Exploration of the Microbial Communities within the Basaltic Subsurface of the Volcanic Island Surtsey in Iceland

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2022

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Rannsókn á neðanjarðar örversamfélögum í basalti
á eldfjallaeyjunni Surtsey við Ísland

Pauline Bergsten

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

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Reykjavik, September 2022

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Bibliographic information:
Pauline Bergsten, 2022, Exploration of the Microbial Communities within the Basaltic Subsurface of the Volcanic Island Surtsey in Iceland, Ph.D. dissertation, Faculty of Life and Environmental Sciences, University of Iceland, 233 pp.

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Printing: Háskólaprent
Reykjavik, Iceland, September 2022

Abstract

Surtsey is a volcanic island located on the south-east offshore extension of the Icelandic rift zone. It was formed during successive eruptions from the seafloor in 1963-1967 and has been officially protected and studied ever since. It represents an exceptional natural setting for studying colonization and succession of life on land. Also for subsurface microbial communities associated with newly formed basaltic tuff deposits in a seawater-hydrothermal system that is still active and at temperatures approaching the presumed thermal limit for functional life. During an international drilling operation at Surtsey in 2017, drill core samples at successive depths as well as associated hot fluids and surface fumes from fumaroles were collected for microbial investigations. This thesis presents the first and most comprehensive research of the Surtsey subsurface biosphere. Multiple approaches were combined on the rare and unique samples to increase the knowledge of microbial communities inhabiting the oceanic subsurface and of the processes that sustain such life. These included molecular analyses of environmental DNA through 16S rRNA gene amplicon and metagenome sequencing, isolation and characterization of bacterial strains and microscopic investigations. Based on the DNA concentration, the microbial cell numbers present in the drill cores were estimated to range from about 5×10^4 to 1×10^6 cells per gram of sample. The Surtsey subsurface is therefore a low biomass environment, making the samples extremely sensitive to external contamination. It is nevertheless a diverse habitat that hosts bacterial and archaeal clades, including extremophiles, that have been previously detected in other terrestrial and marine environments. Yet, many clades belonged to unknown lineages. Predictive functional analyses based on taxonomic identifications revealed that the Surtsey subsurface biosphere is composed of heterotrophic microorganisms as well as chemoautotrophs involved in the sulfur, nitrogen, and methane cycles. However, these results could not be strengthened by the functional metagenomic investigations as they were inconclusive. Numerous enrichment cultures were initiated using different conditions and media and resulted in nearly 200 isolated bacterial strains, which included several novel species. One novel thermophilic bacterial species, *Rhodothermus bifroesti*, was fully characterized and its genome was sequenced and compared with those of the two other described *Rhodothermus* species. Comparative analyses revealed that 2.15% of the amplicon sequence variants from the 16S rRNA gene amplicon sequence datasets were represented by cultivated strains using standard methods. Finally, putative microbial structures adhering to the basaltic tuff were discovered inside the numerous interconnected vesicles found in the basaltic glass. All of the findings point to an active microbial colonization of the Surtsey deposits within 50 years after the eruptions ended, with possible sources of colonization coming from the surrounding ecosystems via microbial dissemination and possible adaptations. The thesis establishes a foundation for future research on the microbial communities that inhabit the Surtsey subsurface and their temporal succession in the face of a cooling and changing hydrothermal environment.

Útdráttur

Surtsey er eldfjallaeyja sem staðsett er á rekbelti suðaustan við Ísland. Hún myndaðist í röð neðansjávargosa á árunum 1963-1967 og hefur verið friðuð síðan. Surtsey hefur þá sérstöðu að þar hefur verið mögulegt að rannsaka landnám lífvera og framvindu vistkerfa frá upphafi. Rannsóknin sem hér er lýst snýr að örverusamfélögum í eynni, í umhverfi þar sem nýmyndað basalt mætir jarðhita og sjó. Þar er að finna breytilegt hitastig sem nálgast efri mörk þess er talið er lífvænlegt. Alþjóðlegur rannsóknarleiðangur var farinn til Surtseyjar árið 2017 til að afla sýna með borunum. Kjarnasýnum var safnað á mismunandi dýpi ásamt vatnssýnum og heitri gufu úr sprungum á yfirborði eyjarinnar. Þessi doktorsritgerð er fyrsta heildstæða rannsóknin á örverusamfélögum neðanjarðar í Surtsey. Margvíslegum aðferðum var beitt til að rannsaka þau einstöku sýni sem aflað var. Markmiðið var að auka þekkingu á samfélögunum og efnaferlunum er þau byggja á. Samsetning örvera var greind með því að raðgreina tegundaákvarðandi 16S rRNA gen og annað erfðaefni sem einangrað var úr umhverfinu. Að auki voru bakterístofnar einangraðir og þeim lýst og smásjáraðferðum beitt. Magn DNA var metið í borkjörnum en samkvæmt því var fjöldi örvera áætlaður á bilinu 5×10^4 til 1×10^6 á gramm. Niðurstöðurnar bentu til þess að afar lítinn lífmassa væri að finna í umhverfinu. Slíkt eykur áhrif mengunar sem getur orðið við sýnatökur. Þrátt fyrir lítinn lífmassa eru örverusamfélög neðanjarðar í Surtsey mjög fjölbreytt og samanstanda bæði af bakteríum og arkeum. Sumar þeirra eru jaðarörverur eða hafa áður greinst í jarðvegi og sjó en aðrir hópar eru lítt þekktir. Greiningar á ættartengslum og lífsháttum benda til þess að sumar fái orku úr lífrænum efnum en aðrar úr ólífrænum og tengist hringrásum brennisteins, köfnunarefnis og metans. Þó tókst ekki að styðja þessar niðurstöður með greiningum á umhverfiserfðamengjum. Fjölmargar auðgunarræktir voru framkvæmdar við ólík skilyrði og með mismunandi ætum. Um 200 bakteríustofnar voru einangraðir. Þar af voru nokkrar nýjar tegundir og einni þeirra, hitakæru bakteríunni *Rhodothermus bifroesti* lýst að fullu, erfðamengi hennar raðgreint og samanburður gerður við tvær þekktar tegundir sömu ættkvíslar. Í ljós kom að við aðstæðurnar sem notaðar voru tókst aðeins að einangra um 2.15% af þeim örverum sem kennsl voru borin á með 16S rRNA raðgreiningum. Smásjárgreiningar á hraunsýnum sýndu ýmis form sem líktust örverum í samtengdum blöðrum í basaltgleri. Niðurstöður rannsóknarinnar í heild bentu til mikils landnáms örvera í Surtsey á þeim 50 árum sem liðin eru frá goslokum. Örverurnar gætu hafa dreifst frá nálægum vistkerfum og aðlagast umhverfinu í hrauninu. Niðurstöðurnar sem lýst er í þessari ritgerð mynda grunn að framtíðar rannsóknum á örverusamfélögum neðanjarðar í Surtsey og framvindu samfara breyttum aðstæðum eins og lækkandi jarðhita.

I dedicate this work to my family

and friends for their love and support

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List of Original Papers

This thesis is based on the following papers, three of which are published in peer-reviewed journals and one is presented as a manuscript. They are referred to by their numbers throughout the text. The papers and supplementary material are included at the end of this thesis.

Paper I: **Bergsten, P.**, Vannier, P., Klonowski, A.M., Knobloch, S., Gudmundsson, M.T., Jackson, M.D., and Marteinsson, V.T. (2021) Basalt-Hosted Microbial Communities in the Subsurface of the Young Volcanic Island of Surtsey, Iceland. *Frontiers in Microbiology* 12: 2789. doi: 10.3389/fmicb.2021.728977.

Paper II: **Bergsten, P.**, Vannier, P., Frion, J., Mougeolle, A., and Marteinsson, V.T. (2022) Culturable Bacterial Diversity from the Basaltic Subsurface of the Young Volcanic Island of Surtsey, Iceland. *Microorganisms*. doi: 10.3390/microorganisms10061177.

Paper III: **Bergsten, P.**, Vannier, P., Mougeolle, A., Rigaud, L., and Marteinsson, V.T. (2022) *Rhodothermus bifroesti* sp. nov., a thermophilic bacterium isolated from the basaltic subsurface of the volcanic island Surtsey. *International Journal of Systematic and Evolutionary Microbiology*. doi: 10.1099/ijsem.0.005214.

Paper IV: **Bergsten, P.**, and Marteinsson, V.T. Microscopic investigations revealed putative biotic structures attached to basaltic drill cores collected from the subsurface of the Surtsey volcano. Manuscript.

Other paper published during the course of this study:

Jackson, M.D., Gudmundsson, M.T., Weisenberger, T.B., Rhodes, J.M., Stefánsson, A., Kleine, B.I., Lippert, P.C., Marquardt, J.M., Reynolds, H.I., Kück, J., Marteinsson, V.T., Vannier, P., Bach, W., Barich, A., **Bergsten, P.**, Bryce, J.G., Cappelletti, P., Couper, S., Fahnestock, M.F., Gorny, C., Grimaldi, C., Groh, M., Gudmundsson, A., Gunnlaugsson, A., Hamlin, C., Högnadóttir., T., Jónasson, K., Jónsson, S.S., Jørgensen, S.L., Klonowski., A.M., Marshall, B., Massey, E., McPhie, J., Moore, J.G., Ólafsson, E.S., Onstad, S.L., Perez, V., Prause, S., Snorrason, S.P., Türke, A., White, J.D.L., and Zimanowski, B. (2019) SUSTAIN drilling at Surtsey volcano, Iceland, tracks hydrothermal and microbiological interactions in basalt 50 years after eruption. *Scientific Drilling* 25: 35–46. doi: 10.5194/sd-25-35-2019.

List of Published Nucleotide Sequences

In the context of this thesis, nucleotide sequence datasets were made publicly available across the DDBJ/ENA/GenBank, Sequence Read Archives (SRA) databases and the European Nucleotide Archive (ENA) at EMBL-EBI. These include:

The BioProject accession number PRJEB42339 (datasets number ERP126178) containing 59 BioSamples with 16S rRNA gene amplicon sequences, as referred to in **Paper I**.

Partial 16S rRNA gene sequences described in **Paper II** can be found in the DDBJ/ENA/GenBank databases under accession numbers OK534092 to OK534145.

DDBJ/ENA/GenBank accession number MW901484 for the full 16S rRNA gene sequence of strain ISCAR-7401^{T}, as referred to in **Paper III**.

The BioProject accession number PRJNA718815 encompasses one BioSample (SAMN18570965) with the whole genome shotgun project SRR14119291, and the assembled genome JAGKTL000000000, as referred to in **Paper III**.

Abbreviations

ADP Adenosine Diphosphate

ASV Amplicon Sequence Variant

ATP Adenosine Triphosphate

BLAST Basic Local Alignment Search Tool

bp (Nucleic Acid) Base Pairs

CORK Circulation Obviation Retrofit Kit

DAPI 4',6-Diamidino-2-Phenylindole

DIC Dissolved Inorganic Carbon

DNA Deoxyribonucleic Acid

DOC Dissolved Organic Carbon

DOE Department of Energy

DSDP Deep Sea Drilling Project

EPS Extracellular Polymeric Substances

FACS Fluorescence-Activated Cell Sorting

FISH Fluorescence *In Situ* Hybridization

ICDP Integrated Continental Drilling Program

IODP International Ocean Discovery Program

ISCaR Icelandic Strain Collection and Records

JdFR Juan de Fuca Ridge

LCHF Lost City Hydrothermal Field

m b.s.l. meters below sea level

m bs meters below surface

Ma Million years old

n.a. data not available

NCBI National Center for Biotechnology Information

ODP Ocean Drilling Program

OTU Operational Taxonomic Unit

PBS Phosphate Buffered Saline

PCA Principal Component Analysis

PCR Polymerase Chain Reaction

POC Particulate Organic Carbon

QIIME Quantitative Insights Into Microbial Ecology

RNA Ribonucleic Acid

rRNA Ribosomal Ribonucleic Acid

SLiMEs Subsurface Lithoautotrophic Microbial Ecosystems

SSU Small Subunit

Acknowledgments

I would like to thank my supervisors, Viggó Þór Marteinsson for offering me this opportunity, for guidance and support along my Ph.D. studies and Pauline Vannier for her supervision in microbial cultivation and most importantly for sharing her passion and enthusiasm for the world of extreme environmental microbiology. I am grateful to Snædís Huld Björnsdóttir for her kind support and precious advices as part of my doctoral committee and especially during the last steps of the thesis manuscript preparation.

