themselves in the snow, and they may therefore only transiently make up part of the diversity found in sample C.

When pyrosequencing 16S rRNA genes from environmental samples chloroplasts are included due to the evolutionary origin of chloroplasts from ancient *Cyanobacteria* (Raven 1970). Consequently, a number of chloroplast sequences are included in our study. The large amount of sequences from *Cyanobacteria* and chloroplasts found at sites A and B is consistent with previous findings of snow diversity (Bowers et al. 2009; Larose et al. 2010; Liu et al. 2009; Harding et al. 2011; Hell et al. 2013; Møller et al. 2013). While most *Cyanobacteria*/chloroplast OTUs matched uncultured organisms, the ones assigned to known organisms revealed the presence of chloroplasts of marine or halophilic algae, including diatoms (*Synedra*, *Odontella*), cryptophytes (*Rhodomonas*), prasinophytes (*Mamiella, Mantoniella*, *Micromonas*) and xanthophyceans (*Vaucheria*). This further supports that the snow has received input from seawater and/or sea ice.

The community composition in the North Pole snow was compared to the surrounding environment of sea ice and ocean water described in a related study conducted during the LOMROG II expedition (Bowman et al. 2012) using a principal component analysis (Fig. 4). The three snow samples, from sites hundreds of kilometres apart, are more similar to each other than to the underlying sea ice and ocean water. This result again shows that the snow community resembles snow communities at distant sites and, gives further support to the idea of a global microbial community common to snow. These results also suggest that the snow community does not receive a major part of its input of microorganisms from the underlying environment, but more likely from the overlying environment, i.e., the atmosphere. Atmospheric input as a major source of biodiversity is a potential factor behind the global similarities of snow microbial communities.

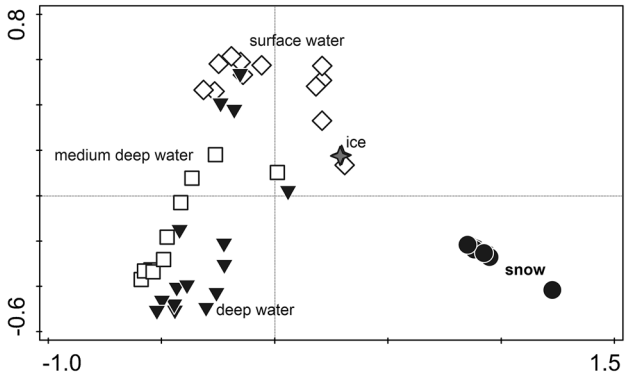

Fig. 4 Principal component analysis (PCA) ordination of the microbial diversity data in the snow from the three North Pole sites, ice and ocean water of three depths. The first axis explains 25.9 % of the variation in the data, and the second axis explains 14.8 % of the variation in the data

Conclusions

This study shows that snow on North Pole ice floes harbors a relatively lower abundance and diversity of microorganisms compared to snow found in the proximity of land and human activity. This indicates that snow at locations remote to terrestrial ground and human activity receives a lower rate of microbial input from the surrounding environment compared to microbial communities in snow at sites closer to terrestrial and anthropogenic sources. Our results also show some importance of local marine sources

for the snow biodiversity, although the closer similarities to other snow habitats than to the underlying environment of ice and ocean suggest a global snow community seeded from the atmosphere.

Acknowledgments This research was supported by The Center for Permafrost CENPERM, DNRF Number 100, funded by the Danish National Research Foundation as well as The V. Kann Rasmussen Foundation and The Lundbeck Foundation. We acknowledge Professor Eske Willerslev, Center for GeoGenetics, University of Copenhagen, for his contributions in the initial phase of the research and Dr. Jens Blom for assisting in sample collection.

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2.2 Freshwater Networks and the Cryosphere

Freshwater networks and in particular riverine networks make good model systems for microbial biogeography, as there is one obvious and dominating direction of transport, along the water flow. This gives a good starting point for making hypotheses and testing these. Consequently, NGS-based studies of freshwater networks linked to the cryosphere have resulted in the formulation of solid hypotheses about biogeography of microbial communities in these systems.

One established hypothesis about freshwater networks is the origin of the riverine microbial community from the upstream terrestrial environment.

The diversity of microbial communities in a freshwater system in Alaska showed a uni-directional decreasing pattern in diversity (Crump, Amaral-Zettler, and Kling 2012). Diversity was highest in soil waters upstream and decreased downstream in larger streams and rivers (Crump, Amaral-Zettler, and Kling 2012). This lead to the hypothesis that freshwater microbial communities originate in upstream terrestrial environments, and the microbes that enter the network at the top are sorted downstream resulting in gradually decreasing diversity. This was confirmed in a recent extensive study of a boreal freshwater system in Eastern Quebec (Ruiz-González, Niño-Garcia, and del Giorgio 2015). This study showed selection by the environment in that microbes from soil were numerically dominant along the freshwater network and microbes that were rare in soil became increasingly abundant downstream, resulting also in a lower diversity downstream (Ruiz-González, Niño-Garcia, and del Giorgio 2015).

Another closely linked hypothesis in freshwater networks is the interaction of two dominating forces that shape microbial communities in riverine systems. Water residence time (WRT) is hypothesized to be a major determinant for the relative influence of hydrology and local sorting (Niño-García, Ruiz-González, and del Giorgio 2016; Nelson, Sadro, and Melack 2009). Hydrology results in a mass-effect through mass-transport of microbial communities and local environmental factors cause selection by the environment (Niño-García, Ruiz-González, and del Giorgio 2016; Nelson, Sadro, and Melack 2009). Longer WRT enables the sorting of species, i.e. selection by the environment. Meanwhile in waters with short WRT local sorting will have less influence

compared to hydrology (Niño-García, Ruiz-González, and del Giorgio 2016; Nelson, Sadro, and Melack 2009).

In summary, NGS studies of freshwater networks have shown that indeed the environment selects and that this force interacts with large-scale transportation in structuring the microbial communities of freshwater networks, which originate from terrestrial microbial communities.

PAPER II: Upstream Freshwater and Terrestrial Sources Are Differentially Reflected in the Bacterial Community Structure along a Small Arctic River and Its Estuary

This research paper deals with the diversity and community composition of a freshwater network on the Disko Island off the West coast of Greenland (69°N). The network consists of a main river, the Red River, and the input from a proglacial lake and a glacier stream as well as the downstream estuary. Samples were assessed through amplicon sequencing of the 16S rRNA gene.

Prior to this paper several recent papers had established the importance of soil microbes in freshwater systems connected to the cryosphere (Niño-García, Ruiz-González, and del Giorgio 2016; Ruiz-González, Niño-Garcia, and del Giorgio 2015; Crump, Amaral-Zettler, and Kling 2012). In this paper we established the addition of microbial communities from the cryosphere to an Arctic freshwater network, with communities coming from a proglacial lake and a glacier stream.

These results had several implications: First, the pattern of diversity found in the Red River freshwater network did not resemble the otherwise established diversity-pattern of freshwater networks. Our results showed a low diversity in the glacier stream coming into the Red River, which contrasted previous results where soil microbes resulted in higher diversity in smaller streams (Ruiz-González, Niño-Garcia, and del Giorgio 2015; Crump, Amaral-Zettler, and Kling 2012).

Second, the results have important implications for the role of freshwater networks connected to the cryosphere. Increasing temperatures and concurring increases in melting

of the cryosphere will result in an even greater influence from these sources on freshwater networks.

The results from Paper II also illustrated the value of assessing Indicator Species, as previously described in part I (Ramette and Tiedje 2007).
Through the calculation of Indicator Species from samples along the river it was possible to identify Indicator OTUs that could be assessed in detail and which gave important information about the distribution of microbes in the Red River network. Through assessment of Indicator OTUs it was possible to clearly visualize the low abundance of these OTUs in the downstream river and the surprising high abundance further into the estuary. Indicator OTUs from the river showed to make up on average a quarter of the microbial community in the estuary. Furthermore, this study highlighted the importance of proper sampling for microbial biogeography studies. Microbial communities in the Red River microbiome that originated from the proglacial lake and glacier stream were visible only close to the input sites and not at the river mouth. If these sites had not been sampled it would not have been possible to know that such a large part of the estuary community was indeed similar to microbes from the river.

Paper II demonstrated the potential use of NGS data from microbial communities as living tracers in ecosystems at a large scale. The genetic signature of microbial communities in the environment can act as both tracers of transport in a large scale and simultaneously give indications of adaptation at a local scale. This has implications for use not only in microbial ecology but also in geomorphology in assessing connectivity in the landscape.

Lastly, this paper is an example of a study in which the use of 16S rRNA gene amplicon sequencing was the optimal choice for testing the hypotheses and research questions. To properly test our hypotheses we needed to be able to compare diversity parameters. This is possible only with amplicon sequencing as discussed in part I. Also we utilized the potential of the signature from 16S rRNA gene sequences as traceable markers.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 September 2016
doi: 10.3389/fmicb.2016.01474

Upstream Freshwater and Terrestrial Sources Are Differentially Reflected in the Bacterial Community Structure along a Small Arctic River and Its Estuary

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OPEN ACCESS

Edited by:
Eva Ortega-Retuerta, Spanish National Research Council, Spain

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Clara Ruiz Gonzalez, Institut de Ciències del Mar (CSIC), Spain

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Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *07 June 2016*
Accepted: *05 September 2016*
Published: *21 September 2016*

Citation:
Hauptmann AL, Markussen TN, Stibal M, Olsen NS, Elberling B, Bælum J, Sicheritz-Pontén T and Jacobsen CS (2016) Upstream Freshwater and Terrestrial Sources Are Differentially Reflected in the Bacterial Community Structure along a Small Arctic River and Its Estuary. Front. Microbiol. 7:1474. doi: 10.3389/fmicb.2016.01474

Glacier melting and altered precipitation patterns influence Arctic freshwater and coastal ecosystems. Arctic rivers are central to Arctic water ecosystems by linking glacier meltwaters and precipitation with the ocean through transport of particulate matter and microorganisms. However, the impact of different water sources on the microbial communities in Arctic rivers and estuaries remains unknown. In this study we used 16S rRNA gene amplicon sequencing to assess a small river and its estuary on the Disko Island, West Greenland (69°N). Samples were taken in August when there is maximum precipitation and temperatures are high in the Disko Bay area. We describe the bacterial community through a river into the estuary, including communities originating in a glacier and a proglacial lake. Our results show that water from the glacier and lake transports distinct communities into the river in terms of diversity and community composition. Bacteria of terrestrial origin were among the dominating OTUs in the main river, while the glacier and lake supplied the river with water containing fewer terrestrial organisms. Also, more psychrophilic taxa were found in the community supplied by the lake. At the river mouth, the presence of dominant bacterial taxa from the lake and glacier was unnoticeable, but these taxa increased their abundances again further into the estuary. On average 23% of the estuary community consisted of indicator OTUs from different sites along the river. Environmental variables showed only weak correlations with community composition, suggesting that hydrology largely influences the observed patterns.

Keywords: biodiversity, bacterial community, freshwater network, Greenland, arctic, polar environments

INTRODUCTION

Arctic river and estuary ecosystems are vulnerable to the ongoing climate change. Increasing temperatures are resulting in negative mass balance of glaciers and increased precipitation, with significant impacts on rivers and estuarine systems (Serreze et al., 2000; Mueller et al., 2003). In addition, Arctic rivers are known to transport significant amounts of organic carbon and biomass

from permafrost and glacier ecosystems into the Arctic oceans and are therefore important factors in global climate change models (Kling et al., 1991; Guo et al., 2007; Lawson et al., 2014; Hawkings et al., 2015). Higher river flow associated with the warming climate may result in a more river-dominated community in the estuaries (Fortunato et al., 2013). Once riverine bacteria reach the estuary, they may influence local nutrient cycling through biofilm formation and forming aggregates (flocs; Decho, 2000). Thus, bacterial communities dispersed through Arctic riverine systems may be important for biogeochemical cycling processes in Arctic estuarine and coastal ecosystems.

There are a number of studies on Arctic estuarine ecosystems focused on biodiversity, biological productivity, seasonal variability, food web interactions, and responses to environmental variables (Galand et al., 2006, 2008; Wells et al., 2006; Vallieres et al., 2008; Fortunato et al., 2012, 2013). However, the river communities have usually been assessed as a whole. How different communities added to the river affect the estuarine community has not been addressed to date. While previous studies have shown that increased river flow alters Arctic river and estuary communities in seasonal patterns (Crump et al., 2009; Fortunato et al., 2012, 2013), it is yet unknown how upstream sources of freshwater microbial communities influence these communities.

A few recent studies on freshwater ecosystems at different spatial scales have greatly increased our understanding of the biogeography of riverine networks (Nelson et al., 2009; Crump et al., 2012, 2007; Ruiz-González et al., 2015; Niño-García et al., 2016). These studies have shown that biogeographic patterns of bacterioplankton communities are a result of the interaction between local environmental variables and mass-effects. Furthermore, that mass-effects are determined by the hydrology as well as the position along the network (Crump et al., 2007, 2012; Nelson et al., 2009; Ruiz-González et al., 2015; Niño-García et al., 2016).

Water residence time (WRT) has shown to be an important factor for determining the relative influence of hydrology vs. local sorting (Niño-García et al., 2016). There seems to be a greater influence from hydrology and mass-effects in systems with short WRT and a greater influence from local sorting in systems with long WRT (Niño-García et al., 2016). Longer WRT in lakes and larger rivers compared to smaller streams consequently results in less diverse communities due to local sorting (Niño-García et al., 2016). In this way, hydrology and local sorting interact and result in a uni-directional pattern of gradually decreasing diversity from smaller streams to larger rivers and lakes (Niño-García et al., 2016). Furthermore, beyond a WRT of 10 days hydrology has been shown to have no additional impact on the structuring of the microbial community (Niño-García et al., 2016). The importance of WRT for selecting lake-specific phylotypes in a freshwater network was also highlighted in another study (Nelson et al., 2009). The study showed less similarity between the microbial community in the inlet and the outlet of a headwater lake compared to the inlets and the outlets of downstream lakes. This indicates that the first lake selects for a lake-specific community, which is then transported downstream in the network (Nelson et al., 2009). These results also illustrated the importance of the position along the network for understanding the bacterial community structure (Nelson et al., 2009). As the position of water bodies in the system might be a key factor for determining the structure of the microbial community at that particular position, the right spatial resolution is important for understanding the structural changes the microbial community undergoes along a freshwater network.

Together with WRT a terrestrial seed bank for freshwater networks also seem to result in a uni-directional structure of the microbial community (Crump et al., 2012; Ruiz-González et al., 2015). In the catchment of the Toolik Lake, Alaska, a clear pattern of decreasing diversity was shown from soil waters farthest upstream with highest species richness through headwater streams and lastly to lowest richness in lake water (Crump et al., 2012). OTUs originating in soil were numerically dominant throughout a freshwater network in the Eastern boreal region of Québec, Canada, and certain OTUs that were rare in soil were shown to increase in number and become dominant in the downstream freshwater environments (Ruiz-González et al., 2015). These studies indicate that an initial inoculation from soil at the beginning of a freshwater network is followed by a species-sorting process downstream (Crump et al., 2012; Ruiz-González et al., 2015).

On a large spatial scale the uni-directional pattern of decreasing microbial diversity along a river might be explained by the common origin from a highly diverse terrestrial community (Ruiz-González et al., 2015) and by increasing local sorting (Niño-García et al., 2016). However, there might be another pattern on a smaller spatial scale revealed with higher resolution. Higher resolution of samples along a freshwater network might reveal the input of new microbial taxa of different origin along the freshwater network. Input of new taxa along a network could result in a different structuring pattern of the microbial community, which is not uni-directional. The addition of new microbial communities along a freshwater network would be particularly clear in smaller networks where input makes up a larger fraction of the downstream water body. This also implies that in larger networks, the downstream community might mask new communities added along the network. Therefore, river communities on a small spatial scale may show not to have a uni-directional structure, explained by seeding with new microbial communities along the network. Furthermore, it is yet unknown whether the spatial directionality described above extends into saline waters or whether the very different environmental conditions met by the riverine community in the estuary result in a different pattern of the microbial network.

We address the question of how the bacterial communities from the Red River, a small river on the Disko Island, West Greenland (69°N) are structured at the small spatial scale, by comparing bacterial communities from five sites along the river including input sites from a glacier and a proglacial lake. We investigate whether the structure of the riverine bacterial communities can help explain the structure of the estuary communities by including 23 samples through three transects of the Red River estuary.

Sampling was done in 2013 in August when precipitation events are common and the permafrost active layer thickness

is maximum resulting in increased erosion along the river (unpublished data). We hypothesize that the river community is composed of organisms from the surrounding terrestrial environment as well as from upstream freshwater sources, such as glaciers and lakes. Furthermore, due to the relatively short WRT we hypothesize that hydrology rather than local sorting is the dominant factor in shaping the community. We assess to which extent the different communities detected along the river structure the bacterial assemblages in the estuary.

Finally, we test and discuss the potential effects of environmental variables based on multivariate statistical analysis.

MATERIALS AND METHODS

Sampling

Sampling was carried out in the Red River and its estuary on the Disko Island, West Greenland (69°N) during August 2013 (**Figure 1**). Around the time of sampling the river flow was 5.7 m^3s^{-1}. The bedrock consists of iron-rich basalt. As the glacier and the stream erode the bedrock the iron precipitates and gives the marked red color of the river. The river drains directly into the Disko Bay and the freshwater and sediments supplied from the river are mixed with the saline bay waters under varying wave influence. A river plume of high concentrations of sediment is often visible indicating how the supplied sediment is dispersed.