This work was supported by the grant of excellence from the Icelandic Research Fund, ICF-RANNÍS (IceSUSTAIN - 163083-051). I would like to thank the Surtsey Research Society, the onsite team and everyone involved with the International Continental Scientific Drilling Program (ICDP) 5059 expedition, SUSTAIN drilling operation at Surtsey, especially Barbara Kleine and Alex Klonowski for all the fun at Surtsey, but also M. T. Gudmundsson, M. D. Jackson, T. Weisenberger, S. Prause, C.F. Gorny, A. Barich, S. Couper, A. Türke, S. L. Onstad and S. Gísli, as well as the drilling team led by B. Marshall, with drillers S. Cole, J. Blouin, A. J. Vecchiarelli, M. Lyon and M. Vinson, and the Icelandic Coast Guard for the helicopter ride. I thank the Icelandic research science fund RANNÍS for having faith in my research by providing me with a one-year doctoral student grant (ELiBSS - 206582-051).

For the financial support and invitation to present my work, I am grateful to the organizing committee of the IPGP PhD student conference (Congres des doctorants IPGP), where I met Aurélien Lecoeuvre, a fellow Ph.D. student, and together we set up a project through the Hubert-Curien Jules Verne exchange program (41098PM). In this context, I thank Campus France, which financially supported the collaboration between Matís and the geomicrobiology group at IPGP (Institut de Physique du Globe de Paris) in Paris. Many thanks to Emmanuelle Gérard and Bénédicte Ménez for their warm welcome and guidance, Stephen Borensztajn for SEM images, and most importantly to Aurélien, who has become a dear friend.

I thank the University of Iceland and the congress organization of Thermophiles 2009 for the travel grants that supported me to attend and present my work at the 15th International Congress on Thermophiles in Japan. I also thank the Census of Deep Life (CoDL) from the Deep Carbon Observatory for the sequencing of the three metagenomes. I would like to thank the organizers of the workshop EBAME, especially Loïs Maignien.

I am grateful to my friends and colleagues at Matís, Justine, Clara, Antoine, Björn, Alex, Aurélien, Brynja, Sigurlaug, Elísabet, Þórdís, René, Mia, Greg, Hörður, Monica, Lilja, Guðbjörg, Ágústa and Steinunn, for your help, support, advice and for making the lab a fun place to work. A special thank goes to Stephen for introducing me to bioinformatic analyses. I also thanks Alan, Julie and Louise for their great work and efforts in cultivation.

Last but not least, I thank my family for their love, infinite support and for always believing in me: to my parents Michèle and Ulrik, my sisters Charlotte, Marie and Poupoune Bergsten, to Véronique, and all the others. Thank you, Lucas, for being the one yesterday, everyday, always and forever. And to finish, I thank Life for giving me a twin. Thanks Emma, tu as toujours été ma force.

1 Introduction

1.1 Definition of the Subsurface Biosphere, Abundance and Limits

The subsurface biosphere, or deep biosphere, is defined as the ecosystems that encompass the habitable region below the surface of continents and the bottom of the ocean, beneath the soil and sediments (Magnabosco *et al.*, 2019). The limits of this habitable region depend on processes such as temperature, pressure, and composition of the system (Edwards *et al.*, 2012b; Magnabosco *et al.*, 2019).

These ecosystems can extend from a few meters to kilometers into the Earth's crust. A recent study revealed the discovery of active microbial communities at depths down to 4.4 km below the surface, indicating the extent of the deep biosphere in continental settings (Purkamo *et al.*, 2020). The habitable volume of the subsurface was recently estimated to range from ~2.0 to 2.3 × 10^9 km^3, which is compared to twice the volume of our oceans (Magnabosco *et al.*, 2019). Over the years, few estimations of the microorganisms inhabiting the subsurface have been made, mainly because of the technical difficulties to acquire subsurface samples without contamination. Yet, some evidence suggests that the subsurface biomass is substantial. In 1998, Whitman and collaborators suggested that the number of subsurface prokaryotes probably exceeds the numbers found in other biomes (Whitman *et al.*, 1998). Twenty years later, Bar-On and collaborators published a paper on the distribution of the biomass on Earth and reiterated that the deep subsurface environments are the world largest microbial habitat, holding nearly 15% of the total biomass on Earth (Bar-On *et al.*, 2018).

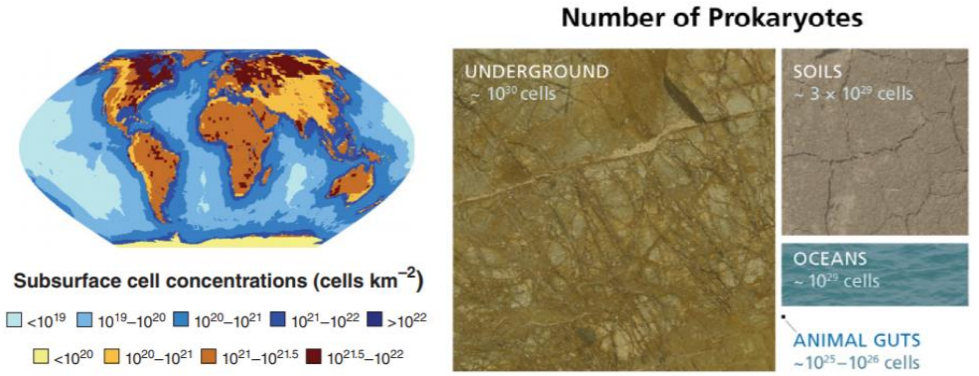

Figure 1. World map distribution of bacterial and archaeal cells in sub-seafloor sediments and continental subsurface (left). Relative size comparison of the prokaryotic cell numbers estimated in the subsurface, soils, oceans, and animal guts (right) (Magnabosco *et al.*, 2019).

These zones, consisting of the continental subsurface, sub-seafloor sediments, and oceanic crust, host all three domains of life and the genetic diversity could rival the one found on the surface. The deep biosphere is mainly composed of bacteria and archaea, from which the great majority belong to the "microbial dark matter" or "rare biosphere", a term used to describe the uncultivated microorganisms, yet to be discovered or characterized. The deep subsurface environments are estimated to contain ~70% of all prokaryotic cells on Earth (Figure 1) and possibly more than 80% of all bacterial and archaeal species (Magnabosco *et al.*, 2018).

Subsurface environments are devoid of light, which implies that most bacteria and archaea on Earth are physically located in environments that exist in permanent darkness. While the absence of light is not a limiting factor for life, extreme conditions of temperature, pressure and pH affect the development of these ecosystems, along with the availability of space, carbon, and energy sources (Orcutt *et al.*, 2011b; Lever *et al.*, 2015a). Discoveries on extremophiles constantly challenge the bounds of habitability and extend the reach of potentially habitable environments. The factors controlling and limiting life are far from being understood, and thus the extent of life remain a major interrogation in the deep biosphere research.

1.2 Beginning of the Subsurface Biosphere Research

The earliest studies that attempted to investigate life in the subsurface started in the 1920s with a team of geologists initially interested in the presence of hydrogen sulfide and bicarbonates in fluids extracted from oil wells (Bastin *et al.*, 1926). Since their presence could not be explained only by chemical processes, they suspected that microbes were responsible for the formation of sulfides, being capable of degrading organic components of oil as a carbon source and reducing sulfur compounds for energy (Bastin and Greer, 1930). They collected fluids some hundred meters below the surface to investigate this hypothesis, whereas the conditions of temperature and pressure occurring in the wells were considered unsuitable to support life at that time. They discovered that cells were present in the fluids from oil reservoirs and succeeded to cultivate anaerobic sulfate-reducing bacteria. They also demonstrated that the chemicals were produced by bacteria (Bastin *et al.*, 1926; Bastin and Greer, 1930). However, due to the sampling procedures, the provenance of these bacteria, which could have been endemic from the oil reservoirs or rather contaminants from the surface, was uncertain.

In the 1930s, another research hinted at the existence of subsurface microorganisms with the investigation of life in deep-sea sediments. Short-length cores (< few meters) were used to sample sediments, and microorganisms were detected at each sampled depth. A switch of microbial communities was observed from aerobes to anaerobes with increasing depth (ZoBell *et al.*, 1936). These early observations demonstrated the existence of life in the oceanic subsurface and generated speculations on how the energy needed to support this subsurface biosphere was produced (Zobell, 1938; ZoBell and Morita, 1957).

A few years later, unexpected microbial degradation of organic matter under deep-sea conditions was observed, when Alvin, a deep-sea submersible, was recovered after remaining ten months on the seafloor at 1540 m depth below the ocean surface. When the

submersible was finally floated again, scientists discovered that the sandwich left by the crewmembers was exceptionally well-preserved. The incident of "Alvin and the sandwich" highlighted the existence of deep-sea microbial communities and their very slow metabolism (ten to 100 times slower than on the surface) (Jannasch *et al.*, 1971). However, researchers believed that no life could persist deeper because of the extreme oligotrophic conditions and again assumed that all the microbes detected at greater depths were contaminants from the surface. Research into subsurface microbiology was inactive for decades until 1977 when a team of geologists made an incredible breakthrough toward our understanding of life on Earth. Diving Alvin, they discovered the first-ever submarine hot spring on the ocean floor – also called hydrothermal vent – near the Galápagos Rift, at 2.5 km depth below the ocean surface. Corliss and collaborators reported in Science that the exploration of the vents revealed the existence of unexpected animal communities around them, that included clams, shrimps, crabs, and tubeworms (Corliss *et al.*, 1979). For a long time, particulate organic carbon (POC) from the surface primary production and its sedimentation to greater depths were considered to be the only food source to fuel life in the dark ocean. Nevertheless, at a depth of 2.5 km, no POC remains to feed the thriving communities. Thus, if these communities composed of large organisms were isolated from the sunlight and all surface energy sources, where did the energy needed to support this life come from? Although the existence of organisms that can synthesize their cell material from inorganic components was previously reported with the study of the bacteria *Beggiatoa* (Winogradsky, 1887), the discovery of communities around hydrothermal vents dramatically changed scientists' thinking about life. The discovery brought evidence that the thriving vent ecosystems were dependent on energy derived from seawater-rock reactions occurring at high temperatures and chemosynthesis by bacteria, the chemical oxidation of reduced compounds rather than photosynthesis.

Later, scientists showed that sulfate, which is abundant in seawater, is converted to hydrogen sulfide as the seawater circulates in the ocean crust, making the warm water flowing out of the vents enriched in hydrogen sulfide and other reduced inorganic compounds. Microorganisms use the hydrogen sulfide from the hydrothermal fluids to support growth, while larger organisms feed on bacteria (Fisher *et al.*, 2007). This deep-sea food chain was unknown before the discovery of ecosystems that only depend on chemosynthetic processes to create organic material. It raised many questions in the scientific community, including the following: Are the ocean vents the only place on Earth where such life can be found, or do they only represent the examples that were discovered first? Are other locations, harder to investigate, also sheltering life based on chemical energy sources?

Thanks to this discovery, investigations into the deep oceanic subsurface biosphere started involving ocean drilling programs such as the Deep Sea Drilling Project (DSDP) and the Ocean Drilling Program (ODP), and abundant prokaryotic communities were identified in deeply buried oceanic sediments, including sulfate-reducing and methane-producing microorganisms (Oremland and Polcin, 1982; Whelan *et al.*, 1986; Parkes *et al.*, 2000; D'Hondt *et al.*, 2002). However, the deep continental subsurface biosphere was only seriously investigated much later than the oceanic subsurface biodiversity. In 1988, a review was published on the microbial ecology of the terrestrial subsurface by considering shallow aquifer sediments and groundwater ecology (Ghiorse and Wilson, 1988). Authors pointed out that numerous studies had previously reported the existence of microorganisms in terrestrial subsurface environments, but microbial ecologists of the time had not thought

seriously about them due to the risks of contamination. To control the main sources of microbiological contamination during sampling, the development of tracers (chemical or other substances placed in or around the borehole to measure fluid movement in injection wells) was a key to providing the credibility needed by the scientific community for studies of life in subsurface environments (Kieft, 2010).

After decades of pieces of evidence and speculations, an astrophysicist published the pioneer paper "The deep, hot biosphere", where it was suggested for the first time that microbial life is widespread through Earth's subsurface (Gold, 1992). Based on Corliss´s discovery, Gold hypothesized that this subsurface life, inhabiting the pore spaces between grains of rocks at depth in the crust of the Earth, is also supported by chemical sources rather than depending on the energy of the sun and photosynthesis. He speculated that the energy and nutrients needed to fuel the subsurface life are delivered by fluid circulation through the rock, which contains oxidized and reduced minerals. Furthermore, he suggested that the extent of this life, which is likely to be all microbial because of the lack of space that could not shelter larger life forms, is comparable in mass and volume to the life on the surface, and likely extends kilometers down below the surface, if the temperature permits. He also pointed out the possibility that life could be found on the inside of other planets.

The question of whether subsurface microorganisms exist and the range of their activity was well addressed when the U.S. Department of Energy (DOE) tried to find a way to bury nuclear waste safely in the ground. They hypothesized that if microorganisms were present in the subsurface, they could either be very beneficial by degrading organic pollutants or highly inconvenient by breaching the closed chamber containing radioactive waste. To search for deep life and study its activity, they formed the "Subsurface Science Program" where subsurface contamination problems were addressed among other challenges, and numerous new techniques were developed to facilitate the collection of sterile samples from deep cores. In 1987, the initial investigation included three drilled boreholes at the Savannah River Plant in South Carolina where scientists applied procedures to avoid microbial contamination from the surface such as processing only the inside portions of the cores (Fliermans and Balkwill, 1989; Phelps *et al.*, 1989; Lehman *et al.*, 1995). The investigations confirmed the existence of subsurface microorganisms and established that they are abundant and diverse at least 500 meters below the surface, the depth of the last core extracted from the boreholes (Fredrickson and Onstott, 1996). Since then, numerous additional studies have undoubtedly demonstrate the great microbial diversity occurring in both the oceanic and the continental subsurface, and nowadays, life in these environments is globally considered ubiquitous and to represent a significant portion of the Earth's biomass.

1.3 Habitat Diversity

Remarkably, deep ecosystems are extremely diverse (Figure 2) and strongly differ from each other in terms of physicochemical characteristics (Edwards *et al.*, 2012b; Lang *et al.*, 2019).

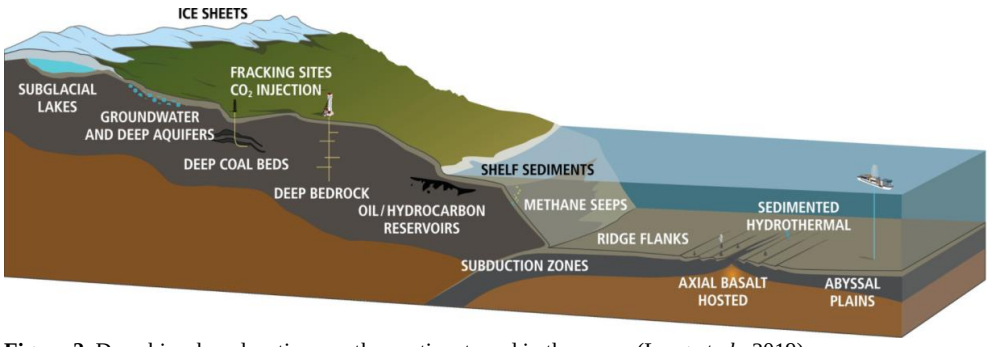

Figure 2. Deep biosphere locations on the continents and in the ocean (Lang *et al.*, 2019).

1.3.1 Continental Crust Biosphere

Only a few drilling projects considering microbiology have sampled the continental subsurface because of the high cost and difficulties of the operations (Zhang *et al.*, 2005; Fernández-Remolar *et al.*, 2008). Instead, many researchers have sampled fluids through "windows" to access deep continental biomes such as wells (Stevens and McKinley, 1995; Chapelle *et al.*, 2002), springs (Suzuki *et al.*, 2013; Magnabosco *et al.*, 2014; Probst *et al.*, 2014), underground research facilities (Murakami *et al.*, 2002; Momper *et al.*, 2017a; b) and deep mines (Onstott *et al.*, 2003; Sahl *et al.*, 2008). However, those "artificial windows" are disturbed by human activities and thus, the microbial communities may not be representative of the indigenous microbial populations of the subsurface. Today, samples can be extracted at great depths from the continental crust e.g., down to 4.4 km depth (Purkamo *et al.*, 2020), thanks to advances in drilling methodologies and by minimizing and quantifying contamination by using tracers (Kieft, 2010). Numerous studies have clearly shown that there is a great microbial diversity in continental subsurface environments including groundwater and deep aquifers, oil and gas reservoirs, deep bedrock, and subglacial ecosystems (Figure 2).

The continental deep subsurface is mainly composed of granitic rocks that are formed by the cooling of magma, resulting in the total absence of organic matter at an early stage. These environments are characterized by the absence of sunlight and oxygen and by an elevation of temperature and pressure with depth (Kieft, 2016). The nature of these biospheres is influenced by the geochemistry of groundwater and hydrogeology. The number of microorganisms living in these environments and their activities are controlled by nutrient and water accessibility (Jones and Bennett, 2017; Rempfert *et al.*, 2017). They also depend on the hydrological flow paths or connectivity with the surrounding environment (e.g., transfer of matter, energy, and/or organisms through water flow), and the rock porosity (e.g., high porosity, the higher water flow and larger physical space for microbial colonization) (Fredrickson and Onstott, 1996; Pedersen, 2000). Continental subsurface environments can be divided between sedimentary and crystalline host rocks even though variations widely occur within and among them (Lang *et al.*, 2019). For instance, radical differences in microbial communities can be observed at different depths of the same borehole (Dutta *et al.*, 2019), which is due to the geological and physicochemical heterogeneity of the studied systems, as well as the origin and composition of the water. Studies suggest that heterotrophic processes dominate in sedimentary systems whereas autotrophic processes are more abundant within crystalline and deep rock aquifers. However, this is not always the case (Stevens, 1997; Fredrickson and Balkwill, 2006).

Most of the studies on the continental deep subsurface biosphere have shown that the number of microorganisms and their diversity decrease with depth (Moser *et al.*, 2005; Cockell *et al.*, 2012; Mcmahon and Parnell, 2014), with one exception (Itävaara *et al.*, 2011). Since these biomes greatly differ among study sites, it is not clear what are the most common microbial groups found in these environments. Usually, bacteria are more abundant and diverse than archaea (Takai *et al.*, 2001; Cockell *et al.*, 2012; Ino *et al.*, 2016; Lau *et al.*, 2016; Rempfert *et al.*, 2017). *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and, mainly *Firmicutes* are often reported in the continental subsurface (Onstott *et al.*, 2003; Moser *et al.*, 2005; Zhang *et al.*, 2005; Lin *et al.*, 2006; Dong *et al.*, 2014). Some other bacterial taxa are also detected with less abundance including *Deinococcus-Thermus*, *Nitrospirae*, *Acidobacteria*, and *Chloroflexi*. *Cyanobacteria* are often detected in the continental subsurface even though they have the potential to carry out photosynthetic metabolism (Onstott *et al.*, 2003; Zhang *et al.*, 2005; Bomberg *et al.*, 2014; Purkamo *et al.*, 2014; Ino *et al.*, 2016; Rempfert *et al.*, 2017). Overall, the archaeal phylum *Crenarchaeota* seems to be more abundant in the upper layers of the subsurface while *Euryarchaeota* seems more common in deeper layers (Takai *et al.*, 2001; Zhao *et al.*, 2006; Nyyssönen *et al.*, 2014; Dutta *et al.*, 2019). Methanogens are recurrently detected in most of the analyzed subsurface environments (Moser *et al.*, 2005; Probst *et al.*, 2014; Purkamo *et al.*, 2014, 2016; Rempfert *et al.*, 2017).

One of the main interest of the study of life in the deep subsurface is the origin of energy needed to sustain it. Does the energy source come from the subsurface itself or rather depend partly on photosynthesis? In the continental subsurface, oxygen is rapidly consumed and both autotrophic and heterotrophic anaerobic metabolisms are dominant. Chemolithoautotrophic microorganisms that can use hydrogen (abiotically produced) as an electron donor were detected (Stevens and McKinley, 1995; Stevens, 1997; Pedersen, 2000), as well as other lithotrophs that can use reduced sulfur compounds (Amend and Teske, 2005; Gihring *et al.*, 2006; Osburn *et al.*, 2014; Lau *et al.*, 2016), iron (Emerson *et al.*, 2007; Sahl *et al.*, 2008; Swanner and Templeton, 2011; Shelobolina *et al.*, 2012; Heim *et al.*, 2017) and nitrogen (Swanner and Templeton, 2011; Nyyssönen *et al.*, 2014; Lau *et al.*, 2016). Early studies on the microorganisms inhabiting the subsurface biomes focused on culture-based measurements of activity (Onstott *et al.*, 1999), and on large collections of clone libraries from amplified regions of the 16S rRNA gene (Fredrickson and Balkwill, 2006; Gihring *et al.*, 2006). Thanks to the development of next-generation sequencing (NGS) technologies, researchers are exploring further the taxonomic and functional profiles of organisms residing in the continental subsurface by 16S rRNA gene amplicon- and shotgun metagenomic sequencing. Such techniques provide a higher depth resolution of the repertoire of the electron acceptors and donors biologically available in these environments. Remarkably, the first metagenome from a deep continental habitat was obtained by extracting DNA from the fluid of a gas-filled fracture 2.8 km beneath Earth's surface in a Gold Mine in South Africa (Chivian *et al.*, 2008). In this study, the authors revealed that the bacterium *Candidatus* Desulforudis audaxviator composed 99.9% of the microorganisms inhabiting the fluid, forming a single-species ecosystem. The thermophilic and strictly anaerobic *Firmicute* is a sulfate-reducing chemoautotroph, capable of fixing nitrogen and carbon. Yet, the diversity of microbial metabolisms detected in the deep continental subsurface is just starting to be understood (Purkamo *et al.*, 2014, 2020; Anantharaman *et al.*, 2016; Momper *et al.*, 2017a).

1.3.2 Oceanic Crust Biosphere

Marine subsurface environments are considerably diverse and can be divided into the oceanic sedimentary subsurface (subseafloor sediments) and the rocky subsurface (e.g., oceanic crust, ultra-basic sites, submarine volcanoes, axial seamounts) (Figure 2).

Since the deep subseafloor basement is equally difficult to sample as the continental subsurface, most microbial surveys on the oceanic subsurface have focused on subseafloor sediments (Cragg and Parkes, 1994; Parkes *et al.*, 2000; D'Hondt *et al.*, 2004, 2019a; Kallmeyer *et al.*, 2012; Inagaki *et al.*, 2015; Wörmer *et al.*, 2019). In deeply buried sediments sampled through scientific drilling programs such as the International Ocean Discovery Program (IODP), the discovered bacteria and archaea are capable of heterotrophic metabolisms, sulfate reduction, and methanogenesis, among other metabolisms. As in a continental subsurface environment, the distribution of microorganisms is heterogeneous in the deep-sea sediments and their numbers decrease significantly with depth (Ciobanu *et al.*, 2014). Metabolic rates appeared to be extremely slow (D'Hondt *et al.*, 2002; Biddle *et al.*, 2012; Orcutt *et al.*, 2013a; Lever *et al.*, 2015a; Braun *et al.*, 2017; Volpi *et al.*, 2017; Reese *et al.*, 2018). As they are compact and thus not very permeable, marine subsurface sediments represent an inhospitable ecosystem. The deep-sea sediments are sometimes described as inhabited by microorganisms in a zombielike state, that are racing to their death as cells are buried deeper and deeper in the sediments over time. The energy and available nutrients become increasingly rare and the metabolic rates are extremely low (Starnawski *et al.*, 2017). However, despite the energy limitation, cells can survive and be active for millions of years at thousands of meters in the sediments (Morono *et al.*, 2011; Orsi *et al.*, 2013; Inagaki *et al.*, 2015; Jorgensen and Marshall, 2016). While the deeply buried sediments can be viewed as energetic deserts, geochemical transition zones might represent oases of microbial life (Zhao *et al.*, 2020). These zones correspond to a redox potential discontinuity layer that marks the transition from oxidizing to reducing conditions in the sediments, where reduced and oxidized chemical compounds converge. This is correlated with an increase in available energy from redox reactions that can be harvested by microbial cells through specific metabolic pathways. These include the sulfate–methane transition zones (Iversen and Jorgensen, 1985; Parkes *et al.*, 2005), nitrate–ammonium transition zones (Zhao *et al.*, 2020) and the oxic–anoxic transition zones (Zhao *et al.*, 2019). The increased energy supply in transition zones allows starved cells to revive and grow locally. Yet, the biosphere in deeply buried sediments is still under investigation and many discoveries are about to be made.