Five locations were sampled in the river with three replicates at each site (**Figure 1**). The top sample (R1) being just upstream of an outlet from an adjacent proglacial lake and the second sample (R2) at the outlet from the proglacial lake. The third sample (R3) being at another outlet to the river supplying water directly from the glacier and the fourth sample (R4) 100 m downstream of R3. Sample R4 was collected at the eastern bank on the opposite site of the upstream outlet from the glacier stream (R3), while all other river samples were collected at the center of the river. The last and fifth sample (R5) was collected close to the river mouth. The distance of each sample to the river mouth is supplied in **Table 1**. In the bay, sampling was done along three transects perpendicular to the coast (**Figure 1**). Each transect consisted of four sampling locations at distances of 100, 300, 700, and 1100 m from the river mouth. At each distance two samples were collected, one surface sample at 0.5 or 1 m from the surface and one deep sample 1 m from the bottom. At water depths above 20 m, the deepest water sample was collected at 20 m depth. In transect 1, 100 m into the estuary (E100) the deep sample is missing so that there are only two replicates (transect 2 and 3) of E100 samples. At E700 one sample, which should have been sampled at 20 m, was sampled at 1 m depth, so that there are 4 replicates of surface samples and 2 replicates of deep samples for E700. Water was sampled by grab sampling using sterile 50 ml syringes (Sarstedt, Germany) either collecting water directly from the river or collected from a 5 L Niskin water sampler (KC Denmark, Denmark) that had been filled at the sampled depth. The 50 ml water samples collected in the syringes were forced through Sterivex™ filters (Merck Millipore, MA, USA) and the filters were afterwards partly dried by forcing air from the syringes through the filters. The filters were frozen and kept at −20°C until analysis.

Temperature, turbidity, and oxygen saturation were measured at all sites using a YSI 6600-V2 CTD sensor with attached probes (YSI, OH, USA). In the bay, the size (in equivalent spherical diameter, ESD), total area and total number of particles were measured in 6 mL water using a laser sheet camera system, the Pcam (Markussen et al., 2016). Individual water samples were taken at all locations and transferred to new 100 ml polyethylene bottles, frozen as quickly as possible and shipped to Copenhagen for further analysis. The total nitrogen (TN) and dissolved organic carbon (DOC) were determined on a Shimadzu TOC-V total organic carbon analyzer (Shimadzu, Japan) with a TNM-1 total nitrogen measuring unit and pH was measured using a Radiometer Analytical SAC90 autosampler (Hach, CO, USA). DOC measurements were based on triplicate measurements. A standard curve using 1000 ppm sodium hydrogen phthalate with concentrations ranging from 0 to 5 ppm were made and a 100 ppm certified Total Organic Carbon (TOC) standard (SCP Science, QC, Canada) was diluted to 1 ppm for use as reference.

DNA Extraction and Sequencing

DNA was extracted from the Sterivex™ filters using the PowerWater© Sterivex™ DNA extraction kit (MO BIO Laboratories, CA, USA), using the protocol provided by the manufacturer. The extracted DNA was stored at −80°C until library preparation.

The nucleic acid concentrations of all samples were assessed by spectrophotometer (Nanodrop® ND-1000, Saveen Werner, SE) to be within the range of 3–5 $ng\,\mu l^{-1}$. DNA was then amplified in triplicate using universal prokaryotic primers targeting the variable region V4 of the 16S rRNA gene (Caporaso et al., 2011), forward primer 515F (GTGCCAGCMGCCGCGGTAA) and reverse primer 806R (GGACTACHVGGGTWTCTAAT), using the HiFi polymerase (PCR-Biosystem, UK). The primers were supplied with 12 distinct barcode sequences of 4–6 bases each and combined as differential sets, thus labeling the samples with individual differently tagged sequences. All PCR runs included triplicate positive (*E. coli*) and negative (dd H_2O) controls. The resulting PCR products (350 bp) were quality controlled by quantification of concentrations using the Qubit® 2.0 dsDNA HS Assay Kit (Life Technologies, CA, USA) and visual inspection of band size following gel electrophoresis. The amplified DNA was then purified using the HighPrep™ PCR size selective carboxyl coted magnetic beads (Magbio, MD, USA). The resulting DNA (average concentration 19.3 $ng\,\mu l^{-1}$) was then pooled and ligation of adaptors was performed according to manufacturer's instructions following the Low Sample (LS) Protocol (TruSeq DNA PCR-Free Sample Preparation Guide, Illumina, CA, USA) with minor modifications. Overhangs on the 3′ ends were removed and 5′ ends filled in by end repair, performed as described in the protocol on 1 μg DNA. Size selection was replaced with a clean-up step with magnetic bead based chemistry (HighPrep™ PCR, CleanNA). A volume of 100 μl from the end repair reaction was purified according to manufacturer's instructions and subsequently eluted in 20 μl

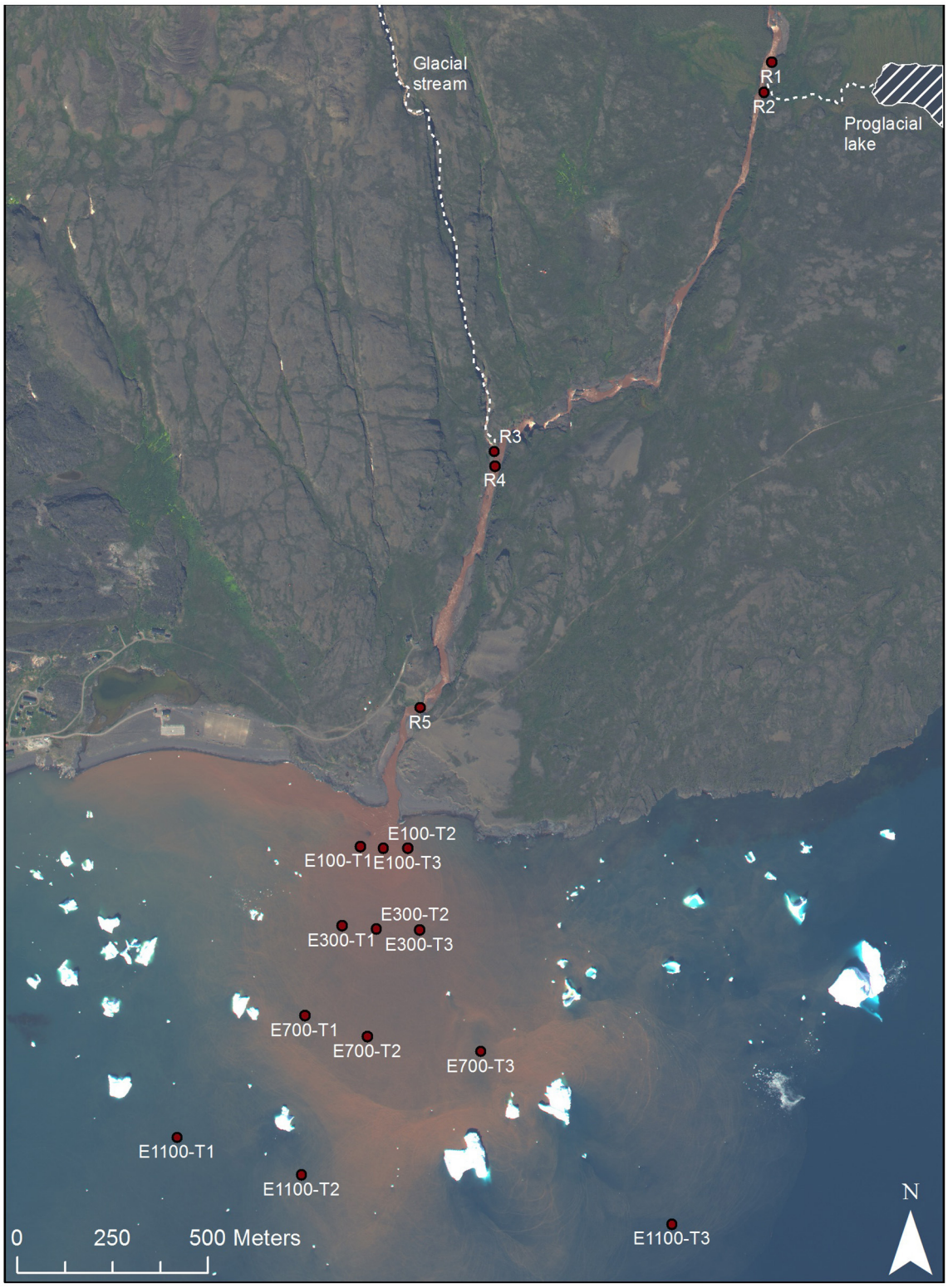

FIGURE 1 | Sample sites, Disko Island, West Greenland, 69°N (Worldview, 2013).

TABLE 1 | Environmental data on Red River and estuary samples.

ID	Transect	Distance m	Depth m	DOC μM	TN μM	pH	Temperature Degrees C	Salinity PSU	Oxygen sat Percent	Turbidity NTU	Particle MD microns	Particle TA pixels/vol	Particle N number/vol
R1	R	−2300	0.5	43.6	2.4	7.4	7.4	0.010	103.980	17.660	NA	NA	NA
R2	R	−2300	0.5	344.1	16.9	8.7	12.6	0.023	108.586	1.200	NA	NA	NA
R3	R	−1000	0.5	21.5	3.7	7.3	5.2	0.010	101.105	15.280	NA	NA	NA
R4	R	−900	0.5	65.6	4.8	7.5	6.0	0.010	108.864	15.036	NA	NA	NA
R5	R	−250	0.5	100.9	6.8	7.9	7.5	0.010	106.555	17.273	NA	NA	NA
E100	T1	100	0.5	84.5	6.5	8.0	7.1	23.679	111.213	10.718	55.752	46,016	296.000
E100	T2	100	0.5	166.0	13.6	8.8	6.9	16.205	109.392	19.774	42.329	25,326	237.000
E100	T2	100	4.5	90.3	11.9	8.7	6.8	32.292	113.223	8.209	38.205	16,397	170.000
E100	T3	100	0.5	135.3	13.4	8.9	7.2	26.068	110.465	10.831	67.652	913	4.000
E100	T3	100	2	62.7	4.5	8.7	7.2	26.909	109.804	11.453	46.778	30,034	241.000
E300	T1	300	0.5	79.9	6.7	8.7	7.3	28.489	110.902	10.320	50.871	40,217	296.000
E300	T1	300	10	78.9	4.2	8.4	6.1	32.513	112.439	8.039	35.656	18,483	262.000
E300	T2	300	0.5	60.0	5.3	8.3	6.8	20.794	111.975	14.174	54.712	61,567	338.000
E300	T2	300	8	109.4	8.1	8.6	6.2	32.477	110.957	11.117	40.354	359,091	830.000
E300	T3	300	0.5	192.4	12.1	8.7	7.1	24.422	111.391	13.374	59.102	42,866	261.000
E300	T3	300	4	61.5	4.4	8.4	6.5	32.409	111.552	8.221	35.014	15,617	209.000
E700	T1	700	1	111.9	8.2	8.8	7.2	31.184	110.461	9.113	44.551	15,743	132.000
E700	T1	700	18	76.7	8.8	8.8	2.3	33.219	108.237	8.024	27.317	10,979	138.000
E700	T2	700	1	69.8	5.2	8.5	7.3	31.145	108.917	11.713	76.069	13,631	49.000
E700	T2	700	20	81.7	5.0	8.4	1.8	33.273	108.753	9.478	33.565	9837	132.000
E700	T3	700	0.5	82.1	5.0	8.5	8.5	11.029	112.471	9.169	60.167	90,634	497.000
E700	XX	700	1	46.1	2.5	8.5	7.4	30.898	112.052	13.635	60.931	30,626	142.000
E1100	T1	1100	1	30.2	3.2	8.5	7.1	31.946	110.700	9.694	69.067	15,343	65.000
E1100	T1	1100	20	97.2	8.1	8.8	2.4	33.203	109.182	9.248	29.010	21,687	292.000
E1100	T2	1100	1	115.6	10.4	8.7	7.1	31.733	110.749	11.127	60.402	17,858	82.000
E1100	T2	1100	20	108.1	9.7	8.8	3.0	33.093	111.896	10.633	27.934	16,471	316.000
E1100	T3	1100	1	73.4	6.0	8.7	8.0	26.516	109.069	13.009	58.248	49,641	280.000
E1100	T3	1100	20	171.8	12.1	8.7	2.2	33.245	111.237	8.392	32.262	14,365	208.000

Distance, Distance to river mouth; Oxygen sat, Oxygen saturation; Particle MD, Particle Mean Diameter; Particle TA, Particle Total Area; Particle N, Particle Number. Particle data was measured in a water volume of 6 mL, vol, measured water volume. Transect XX was sampled outside the three transects.

molecular biology grade water (MO BIO Laboratories, CA, USA). Following this step, the 3′ ends were adenylated (adding an "A" nucleotide) to prevent them from ligating to one another during the ligation reaction. Then adaptors with a "T" overhang were ligated onto the DNA fragments of the two assemblages as described in the protocol. The assemblage was ligated with the AD012 index adaptor (CTTGTA), and then subjected to a clean-up step with purification beads provided in the kit. Quality control of the ligated amplicons (∼400 bp) was performed by PCR amplification using primers targeting the index adaptor followed by gel electrophoresis and visual inspection. Finally, the amplicon assemblage was diluted to a concentration of 3.3 ng μl^{-1} and sequenced with MiSeq 250PE (Illumina), adding 30% PhiX DNA. Demultiplexed merged reads are deposited in the NCBI Sequence Read Archive (SRA) database under SRA accession SRP076603.

Computational Analyses

The sequencing data was quality checked using FastQC (Patel and Jain, 2012) and read pairs were merged with the paired-end read merger PEAR (Zhang et al., 2014). Only properly merged reads were used for downstream analysis. Merged reads were processed using Qiime version 1.8.0 (Caporaso et al., 2010a). Demultiplexing with *split_libraries_fastq.py* was performed with quality filtering at phred threshold $\geq$ 20. Chimeric sequences were removed from demultiplexed data with USEARCH uchime reference-based chimera removal using the Greengenes database from May 2013 as reference (Edgar et al., 2011). Chimera check removed 12.3% of sequences. Operational taxonomic units (OTUs) were subsequently picked based on 97% identity using *de novo* OTU picking, which also includes taxonomy assignment using PyNAST alignment against the Greengenes core set of 16S rRNA sequences (Caporaso et al., 2010b). Sequences only represented once in the dataset were removed, which reduced the dataset with 13.7%.

Shannon indices (Shannon, 1948), Chao1 richness (Chao, 1984) and rarefaction plots were computed using *alpha_rarefaction.py*. Chloroplast sequences were removed and samples were rarefied to the shallowest sample depth of 12,180 sequences per sample with R version 3.1.0 (R Development

Core Team, 2008) and R package Vegan (Oksanen et al., 2015). BIOENV analyses were used to assess how well the community structure was explained by environmental variables using non-factorial metadata (**Table 1**; Clarke and Ainsworth, 1993). For BIOENV analysis the Vegan package was used to create distance matrices of environmental data (Euclidean distances) and community composition (Bray-Curtis distances), which were then compared through Spearman's rank coefficients. DOC, TN, temperature, salinity, oxygen saturation, and turbidity were log transformed prior to analysis. Depth was not included for BIOENV analyses including only river samples as depth was constant and particle data was not included for any analyses including river samples, as the data was not available. LabDSV package in R was used for non-metric multidimensional scaling (NMDS) and indicator species analysis. Indicator species are here denoted indicator OTUs and are defined as OTUs having a higher abundance at one site compared to other sites with indicator values $d \geq 0.3$ at a significance level of $p \leq 0.05$. Indicator values are a product of relative abundance of an OTU in samples from one site (between 0 and 1) and the relative average abundance of that OTU across all sites (Dufrene and Legendre, 1997). The used indicator species and indicator OTU concept in this study are not equal to the Indicator Species concept representing species that are markers for certain environmental variables in an ecosystem. NMDS analyses were conducted using Bray-Curtis distance matrices. NMDS stress values are included in **Figure 3**.

RESULTS

River System Characteristics

Dissolved organic carbon (DOC) and total nitrogen (TN) concentrations in the river were in the same range as those in the estuary (**Table 1**). River site R2 by the lake outlet had the highest DOC and TN concentrations of all samples in the river and estuary. pH values in the river were slightly lower than in the estuary except for R2, which had a higher pH comparable to the estuary samples. Temperature ranged from 1.7°C at 20 m depth 700 m into the estuary to 12.6°C at river site R2. Temperatures were generally lower in the deep water samples from the estuary compared to the surface samples. Salinity in the river samples was 0.01 PSU for all samples except for R2 where it was 0.023 PSU. The higher salinity in the water from the lake can be explained by the accumulation of ions in the lake due to longer WRT in the lake compared to the river allowing for evaporation of water from the lake. The longer WRT may also explain the higher temperature at site R2. In the estuary, salinity was consistently lower in the shallow water samples compared to deep water samples at the same distance from the river mouth. This was expected from the lower density of the freshwater from the river being mixed into the estuary. Turbidity across all samples, excluding river site R2, ranged from 8.0 to 19.8 NTU, while it was remarkably lower at R2 (1.2 NTU). Camera data from the estuary showed that particle mean diameter was generally higher at shallow depths compared to deep water samples.

Alpha Diversity

Illumina sequencing of variable region V4 of the 16S SSU rRNA gene from a total of 38 samples resulted in 462,840 individual sequences after rarefaction to 12,180 sequences per sample, which were binned into 63,624 unique OTUs (97% sequence identity). The number of observed OTUs was not exhausted at this level of rarefaction (**Supplementary Figure 1**). Shannon indices for the river samples ranged from 5.6 to 10.8 and Chao1 richness in the river ranged from 1408 to 19,117 OTUs per sample (**Figure 2**). The alpha diversity of the bacterial community represented by both Shannon indices and Chao1 richness decreased at R2 and R3, the sites at which the lake and the glacier stream drains into the river (**Figures 1**, **2**). The alpha diversity rose again at R4, ca. 100 m from the glacial input site. At the river mouth (R5), the diversity increased again and reached a similar level to the first river site (R1) upstream of the glacier and lake input sites.

Shannon indices for the estuary samples ranged from 5.4 to 10.6 while Chao1 richness varied from 2589 to 19,021 OTUs (**Figure 2**). The diversity and richness were higher in the estuary than in the glacier stream and lake input samples, and slightly lower than in the remaining river samples. There was no apparent pattern in the difference in diversity and richness attributed to different depths of the estuary, sample sites or the distance to the river mouth.

Community Composition Analysis

The samples from the first site of the river (R1), upstream of the lake and glacier stream outlets to the river, clustered with samples from the bottom site of the river (R5; **Figure 3**). These two sites also shared a high number of indicator OTUs (**Figures 4A,E**) and showed similar diversity and richness (**Figure 2**). The river site by the proglacial lake outlet (R2) clustered with the river site at the glacier stream outlet (R3). These sites, R2 and R3, also had lower diversity than the other river sites (**Figure 2**).

In the estuary, the bacterial communities clustered according to sample site for the two sites that were farthest into the estuary (E700 and E1100). The samples from the sites closest to the river mouth (E100 and E300) were dispersed across NMDS 1 and 2 (**Figure 3**). The samples did not cluster according to sample depth. Samples from the sample sites closest to the river mouth (E100 and E300) clustered more closely with river samples than the samples farthest from the river mouth (E700 and E1100).

Environmental Controls

BIOENV analysis showed that the total community as well as the non-indicator and indicator OTUs in the river correlated significantly with turbidity at $p \leq 0.05$. The strongest correlation was found between the river non-indicator OTUs and turbidity with a Spearman's rank correlation coefficient of 0.586.

BIOENV analysis of the estuary community showed no significant correlations with environmental variables (**Table 2**).

Indicator Taxa Analysis

The number of indicator OTUs in the river ranged from 158 at the second-to-last site of the river (R4) to 678 at the input site from the proglacial lake (R2), which also had the highest percentage of top indicator OTUs (Indicator Value = 1; **Table 3**).

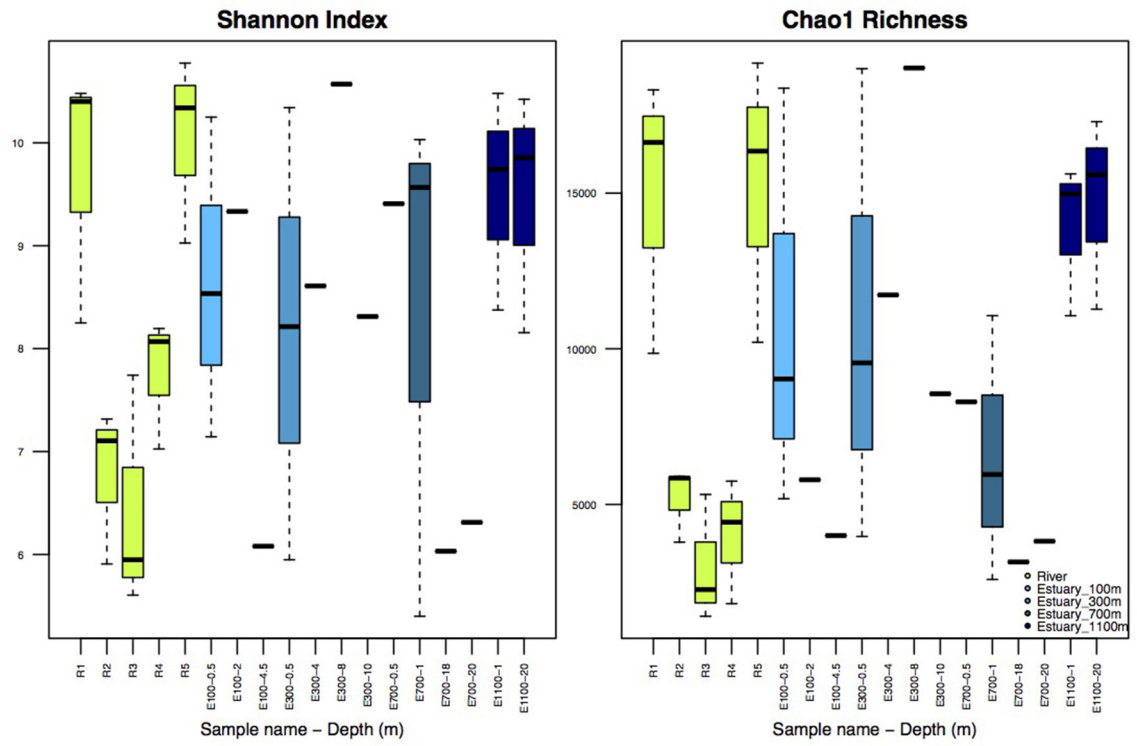

FIGURE 2 | Shannon Index and Chao1 Richness. R1–R5 are river samples ($n = 3$), E100–E1100 are estuary samples from 100 to 1100 m into the estuary with varying number of replicates. Replicates are samples from the three different transects and should not be confused with replicates of the same water mass. Depth is indicated after each site name, such that for example E100-0.5 is taken at 0.5 m depth. Sample sites with no replicates ($n = 1$) are indicated without boxes, for sample sites with boxes $n = 3$.

There was a high number of shared indicator OTUs between the top and bottom of the river (**Figures 4A,E**). Two hundred and eight indicator OTUs from R1 were found at R5 while only 14, 31, and 28 indicator OTUs from R1 were found at R2, R3 and R4 respectively. Taxonomic composition of indicator OTUs at Class level showed similar fractions of Flavobacteria and Gammaproteobacteria across river samples. A greater fraction of Actinobacteria were found in R2-R4 while very few Acidobacteria were found in these samples compared to R1 and R5 where also a higher fraction of Unknown were found (**Figure 5**). A number of indicator OTU sequences at the uppermost river site (R1) showed similarity to members of *Rhizobiales* isolated from plant roots and soil (Lee et al., 2005) as well as to strict anaerobes such as *Caldilinea*, *Anaerolineaceae* (Yamada et al., 2006), and *Desulfobacteraceae* (Garrity et al., 2006; **Figure 5**).

Indicator OTUs identified at the outlet from the lake and glacier stream were found in low numbers at the other river sites (**Figures 4B,C**). The lake outlet site (R2) had the highest number of indicator OTUs and percentage of top indicator OTUs (**Table 3**). A number of taxa known to be psychrophilic, such as *Moritella* (Urakawa et al., 1998), *Polaribacter* (Gosink et al., 1998), *Oleispira* (Yakimov et al., 2003), *Crocinitomix* (Bowman et al., 2003), and *Psychromonas* (Mountfort et al., 1998) were found among the best matches for the indicator OTUs from the lake outlet, unlike at the other river sites.

The distribution of estuary indicator OTUs showed a different pattern than the river indicator OTUs (**Figures 4F–I**). The number of indicator OTUs in the estuary was generally lower than at the river sites. An exception to this was the outermost estuary sample (E1100), which had a number of indicator OTUs comparable to the river sites (**Table 3**). No top indicator OTUs were found in any of the estuary samples, meaning that no OTUs from the estuary were unique to any of the sample sites. The indicator OTUs for each sample site in the estuary were found only in low numbers at the other sites both in the river and the estuary (**Figure 4**) and the taxonomic composition at Class level was less similar among the estuary samples than among the river samples (**Figure 5**).

On average, the bacterial communities in the estuary were made up of 23% river indicator OTU sequences (**Figure 6**). There was an overall decreasing contribution of river indicator OTUs in the estuary sites with 26–27% river indicator OTUs closest to the river mouth at E100 sites, 17–25% at E300 sites and 8–10% at E1100 sites. E700 sites were exceptions with 22–52% of the community being river indicator OTUs (**Figure 6**). Closer to the river mouth at sites E100 and E300 there was a larger fraction of the indicator OTUs from the top of the river (R1) and river mouth (R5), except for the deep sample at E100, where the distribution of river indicator OTUs was similar to the E700 estuary sites (**Figure 6**). In E700 both in the deep and surface

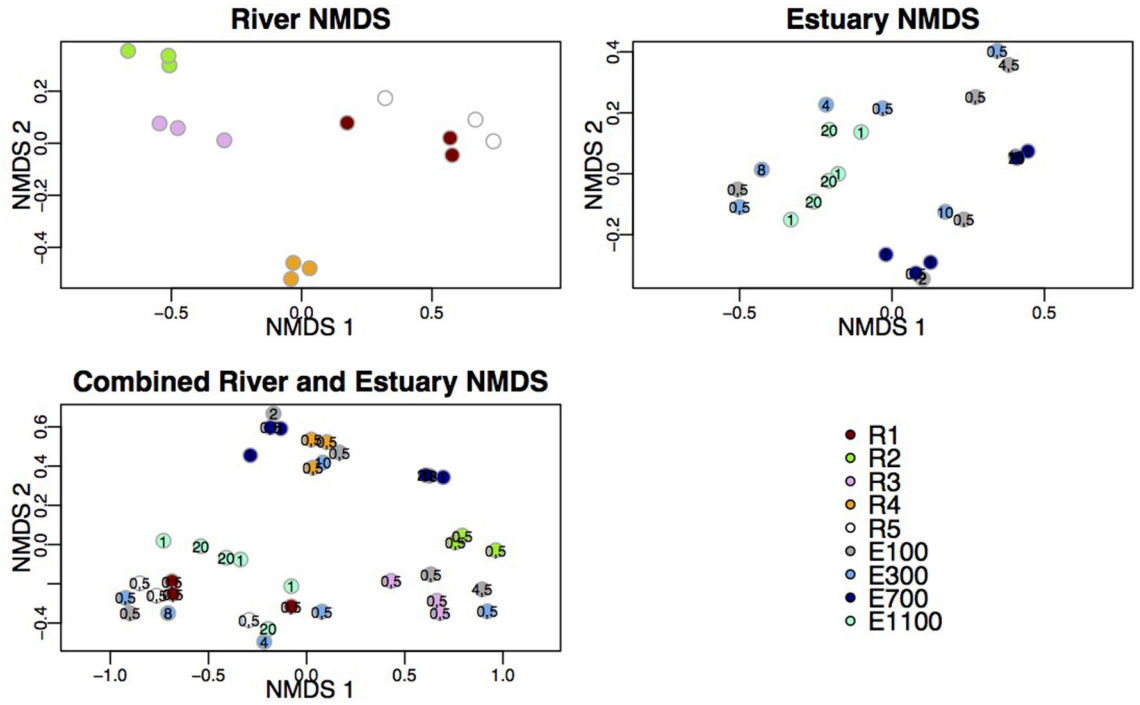

FIGURE 3 | NMDS plots of river data, estuary data and combined river and estuary data. Following stress values were obtained: river = 6.49%, estuary = 8.75%, combined = 10.29%. Depths are indicated at the estuary points.

samples R2 (lake outlet) indicator OTUs were more abundant than in the other estuary samples, and were more abundant than indicator OTUs from any other river sites (**Figure 6**). At the estuary sites farthest from the river mouth (E1100) the samples had the highest fraction of non-river indicator OTU sequences (**Figure 6**).

DISCUSSION

Alpha Diversity

Shannon indices for the river samples at the lake outlet (R2) and glacier stream outlet (R3) were comparable to a recent study of 87 small streams and rivers in the La Côte-Nord region of Québec, Canada, where OTUs were clustered with the same method as in the present study (Ruiz-González et al., 2015). The remaining river samples had slightly higher diversity than found in previous studies (Galand et al., 2006, 2008; Crump et al., 2009). The difference from less recent studies is likely due to the difference in the technologies applied and the resulting lower number of sequences in the previous studies. Together with sequencing technologies, which have changed dramatically in the last decade, OTU clustering has shown to have a great impact on the detected alpha diversity (Sinclair et al., 2015). Therefore, the comparison of alpha diversity among studies should be interpreted with care. Our results were obtained with the use of the Qiime pipeline (Caporaso et al., 2010a), which has shown to create a larger number of OTUs when compared to other popular clustering methods (Sinclair et al., 2015). Consequently, we might detect a higher diversity because of the clustering method used.

In an extensive study of freshwater networks, small streams were shown to have higher Shannon indices than larger rivers (Ruiz-González et al., 2015). This was attributed to the common terrestrial origin of the microbial community resulting in an initially high diversity in the small streams originating from the surrounding soil (Ruiz-González et al., 2015). This is in contrast with our results showing less diversity in the glacier stream compared to the main river (**Figure 2**). The lower diversity might be due to the origin of this stream in a glacier rather than subsurface groundwater and surface runoff as described in the above-mentioned study (Ruiz-González et al., 2015). This notion is supported by the fact that the diversity of the glacier stream outlet (R3) is comparable to those recently described for proglacial lakes (Peter and Sommaruga, 2016). Also, the indicator OTUs from the glacier stream were similar to taxa commonly found in freshwater and marine environments (details not shown). These results emphasize the importance of high spatial resolution for assessing the origin of the metacommunity in a complex freshwater network. In this study, the diversity along the network does not follow a uni-directional pattern (**Figure 2**). Our results illustrate that the origin and structuring of the microbial community might be very different from one network to another. How glaciers and glacier streams affect the metacommunity of freshwater networks is a highly relevant topic yet to be investigated.

The Chao1 richness in the river samples was higher than in the previous papers focused on large Arctic rivers (Galand et al., 2006, 2008) but comparable to that described in a recent paper using the same sequencing platform (Niño-García et al., 2016).

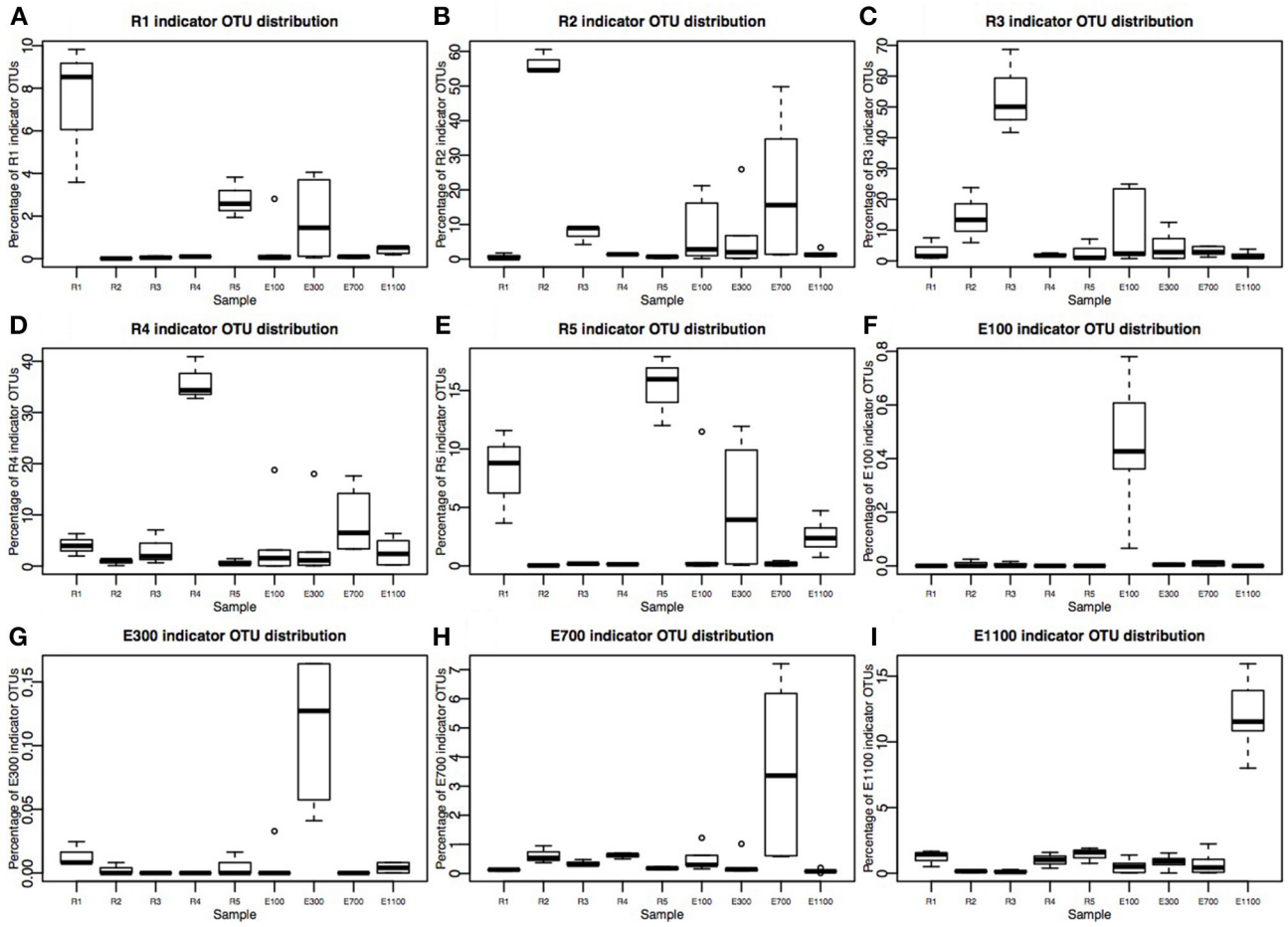

FIGURE 4 | Percentage of indicator OTU sequences distribution across sample sites. Please note that Y-axes are different in each plot. **(A–E)** River sites (R1–R5) consist of three replicates corresponding to 36,540 sequences total for each site after rarefaction to 12,180 sequences per sample. **(F–I)** Estuary sample sites E300–E1100 are each sampled in three transects at two depths, making up six samples per distance from the river mouth, corresponding to 73,080 sequences. E100 lacks one sample at transect 1, deep sample, therefore it consists of 60,900 sequences.

The drop in alpha diversity at the input sites from the lake and the glacier (R2 and R3, **Figure 2**) shows that the lake and the glacier stream input less diverse bacterial communities into the main river. Lower diversity in lakes compared with the connected rivers has been attributed to longer WRT in lakes (Crump et al., 2012; Ruiz-González et al., 2015; Niño-García et al., 2016). This is especially pronounced in small streams and rivers, where WRT is too short to allow for local sorting of the bacterial community (Crump et al., 2012; Ruiz-González et al., 2015; Niño-García et al., 2016). Downstream of the input sites, the alpha diversity rises again and by the river mouth reaches a level similar to the first river site (R1) upstream of the input sites (R2 and R3). This shows that the volume of water from the lake and glacier outlets does not dilute the downstream river community. Importantly, it also suggests that the less diverse communities from the lake and the glacier stream are concealed downstream of the input sites by the higher diversity of the main river.

Shannon indices in the estuary samples were higher than those previously described for Arctic estuaries (Galand et al., 2006, 2008). Previous studies of large Arctic rivers show that bacterial diversity and abundance decrease from rivers to estuaries probably due to upstream input from terrestrial sources (Meon and Amon, 2004; Galand et al., 2006, 2008). Our results from a small river support this conclusion by showing a slight decrease in diversity from the main river sites (R1 and R5) to the estuary (**Figure 2**). The diversity in the estuary sites closer to the river and in the shallow samples could be expected to be higher than more distant and deep estuary samples due to a higher concentration of the river bacterial community, which is not evident from our results (**Figures 2**, **6**). This indicates that although the community structure from the river to the estuary aligns with previous results by showing a directional decrease in diversity, this directional structure cannot be detected further down the network, in the estuary transects. The lack of pattern in diversity and richness attributed to different depths of the estuary samples or the distance to the river mouth could partly be attributed to an insufficient resolution in sample depth. The low resolution might not allow for the detection of a clear plume

TABLE 2 | BIOENV analysis of the total, river ($n = 15$) and estuary ($n = 23$) communities and the indicator OTUs and non-indicator OTUs.