Despite over 50 years of deep-sea drilling, less is known about the microbial diversity of the oceanic crust because it is also a heterogeneous environment and it is even more difficult to access. The upper layer of the igneous oceanic crust is mainly composed of porous and permeable basaltic lava (Edwards *et al.*, 2005). It is continuously created at mid-ocean ridges by eruptions of lava and its solidification occurs by contact with the cold seawater. The oceanic crust encompasses not just the host rocks, but the fluids that circulate vigorously through them (Furnes and Staudigel, 1999; Edwards *et al.*, 2005; Orcutt *et al.*, 2011b). Basalt-seawater reactions occur at high temperatures and produce highly reduced, metal-rich fluids that are in disequilibrium with the surrounding seawater. These spontaneous oxidation-reduction reactions cause the weathering of basalts altering significantly the oceanic crust and provide potential energy sources that allow chemolithotrophic foundation for basalt-hosted biomes (e.g., through Fe and S oxidation) (Bach and Edwards, 2003; Amend and Teske, 2005; Staudigel *et al.*, 2008; Santelli *et al.*,

2009). A distinction can be made between the warm, anoxic basement (vigorous circulation of highly reduced fluids and high temperature) and the cold, oxic basement (passive circulation, <20°C).

Subseafloor borehole observatories, or CORKs (Circulation Obviation Retrofit Kit observatories), were installed in the subseafloor basement to collect and study the fluid circulation in the oceanic crust (Davis *et al.*, 1992; Becker and Davis, 2005; Wheat *et al.*, 2011). The first investigation of the crustal biosphere was conducted on fluids (~65°C) extracted hundreds of meters below the seafloor through a CORK observatory located in the 3.5 million-year-old ocean crust at the Juan de Fuca Ridge (JdFR) flank (Cowen *et al.*, 2003). In this pioneer publication, the authors gave the first confirmation of microbial life in the deep marine igneous basement. Using 16S rRNA gene cloning and sequencing, they revealed the presence of diverse thermophilic bacteria and archaea capable of nitrate and sulfate reduction, as well as heterotrophic fermentation. They also discovered a *Firmicute* lineage that was later found to be closely related to the terrestrial subsurface lineage *Candidatus* Desulforudis audaxviator (Chivian *et al.*, 2008; Jungbluth *et al.*, 2017). A decade after that, a study at JdFR flank revealed a temporal dynamic of this biosphere showing significant variation in microbial community structure of the fluids over three years of sampling (Jungbluth *et al.*, 2013). Studies on the rocky basement of JdFR flank identified bacteria and archaea involved in methane- and sulfur-cycling (Lever *et al.*, 2013), as well as iron- and nitrogen-cycling (Orcutt *et al.*, 2011a; Smith *et al.*, 2011). Using 16S rRNA gene amplicon sequencing, lineages such as *Archaeoglobi*, *Aminicenantes*, and *Acetothermia* were discovered in the crustal fluids recovered from these CORKs observatories (Robador *et al.*, 2015), and their presence was later confirmed by metagenome sequencing and genome binning (Jungbluth *et al.*, 2017). CORKs observatories were also installed in other places in the world. For example, one is located on the Costa Rica ridge flank and allowed the study of the warm and anoxic basement crust in another location. The microbial communities detected there were different than the one at JdFR flank, and revealed lineages of sulfur-oxidizing bacteria (Nigro *et al.*, 2012).

It has been shown that a large portion of the oceanic crust is oxic, due to seafloor hydrothermal circulation refilling oxygen at depth (Røy *et al.*, 2012; Ziebis *et al.*, 2012; Orcutt *et al.*, 2013b; Braun *et al.*, 2017). Several CORKs observatories were installed on the western flank of the Mid-Atlantic Ridge, at North Pond, where the ocean crust is colder, still relatively young (~8 Ma), and oxygenated (Edwards *et al.*, 2012a). The microbial communities were dominated by the bacterial phylum *Proteobacteria* and were involved in both heterotrophic and autotrophic metabolisms (Meyer *et al.*, 2016). A study revealed that the microbial communities detected in the subsurface oceanic crust in the young and cool ridge flank system of North Pond were closely intertwined with the microorganisms inhabiting the overlying sediments (Jørgensen and Zhao, 2016). In addition, members of these microbial communities could use hypoxic or anoxic conditions (Tully *et al.*, 2018).

Overall, diverse microbial communities from crustal environments have been detected with a large range of bacterial phyla. This includes, for example, the presence of *Deltaproteobacteria*, *Firmicutes*, *Gammaproteobacteria*, and *Bacteroidetes* in the JdFR and the Costa Rica Rift (Nigro *et al.*, 2012; Jungbluth *et al.*, 2013, 2014), or dominance of *Gammaproteobacteria* and *Alphaproteobacteria* in seafloor basaltic glass from the East Pacific Rise (Santelli *et al.*, 2008, 2009), the Arctic spreading ridges (Lysnes *et al.*, 2004), altered basalts from the Hawaiian Loihi Seamount (Templeton *et al.*, 2005; Santelli *et al.*,

2008; Jacobson Meyers *et al.*, 2014) and the Mid-Atlantic Ridge (Rathsack *et al.*, 2009; Mason *et al.*, 2010).

After many drillings and comparisons between the warm/anoxic and cold/oxic oceanic crust, it seems that *Alpha-* and *Gammaproteobacteria* are more often detected in cold basaltic habitats (<10°C) that are exposed to oxic seawater, while microorganisms involved in sulfate reduction are more often detected in the warm and anoxic crust (Orcutt *et al.*, 2020). In addition, the comparison of biomes hosted in young (<~3 Ma) and old (80 Ma) oceanic crust revealed a significant correlation between microbial community and basalt age. Microbial diversity increases with the age of the basalt (Orcutt *et al.*, 2020). Moreover, the community composition detected in old basalt converges towards similar profiles over time (Lee *et al.*, 2015) in comparison with the great differences observed between young basaltic crusts.

Other types of marine subsurface environments exist, such as ultra-basic sites, submarine volcanos and seamounts. Ultra-basic sites, such as Lost City hydrothermal field (LCHF) and Prony, are influenced by marine serpentinization, a geochemical process that produces fluids with a very high pH by the reaction on minerals within the rocks and seawater (Charlou *et al.*, 2002; Schrenk *et al.*, 2013; Lecoeuvre *et al.*, 2021). It abiotically produces hydrogen, methane, and small-chain hydrocarbons, which can be used as energy for microbial metabolisms. Differences in microbial diversity have been shown between the actively and inactively venting carbonate chimneys of LCHF (Schrenk *et al.*, 2004; Brazelton *et al.*, 2006). While the active ones are dominated by methane-cycling archaea in their anoxic interior and by methanotrophic and sulfur-oxidizing bacteria in the exterior, inactive chimneys are much more diverse (Brazelton *et al.*, 2010). The chimney from Prony also exhibits diverse microbial communities, which are dominated by *Firmicutes*, *Euryarchaeota* (*Methanosarcinales*), and *Chloroflexi* (Quéméneur *et al.*, 2014; Postec *et al.*, 2015). On the contrary, submarine volcanos such as the Suiyo and seamounts emit fluids with a high concentration of H_2S with a pH lower than the seawater. Microbial communities from these volcanos often involved sulfur-oxidizing *Epsilonbacteraeota* (Higashi *et al.*, 2004; Opatkiewicz *et al.*, 2009). However, more microbial surveys on different locations and crustal types are needed to globally understand the oceanic crust biosphere.

1.4 Subsurface Microbial Metabolisms

Most microorganisms obtain their energy from nutrients they absorb from the environment. After entering the cell, the nutrients are chemically processed to be transformed into energy. This involves a series of chemical reactions, called the metabolic pathway, where large molecules are broken down into smaller molecules that can be used for the synthesis of new cellular components and the production of energy in the form of ATP. The metabolism is the sum of all the chemical reactions that occur in the living organism and all life forms require a source of energy for growth, repair, and maintenance of the chemical and physiological processes. Anabolic reactions of the metabolism use ATP for the synthesis of large molecules from smaller constituents, while catabolic reactions break down those large molecules into smaller ones enabling the production of energy.

Microorganisms are categorized based on how they obtain energy and carbon from the environment. Heterotrophic and autotropic microorganisms can be distinguished according to their source of carbon which can be of organic or inorganic origin (carbon dioxide, CO_2). They are called chemotrophs or phototrophs if the energy comes from a chemical source or the sunlight, respectively. Therefore, we count chemoautotrophs (e.g., hydrogen-, sulfur-, iron-, nitrogen-, and carbon monoxide-oxidizing bacteria), chemoheterotrophs (all animals, most fungi, protozoa, and bacteria), photoautotrophs (plants, algae, cyanobacteria, and green and purple sulfur bacteria), and photoheterotrophs (green and purple non-sulfur bacteria and heliobacteria) based on this classification by energy and carbon sources. In addition, a distinction is made within the chemotrophs according to the nature of the electron donors that can be from organic (chemoorganotrophs) or inorganic (chemolithotrophs) sources.

Catabolic metabolism includes respiration and fermentation. Respiration uses an electron donor (reducing agent) and an electron acceptor (oxidant). The transfer of electrons creates a gradient of protons that powers the synthesis of ATP through the oxidative phosphorylation of the ADP. This process called chemiosmosis is performed by the action of a large enzyme called ATP synthase. Through respiration, microorganisms can use many inorganic compounds as electron acceptors (oxygen, O_2; sulfate, SO_4^{2-}; nitrate, NO^{3-}; etc.). When O_2 is used as the electron acceptor, microorganisms are aerobic and when they use another type of electron acceptor they are anaerobic. The O_2 is an oxidizing agent with high energy, making this molecule a good electron acceptor. However, other molecules such as SO_4^{2-}, NO^{3-} or sulfur, S, as terminal electron acceptors have less potential for reduction thereby releasing less energy, which makes the anaerobic respiration less efficient than aerobic respiration. Microorganisms use these redox reactions for the acquisition of their energy. Since the energy released changes depending on the redox reaction, some metabolic pathways are more or less energetic than others (Amend and Shock, 2001; McCollom and Amend, 2005; LaRowe and Amend, 2015). Fermentation is an alternative metabolism to respiration, occurring in absence of O_2. Fermentation and respiration start in the same way while during fermentation, the electron transport chain is not functional due to the absence of O_2, and therefore the energy is not released. Instead, ATP is generated by substrate-level phosphorylation, a reaction that transfers a phosphate group from a substrate directly to ADP. Fermentation breaks down organic molecules into lactate (lactic acid fermentation) or alcohol (alcohol fermentation) to produce energy. During fermentation, ATP is produced by a process that is less energetic than the oxidative phosphorylation associated with respiration. In general, aerobic metabolisms require more energy for the fixation of carbon.

Although the subsurface is a heterogeneous environment in terms of physical and chemical conditions, some themes are common, such as the absence of light and consequently the complete absence or rare occurrence of light-derived biomass and the insufficiency of nutrients (Hoehler and Jørgensen, 2013; Lever *et al.*, 2015a). Contrary to the deep-sea sediments, the crust is a porous and permeable environment (Magnabosco *et al.*, 2019). Seawater or meteoric water circulates in and out of the crust through the cracks and fissures, where it undergoes fluid-rock reactions with silicates and other minerals in the rock, changing the chemical content of the fluid over time and creating redox gradients. The oxygenated seawater mixes with reduced hydrothermal fluids or minerals, which create chemical disequilibria that microorganisms can exploit for metabolic energy and biomass production (McCollom and Shock, 1997; Orcutt *et al.*, 2011b; Edwards *et al.*,

2012c). These reactions need to free enough energy for the cell to synthesize ATP (Amend and Shock, 2001; McCollom and Amend, 2005; LaRowe and Amend, 2015).