Community composition subsamples	Spearman's rho	Environmental variables
River, Non-indicator OTUs	0.5864	Turbidity
River, Indicator OTUs	0.5473	Turbidity
River, all OTUs	0.5559	Turbidity
Total community, Non-indicator OTUs	0.2021	Temperature, turbidity
Total community, Indicator OTUs	0.1976	DOC, turbidity
Estuary, Indicator OTUs	0.1480	Distance from river outlet, DOC, Salinity, Turbidity
Estuary, all OTUs	0.1015	Distance from river outlet, turbidity, TN, DOC, Total area of particles
Estuary, Non-indicator OTUs	0.0756	Distance from river outlet, turbidity, Total area of particles

TABLE 3 | Number of Indicator OTUs and Top Indicator OTUs across sample sites.

Sample	Number of Indicator OTUs*	Number of Top Indicator OTUs**
R1	352	1 (0.28%)
R2	678	34 (5%)
R3	194	5 (2.58%)
R4	158	1 (0.63%)
R5	560	1 (0.18%)
E100	99	0
E300	18	0
E700	43	0
E1100	346	0

** Including only indicator OTUs with Indicator Value ≥ 0.3 and P-value ≤ 0.05.*
*** Indicator Value = 1.*

and different depth zones. While distinct bacterial communities have been found to be associated with the plume and different oceanic zones in an estuary, these results are from sampled oceanic zones several kilometers farther into the ocean than our samples (Fortunato et al., 2012). Estuary samples were previously discussed as harboring a mix of bacterial communities from the river and the coastal ocean with no distinct autochthonous estuary-community (Fortunato et al., 2012), which resembles our results. The high variability in diversity found among the estuary samples suggests a highly heterogeneous community, also expected in such a region where waters of very different chemistry and origin meet. The large variability in diversity and richness seen at the different estuarine sites (**Figure 2**) could be explained by the sampling of the different water masses, since sequences from the three different transects were pooled together for each estuarine site and depth. The variability of diversity as well as environmental variables seem to lessen farther into the estuary, which could be expected as homogeneity increases as a greater fraction of the estuary is made up of marine waters (**Figure 2** and **Supplementary Table 1**).

Community Composition Analysis

Samples from the first site of the river (R1), upstream of the lake and glacier stream outlets, clustered with samples from the bottom site of the river (R5) while the river site by the proglacial lake outlet (R2) clustered with the river site at the glacier stream outlet (R3) as shown by the NMDS plot (**Figure 3**). R1 and R5 also shared a high number of indicator OTUs (**Figures 4A,E**) as well as similar diversity and richness (**Figure 2**). In contrast, R2 and R3 had lower diversity than the other river sites. The NMDS plots, indicator OTU analysis and alpha diversity results imply that waters sourced from the lake and the glacier stream carry different bacterial communities than that of the main river. The larger volume of the main river community then probably masks the lake and glacier stream communities, thus resulting in the close similarity between the sites R1 and R5. The isolation of the R4 samples from other river samples in the NMDS plots may be explained by the difference in sampling at this site, which was closer to the river bank compared to the other river samples. Another explanation might be the imperfect mixing of water from the upstream lake and glacier outlets with that of the main river at this site. The latter seems to be the best explanation since the comparably low number of indicator OTUs found at R4 suggests that this site contains a mixture of the upstream communities rather than a distinct community from the sampling site (**Table 3**). This agrees with results from a study on an Arctic tundra catchment, showing that streams leaving lakes have decreasing similarity to the lake microbial community as a function of distance (Crump et al., 2007).

The dispersal of estuary samples on the NMDS plots was in accordance with the diversity measures, which were similar within individual sampling sites independent of sample depth (**Figures 2**, **3**). Samples from the sites closest to the river mouth (E100 and E300) clustered more closely with river samples than the samples farthest from the river mouth (E700 and E1100), consistent with a gradual mixing of the river community with a marine community within the estuary environment. Remarkably, R4 river samples clustered more closely with estuary samples than with the other river samples. Indicator OTUs from R4 are present throughout the estuary transects (**Figure 6**) and the taxonomic composition of samples from R4 has the largest resemblance to that of estuary site E1100 (**Figure 5**), which might explain the NMDS results (**Figure 3**). Furthermore, the bacterial community at this site seems to be a mixture of the different river communities as suggested by the indicator OTU results (**Table 3**). Therefore, the clustering of R4 samples with estuary samples might also reflect the resemblance to the estuary, in which the river communities are also mixed (**Figures 4F**–I, **6**).

Samples from E100 and E300 were more widely dispersed across the NMDS plots than the samples from farther into the estuary, indicating greater heterogeneity of the bacterial communities. This is not unexpected from a region of mixing of largely different water bodies both in terms of physical and chemical variables as well as origin. The NMDS plot did not indicate that the bacterial communities were stratified according to sample depth. The low resolution of samples through the water column might be part of the explanation. However, the results

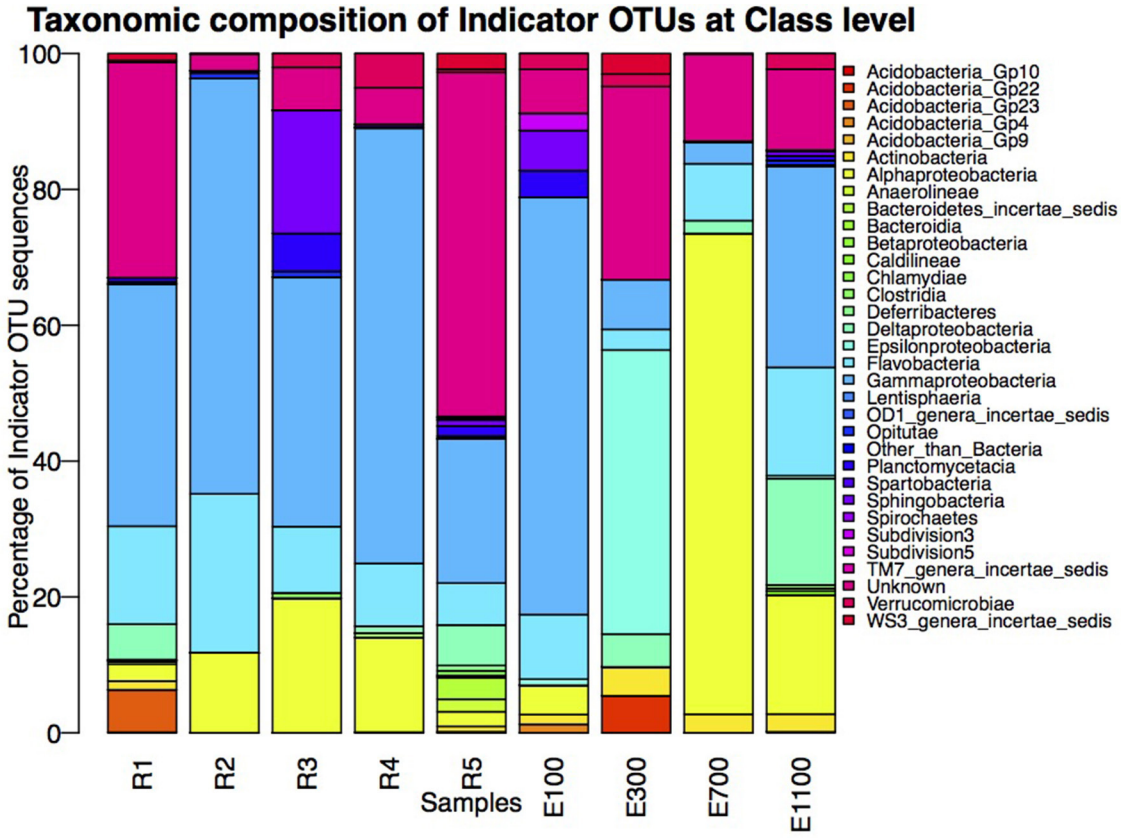

FIGURE 5 | Taxonomic composition of indicator OTUs at Class level. Percentages of indicator OTU sequences of each Class are calculated as percentage of the total number of sequences of indicator OTUs from the individual samples.

might also indicate a high degree of mixing through the water column of the Red River estuary at the time of sampling. As previously discussed, this may also be explained by the proximity to the river of the estuary samples in this study compared to other studies, where bacterial communities in the estuary were shown to be stratified according to depth (Fortunato et al., 2012).

Environmental Controls

The BIOENV analysis did not show strong correlations between environmental variables and community composition but it did highlight turbidity as a community-shaping factor in the river (**Table 2**). The bacterial community in a freshwater network fed by glaciers has recently been shown to be structured along the turbidity gradient (Peter and Sommaruga, 2016). The BIOENV results support the idea that the bacterial community in the Red River freshwater network is partly sourced from the glacier. The lower turbidity at R2 (1.2 NTU) compared to an average of 16.3 NTU ($SD = 1.2$) at the other river sites is noteworthy since proglacial lakes are known to have high turbidity (Peter and Sommaruga, 2016). While turbidity of the proglacial lake outlet (R2) is higher than that shown for a non-glacier fed lake in the Austrian Central Alps, it is remarkably low compared to other glacier-fed lakes (Peter and Sommaruga, 2016). This might indicate that the proglacial lake is losing hydrological connectivity to the glacier (Peter and Sommaruga, 2016). It should be taken into consideration that the samples are not taken from the actual lake but several 100m downstream (**Figure 1**).

The Red River is small in size compared to large rivers previously described such as the Mackenzie River (Galand et al., 2008; Garneau et al., 2009) and the Columbia River (Fortunato et al., 2013). For comparison, the average water flow from August to November in the Columbia River was 2988 m^3s^{-1} (Fortunato et al., 2013), while the river flow in the Red River around sampling time was 5.7 m^3s^{-1}. An estimated time from top sampling site R1 to the river mouth at R5 is 40 min for the moving water body where the samples are taken. It has been shown that at sites with shorter WRT than 10 days the bacterial community composition was predominantly structured by hydrology (Niño-García et al., 2016). Accordingly, we hypothesized that hydrology would be dominant in shaping the bacterial community in the relatively small Red River with short WRT. Consequently, we did not expect strong correlations between community composition and environmental variables in the river samples. Our study represents a single catchment with short WRT and the results of the BIOENV analysis agrees with previous results by showing weak correlations between the bacterial community and environmental variables (Niño-García et al., 2016).

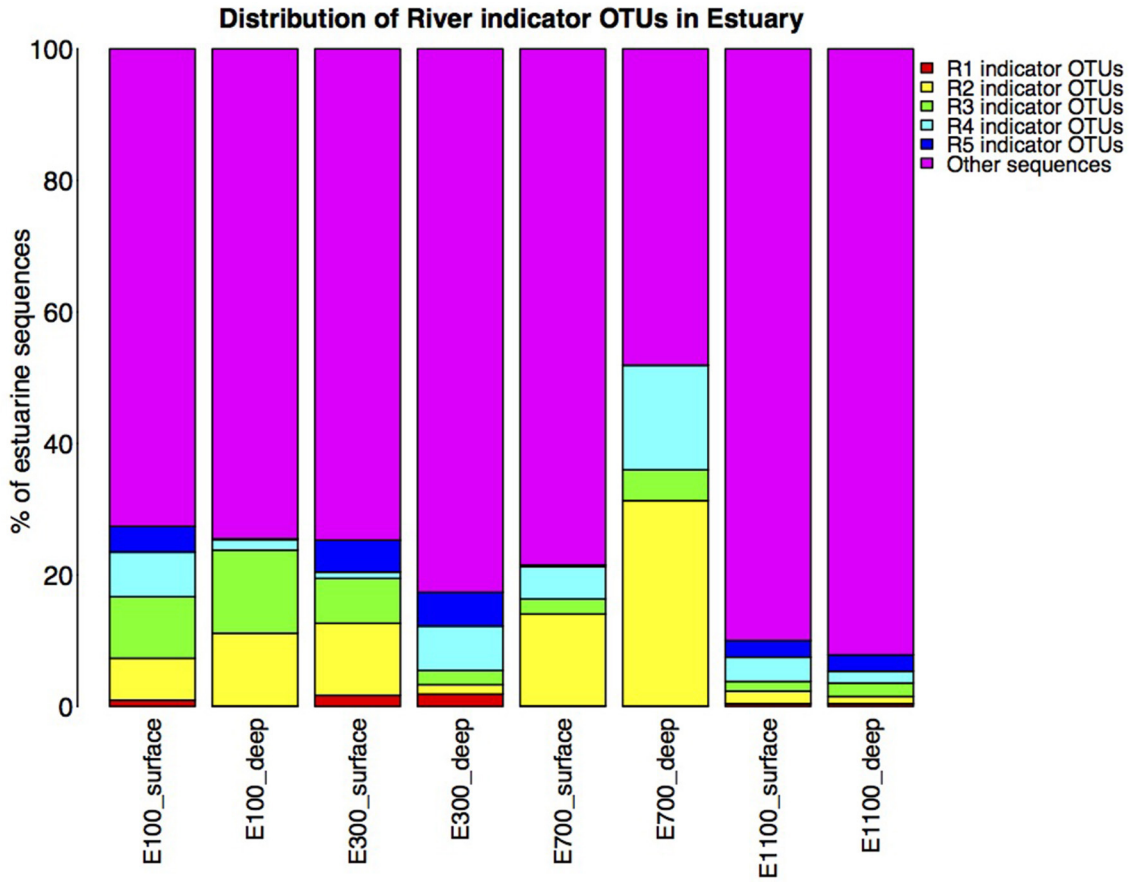

FIGURE 6 | Distribution of river indicator OTUs in the estuary. Calculated as percentage of river indicator OTU sequences of total estuary sequences of the estuary site in question. Note that the total number of sequences in each estuary site may differ due to different number of replicates as described in Section Materials and Methods.

Salinity has previously been highlighted as a community-shaping factor in estuaries and rivers. For example, the abundance of Alphaproteobacteria, Betaproteobacteria, and Actinobacteria correlated strongly with salinity in the Delaware estuary where a strong negative correlation between Betaproteobacteria and Actinobacteria was shown together with a positive correlation between salinity and Alphaproteobacteria (Kirchman et al., 2005). Salinity, together with temperature, explained 45% of the variation in the community composition in a study of the Mackenzie Shelf (Garneau et al., 2009). Salinity was not identified as a significant factor in the BIOENV analysis. The lack of correlation to salinity in our study is also evident from the NMDS analysis (**Figure 3**), where R4 river samples cluster with estuary samples despite the large difference in salinity between these environments (**Table 1**). These results suggest that there may be environmental or hydrological factors other than salinity that explain the observed patterns in taxonomic composition in the study site.

BIOENV analysis of the estuary community showed no significant correlations with environmental variables (**Table 2**). Also no correlation was found between the bacterial community and spatial variables including distance from the river mouth and depth. The results from the BIOENV analyses indicate that the bacterial community in the estuary is not dispersed according to environmental variables or stratified according to distinct water bodies of riverine or oceanic origin, supporting the results from the diversity assessments (**Figure 2**) as well as the NMDS plots (**Figure 3**). Our samples represent a very small fraction of the total estuary; a higher resolution of samples in the estuary might result in more conclusive results.

Indicator Taxa Analysis

Indicator OTUs identified at the input sites from the lake and glacier stream were found in low numbers at the other river sites and, therefore, seem to be specific to their respective sources (**Figures 4B,C**). Notably, of the 678 indicator OTUs from the lake outlet (R2, **Table 3**), 570 OTUs were not found in the upstream river site (R1) and seem to originate from the proglacial lake. The lake outlet site (R2) had a particularly high number of indicator OTUs and percentage of top indicator OTUs (**Table 3**). Water bodies with longer WRT have been shown to harbor a less diverse and more differentiated community explained by local sorting of

the microbial community (Niño-García et al., 2016). Results from the indicator OTU analysis and diversity of the lake outlet site (R2) show that the lake with a longer WRT has a less diverse and more specialized community compared with the river. A number of taxa known to be psychrophilic, such as *Moritella* (Urakawa et al., 1998), *Polaribacter* (Gosink et al., 1998), *Oleispira* (Yakimov et al., 2003), *Crocinitomix* (Bowman et al., 2003), and *Psychromonas* (Mountfort et al., 1998), were found among the best matches for the indicator OTUs from the lake outlet, unlike at the other river sites.

The short WRT in the Red River network should accordingly result in a low degree of differentiation, which is confirmed by the low number of top indicator OTUs (i.e., OTUs unique for a particular site), which average <2% in present study (**Table 3**). Another study of freshwater networks highlighted an average of 11% unique OTUs between different ecosystems as representing a low number (Ruiz-González et al., 2015). These results differ from our study in that they considered many different lakes while our results are obtained from one lake only (Ruiz-González et al., 2015).

Samples from the lake and glacier outlets (R2 and R3) as well as the sample site just after the glacier outlet (R4) had very few Acidobacterial classes compared to the top and bottom site of the river (**Figure 5**). Acidobacterial classes were shown to be most common in soil compared to the adjacent freshwater network and Acidobacteria in rivers seem to be sourced from the surrounding terrestrial environment (Ruiz-González et al., 2015). The more differentiated lake community thus seems to harbor a lower fraction of organisms from the surrounding soil community compared to the main river. This might partly be explained by the local sorting of the bacterial community in the lake. It could potentially also to be explained by the presence of taxa with different origin than the main river community, as indicated by the large number of indicator OTUs, which are not present in the upstream site. The lower turbidity at the lake outlet site (R2) might also indicate that there is less input of soil to the lake than to the main river, which causes less mass dispersal effect from the surrounding terrestrial environment. The lower turbidity might, however, also be explained by less suspended particles in the lake because of increased sedimentation due to the longer WRT.

A great number of previous studies of riverine microbial communities have suggested and shown that the river communities are influenced by input of microorganisms from surrounding soil environments (Crump and Baross, 2000; Galand et al., 2006, 2008; Crump et al., 2012, 2007; Ruiz-González et al., 2015; Niño-García et al., 2016). Our results support this by showing that potentially soil-related taxa make up a significantly large fraction of the bacterial community making them part of the indicator OTUs of the main riverine bacterial community (**Figure 5**). Notably, the results also indicate that, along the river, distinct communities may not have the same degree of influence from the terrestrial surroundings. August is a month of high precipitation and increased erosion around the Red River, which would result in a relatively high influence from the surrounding soil community. The influence from soil may be less pronounced in other months as water flow and erosion levels change.

The dominance of soil microbes in freshwater networks has been established in several recent studies, highlighting soil as the origin of the network metacommunities (Ruiz-González et al., 2015; Niño-García et al., 2016). A gradual differentiation of a stream from an upstream lake as a function of distance has been attributed to the origin of the freshwater communities from a terrestrial metacommunity (Crump et al., 2012, 2007). Our results suggest that glaciers may also supply part of the metacommunity resulting in a different structuring pattern of the network. In our case the structuring pattern was not unidirectional throughout the network but rather showed local changes as different bacterial communities were added to the river. This is illustrated in the diversity results (**Figure 2**) as well as the NMDS plots of the community composition (**Figure 3**). These results together with the indicator OTU analysis highlight the importance of additional sources of the metacommunity such as glaciers.

The indicator OTUs from the lake-sourced water (R2) can be found in the second highest abundance in the estuary site 700 m into the estuary (E700) (**Figures 4B**, **6**). The taxonomic composition of E700 differs from the other estuary sites and this site contains a high fraction of Alphaproteobacteria (71%) and a relatively small fraction of Gammaproteobacteria (3%) (**Figure 5**). Of the 678 R2 indicator OTUs 161 are found at the estuary site 700 m into the estuary, where they make up 20% of the sequences at E700 with a higher fraction in the deep samples compared to the surface samples (**Figures 4B**, **6**). This resembles results from the Columbia River, where the estuary samples were comprised of just over 20% riverine community (Fortunato et al., 2012). The distribution of R2 indicator OTUs suggests that although the organisms from the lake do not form a large enough fraction of the community to be notable along the downstream river, they are transported into the estuary where they form a larger fraction of the community. Our results align with the "landscape reservoir" concept proposed for the Toolik lake, Alaska, where rare organisms from the upslope landscape influence downslope bacterial diversity and become dominant in environments with favorable conditions (Crump et al., 2012).

The taxonomy of the nine indicator OTUs from R2 found in high numbers (>100 sequences) in the E700 samples were mostly related to organisms isolated from oceanic environments such as *Marinomonas* (Van Landschoot and De Ley, 1983), *Oleispira* (Yakimov et al., 2003), *Pseudoalteromonas* (Bowman, 2007), *Polaribacter* (Gosink et al., 1998), and *Sulfitobacter* (Sorokin, 1995). Of related non-marine organisms were *Glaciecola*, which was first described as a Gammaproteobacterium isolated from Antarctic sea ice (Shivaji and Reddy, 2014) and *Rhodobacteraceae* known from aquatic environments (Pujalte et al., 2014). The fact that indicator OTUs from the proglacial lake outlet to the river are similar to known marine organisms suggests that these organisms are commonly found in marine environments and that they are not originally known from terrestrial environments. Since it is unlikely that organisms are transported from the estuary to the proglacial lake over 2 km upstream, these organisms in the estuary more likely originate from the upstream freshwater network. Possibly, they become such common organisms in the estuarine and marine environments, that these

are the environments from which they have become known. It is well established that bacterial communities found in freshwater networks can be traced from upstream positions in the network (Crump et al., 2012; Ruiz-González et al., 2015; Niño-García et al., 2016). We show that in the Red River estuary the river community can be found in the estuary with an overall decreasing fraction from the river mouth toward the ocean (**Figure 6**). Interestingly, communities that are not notable throughout the river are transported to the estuary where they seem to become an equally large fraction of the estuary as the main river community (**Figures 4B**, **6**). Distinct communities from the river seem to influence the estuary to different extend, so that communities from certain parts of the river make up notably larger fractions of the estuary at some sites (**Figures 4**, **6**).

"Seed bank" is a term proposed for the fraction of dormant organisms that may be resuscitated when met with different environmental conditions through e.g., dispersal to other ecosystems (Lennon and Jones, 2011). The concept of seed banks was recently extended to freshwater networks where organisms originating in a soil community were proposed as the seed bank for boreal freshwater networks (Ruiz-González et al., 2015). For freshwater networks it was discussed that shallower sequencing depth might lead to the erroneous conclusion that freshwater communities do not derive from a shared pool of terrestrial microbes (Ruiz-González et al., 2015). This could lead to an incomplete understanding of the mechanisms of assembly and the actual linkages and dispersal of microbes between connected ecosystems (Ruiz-González et al., 2015). We show that sampling resolution not only in terms of sequencing depth but also resolution along the network may result in overlooking distinct microbial communities and how these are distributed and linked to the downstream estuary. Our results indicate that not only the terrestrial surroundings but also upstream glaciers may act as seed banks for freshwater networks. While the uni-directional structure in freshwater networks might be a consequence of the numerical dominance of terrestrial OTUs as shown previously (Crump et al., 2012; Ruiz-González et al., 2015) our results suggest that this does not necessarily imply that the bacterial community in a freshwater network has a common origin from microbes from soil. A higher resolution along the river might reveal distinct bacterial communities of different origin and with different composition, which are introduced downstream in the network. These distinct communities, which might be concealed by the numerically dominant terrestrial community along the river, are able to act as seed banks for downstream environments. The different composition of inputs along the river affects the structure of the community, which is not necessarily uni-directional for all freshwater networks as shown in the present study.

Therefore, sampling with the right resolution, both in terms of sequencing depth and the distance between samples along the network, is crucial for understanding the source of microbial communities found in the estuary. This is especially true at times with high precipitation and erosion. Our study shows that with the right resolution, microbial communities can be valuable in understanding transport pathways of meltwater and matter from source to oceans in that they can serve as both tracers as well as indicators of origin in their adaptation to the environment.

The indicator OTUs for each sample site in the estuary were found only in low numbers at the other sites both in the river and the estuary. This pattern is in contrast with the results from the Columbia River, USA, where indicator OTUs from the upper water-column of the estuary (<56 m depth) showed generalist taxa qualities by having high relative abundance and occurrence in a high number of samples outside their indicator environment when compared to the river environment (Fortunato et al., 2013). Ocean-specific taxa are not expected to be found upstream in the river, which is also apparent from the distribution of estuary indicator OTUs (**Figures 4F–I**). The low number of indicator OTUs and lack of top-indicators in the estuary show that the different sites in the estuary do not hold distinct communities. These results are in accordance with the results of the BIOENV analysis and NMDS plots, which suggest that the bacterial communities are not dispersed according to environmental or spatial variables, as well as the highly variable diversity measures in the estuary. As previously discussed, samples in the present study are sampled relatively close to the river mouth and a more distinct stratification of the bacterial communities might become visible farther into the estuary.

We expected the estuary sites to contain a mixture of the communities found in the river and the ocean, with more environmental variability closer to the river mouth due to the mixing of river- and sea-water. This is supported by our data, which show greater variance in environmental data closest to the river mouth (**Supplementary Table 1**) as well as river indicator OTUs from more of the river sites closer to the river mouth at E100 and E300 (**Figure 6**). Mixing of river and ocean water may result in an allochthonously dominated community shaped by hydrology rather than by environmental selection, also indicated by the NMDS plot (**Figure 3**) and BIOENV analysis (**Table 2**). This is supported by the fact that the outermost sample site of the estuary (E1100) had a higher number of indicator OTUs (**Table 3**) as variability is expected to decrease with increasing distance from the river mouth and the most distant estuary site is expected to contain a higher number of ocean indicator OTUs. This was supported by the taxonomy of the indicator OTUs that were all similar to marine-related taxa at E1100 (details not shown). This site also had a lower fraction of river indicator OTUs compared to the estuary sites closer to the river mouth (**Figures 4**, **6**).

CONCLUSIONS

The bacterial community in the Red River, a small river on the Disko Island, West Greenland, is sourced partly from the surrounding terrestrial environment but also receives distinct microbial communities from a proglacial lake and a glacier stream that harbor lower diversity and different composition than the main river. These input communities are less influenced by terrestrial sources than the main river and the proglacial lake input has a higher fraction of OTUs resembling psychrophilic taxa. The combined community in the river is then mixed with oceanic waters in the estuary, where the indicator OTUs of the river communities made up on average 23% of the estuary community at different sites. While the indicator OTUs from the lake and glacier outlets are not notable in

the downstream river they make up large fractions of the community at some sites in the estuary. The bacterial community of the river showed a weak correlation to turbidity while the estuarine bacterial community showed no correlation to environmental or spatial variables. Our results illustrate the added value of examining bacterial communities to better understand and trace the transport of meltwaters from their source to the oceans. Lastly the results show that sampling resolution along the river is crucial for understanding the source of different bacterial communities in a river and estuary system.

AUTHOR CONTRIBUTIONS

CJ and TM designed the study and sampled, NO performed sample preparations and DNA sequencing, AH performed bioinformatical analyses with contributions from JB and TS and statistical analyses with contributions from MS, AH wrote the manuscript with contributions from MS, TM, NO, BE, and CJ. All authors discussed the results and reviewed the manuscript.

FUNDING

This work was supported by the Center for Permafrost (CENPERM) Center no 100 from the Danish National Research Foundation (DNRF100) as well as the Novo Nordisk Foundation Center for Biosustainability.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01474

Supplementary Figure 1 | Rarefaction curve showing number of observed species against sequences per sample.

Supplementary Table 1 | Statistics of environmental data for estuary samples.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.3 Cryoconite

Cryoconite debris consists of microbe-mineral aggregates on glacial surfaces (Takeuchi, Kohshima, and Seko 2001). These aggregates reduce albedo locally and melting of the ice surrounding the cryoconite results in the phenomenon of cryoconite holes (Takeuchi, Kohshima, and Seko 2001). Cryoconite microbial communities are popularly known as hot-spots of microbial diversity and activity in the cryosphere (Edwards et al. 2011; Anesio et al. 2009).

Cryoconite microbial communities are interesting from a biogeographical point of view in that they are well defined and similar habitats found in very different geographical regions. It has been discussed to which degree cryoconite microbial communities are endemic (Cameron et al. 2016) and whether they vary on a temporal scale (Musilova et al. 2015). Studies have shown that microbial communities in cryoconite are different in alpine and glacial ice (Edwards et al. 2014) and within a region they vary at a scale of tens of kilometers (Cameron et al. 2016). The microbial communities have shown to be stable over an ablation season (Musilova et al. 2015).

Cryoconite habitats share many features with snow habitats and yet the microbial communities of these two habitats have shown to be very different (Cameron et al. 2015; Musilova et al. 2015). The discussed origin of cryoconite microbial communities are long-distance transported microbes from other cold habitats and atmospheric deposition from proximate terrestrial environments (Musilova et al. 2015; Stibal et al. 2015; Edwards et al. 2014).

There are clear signs that the cryoconite microbial community is adapted to its habitat, indicating a selection by the environment, and interestingly that the microbes in turn also shape their environment. An important hypothesis in cryoconite microbial ecology is the concept of ecosystem engineering, that is the active shaping of the ecosystem by the microbial community (Langford et al. 2010). Filamentous and extracellular polymeric substance-forming Cyanobacteria are abundant in cryoconite. It has been hypothesized that the filamentous and sticky character of these microbes help aggregate particles and thus initiate the formation of cryoconite (Langford et al. 2010; Gokul et al. 2016).

Studies of cryoconite have shown very different fractions of Cyanobacteria in these communities (Franzetti et al. 2016; Cameron et al. 2016; Edwards et al. 2014).
This is also interesting as Cyanobacteria could be important for producing bio-available carbon in these isolated and resource scarce ecosystems (Cameron et al. 2016). This Cyanobacteria discrepancy was also discussed for the snow environment and has yet to be resolved.

PAPER III: Contamination of the Arctic reflected in microbial metagenomes from the Greenland ice sheet

Paper III illustrates the global context of a cryosphere community and how extensive data from the newest NGS technologies enabled the proposal of new hypotheses. The idea of contamination of the cryosphere is not new (Edwards et al. 2013; Møller et al. 2010), but through broad and deep sequencing and through the extraction of full genomes from shotgun metagenomes Paper III paints a picture of a community adapted to contamination to a previously unacknowledged degree. While the results are obvious, they could easily have been overlooked if the right question had not been asked. The large amounts of data only revealed the presence of genes and genomes related to contamination through in-depth assessments of the data. Had we not posed an initial hypothesis regarding the potential adaptation of the cryoconite community to a contaminated habitat, we would not have obtained this result. This shows the importance of hypotheses in cryosphere biogeography, while also illustrating the potential for NGS data to test stated hypotheses.
There are several interesting implications of the results from Paper III.

First, it is likely that the cryoconite microbial community has adapted not only to currently deposited contaminants but also to the accumulated contaminants trapped in the ice deposited through decades. In this way, the microbial community of this seemingly isolated environment is shaped not only by global influences, but influences from an extensive temporal dimension in addition to the great spatial dimension. This supports the suggestions for future biogeography research described in the Microbial Biogeography section of part I of this thesis. According to this, biogeography research should aim at sampling systematically to better distinguish between contemporary and historical factors (Martiny et al. 2006).

Secondly, there was a surprising similarity between patterns in human contamination around the coast of Greenland and microbial contamination expressed by genes associated with contaminants corresponding to those found in Inuit. This encourages further studies in the link between deposited contaminants in the cryosphere, the entry of contaminants at the bottom of the food chain, and the bioaccumulation of contaminants all the way through the food chain.

The notable presence of contamination-related genes and genomes suggests that contaminants are a major factor in shaping the microbial community in cryoconite. In a community with higher diversity of microbes and with less limited resources the signature of contamination might not have been as strong as seen for cryoconite. Also therefore, cryoconite microbial communities might serve as a good indicator of entry of contaminants into an ecosystem and potentially the food chain.

In Paper III it was possible to extract 29 genomes from shotgun metagenomic data, of which 16 genomes were complete according to their content of single copy housekeeping genes. The method used for extracting genomes (Nielsen et al. 2014) performed well on this relatively simple microbial community, while the same method tested on the complex community of permafrost samples prior to this study did not perform equally well (unpublished data). This illustrates well how the large amounts of data generated by NGS technologies poses difficulties and how we are gradually increasing our potential for exploring complex microbial communities through new bioinformatical tools.

For 56% of the binned genomes the closest neighbors were organisms known to be able to grow filamentously. As described above, previously ecosystem engineering of cryoconite ecosystems has been attributed to the Cyanobacterial fraction of the community alone. The binned genomes suggest that members of the cryoconite community other than Cyanobacteria, including also heterotrophs, may play a role in the function of ecosystem engineers. These results were not included in the study as this was not part of the focus.

Contamination of the Arctic reflected in microbial metagenomes from the Greenland ice sheet

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Abstract

Globally emitted contaminants accumulate in the Arctic and are stored in the frozen environments of the cryosphere. Climate change influences the release of these contaminants through elevated melt rates, resulting in increased contamination locally. Our understanding of how biological processes impact Arctic contamination is limited. Through shotgun metagenomic data and binned genomes from metagenomes we show that microbial communities, sampled from multiple surface ice locations on the Greenland ice sheet, have the potential for degradation of, and resistance to contaminants, which is suggestive of their adaptation to contaminated habitats. Bacterial potential to degrade anthropogenically-sourced contaminants, such as toxic and persistent polychlorinated biphenyls, was found to be spatially variable. These results indicate that microbial communities of the Greenland ice sheet might serve as a signature for first entry of contaminants into the Arctic food chain. We

propose that cryoconite communities may act as first-response indicators of Greenland ice sheet contamination.

Introduction

The Arctic ecosystem and food chain is strongly influenced by anthropogenic contaminants[1]. Long-range atmospheric transport is the major determinant in the global distribution of contaminants[2]. Contaminants from around the globe become trapped within the snow and ice of the cryosphere, resulting in these regions acting as reservoirs of toxic chemicals[2].

Climate change and concurrent increased melting of the cryosphere has resulted in increased release of contaminants to the polar regions[2,3]. Melting glaciers are becoming secondary sources of contaminants such as persistent organic pollutants (POPs). Specifically, melting mountain glaciers are considered local sources for POPs such as polychlorinated biphenyls (PCBs)[2,4]. Dynamic interconnections exists between historic and current emissions for POPs, the cryosphere and climate change, and it has been shown that climate change has lead to increased release of POPs from Alpine glaciers since the 1990s[3].

The role that microbial communities play in the fate of Arctic contaminants has been identified as a knowledge-gap[2]. Microbial communities sampled from cryoconite holes, that is supraglacial holes containing mineral-microbe aggregates, have been shown to harbor genes associated with heavy metal resistance[5]. Cultured isolates from cryoconite communities are known to be able to degrade xenobiotics including PCBs[6]. Cryoconite microbial communities from the Greenland ice sheet incubated under *in situ* conditions are able to degrade pesticides, suggesting that the supraglacial microbial community might remove a fraction of the contaminants deposited to this environment before they can be released to downstream ecosystems[7]. Similarly, it has been suggested that bacteria in snow might lower the risk of bio-available mercury being incorporated into Arctic food chains[8]. The potential for mercury reduction among microbes in Arctic coastal seawater has been found to be able to account for most of the Hg(0) that is produced in high Arctic waters[9]. Mercury concentrations in snow have been found to correlate with mercury resistance gene copy numbers of snow microbiota[10]. In the culturable fraction of Bacteria in snow from the high Arctic, 31% of the community were shown to be mercury-resistant, compared to less than 2% in proximate environments of freshwater and brine[8].

In this study we address the potential for degradation and resistance to contamination by microbial communities sampled from cryoconite around the Greenland ice sheet. Industrial emissions have resulted in deposited contaminants such as black carbon on the Greenland ice sheet since the 1850s[11]. We hypothesized that contaminants deposited on the nutrient-limited Greenland ice sheet influence the microbial community composition, resulting in microbiota with the ability to utilize and resist long-distance transported contaminants including PCBs, polycyclic aromatic hydrocarbons (PAHs) and heavy metals such as mercury and lead. To the best of our knowledge this is the largest shotgun metagenomic dataset of cryoconite to date, and the first investigation that puts focus on cryoconite as a contaminated habitat.