In oxic subsurface environments such as the oxic oceanic crust, seawater circulation brings energetic oxidants such as O_2 and NO_3^- that are consumed in the deeper levels (D'Hondt *et al.*, 2015). Less energetic oxidizing compounds such as CO_2 and SO_4^{2-} are found in greater abundance in the deep subsurface, influencing the metabolisms of the microbial communities involved. In hydrothermal vents or fractured rock aquifers such as the permeable oceanic crust, oxidized water mix with reduced subsurface fluids (Campbell et al. 2013; Meyer et al. 2016; Zinke et al. 2018; Dick 2019; Lang et al. 2019). In these environments, chemolithoautotrophs could be the foundation of ecosystem development, producing complex organic molecules and giving a source of energy and carbon to sustain chemoheterotrophs (Edwards *et al.*, 2012b). Whereas heterotrophs can also use dissolved organic carbon (DOC) transported from the surface in addition to the one produced *in situ*, chemolithoautotrophs fix dissolved inorganic carbon (DIC), such as CO_2, bicarbonate; HCO^{3-} and carbonate ion; CO_3^{2-}. Hydrogen-driven Subsurface Lithoautotrophic Microbial Ecosystems (SLiMEs) often inhabit oligotrophic subsurface environments (Stevens and McKinley, 1995). The electron donor and electron acceptor will be derived from an abiotic geochemical source (Andreani and Ménez, 2019). These ecosystems, fuelled by H_2, can support the occurrence of acetogens, autotrophic methanogens, and sulfate reducers (Lin *et al.*, 1961; Lever *et al.*, 2013; Magnabosco *et al.*, 2016). In those oligotrophic environments, the subsurface inhabitants with diverse functional traits cooperate to maximize the energy yield and growth because of the limitation of available nutrients and energy substrates (Biebl and Pfennig, 1978; Morris *et al.*, 2013). These trophic relationships and energy flow between and among the members of an ecosystem, allowing a syntrophic partnership, are based on obligate mutualistic metabolisms that benefit both partners (Morris *et al.*, 2013). For example, in deep sediments, anaerobic methanotrophic archaea are in syntrophic partnership with sulfate-reducing bacteria (Skennerton *et al.*, 2017). The anaerobic oxidation of the methane, CH_4, by the archaea constitutes the primary mechanism for methane removal in ocean sediments (Skennerton *et al.*, 2017) and has a significant ecological impact on a large diversity of biogeochemical cycles (Anantharaman *et al.*, 2016; Lau *et al.*, 2016). Indeed, through their metabolism, by degrading or synthesizing molecules to produce energy and biomass, microorganisms interact with their environment and influence it, perhaps even controlling elemental cycles on a global scale (LaRowe and Amend, 2015). The microbial activity in the subsurface can change the fluid's geochemical composition (Wankel et al. 2011, 2012), for example by increasing the concentration of CH_4 or decreasing the concentration of H_2S (Butterfield *et al.*, 2004; Proskurowski *et al.*, 2008). It can also change the degree of rock alteration (Ménez et al. 2018b), generating a cascade effect by having an impact on the fluid circulation and therefore on the microbial communities.

Although subsurface microbial activity potentially has a massive impact on the global biogeochemical cycles, the diversity of microbial metabolisms in the subsurface remains largely unknown. Many efforts are still needed to understand the global impact. And to do so, it is of fundamental importance to cultivate more microorganisms that are involved in these processes to describe their metabolisms and the different energy and carbon sources they require.

1.5 Influence of the Physico-Chemical Factors

The hypothesis "everything is everywhere, but, the environment selects" was first published in 1934 (Baas-Becking, 1934). While "everything is everywhere" indicates that microorganisms have a remarkable potential for dispersion, "the environment selects" implies that only some of them are adapted to thrive and proliferate in a specific environment. For a long time, microbial communities were assumed to have unlimited dispersal capacities throughout all environments. Currently, it is largely considered that even generalist microbial species may not be so widespread and tolerant when their habitats are considered. Although the "everything is everywhere" assumption is not correct, it has been used over the century as a null hypothesis offering valuable insights into microbial biogeography, evolution, and ecology. The second assumption implies that the ecological range of microbial life is constrained by the environment. Physical and chemical conditions of the environment control the establishment, abundance, diversity, and evolution of the microbial communities (Magnabosco *et al.*, 2019). Consequently, their physiology and metabolisms must be adapted to these environmental conditions. In subsurface environments, the physico-chemical conditions such as temperature, pressure, pH, and salinity are not homogeneous, continuously changing over time, and are sometimes extreme (Figure 3). Studying the correlation between these environmental factors and subsurface life has proven difficult to quantify, partly due to the lack of data and the problem of measuring environmental variables at appropriate resolutions. Only a few studies include subsurface samples near these extremes and the adaptation strategies of subsurface microbial communities remain poorly studied (Magnabosco et al. 2019).

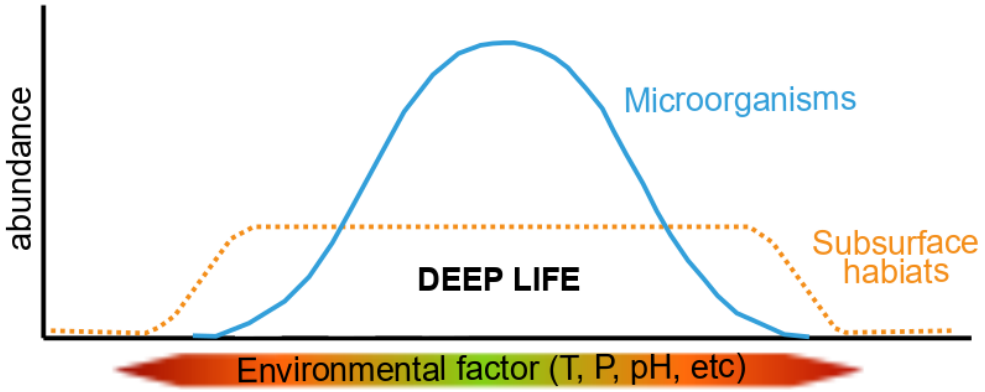

Figure 3. Distribution of the microbial abundance across subsurface habitats, which depends on environmental factors.The green color corresponds to the habitable region of the subsurface, while the red corresponds to the uninhabitable. The distribution of the subsurface life is represented by the overlap of the two curves (Magnabosco *et al.*, 2019).

Physical extremes such as high pressure as well as small particle sizes (~1 μm) of the rocks can be a limit to life in the subsurface environment (Fredrickson *et al.*, 1997). For example, the low porosity limits the physical space and fluid movement needed for the establishment and dissemination of microorganisms, also limiting the transport of nutrients (Kieft *et al.*, 1998; Zhang *et al.*, 1998; Rebata-Landa and Santamarina, 2006). As subsurface microorganisms face extreme environmental conditions, cell abundance decreases with depth (Cockell *et al.*, 2012; Mcmahon and Parnell, 2014). The subsurface environments

hold possibly more than 80% of all bacterial and archaeal species on Earth (Magnabosco *et al.*, 2018), thus most of the prokaryotes live under high-pressure conditions. Therefore, the distribution of life in the subsurface must be constrained by pressure. Although some well-adapted surface microorganisms are capable to grow under high-pressure conditions (piezotolerant), most of them deteriorate in these conditions. Likewise, metabolically active microorganisms isolated from pressurized subsurface environments often show optimal growth at high pressure but are incapable of growth at the surface, under ambient pressure conditions (strict piezophiles) (Cario *et al.*, 2019). The most piezophilic microorganism known to date was isolated from the deep marine environment, with optimum growth at a pressure of 120 MPa (Kusube *et al.*, 2017). Although the very limited number of piezophilic microorganisms observed by culture-independent techniques fails to represent the high diversity inhabiting subsurface ecosystems, different adaptation strategies to live under elevated pressure conditions were identified (Bartlett, 2002; Abe, 2007; Oger and Jebbar, 2010). These include biochemical modifications in the cell wall and membrane composition (Cario *et al.*, 2015) and specific high-pressure gene expressions (Vannier *et al.*, 2015; Michoud and Jebbar, 2016). Therefore, it is unlikely that microbial life in the subsurface is limited by pressure since cells have been shown to thrive at pressures far higher than those encountered in the Earth's habitable crust (Sharma *et al.*, 2002). High pressure comes often in addition to other stress factors such as temperature.

In the subsurface, geothermal gradients can be extreme and the variation of temperature can occur within centimeters. The temperature increases with depth, as a function of the local heat flux and thermal conductivity. It can exceed the presumed upper-temperature limit for a functional life, which is currently at 122°C for laboratory-grown strains (Takai *et al.*, 2008). Thus, our current understanding of the extent of the subsurface biosphere is limited to depths shallower than a theoretical ~120°C isotherm estimated at around 17 km (Colwell and Smith, 2004). Since the upper-temperature limit of microbial communities in their environment is still unknown (Amend and Teske, 2005; Magnabosco *et al.*, 2019), this theoretical depth at 17 km might change if microorganisms that can grow beyond this temperature are discovered. Furthermore, geothermal gradients in the subsurface are extremely variable over the globe and thus the theoretical ~120°C isotherm depth greatly varies by location. For instance, the temperature at geothermal active sites near the surface (e.g., hot springs, hydrothermal vents, rift zones) can be high enough to limit the growth of microorganisms. At the same time in other locations (e.g., at subduction zones), the temperature can decrease very slowly with depth, increasing the theoretical ~120°C isotherm depth to much deeper. Nevertheless, numerous studies show that cell abundance and temperature are negatively correlated in subsurface environments, the more the temperature increases the fewer cells are detected. This brings evidence that temperature is a determining factor for the structure and diversity of subsurface microbial communities (Baquiran *et al.*, 2016; Ramírez *et al.*, 2019). Adaptation strategies to elevated temperature include the stability of protein structures (Cacciapuoti *et al.*, 2004), genome reduction and gene loss (Burra *et al.*, 2010).

Many of the physical and chemical extremes that influence and limit life are still poorly studied in subsurface environments. In addition to space, nutrients, and temperature, other of these extremes may be permissive for growth. As with the temperature, large gradients of pH can be observed in the subsurface and this heterogeneity can be observed at the global scale as well as at the microscale (µm–cm). It can also change over time and influence the biochemistry of the microbial communities (Fones *et al.*, 2019). For example,

fluid-rock interactions influence the solubility of minerals, impacting the pH and the availability of nutrients and energy sources (Jin and Kirk, 2018). The archaeon *Aciduliprofundum boonei*, isolated from hydrothermal fluids, owns the record of growth at the lowest pH (pH 3.3) for a subsurface isolate (Reysenbach *et al.*, 2006). In comparison, the highest pH of 12.5 was observed for the bacterium *Serpentinomonas* sp. B1, isolated from a continental serpentinizing site (Suzuki *et al.*, 2014). Despite this, the pH limits for subsurface life in the environment is still unknown. Although some microorganisms or microbial communities can thrive at extreme pHs (Takai *et al.*, 2005; Blank *et al.*, 2009), no studies show that subsurface microbial life is limited by pH as the sole stressor. Maintaining a cytoplasmic pH lower than the outside pH, for example, is an adaptation strategy to high pH.

As another chemical extreme, the salinity can be a limiting factor for subsurface microbial life due to the high energetic costs related to osmotic adaptation (Boetius and Joye, 2009), the limitation of available water (Stevenson *et al.*, 2015), and the chaotropicity affecting the structure of the molecules (Hallsworth *et al.*, 2007; Magnabosco *et al.*, 2019). High salt concentrations can be found in some subsurface environments such as in the deep sea, where halophilic microorganisms were isolated (Antunes *et al.*, 2008). However, some studies showed that a high concentration of dissolved ions such as magnesium and chloride can inhibit microbial growth (e.g., evaporites in deep mine (Payler *et al.*, 2019).

In addition, the redox conditions of the environment can greatly influence the diversity of subsurface microbial communities (Ramírez-Flandes *et al.*, 2019). Oxygen is most often lacking in subsurface environments (except the oxic oceanic crust) and its presence is a limiting factor for some microorganisms. Indeed, strict anaerobic microorganisms are unable to grow in the presence of oxygen. However, adaptation strategies exist to overcome the variation of redox conditions in the subsurface, including the formation of biofilms (Flemming and Wuertz, 2019). Production of extracellular polymeric substances (EPS) around the cells could protect them from the variation in oxygen concentrations from the outside, creating an anoxic micro-environment for optimal carbon fixation (Stokke *et al.*, 2015; Meier *et al.*, 2017). It has been shown that a significant difference exists between the microbial communities detected in rock-attached biofilms and the ones that inhabit the circulating fluids within the oceanic crust (Ramírez *et al.*, 2019). Finally, it has been shown that the minerals present in the rock could influence the structure of the microbial communities in the oceanic crust (Toner *et al.*, 2013; Smith *et al.*, 2017). However, this has been contradicted by studies suggesting that temperature and/or redox conditions have a much more structuring influence on the communities than minerals (Baquiran *et al.*, 2016; Ramírez *et al.*, 2019). The limits and boundaries of life as we know could change anytime with discoveries of still unknown microorganisms and new microbial adaption strategies.

1.6 Study Site: The Surtsey Volcano Geothermal System

1.6.1 Location, Formation and Protection

Surtsey is a volcanic island formed by 3.5 years of basaltic eruptions between November 14th, 1963 and June 5th, 1967. The Surtsey eruption is one of Iceland's longest eruptions in recorded history. Surtsey, or Surt's island, is named after Surt, a fire giant in Norse mythology, “ey” meaning “island” in Icelandic. It is part of the Vestmannaeyjar (Vestmann Islands) archipelago (63°18'10.8'N; 20°36'16.9'W), a volcanic system within the southern offshore extension of Iceland’s Eastern Volcanic Zone (Figure 4). The island is located 32 kilometers from Iceland's south coast (Thorarinsson *et al.*, 1964).