Materials and Methods

Sampling

Sampling has been described previously [12,13]. In short, 34 cryoconite samples were collected from the Greenland ice sheet in five different locations between May and September 2013. Locations were Tasiilaq (TAS) in Southeast Greenland, Qassimiut (QAS) in Southwest Greenland, Kangerlussuaq (KAN) in West Greenland and Dark Site (DS) also in West Greenland, just inland from the Disko Bay area, and lastly Thule (THU) in Northwest Greenland. Sample details and map of sampling sites are available from previously published results [12,13]. Within sampling regions A,B and C samples were sampled 500 meters apart, within these samples 1,2 and 3 were sampled 10 meters apart and finally within these samples a and b were sampled 20 cm apart.

Library preparation and Sequencing

DNA extraction was performed as previously described in Cameron *et al.*, 2016[12]. DNA shearing and library preparations were performed according to the NEXTflex Rapid DNA-Seq Kit**,** V13.08 (Bioo Scientific, Austin, TX, USA). Briefly, 250ng genomic DNA was sheared by Covaris E210 System using 10% duty cycle, intensity of 5 cycles per burst of 200 for 300 seconds to create 200bp fragments. The samples were end-repaired and adenylated to produce an A-overhang. Adapters containing unique barcodes were ligated on to the DNA. Samples were then

purified using bead size selection for a selection range around 300-400bp with the Agercount AMPure XP beads (Beckman Culter, Beverly, MA, USA).
The purified DNA libraries were amplified with a denaturation time of 2 minutes at 98°C, followed by 12 cycles of denaturation at 98°C for 30 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 1 minute according to the protocol. The final extension was performed at 72°C for 4 minutes. Amplification was followed by DNA quantification using NanoDrop ND-1000 UV-VIS Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the quality was checked on an agilent 2100 Bioanalyzer using the Bioanalyser DNA High sensitivity (Agilent Technologies, Santa Clara, CA, USA). Library preparation was performed by in-house facilities (DTU Multi-Assay Core (DMAC), Technical University of Denmark). DNA libraries were mixed into 9 pools in equimolar ratios, resulting in 5 samples per sequencing lane. Sequencing was performed as a 100 bp paired-end run on HiSeq 2000 (Illumina Int., San Diego, CA, USA) following the manufacturer's recommendations.

Demultiplexing and Data Quality Check

The number of different barcodes in raw sequencing files were counted prior to demultiplexing into individual sample files. This was done in order to confirm that all barcodes were represented in roughly equal numbers in the sequencing output, and to check for contamination. Demultiplexed files for each of the samples from nine lanes of Illumina HiSeq sequencing were concatenated to obtain one complete sample file in fastq format[14]. Data quality was assessed using FastQC version 0.11.2[15] and reads were trimmed for bad quality bases and Illumina adaptors using Cutadapt[16] through MGmapper version 2.5[17]. Cutadapt settings were used with a minimum phred score for base quality of 30, and minimum read length of 30bp.

Scaffold Assembly and Gene Calling

Quality trimmed fastq files were assembled using idba_ud assembler version 1.1.1. with pre-correction[18]. Prokaryotic open reading frames were called using Prodigal version 2.6.2[19].

Binning of genomes

Files with genes called by Prodigal for each sample were concatenated into a gene catalogue and each entry was given a unique header. The gene catalogue was clustered to reduce homology at 95% identity using USEARCH package cluster_fast version 8.1.1861[20]. Centroids from the clustering were used in downstream analyses. For each sample raw reads were mapped against the indexed gene catalogue using bwa mem version 0.7.12[21]. Samtools version 0.1.18[22] was used to sort and convert sam format files from mapping to bam format files. An in house python script was used to count number of reads mapping to genes in the gene catalogue and the read counts were assembled into a count matrix with an in house perl script. The count matrix was rarefied to the depth of the shallowest sample with a depth of 49,671,209 read counts per sample. This was done using the Vegan package *rrarefy*[23] in R Studio version 0.99.903[24]. Genome bins were clustered based on gene co-abundance with the Canopy Clustering method with max canopy distance 0.1 and max merge distance 0.05[25]. A total of 29 bins with correlation to profile > 0.95 and between 3000 and 8000 genes were assembled from contigs to which binned genes belonged. The quality of the binned genomes was tested with Checkm[26]. The genomes were annotated and further assessed in RAST[27]. When comparing binned genomes with control genomes in RAST the control genomes used were *Escherichia coli* 536 publicly available in RAST (control 1), *Lactobacillus casei* ATCC 334 also publicly available in RAST (control 2) as well as *Belliella baltica* DSM 15883[28] (control 3).

MGmapper – taxonomic composition

MGmapper version 2.5[17] was used to remove PhiX and map reads from each sample against genome databases of Bacteria and Bacteria draft genomes. Results from mapping to the bacterial databases were used for making stacked barplot of taxonomic composition. Parameters for mapped reads included in taxonomic composition were properly paired reads, alignment score >30, minimum coverage 80% resulting in minimum identity of 86%, minimum 10 reads mapped to a strain for it to be evaluated as true.

Differential potential for degradation of xenobiotics and heavy metal resistance

Genes in the rarefied count matrix were annotated by blasting against KEGG database[29] using Diamond blastx version 0.7.9[30] resulting in BLAST tabular m8 format sequence IDs. Only genes from the original gene catalogue with blast hits E-value < 1e-50 were included in counts. There were several genes in the count matrix with identical sequence IDs although the count matrix is based on homology-reduced genes. This can be explained by the fact that homology reduction is sequence based and entries in the count matrix from different parts of a gene would have the same annotation but would not be combined in the homology reduction step (USEARCH cluster_fast). The counts of identical sequence IDs were combined and information on gene names and EC-numbers were added to each entry in the annotated and combined count matrix using in house perl scripts. Entries in the count matrix with identical gene names and EC-numbers were again combined (scripts are available upon request).

The genetic potential for degradation or resistance to a number of contaminants were selected based on known contaminants in the Arctic food chain[1]. Based on KEGG[29] K0 numbers or gene names the number of reads mapping to responsible genes were extracted from the rarefied count matrix.

For PCB degradation K08689, K08690, K00462 and K10222 were found in the count matrix[29]. For PAH degradation EC-numbers 1.1.1.256, 1.13.11.3, 1.13.11.38, 1.13.11.8, 1.14.12.12, 1.14.12.15, 1.14.12.7, 1.14.13.1, 1.14.13.23, 1.2.1.78, 1.3.1.19, 1.3.1.29, 1.3.1.49, 1.3.1.53, 1.3.1.64, 3.1.1.35, 4.1.1.55, 4.1.1.69, 4.1.2.34 were searched for[29]. For heavy metal resistance genes czc, chr, ncc and mer[31] as well as *Pbr*[32] genes were extracted from the count matrix.

Of other contaminants listed as important for the Arctic food chain[1] PFOS were not assessed as evidence suggests that these are not degraded by bacteria[33]. PBDEs were also not assessed as it is still unknown which genes are responsible for degradation[34].

For plotting the number of reads mapped to degradation and resistance genes and for comparison between samples, the total number of reads mapped to target genes were divided by the total number of reads mapped to 14 genes that have been found to be present in most bacterial genomes[35]. This was used as a measure of an internal constant, since the number of these genes could be expected not to differ between organisms. In this way, we obtained an approximation of the fraction of degradation and resistance genes in a sample.

Two metagenomic samples of healthy human gut were run through the same pipeline as described above. These samples were used as negative controls, and in particular, to confirm that genes detected for contaminant degradation and resistance were not background artifacts. Human gut metagenomes were downloaded from the European Nucleotide Archive study PRJEB1220[25]. We assume that the healthy human guts were non-contaminated. None of the contaminant related genes were found in these negative controls.

Results and Discussion

Table 1: Genome bin overview

Bin ID	Complete-ness	Contamina-tion	Strain heterogeneity	Contigs (number)	Size (bp)	GC content (%)	Closest neighbor in RAST (score)	Phylum	Family
81	100	548,45	99,52	2298	23062772	64.5	*Acidiphilium cryptum* JF-5 (501)	Proteobacteria	Acetobacteraceae
59	100	384,08	95,25	2700	15614510	58.8	*Acidobacterium* sp. MP5ACTX9 (516)	Acidobacteria	Acidobacteriaceae
58	100	910,4	99,08	2933	34698014	66	*Frankia* sp. EuI1c (532)	Actinobacteria	Frankiaceae
51	100	116,65	79,66	2481	8649830	44.5	*Chitinophaga pinensis* DSM 2588 (581)	Bacteriodetes	Chitinophagaceae
50	100	707,64	97,73	3490	26578570	35.4	*Chitinophaga pinensis* DSM 2588 (542)	Bacteriodetes	Chitinophagaceae
47	100	1305,01	95,36	4087	38823998	65.4	*Caulobacter* sp. K31 (513)	Proteobacteria	Caulobacteraceae
45	100	571,82	91,33	4737	26076195	58.7	*Acidiphilium cryptum* JF-5 (525)	Proteobacteria	Acetobacteraceae
41	100	782,94	98,52	3809	33343276	57.9	*Acidobacterium* sp. MP5ACTX9 (511)	Acidobacteria	Acidobacteriaceae
35	100	1051,64	99,63	4986	38873183	65.2	*Chloroflexus aggregans* DSM 9485 (393)	Chloroflexi	Chloroflexaceae
34	100	728,28	97,53	4400	31824964	36.8	*Chitinophaga pinensis* DSM 2588 (613)	Bacteriodetes	Chitinophagaceae
28	100	767,26	52,11	3772	27944005	64.8	*Clavibacter michiganensis* subsp. *michiganensis* NCPPB 382 (526)	Actinobacteria	Microbacteriaceae
22	100	651,67	98,87	5874	37385964	49.2	*Nostoc* sp. PCC 7120 (648)	Cyanobacteria	Nostocaceae
21	100	1117,12	94,33	6004	48259586	63.3	*Streptosporangium roseum* DSM 43021 (503)	Actinobacteria	Streptosporangiaceae
77	98,28	300,52	80,03	1909	9563229	63.4	*Mucilaginibacter paludis* DSM 18603 (838)	Bacteriodetes	Sphingobacteriacea
18	98,09	376,32	93,83	6064	23602306	56.8	*Ktedonobacter racemifer* DSM 44963 (549)	Chloroflexi	Ktedonobacteraceae
63	96,55	164,54	90,64	3102	9599541	64.7	*Acidiphilium cryptum* JF-5 (522)	Proteobacteria	Acetobacteraceae
73	95,83	463,02	98,19	3048	18407868	66.2	*Acidiphilium cryptum* JF-5 (514)	Proteobacteria	Acetobacteraceae
48	95,83	348,87	98,45	3134	17680617	65.4	*Gluconacetobacter diazotrophicus* PAl 5 (522)	Proteobacteria	Acetobacteraceae
29	95,83	668,8	99,58	5625	26933316	63.3	*Solibacter usitatus* Ellin6076 (501)	Acidobacteria	Solibacteraceae
09	95,83	432,39	64,45	6003	36290647	62.4	*Leptothrix cholodnii* SP-6 (507)	Proteobacteria	Unclassified
84	95,61	107,35	80,77	2010	5422601	60.7	marine actinobacterium PHSC20C1 (537)	Actinobacteria	Unclassified
30	92,11	75,41	93,02	4885	10047939	47.3	*Desulfuromonas acetoxidans* (501)	Proteobacteria	Desulforomonadaceae
40	88,95	95,22	6,67	5050	8729345	40.7	*Pedobacter heparinus* DSM 2366 (583)	Bacteriodetes	Sphingobacteriacea
69	67,46	43,03	9,09	3522	3811046	62.9	*Leifsonia xyli* subsp. *xyli* str. CTCB07 (517)	Actinobacteria	Microbacteriaceae

Clustering of genomes from metagenomes reveal adaptation to contamination

Metagenomic samples were binned into 29 genomes through *de novo* clustering by co-abundance genes (Supplementary Figure 1). Of the 29 genome bins, 24 were successfully annotated in RAST (Table 1). 13 bins were complete genomes based on their content of marker genes (Table 1; Supplementary Figure 1). The strain heterogeneity of detected marker genes were above 90% for 11 of the 13 bins that were 100% complete (Table 1). GC content ranged between 35.4% and 66.2% (mean=57.7±9.8).

The closest neighbors of resulting putative genomes were one Cyanobacterium (*Nostoc* sp. PCC 7120), five Proteobacteria, five Actinobacteria, three Bacteroidetes, two Acidobacteria and two Chloroflexi (Table 1). These phyla were all among the more abundant (>1%) in the metagenomic samples (Figure 1). Half of the genome bins belong to families shown to represent above 1% of OTUs in the sampled sites according to a previous study based on 16S rRNA gene amplicon sequencing[12]. These were Acetobacteraceae, Acidobacteriaceae, Solibacteraceae, Microbacteriaceae, Chitinophagaceae, Sphingobacteriaceae, and Nostocaceae (Table 1). The mean richness calculated as CatchAll alpha diversity of the samples grouped by area were previously estimated to be between 31 and 116 species in these samples based on 16S rRNA gene sequencing[12]. The 18 different species found among the binned genomes therefore potentially makes up on average between 58% and 15.5% of the community.

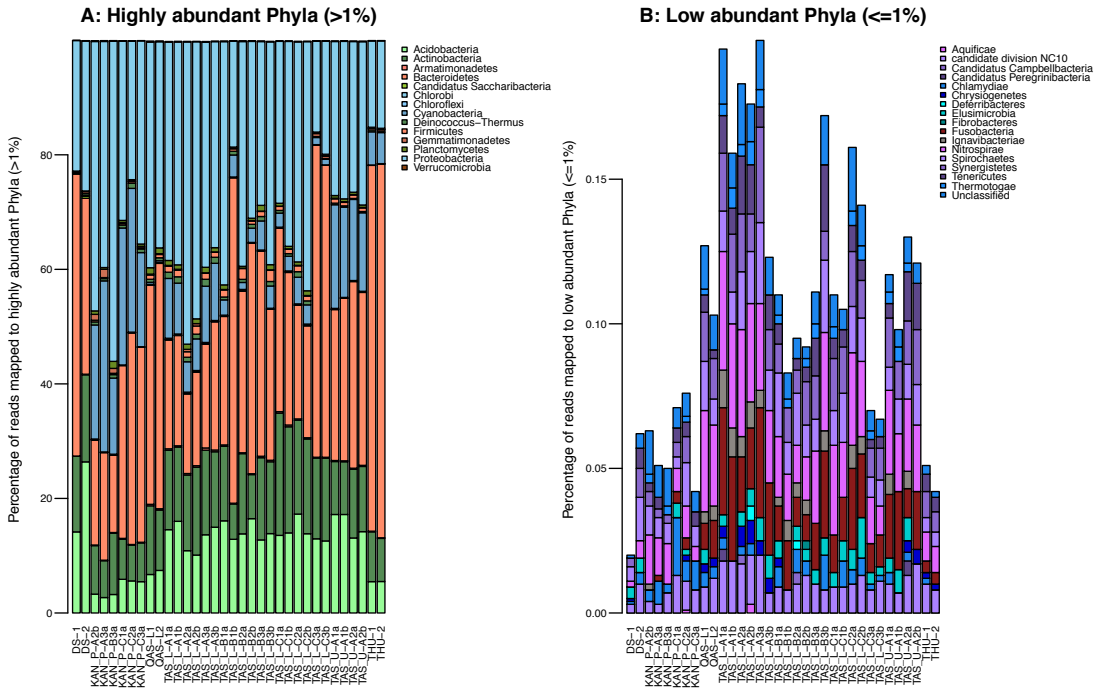

Figure 1: Taxonomic composition across samples of A) most abundant Phyla (>1%) and B) low abundant Phyla (≤1%)

Among the closest neighbors to the binned genomes were a number of organisms isolated from contaminated habitats or known to be resistant to contaminants. The closest neighbor of bin 9, *Leptothrix cholodnii* SP-6, has received interest as a potential bioremediation organism due to its ability to oxidize iron and manganese and potential ability to sequester heavy metals[36]. *Frankia* sp. strain EuI1c, the closest neighbor of bin 58, belongs to a group of organisms known to be less sensitive to heavy metals such as lead and arsenic[37] and this strain in particular has been shown to have cellular mechanisms for increased copper tolerance[38]. The closest neighbor of bin 47, *Caulobacter* sp. K31, was isolated from chlorophenol-contaminated groundwater and has the ability to tolerate copper and chlorophenols[39]. Four of the bin genomes (bins 81, 45, 73 and 63) were closest neighbors to *Acidiphilium cryptum* JF-5, which was isolated from acidic coal mine lake in eastern Germany and was shown to be able to reduce Fe(III)[40]. These binned putative genomes indicate that cryoconite holes on the Greenland ice sheet are potentially contaminated habitats that contain organisms with the ability to survive and utilize long-distance transported contaminants.

The percentage of genes in different contamination-related active subsystems were compared among the genome bins listed above. These were compared to three controls, genomes of known organisms that are not from cryoconite, namely *Escherichia coli*, *Lactobaccillus casei,* and *Belliella baltica*, an isolate from the Baltic Sea[28].

There were a number of active subsystems associated with contaminants, for which some binned genomes had a comparably high percentage of genes (Figure 2). Bin 9 (nearest neighbor *Leptothrix cholodnii* SP-6) had a notably high percentage of genes for zinc resistance (Figure 2A). Bin 73 (nearest neighbor *Acidiphilium cryptum* JF-5) had high percentages of genes in mercuric reductase, copper tolerance, and resistance to chromium compounds as well as metabolism of aromatic compounds (Figure 2B-E). In general, the binned genomes had relatively high percentages of genes in metabolism of aromatic compounds when compared to the controls (Figure 2E).

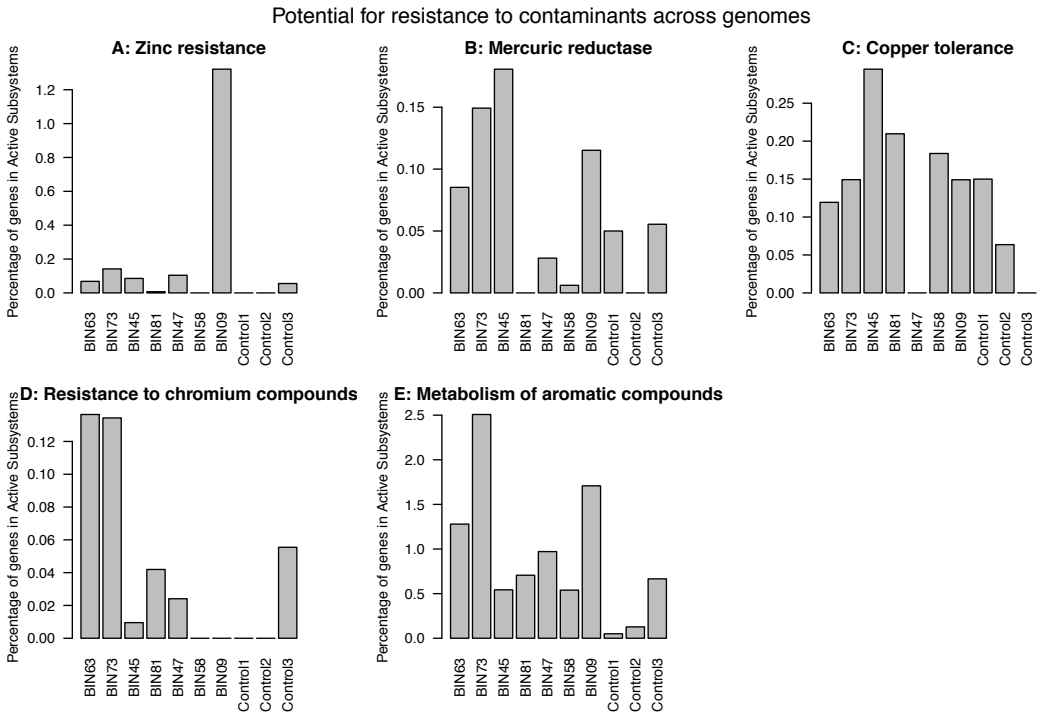

Figure 2: Percentage of genes in contamination-associated active subsystems of the total number of genes in active subsystems within each genome (count data from RAST). Control genomes were organisms known from other habitats: *Escherichia coli* 536 (control 1), *Lactobacillus casei* ATCC (control 2) as well as *Belliella baltica* DSM 15883 (control 3).

Heavy metals mercury and lead, and persistent toxic compounds such as dichloro-diphenyl-trichloroethan (DDT) and polychlorinated biphenyls (PCB) are well-known contaminants in Greenland and have been found to bio-accumulate in the food chain[1]. Polycyclic aromatic hydrocarbons (PAHs) have been found on the Greenland ice sheet[41]. Raw reads from each cryoconite community were mapped to microbial genes associated with degradation or resistance to Arctic contaminants, including genes involved in PCB, PAH and DDT degradation[29] and genes for resistance to mercury and lead[31,32]. Read counts were normalized to the number of reads mapped to housekeeping genes in each sample (Figure 3A-E).

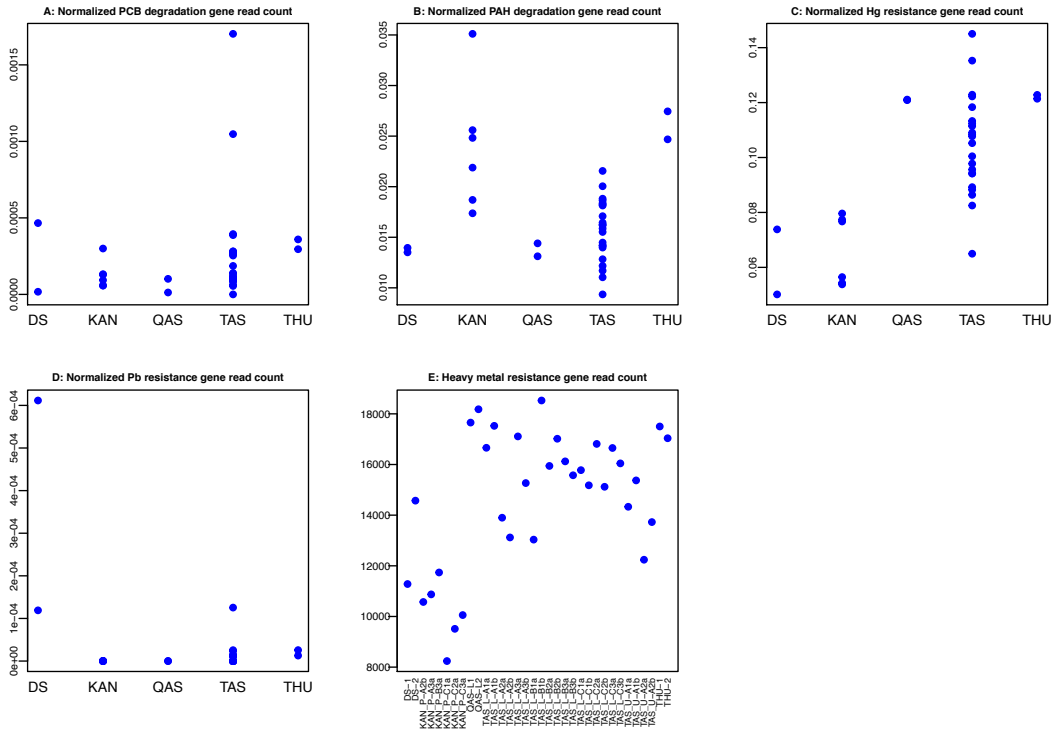

Figure 3: Read counts normalized to counts of reads mapped to housekeeping genes for reads mapped to A) PCB degradation genes, B) PAH degradation genes C) Mercury resistance genes, D) Lead resistance genes and E) general heavy metal resistance genes. Note that counts in panel E have not been normalized to housekeeping genes.

All sample sites had reads that mapped to PCB degradation genes (Figure 3A). Counts of reads mapped to PCB degradation genes were variable between samples taken from each region. The highest number of reads mapped to PCB degradation genes were found among the TAS samples from Tasiilaq in Southeast Greenland,

ranging from 0 (TAS-U-A1b) to 145 (TAS-L-C3b) reads per sample (normalized counts $2.8e^{-4} \pm 3.8e^{-4}$) (Figure 3A, Supplementary Table 1). Two TAS samples had over twice as high counts of PCB degradation genes compared to all of the other samples (1.70e-03 and 1.05e-03 PCB degradation reads/housekeeping reads).

The KAN samples from Kangerlussuaq had the most reads mapped to degradation of PAHs followed by the THU (Thule) samples (Figure 3B). The relatively high abundance in the KAN samples contrasts the pattern from the other contaminants, where the KAN samples had relatively low abundance of reads (Figure 3A,C,D,E). PAHs are generated primarily through the incomplete combustion of organic materials such as oil, petrol and wood[42]. The PAH content in snow from the Kangerlussuaq region was assessed previously under the hypothesis that the proximity of the site to the largest airport of Greenland could cause PAH contamination[41]. While PAH contamination was found to be below detection limit in the study, the results were inconsistent with previous results and well below the concentrations found in near-by cryosphere environments[41,43,44]. The relatively high abundance of reads mapping to PAH degradation genes in first Kangerlussuaq and next Thule samples might be explained by the proximity of these sites to the largest commercial airport of Greenland (Kangerlussuaq) and the Thule air base.

The number of reads mapped to mercury resistance genes ranged from 4644 (KAN-P-A2b) to 10283 (TAS-L-B1b) (unnormalized counts 7671±1586.5). The highest normalized read count of mercury resistance genes were also found in the TAS samples (Figure 3C). The two QAS and the two THU samples had similar counts, which were intermediate between the high TAS samples and the lower counts in the DS and KAN samples (Figure 3C, Supplementary Table 1). Mercury concentrations have been measured in the KAN, QAS, TAS and THU regions[41]. Concentrations were lower in snow from Kangerlussuaq (KAN) and higher in the South (QAS) and Southeast (TAS) (Supplementary Figure 2A), as seen for read counts (Figure 3C). Interestingly, Inuit from Tasiilaq in Southeast Greenland have also shown to have higher contamination with PCBs and mercury in recent years[1]. In contrast, no reads from any of the samples mapped to DDT degradation. In the Arctic regions concentrations of DDT have been declining since 1979 while PCB concentrations started decreasing in 1986[1].

Lead resistance read counts were found to have a distinct pattern of abundance, with very high numbers in one of the DS samples in West Greenland and

no reads in KAN and QAS samples in Southwest and South, respectively. The number of reads mapped to lead resistance genes ranged between several samples with 0 reads to 71 reads in the DS-1 sample (unnormalized counts 3.18±12.32). The DS-1 sample had almost six times as many reads mapped to lead resistance genes as the other samples (Figure 3D). In general, the KAN samples had lower numbers of reads mapped to the combined set of heavy metal resistance genes (Figure 3E). Lead concentrations on the Greenland ice sheet has been measured to be slightly higher in the Thule (THU) region (Supplementary Figure 2B)[41]. The pattern for lead concentrations (Supplementary Figure 2B) do not seem to resemple the pattern for read counts of lead resistance genes (Figure 3D).

Inuit lead contamination has been shown to be similar across Greenland with slightly lower contamination in Tasiilaq in Southeast Greenland[1], in contrast to other contaminants (Figure 3A-C,E), and in resemblance to the pattern observed for reads mapped to lead resistance genes in cryoconite (Figure 3D).

Our results show that microbial communities on the Greenland ice sheet have differential potential for degradation and resistance to long-distance transported contaminants known to the Arctic. These results suggest that deposition of bioavailable compounds is one factor that shapes microbial communities in cryoconite. The notion that supraglacial environments are innoculated through local and long-range transport through the atmosphere has been discussed previously[5,12,45–47]. Previous reports from these sampling sites showed that area was the strongest explanatory variable for community composition[12]. If microbial deposition from the atmosphere solely shaped the supraglacial communities, factors such as altitude, distance from the margin, position along the N-S transect as well as sample type (such as ice and cryoconite) would have negligable effects, which is not the case[12].

Contamination of supraglacial environments is constantly occurring and contaminants of previous times are locked into the ice at certain depths[48]. Temporal variability in contaminant release should therefore be considered, and bioavailable contaminant concentrations within cryoconite holes may indeed be dependent on the historic deposition events of the exposed ice surfaces. In this respect, hundreds of years of anthropogenic influence might be shaping cryosphere communities. It has been established that current contaminations in higher trophic levels are largely caused by

375 past events[49]. This might also hold true for microbial communities, particularly in
376 supraglacial habitats where contaminants are locked in the ice.
377
378 The observed pattern for microbial potential for degradation and resistance resembles
379 the one observed for the top of the food chain, the Inuit of Greenland. Our results
380 suggest that cryoconite microbial communities may serve as indicators for first entry
381 of contaminants into the Arctic food chain. This notion is not unlikely considering
382 that gene copy number of resistance genes is known to correlate with contaminant
383 concentrations in snow[10], and melting of the cryosphere has been shown to release
384 contaminants into the downstream ecosystems[2–4]. Cryoconite microbial communities
385 may adapt to historic contaminants released from the melt of frozen waters, which
386 will be transported to downstream locations. Further studies are required in order to
387 better understand the biogeochemical relationships between microbial communities
388 and contaminants. This has important implications for the release of contaminants
389 into the ecosystem as a result of climate warming in the Arctic.
390
391 **Conclusions**
392 The metagenome of the microbial community in cryoconite holes on the Greenland
393 ice sheet show clear potential for degradation and resistance to long-distance
394 transported contaminants including polychlorinated biphenyls (PCB), polycyclic
395 aromatic hydrocarbons (PAHs) and heavy metals including mercury and lead. Binned
396 genomes showed indications that the genomes and the closest neighbors of the
397 genomes have potential for degradation and resistance to contaminants, supporting the
398 idea that cryoconite is a polluted habitat.
399
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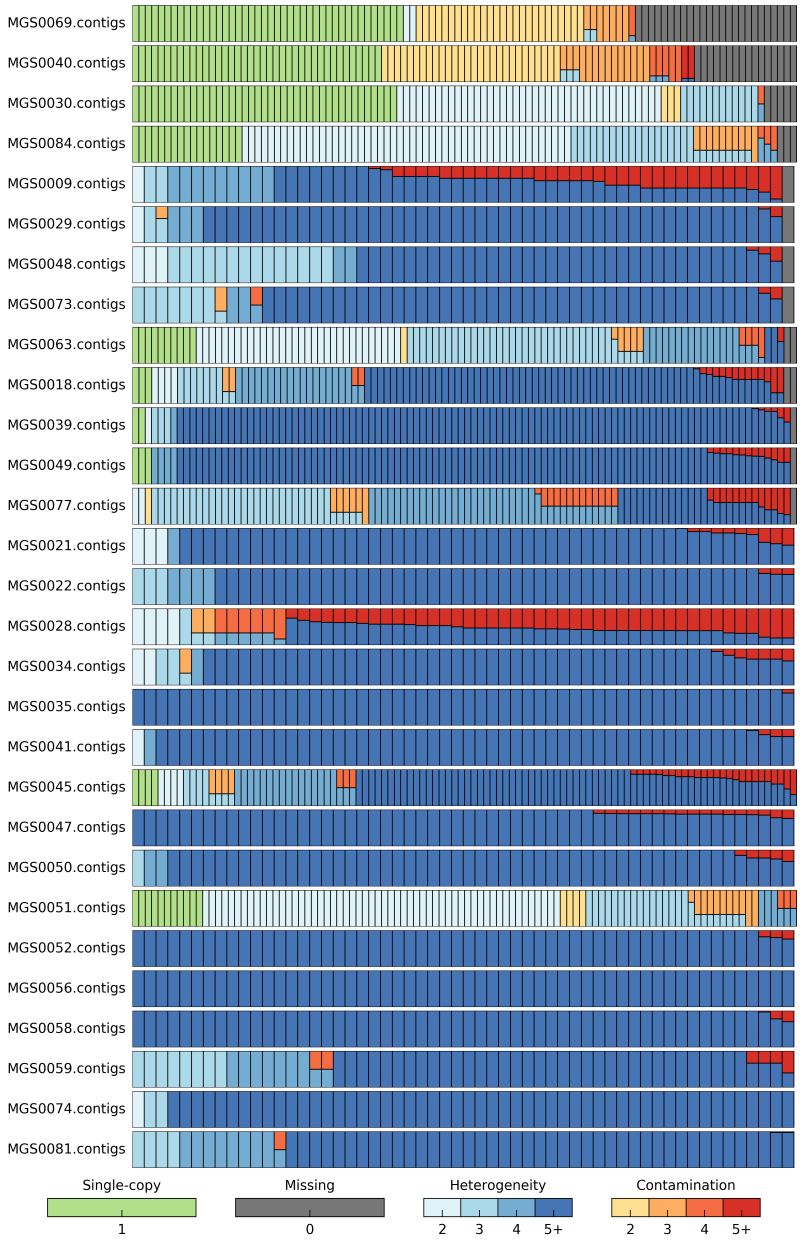
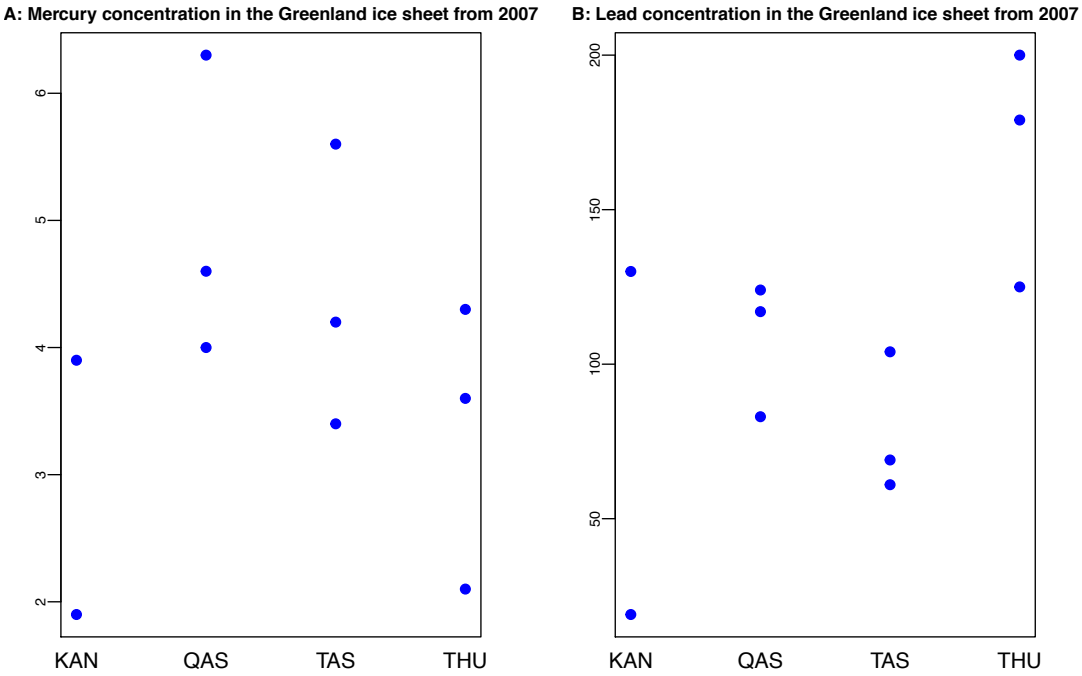
Supplementary Figure 1: Binned genomes from metagenomic dataset assessed in Checkm. Numbers of bin-names correspond to Bin ID number in Table 2 such that MGS0009 equals bin ID 09 in Table 2. Each line in the bins represents a single-copy housekeeping gene. Green lines are single-copy genes found once while blue and red nuances are single-copy genes found multiple times. Blue nuances represent multiple single-copy genes with ≥90% amino acid identity, while red nuances represent single-copy genes with less than 90% amino acid identity, suggesting contamination from not closely related organisms.



Supplementary Figure 2: Concentration in ng/kg melted snow of mercury (A) and lead (B) in the Greenland ice sheet according to technical report (Jacobsen *et al.*, 2012. Global contamination of the Greenlandic icecap with organic contaminants – distribution of contaminants and studies of the microbiological and photochemical degradation)



Supplementary Table 1: Means and standard deviations of counts of reads mapped to PCB degradation genes, PAH degradation genes, Hg resistance genes or Pb resistance genes respectively, divided by the number of reads mapped to housekeeping genes within each sample.