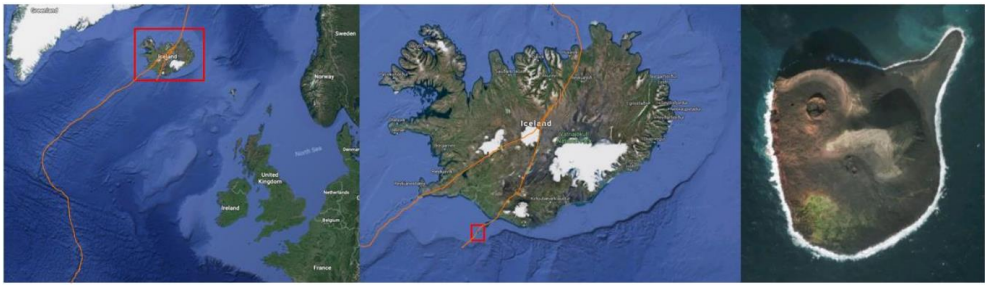

Figure 4. Location of Surtsey island on the southern offshore extension of Iceland’s Eastern Volcanic Zone (illustrated in orange).

The ocean depth was 130 m below sea level before the eruption. At the end of the eruption, in June 1967, Surtsey had a surface area of 2.65 km^2, and its highest point was 174 meters above sea level. The entire erupted volume was roughly 1 km^3 (Schipper *et al.*, 2015). Explosive eruptions followed by basaltic lava flows were extremely well documented, and this occurrence constituted a new form of volcanic eruption (Thorarinsson *et al.*, 1964; Thorarinsson and Þórarinsson, 1965; Kokelaar, 1983). Surtseyan volcanism is a kind of shallow subaqueous explosive volcanism in which magma-water interaction has a significant impact on plume and fragmentation dynamics, resulting in characteristic steam-rich tephra jets and fine-grained deposits (Walker, 1973). As the volcanic ash from which they are created quickly solidifies into a hard rock known as 'tuff,' they pile around the crater to form a cone known as a 'tuff cone.' The Surtsey eruptions formed two semicircular-shaped tephra cones and two half lava shields formed, one in the Surtungur cone and the other in the Surtur cone (Figure 5). In 2020, the island has eroded to less than 1.2 km^2 and a height of 150 m above sea level due to sea erosion (Baldursson & Ingadóttir, 2007; Óskarsson *et al.*, 2020).

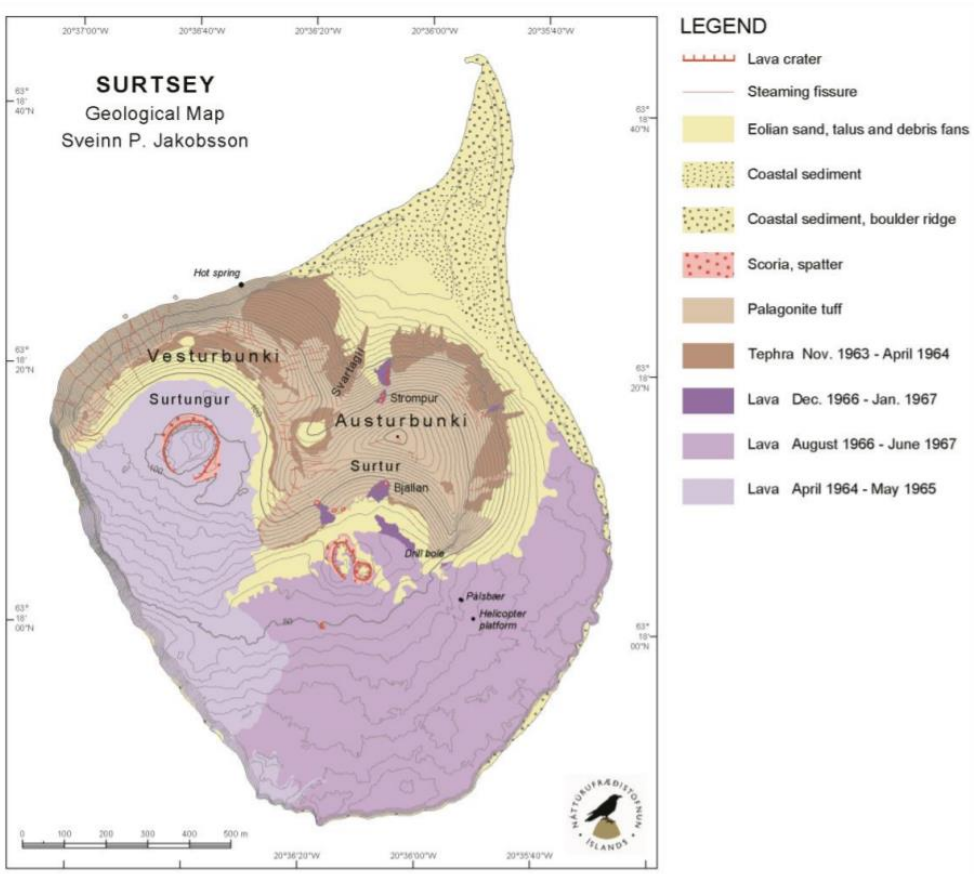

Figure 5. Geological map of Surtsey (Baldursson & Ingadóttir, 2007). Colors refer to basaltic rock compositions and time of formation.

The island has been protected since the initiation of eruptive activity and, since then, has been accessible only to scientific investigations. Long-term longitudinal studies have provided a unique record of pioneering species of microorganisms, plants and animals colonizing the surface of the basaltic deposits (Brock, 1966; Schwartz *et al.*, 1972; Henriksson & Henriksson, 1974, 1982; Baldursson & Ingadóttir, 2007; Magnússon *et al.*, 2014). The Surtsey island and its underwater surroundings were designated a World Heritage Site by the United Nations Educational, Scientific and Cultural Organization (UNESCO) (https://whc.unesco.org/en/list/1267) in 2008 (Baldursson & Ingadóttir, 2007). The Surtsey research society was created to organize and promote research relating to the island of Surtsey in the earth and biological sciences and has published many scientific reports over the years (https://english.surtsey.is/).

1.6.2 Geothermal System

In 1979, a 181 m core (SE-01) was drilled through the eastern sector of the Surtur vent (Figure 6). The top of the core is situated at about 58 m above the sea level and is assumed to end a few meters above the pre-eruption sea floor (Jakobsson and Moore, 1982).

Investigations of the core described volcanic structures in the subaerial and submarine deposits – above and below coastal sea level, respectively, as well as the hydrothermal system, and the composition and rapid alteration of the basaltic tephra that occurred through palagonitization processes (Jakobsson, 1978; Jakobsson and Moore, 1982, 1986; Jackson *et al.*, 2019a). In 2017, three new cored boreholes (SE-02a, SE-02b and SE-03; Figure 6) were acquired through the International Continental Scientific Drilling Program (ICDP) 5,059 expedition, the SUSTAIN drilling operation (Jackson *et al.*, 2015, 2019b; Weisenberger *et al.*, 2019).

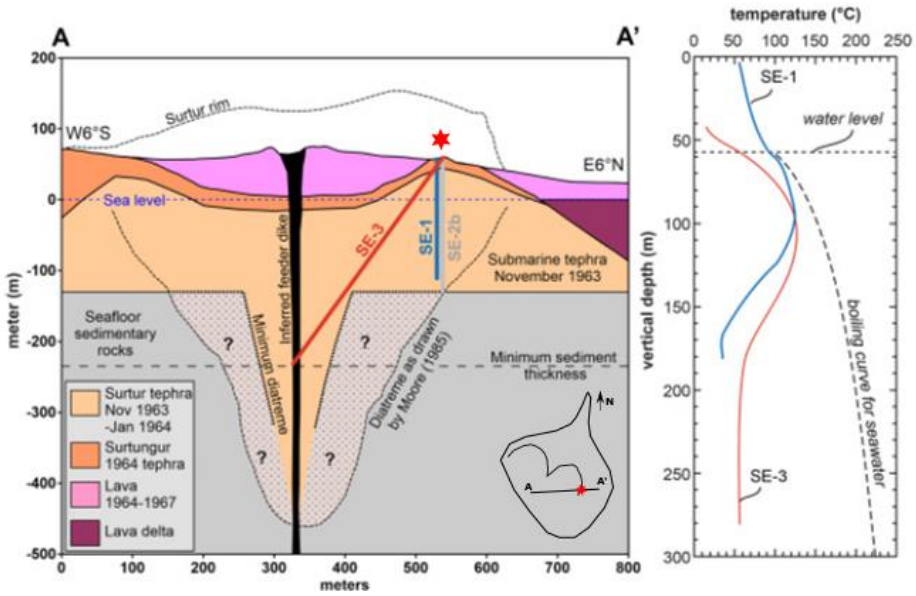

Figure 6. Cross-section of the Surtur vent, the eastern crater of Surtsey, showing eruptive deposits (subaerial, submarine tuff and lava shield in the central crater), seafloor sediments and sedimentary rocks, a hypothesized sub-seafloor diatreme as deduced from eruptive history by Moore (1985), location of the 1979 borehole, SE-01, and the 2017 boreholes, SE-2B and SE-03 (red star), and corresponding temperature profiles from 2017 (Jackson *et al.*, 2019b; Kleine *et al.*, 2020).

The hydrothermal system has cooled down over the years, according to annual temperature monitoring in the SE-01 borehole. At 100 m depth, the maximum temperature was 141.3°C in 1980, and it progressively fell to 124.6°C in 2017 (Figure 6) (Jakobsson and Moore, 1986; Ólafsson and Jakobsson, 2009; Marteinsson *et al.*, 2015; Jackson *et al.*, 2019b). Seawater infiltrates the tephra deposits in a submarine inflow zone at 144–155 m b.s (Jakobsson and Moore, 1986; Jackson *et al.*, 2019b; Kleine *et al.*, 2020). The subsurface geochemistry and mineralogical changes in the altered basaltic deposits have been widely described over time (Jakobsson and Moore, 1982, 1986; Schipper *et al.*, 2016; McPhie *et al.*, 2020; Moore and Jackson, 2020; Prause *et al.*, 2020). The basaltic deposits of Surtsey host a seawater-dominated geothermal system with a gradient of temperatures varying between 40 and 141°C. Chemical analyses of borehole fluids showed depletion in elements, such as Mg, SO_4, and dissolved inorganic carbon (Figure 7) indicating that the associated geothermal waters in submarine deposits originate from seawater. Basalt alterations at those temperatures modified the chemical concentration of the seawater that transformed into geothermal waters (Kleine *et al.*, 2020, 2022).