Site	PCB Mean	PCB Std.dev.	PAH Mean	PAH Std.dev.	Hg Mean	Hg Std.dev.	Pb Mean	Pb Std.Dev.
DS	2,42E-04	3,17E-04	1,37E-02	3,14E-04	6,20E-02	1,67E-02	3,65E-04	3,48E-04
KAN	1,28E-04	9,01E-05	2,39E-02	6,37E-03	6,64E-02	1,27E-02	0,00E+00	0,00E+00
QAS	5,70E-05	6,30E-05	1,38E-02	9,05E-04	1,21E-01	1,46E-04	0,00E+00	0,00E+00
TAS	2,81E-04	3,84E-04	1,57E-02	3,10E-03	1,05E-01	1,81E-02	1,14E-05	2,68E-05
THU	3,27E-04	4,52E-05	2,61E-02	1,96E-03	1,22E-01	9,92E-04	1,93E-05	9,09E-06

Part III

3.1 Conclusions

The cryosphere is a biome

In part I it was argued that next-generation sequencing technologies has enabled the acknowledgement of the cryosphere as a biome (Boetius et al. 2015; Anesio and Laybourn-Parry 2012). In part II this was illustrated through two studies utilizing 16S rRNA gene amplicon sequencing (Paper II) and shotgun metagenomics (Paper III) respectively.

Paper II showed that the microbial community from cryosphere-associated habitats, a proglacial lake and a glacier stream, are different from the microbial community of the main freshwater habitat in terms of community composition and diversity (Hauptmann et al. 2016). The diversity of microbial communities from these cryosphere-associated habitats indicated a greater fraction of cold-adapted microbes and less microbes from soil (Hauptmann et al. 2016).

Paper III showed that the cryoconite microbial community has the potential for degradation and resistance to contaminants, indicating that this community is highly adapted to the resource-scarce and potentially contaminated cryoconite habitat.

In conclusion, through the utilization of next-generation sequencing technologies Paper II and III showed cryosphere-associated microbial communities adapted to their respective cryosphere habitats, which illustrated the role of the cryosphere as a biome.

Data-intensive research can be hypothesis-driven

A quantitative assessment of hypothesis-driven versus data-driven research in part I concluded in favor of the opinion that data-intensive research can go hand-in-hand with hypothesis-driven research. Papers II and III showed that data-intensive research can yield informative results if the data is used in a hypothesis-driven manner.

In Paper II 16S rRNA gene amplicon sequencing was used to assess the pattern in biodiversity of a freshwater network in West Greenland (Hauptmann et al. 2016). In contrast to conclusions from previous studies we had the hypothesis that the microbial community in cryosphere-associated freshwater networks receive microbial input from the cryosphere and not only from the terrestrial surroundings. This hypothesis was

confirmed through NGS technologies enabling in-depth assessment of biodiversity as well as community composition. In addition, the utilization of Indicator Species analysis, as suggested in part I, enabled the tracing of the distinct microbial communities from the cryosphere-associated habitats into the estuary.

In Paper III shotgun metagenomics from Greenland ice sheet cryoconite holes was used to confirm the initial hypothesis that cryoconite holes present a contaminated habitat with a microbial community adapted to handling various sources of contamination. It is noteworthy that without having first identified this hypothesis, this result would not have been apparent in the large amount of data. Thus, hypothesis generation prior to conducting data-intensive studies is crucial for obtaining clear and interesting results. The extensive amount of data in Paper III also enabled the binning of genomes from metagenomes, which lead to the new hypothesis that not only Cyanobacteria but also other Bacterial phyla are part of cryoconite ecosystem engineering.

In conclusion, data-intensive research benefits from identification of hypotheses and can also lead to novel hypotheses.

The results from the quantitative assessment of hypothesis-driven versus data-driven research also suggested that in the initial phase of implementing novel NGS technologies in cryosphere biogeography around year 2012-2013, there might have been a lag-phase where research was less hypothesis-driven. The oldest study included in this thesis, Paper I (Hauptmann et al. 2014), was conducted in 2013 while the first NGS-studies of snow were published (Møller et al. 2013; Hell et al. 2013). This study was less hypothesis-driven and its strength was in its example of an extremely remote sampling site. This Paper illustrates a descriptive approach to cryosphere biogeography, which shows support of previous notions and encourages further studies.

It is important that in future implementations of novel technologies in cryosphere biogeography we are aware of our potential lack of hypotheses. In this phase we must design studies that optimize opportunities for generating novel hypotheses based on results from descriptive studies.

Amplicon sequencing is not per definition inferior to shotgun metagenomics

As described in part I NGS resulted in a greatly increased biodiversity of the cryosphere when compared to pre-NGS studies. As microbial biogeography deals with the distribution of microbial diversity, biodiversity is a central measure in this field of research. As argued in part I shotgun metagenomics does not at this point in time present the optimal method for assessing biodiversity. 16S rRNA gene amplicon sequencing has the advantage of being optimized for assessing biodiversity and when applied to the right studies therefore has its merits particularly in microbial biogeography.

Paper II presents such a study, where the use of amplicon sequencing was the superior choice over shotgun metagenomics. In this study, the pattern of diversity, which could be compared between samples, resulted in a conclusion in contrast to previously established hypotheses based on the same measures of diversity (Hauptmann et al. 2016). The biodiversity of the Red River freshwater network of the Disko Island in Greenland did not show a uni-directional decrease in diversity along the river (Hauptmann et al. 2016) as had been shown in previous studies (Ruiz-González, Niño-Garcia, and del Giorgio 2015; Crump, Amaral-Zettler, and Kling 2012). This result supported other results from the study by indicating that the microbial community in the streams from the glacier and the proglacial lake had low diversity because of their origin in the less diverse cryosphere compared to soil (Hauptmann et al. 2016).

Therefore, in microbial biogeography studies where measures of diversity are crucial for testing stated hypotheses amplicon sequencing can be the optimal choice.

Sampling strategy is an important future focus point in cryosphere biogeography – *think before you sequence*

In part I better sampling was highlighted as important for avoiding that microbial biogeography become mired in phenomenological description (Martiny et al. 2006).

Research papers of microbial biogeography of the cryosphere described in part I showed a tendency to refer to additional sampling in conclusions for future research. In part II different kinds of sampling could also have advanced the studies.

Paper I regarding snow included three sample sites from North Pole ice floes (Hauptmann et al. 2014). These samples showed notable difference between the

microbial communities at the three samples sites (Hauptmann et al. 2014). Additional spatial sampling could have helped an understanding of the geographic range to which the microbial communities on North Pole ice floes differ. Furthermore, sampling of additional metadata could have helped give suggestions for explanations of the differences observed in the microbial community composition.

Paper III includes extensive sampling of the vast Greenland ice sheet. The extensive dataset yielded interesting results as described above but additional sampling of chemical data could have helped link the potential for degradation of and resistance to contaminants to the contaminants themselves.

Paper II concluded that the right spatial resolution of samples in a freshwater network enabled the identification of Indicator OTUs from the proglacial lake and glacier stream and their distribution in the estuary (Hauptmann et al. 2016). These Indicator OTUs could not be observed upstream or at the river mouth and would not have been known to originate from the river if only these sites of the river had been sampled (Hauptmann et al. 2016). As the river Indicator OTUs made up on average a quarter of the estuary community, our understanding of the origin of the estuary community would be greatly limited had the river not been sampled in the right spatial resolution (Hauptmann et al. 2016).

In conclusion, based on the results from part I and part II of this thesis it is my recommendation that increased focus on planning of sampling is important to advance our understanding of microbial biogeography of the cryosphere.

To get a better understanding of microbial biogeography it was also suggested in part I that we need to put focus on distinguishing between contemporary and historical factors (Martiny et al. 2006). The results of Paper III showed just how important this distinction might be in particular for cryosphere biogeography. Paper III showed that the microbial community in cryoconite holes of the Greenland ice sheet are adapted to contaminants, which have been emitted in present and even more in past times. These contaminants are known to be trapped in the ice. The microbial community in cryoconite holes slowly melts through the ice and will encounter contamination from past times. As the microbial community of cryoconite seem to be highly adapted to contamination, the microbial

community and their biogeography will be shaped in part by the availability of contaminants from current and past times.

In this way, Paper III concludes in great favor of the recommendation that we need to consider both contemporary and historical factors to advance our understanding of microbial biogeography.

Microbial biogeography is more than testing the Baas-Becking hypothesis

The historical and central role that the Baas-Becking hypothesis plays in microbial biogeography was described in part I. It was discussed that despite the great advances that NGS technologies have enabled, there is still dispute about this one dominating hypothesis in microbial biogeography. This is true also in the field of cryosphere microbial biogeography, which has been suggested as the ideal habitat for studying microbial biogeography (Staley and Gosink 1999).

Cryosphere microbial biogeography has become an established field of research but based on the results of this thesis I do not believe that we can say something useful on a general level about microbial biogeography of the cryosphere. Instead, we need to make hypotheses about defined parts of the cryosphere. Very importantly, based on our hypothesis about the microbial biogeography of our favorite cryosphere habitat, we need to make informed choices about our methodology and not least about what data we need. By focusing our research and using the right methodology we might do much more than confirm or reject the Baas-Becking hypothesis.

In Paper II amplicon sequencing of microbes in a freshwater network enabled the tracing of OTUs from the cryosphere to the ocean (Hauptmann et al. 2016). In this way, the microbial community acted as tracers of dispersal from the cryosphere. Something that is highly relevant to ongoing climate research.

In Paper III shotgun metagenomic sequencing revealed a microbial community with potential for degradation and resistance to anthropogenic contaminants. Surprisingly, the results also indicated that the level of potential align with the level of contamination at the top of the food chain, among the Inuit. This encourages further research into the use of microbial metagenomics as tracers for contamination. Particularly

in the cryosphere where contaminants are trapped in frozen environments and where bioaccumulation of contaminants is of high concern (AMAP 2015).

In conclusion, microbial biogeography of the cryosphere through a next-generation sequencing approach can be relevant not only to microbial biogeographers but also to the wider scientific community and finally to the inhabitants of the Arctic.

3.2 Perspectives

The freezing habitats of snow on the polar ice cap and the Greenland ice sheet seem like barren places to the untrained eye. With a microbial perspective enabled by next-generation sequencing technologies suddenly these extreme habitats of the Arctic cryosphere become places of diverse bacterial communities.

These communities are important for our understanding of the impacts of the changing climate. The melting cryosphere seeds microbial communities into downstream ecosystems such as freshwater networks, which again transport microbes further on to the oceans (Hauptmann et al. 2016). Here, the communities, which originated in the cryosphere, may become part of a whole new ecosystem and their function may have profoundly different impact on the ecosystem and the exchange of nutrients and gases.

The microbial communities of the cryosphere also seem to hold promise for the inhabitants of the Arctic. Contamination of the food chain in the Arctic is of great concern to local peoples such as the Inuit in Greenland, whose economy is based on the fishing industry. The microbial community might serve as tracers of contamination and help our understanding of dispersal of contamination through the Arctic food chain (Paper III, unpublished).

Another potential that cryosphere microbiology holds for the inhabitants of the Arctic is the identification of biotechnologically relevant microorganisms. Sustainable development of the Arctic is a global concern, particularly in a time where the search for oil and minerals in the Arctic is intensifying. In parallel to the work on this thesis this has been a theme that I have dealt with extensively through the establishment of the first biotechnological company of Greenland, through blogging and debates as well as through interacting with the legislative system of Greenland. The original incentive to go Disko Island in 2013, where sampling for Paper II was conducted, was based on local stories about homothermal springs that might harbor cold-adapted microorganisms with potential for industrial enzymes. The results from Paper II did indicate a higher fraction of cold-adapted microorganisms in certain parts of the freshwater system of the Red River.

In this way microbial biogeography might help steer more efficient biotechnological research from Greenland. This is also indicated by Paper III, in which a high number of genomes from organisms with ability to degrade contaminants were found (Paper III, unpublished). Some of these organisms showed resemblance to organisms known as bioremediators, which are also of high biotechnological relevance.

An important conclusion of this thesis is that future cryosphere biogeography research must put emphasis on the correct sampling strategy. An aspect of sampling which is absent from the cryosphere biogeography literature and research society is the legislative framework that researchers are subject to in remote regions such as the Greenland ice sheet or the Disko Island. Moreover, the ethical concerns that needs to be considered when sampling.

When sampling in Greenland investigators are obligated to acquire a sampling permission from the self-rule government of Greenland according to the newly updated Greenlandic law of June 2016, number 3 of 3 on the utilization of genetic resources. This is not well known in the research community and researchers rarely follow this law. The law is an implementation of the Convention on Biological Diversity and the Nagoya Protocol. The Convention's main objectives are to protect biological diversity, ensure sustainable use of biological diversity as well as help the fair and equitable sharing of the benefits that may arise from the utilization of biological diversity.

While this might seem to be mostly relevant to biotechnological research from which researchers might gain commercially, it is also important for researchers in microbial biogeography of the cryosphere. As a central part of the culture the Inuit depend on the prey for their hunt just as they also depend on the ice, to which they have adapted their hunting methods for hundreds of years. But the ice is changing and part of that change is the microbes. Therefore, knowledge of microbes in the cryosphere has great value to the Inuit living in this environment as illustrated by the introductory citation by H. C. Petersen (Petersen 2001).

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Appendices

Appendix 1:

Words used for quantitative assessment of hypothesis-driven (Ecology related) versus data-driven (Technology related) research. Words related to the term "hypothesis" are highlighted in bold.

Ecology related	**Technology related**
ecological	genomic
ecology	genomics
ecologists	metagenomic
ecologically	sequenced
ecologist	sequencing
biogeography	sequencebased
biogeographic	pyrosequencing
biogeographical	illumina
biogeographies	pairedend
baasbecking	16s
becking	amplicon
baas	amplicons
cosmopolitan	ngs
cosmopolitanism	highthroughput
endemic	bioinformatics
endemism	bioinformatical
endemicity	bioinformatic
hypothesis	otu
hypotheses	otus
hypothesized	
hypothesize	
hypothetical	
theory	
theoretical	
theories	
idea	

Appendix 2

Perl script for counting words listed in Appendix 1

```
#!/usr/bin/perl
use strict;
use warnings;

##########################################################################
# Script for extracting indicator words
# This script results in a tab-separated text file listing the following
# year, indicator_word, number_of_papers_total, number_of_papers_with_word, and
# average_number_of_uses_in_each_paper
# usage: perl program.pl indicator_words.txt papers.txt
# Indicator words should be in small letters and with no non-word characters, remove
# e.g. "-" from Baas-Becking
# and they should be followed by their classification such as either "ecology" or
# "technology" in a tab-separated file
# Text file containing papers should have papers from different years separated by
# "YEAR: XXXX"
# each new paper should start with a line stating "NEWPAPER"
# file should end with "YEAR: 0000"
# Indicator words cannot contain Danish special characters such as æ,ø,å
##########################################################################

# get names of file containing list of indicator words and file containing text from
# papers

if (scalar(@ARGV) != 2) {
        die "There are too few or too many command line options. Usage: perl
program.pl indicator_words.txt papers.txt\n";
}

my $indicator_words_file = $ARGV[0];
```

```
my $papers = $ARGV[1];

# read in indicator words into look-up hash
my %indicator_words;
my %classifications;
open (iw_IN, "<", "$indicator_words_file") or die "File containing indicator words
could not be opened.\n$!\n";
while (defined (my $iw_line = <iw_IN>)) {
        chomp $iw_line;
        my @temp = split/      /, $iw_line;
        $classifications{$temp[0]} = $temp[1];
        $indicator_words{$temp[0]} = 1;
}

close iw_IN;

# for each year count number of papers with each indicator word (%count_papers
hash)
# and count use of indicator word (%count_words hash)

my $year = "";
my $first_iteration = "ON";
my $paper_count_total = 0;
my %count_papers;
my %count_words;

# while loop over the entire text file for all years
open(IN, "<", "$papers") or die "The file containing papers to be assessed cannot be
opened.\n$!\n";
open(OUT, ">", "Indicator_word_statistics.txt");
print OUT
"year\tindicator_word\tnumber_of_papers_total\tnumber_of_papers_with_word\tcoun
t_of_word\taverage_number_of_uses_in_each_paper\tclassification\n";
```

```
while (defined (my $line = <IN>)) {
	chomp $line;
	if ($line =~ m/^YEAR: (\d+)$/) {
		if ($first_iteration eq "OFF") {
			foreach my $keys (sort keys %indicator_words) {
				if (exists $count_papers{$keys}) {
					print OUT
"$year\t"."$keys\t"."$paper_count_total\t".$count_papers{$keys}."\t".$count_words{
$keys}."\t".$count_words{$keys}/$paper_count_total."\t".$classifications{$keys}."\n
";
				}
			}
		} else {
			$first_iteration = "OFF";
		}
# for each new year the paper count should be reset, after it has been written to output,
# same with year and paper- and word-counts
		$paper_count_total = 0;
		$year = $1;
		%count_papers = ();
		%count_words = ();
	} else {
		if ($line =~ m/^NEWPAPER$/) {
			$paper_count_total += 1;
 # if it is a new paper the indicator for having counted a word in the paper should be
# reset
			foreach my $keys (keys %indicator_words) {
				$indicator_words{$keys} = 1;
			}
		} else {
			$line =~ s/- //g;			# remove "- " which
occurs at line ends when text has been copied
			my @words = split / /, $line;
			foreach my $word (@words) {
```

```
                        $word =~ tr/A-Z/a-z/; # makes all letters small
                        $word =~ s/\W//g;           # removes all non-word
characters (including "-")
                        # for words that are defined as indicator words
                        if (exists $indicator_words{$word}) {
                                if (exists $count_papers{$word}) {
                                                # the word is known
                                        if ($indicator_words{$word} == 1) {
                                                # the word is known but
not in this paper
                                                $count_papers{$word} += 1;
                                                $indicator_words{$word} = 2;

                                        }
                                } else {
                                                                        #
the first time the word is met in all papers
                                                $count_papers{$word} = 1;
                                                $indicator_words{$word} = 2;
                                }
                                if (exists $count_words{$word}) {
                                                $count_words{$word} += 1;
                                } else {
                                                $count_words{$word} = 1;

                                }
                        }
                }
        }
}

close IN;
close OUT;
```