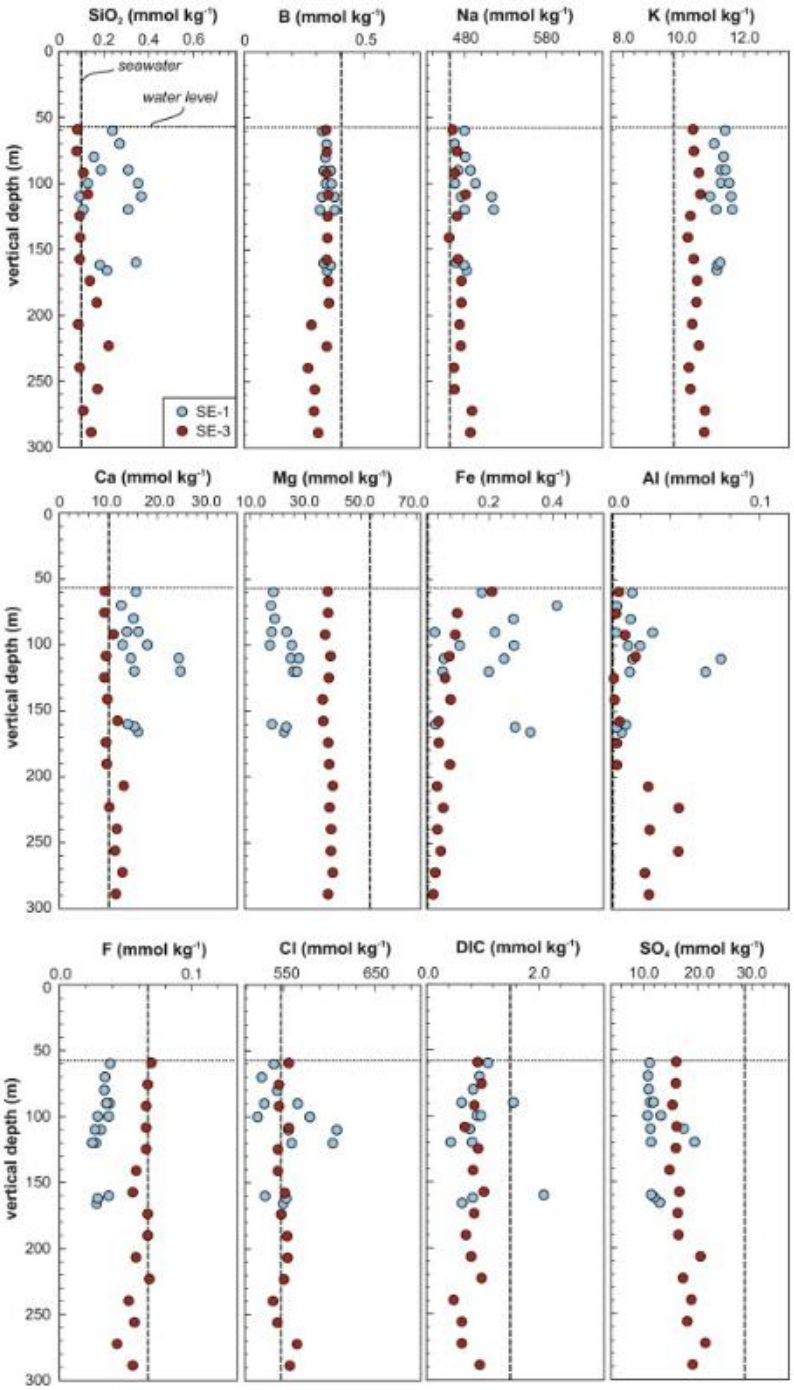

Figure 7. Elemental concentrations from SE-01 (blue) and SE-03 (red) borehole fluid samples as a function of the depth. Horizontal dotted line: costal seawater level in the boreholes. Vertical dashed line: average concentration of the corresponding element in seawater. DIC = dissolved inorganic carbon. From (Kleine *et al.*, 2020).

1.6.3 Previous Microbial Investigation

During a pioneering study that aimed to explore the subsurface microbial communities inhabiting the Surtsey volcano, five borehole water samples were collected in 2009 using a stainless-steel bailer connected to a slickline (Marteinsson *et al.*, 2015). The sampling procedure consists of lowering the open bailer into the borehole to the desired sampling depth, sending a glass fiber jar that moves freely along the slickline and hit the top of the bailer to mechanically close it. The sealed bailer that contains the sample is then retrieved to the surface (Figure 8) (Kleine *et al.*, 2020).

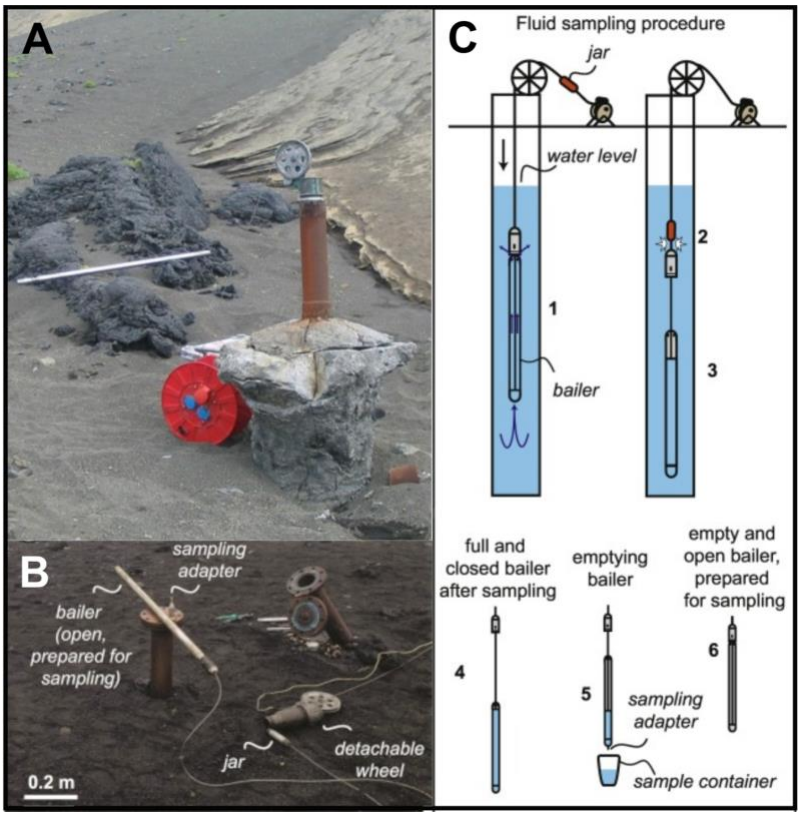

Figure 8. Sampling procedure to collect borehole fluid samples. A) Top of borehole SE-01 drilled in 1979. B) Stainless-steel bailer connected to a slickline. C) Sampling procedure modified from (Kleine *et al.*, 2020).

The total capacity of the one meter-long, 5 cm-diameter, cylindrical sampler is about 1.3 L. The samples SB1, SB2, SB4, SB5 and SB6 were collected at 57, 58, 145, 168 and 170 m depth, respectively. The *in-situ* temperature measured 100°C for both SB1 and SB2, 80°C for SB4 and 54-55°C for SB5 and SB6. The pH and salinity of the samples were also measured and showed values around 8 and 3.7%, respectively (Marteinsson *et al.*, 2015). Samples SB1 and SB2 correspond to the zone of daily intertidal fluctuations at coastal sea level, located at 58 m below surface. Aliquots of fluid samples were reduced using a Na_2S solution (0.05% *w/v* final concentration) and kept under anaerobic conditions at 4°C until cultivation experiments. Different media were tested (e.g., R2A, 162, "Thermotoga" media and YPS) at 40, 60 and 80°C but no growth was observed after 6 weeks of enrichment

(Marteinsson *et al.*, 2015). Fluids were also filtered through 0.22 µm pore size cellulose membrane filters and DNA was extracted from the biomass. After PCR amplification, both the construction of clone libraries and pyrosequencing were successful for some of the samples.

Bacterial clone libraries from samples SB5 and SB6 (168 and 170 m b. s., respectively) were dominated by bacterial 16S rRNA genes sequences that showed high percentages of sequence similarity with sequences of the uncultured *Deltaproteobacterium* ANOX-077 (15 clones) received from hydrocarbon polluted subtidal marine sediments (Genbank: JF344639) and the uncultured bacterium (most likely *actinobacteria*) clone MD08f7_11419 (11 clones) retrieved from soil exposed to elevated atmospheric CO_2 (JQ369412). Other bacterial clones were closely related to sequences retrieved from intertidal marine sediments (AY568900; *Deltaproteobacteria*), contaminated soil (EU517548, *Desulfobacteraceae*), borehole fluid from a gold mine (AY741689, *Candidatus* Desulforudis) and marine sediments (FJ455891) (Marteinsson *et al.*, 2015).

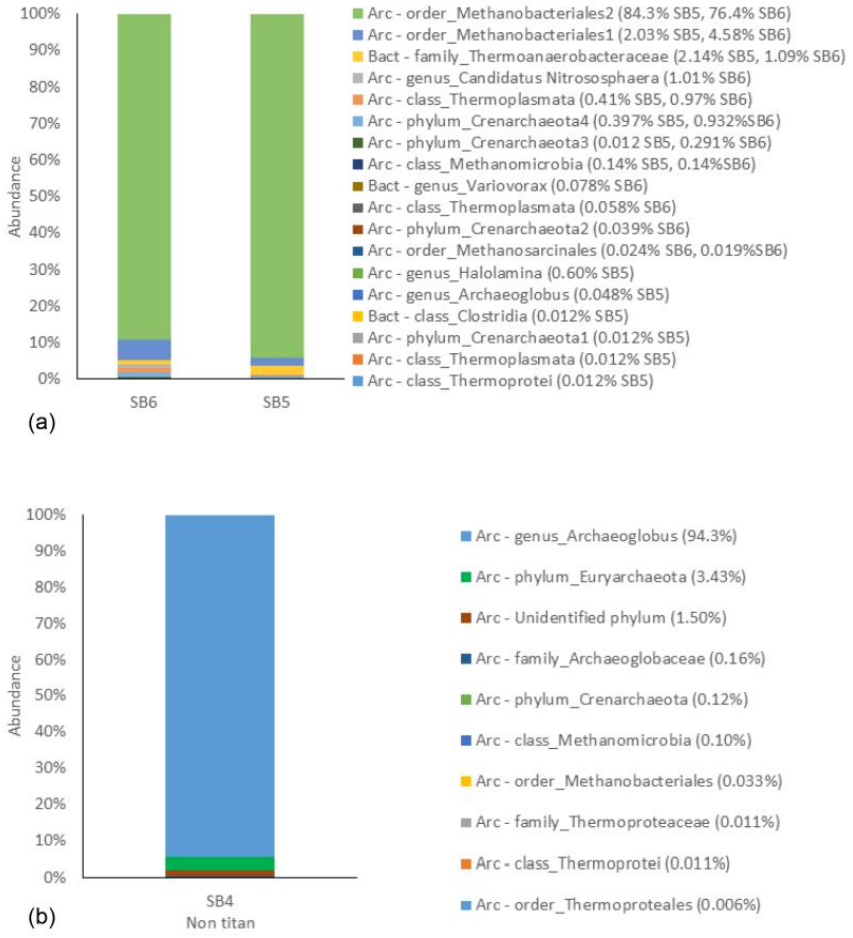

Figure 9. 16S rRNA gene sequences obtained by pyrosequencing from borehole fluid samples (SB4, SB5 and SB6) collected from SE-01 (Marteinsson *et al.*, 2015).

Archaeal clone libraries from sample SB6 (180 m b.s.) were dominated by 16S rRNA gene sequences that showed high percentages of similarity with sequences of an uncultured archaeon retrieved from the deep continental crust (DQ354739). Out of the 40 clone sequences, two were closely related to an uncultured archaeon retrieved from marine sediments (DQ988142) and deep-sea hydrothermal sediments (AB301979). Pyrosequencing analyses from the same study showed that the SB4 v6 library consisted mostly of an Operating Taxonomic Unit (OTU) assigned to the (hyper)thermophilic and sulfate-reducing archaea *Archaeoglobus* from the phylum *Euryarchaeota* (94.5%). However, libraries from longer reads of the v4– v6 regions on samples SB5 and SB6 showed the main OTU assigned to a taxon from the archaeal order *Methanobacteriales* (SB6; 76.5% and SB5; 84.2%) (Figure 9).

Overall, the taxa detected in this study were closely related to sequences retrieved from both terrestrial and marine habitats, and their taxonomic affiliations suggest that they could be involved in the sulfur cycle and methanogenesis.

Furthermore, recent studies of 1979 and 2017 drill core samples showed abundant microtubule structures in basaltic glass that resemble endolithic micro-borings (Jackson *et al.*, 2019a; Jackson, 2020). The observation of these structures in drill core samples retrieved from the subsurface of Surtsey could indicate biotic alteration of the basalt (microbial microboring into the glass), as has previously been observed in other basalt samples (Fisk *et al.*, 2003; Staudigel *et al.*, 2008; Walton, 2008; Mcloughlin *et al.*, 2010).

2 Objectives

This study explores the microbial communities inhabiting the basaltic subsurface of the Surtsey volcano in Iceland. It employed a collection of samples that included drill cores originating from successive depths, associated borehole fluids and fumarole samples. The objectives were to investigate the structure of the microbial communities as well as their taxonomic diversity and distribution among the sample types. Also to evaluate the functional potential of the microbial communities and the colonization of the newly formed basaltic crust. A further objective was to cultivate subsurface microorganisms from Surtsey island in the laboratory to discover and characterize novel species and to assess the culturable diversity in comparison to in-situ diversity estimated by molecular methods. Finally, to estimate the cell abundance as a function of depth and to investigate their organization towards the basaltic substrate. Specific research questions were outlined in the papers, which form the basis of the thesis, and are summarized below.

In **Paper I**, the following research questions were addressed: Has microbial life colonized the basaltic cores only 50 years after the eruptions terminated? Does life persist in the subsurface zones crossing the presumed thermal limit for life? How can we distinguish the true residents of the subsurface from the marine microbial taxa derived from the drilling fluid? Which microorganisms inhabit Surtsey's basalt and fluids and what is their metabolic potential? Do the microbial communities differ between the subaerial and submarine deposits?

Paper II examines the culturable microbial diversity of the subsurface of the Surtsey volcano estimated using diverse culture conditions. The following questions were inquired: What portion of the Surstey subsurface biosphere can we cultivate in the laboratory using standard methods? Can we isolate new species? From where do the closest known relatives of the isolates originate and what are the phylogenetic relationships? Were the isolates detected in the Surtsey subsurface by culture-independent methods?

Paper III describes a novel thermophilic bacterium isolated from the subsurface of the Surtsey volcano. The following questions were evaluated: How does the novel bacterium differ from its closest cultured relatives in terms of ecology, physiology, chemotaxonomy and genome features? What are the phylogenetic relationships between the microorganisms and do the Surstey isolates represent a novel taxonomic group?

The following questions were assessed in **Paper IV:** Can we estimate the cell numbers in the drill core samples as a function of depth? Can we detect cells, actual microbial structures, or biotic signatures in the basaltic cores only 50 years after the eruptions terminated? And if so, how organized are the cells towards the microstructures of the subtract (e.g. cavities, vesicles, tubules, cracks, etc.) and the minerals?

3 Methods

3.1 Experimental Settings on Surtsey Volcano

3.1.1 Drilling Operation

During the ICDP SUSTAIN drilling operation at Surtsey, from August 10th to September 4th 2017, an Atlas Copco CS1000 drill rig was used to drill through the volcano, with its components flown by helicopter to Surtsey and reassembled on-site (Jackson *et al.*, 2019b; Weisenberger *et al.*, 2019). In addition to the SE-01 vertical borehole drilled in 1979, three other new boreholes were drilled in 2017.

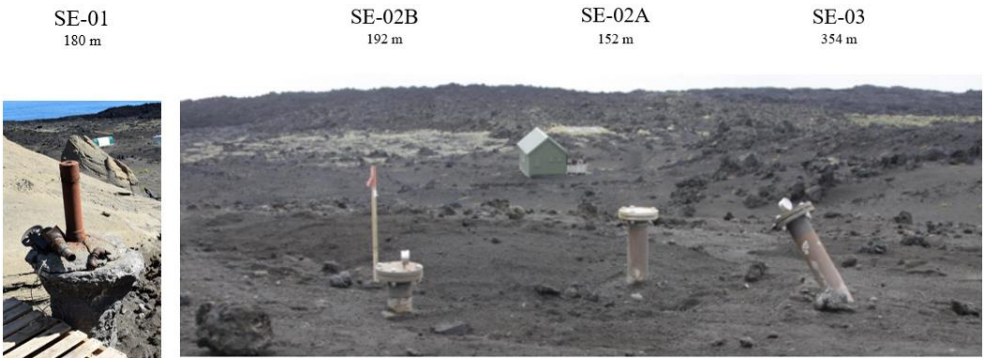

Figure 10. Pictures of the four well head boreholes and their respective depths.

The first vertical borehole, SE-02A, was cored to 152 m b.s. until the borehole collapsed. The second vertical, SE-02B, the cored borehole was drilled to 192 m. The third borehole, SE-03, was drilled under the eastern crater, angled at 35° from the vertical and reached a measured depth of 354 m (290 m vertical depth) (Figure 10).

At the drill site, the seawater drilling fluid for boreholes SE-02A and SE-02B was filtered and UV-sterilized twice. When coring SE-02A, no mud products were utilized, however, minor volumes of attapulgite mud were used in SE-02B and SE-03. The drill cores were processed on-site and samples were taken for pore water geochemical analysis and microbiological examinations (Figure 11). A helicopter brought about 650 meters of the core to Heimaey stored in core boxes, where it was scanned, documented, and described. Today, both the 1979 and 2017 cores are stored at the Icelandic Institute of Natural History (Náttúrufræðistofnun íslands; https://www.ni.is/).

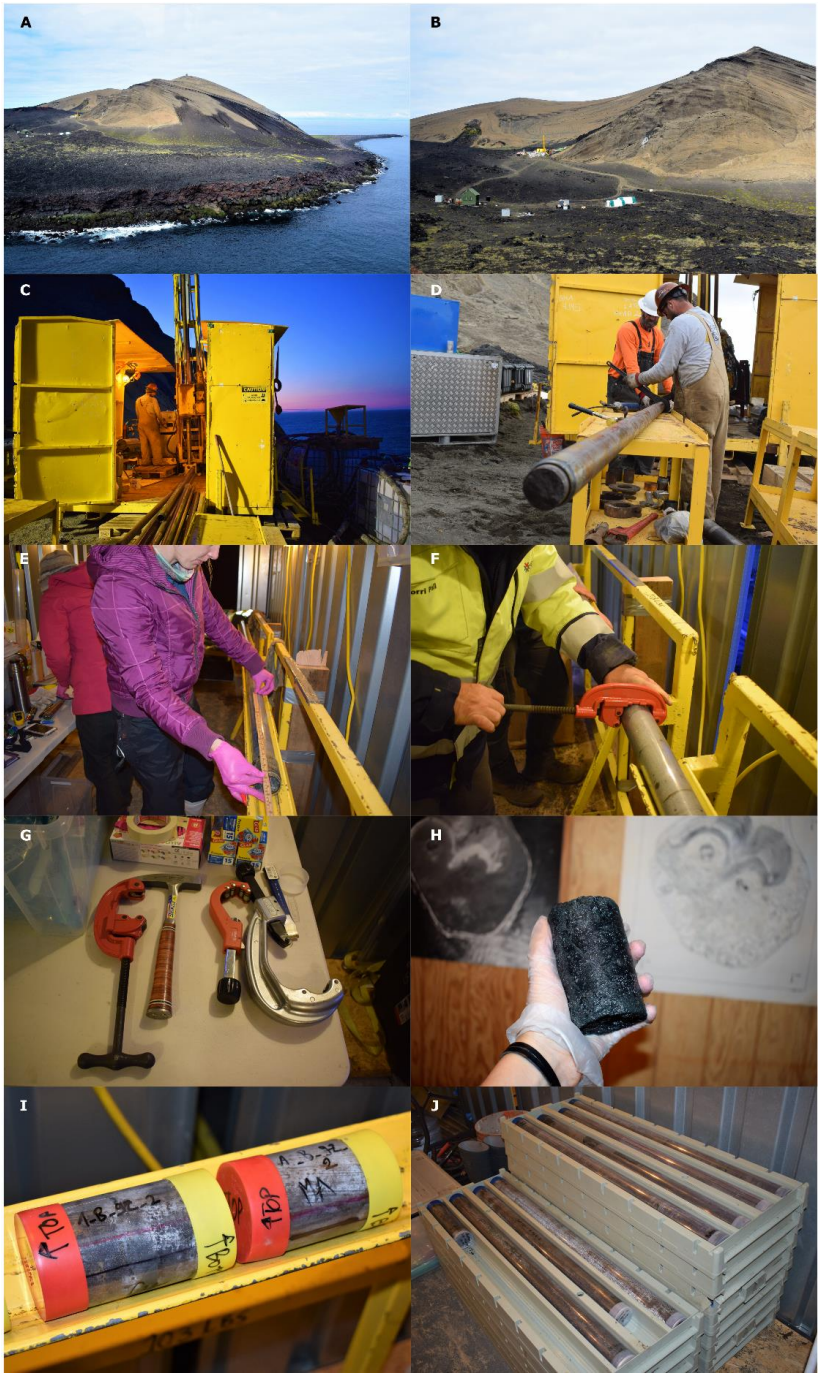

Figure 11. ICDP SUSTAIN drilling operation. A) South-east view of Surtsey island. B, C) Drilling site in August 2017. D) Core being extracted from SE-02A. E, F) Measurements and cutting the core though the core liner. G) Tools used to cut and core were regularly sterilized using 70% ethanol. H) Drill core sample removed from the core liner. I) Core samples kept in liner with cap at both end for future microbial analyses. J) Storage of the core in core boxes.

The paper entitled “SUSTAIN drilling at Surtsey volcano, Iceland, tracks hydrothermal and microbiological interactions in basalt 50 years after eruption” was published in 2019 by Jackson and collaborators in the peer-reviewed journal Scientific Drilling. It describes the work achieved during the SUSTAIN drilling project at Surtsey volcano (ICDP Expedition 5059), which resulted in the three cored boreholes. The paper also goes over the different preparations needed for the sampling and a zero-impact drilling operation, as well as the system set up to minimize microbial contamination in the drilling fluid (Jackson *et al.*, 2019b). In addition, the ICDP Operational Report has been published in the GFZ publication database by Weisenberger and collaborators and entitled “Operational report for the 2017 Surtsey Underwater volcanic System for Thermophiles, Alteration processes and INnovative concretes (SUSTAIN) drilling project at Surtsey Volcano, Iceland” and report all information about the drilling operation (Weisenberger *et al.*, 2019).

3.1.2 Drilling Fluid

Seawater was pumped, through a 1.4 km-long water pipe, from the western shore of the northern peninsula – providing a better shelter from wave action - into a 1000 L reservoir tank. Then, the seawater was sterilized before injection into the drilling head with filter units (PENTEK Big Blue, 30 µm cartridge filters) and a UV light system (AQUA4ALT, WEDECO). Unfortunately, the drilling fluids were not sterile due to the repeated clogging of the two filter units that were used to reduce debris from the seawater before entering the UV light system (Figure 12a). Consequently, it decreased the water flow rate, which was not enough to allow the drilling operation to keep going without interruption. The two vertical drill holes were cored using semi-sterilized seawater drilling fluid and occasionally using non-sterile attapulgite mud (Florigel High-Yield Salt Water Stable Drilling Clay) and the inclined drill hole was cored with untreated seawater and occasional attapulgite mud.

Figure 12. Pictures from the operational report of the SUSTAIN drilling project showing filtration and sterilization of seawater at the drill site. a) Water storage at the rig site and geologist, managing the water sterilization system. b) Used water filter. From (Weisenberger *et al.*, 2019).

As the drilling fluids were suspected to be contaminated, samples were collected to track the microbial contaminants injected into the boreholes during the process (Table 1). At each depth where core samples were collected for microbial investigations; 1 liter of drilling fluid was collected and filtrated through Sterivex™ filters (Millipore) to keep only the microbial fraction. These samples were stored in liquid nitrogen at -196°C on-site and then transferred at -80°C to the laboratory for future molecular analyses.

Table 1. Drilling fluids collected in 2017 were annotated by the ICDP labeling system and corresponding depths.

Borehole	Sample ID	Core run number	Corresponding depth (m)	Sampling date
SE-02A	1B-0Z-C	0	0	07/08/2017
	1B-3Z-C	3	14.32	10/08/2017
	1B-25Z-C	25	71.62	12/08/2017
	1B-49Z-C	49	139.37	16/08/2017
SE-02B	1C-17Z-C	17	54.06	23/08/2017
	1C-39Z-C	39	109.29	24/08/2017
	1C-51Z-C	51	142	25/08/2017
	1C-59Z-C	59	164.19	25/08/2017

3.1.3 Sampling for Microbial Investigation

Four categories of samples were collected on the island: borehole fluids, drill cores, drilling fluids and condensed steam from fumarole. Sample information, sampling method and storage are described for each of these sample types.

Borehole fluid samples

Using the stainless-steel bailer, subsurface fluid samples were collected in 2016, 2017 (before and after drilling) and 2018 from the drill holes. Several washes (70% ethanol) of the sampler were performed between each sampling depth to avoid contamination. A total of 18 samples were collected from SE-01 (11 in 2016, 7 in 2017 – including 2 collected after the end of the drillings), 9 samples were collected from SE-02b in 2017, 4 samples from SE-02a in 2018 and 6 samples from SE-03 (5 in 2017, 1 in 2018) (Table 2).

Table 2. Borehole fluid samples were collected from Surtsey boreholes in 2016, 2017 and 2018.

Borehole fluids collected in 2016					
Borehole	**Sampling date**	**Depth (m)**	**Volume (ml)**	**Sample ID**	**Cultivation ID**
SE-01	09/06/2016	164	500	2016_1	1
SE-01	09/06/2016	164	300	2016_2	/
SE-01	09/06/2016	166	500	2016_3	2
SE-01	09/06/2016	162	400	2016_4	3
SE-01	09/06/2016	70	250	2016_5	4

SE-01	09/06/2016	100	500	2016_6	5
SE-01	09/06/2016	100	250	2016_7	/
SE-01	09/06/2016	120	550	2016_8	6
SE-01	09/06/2016	160	500	2016_9	/
SE-01	09/06/2016	160	150	2016_10	7
SE-01	09/06/2016	Mix	1400	2016_11	8
Borehole fluids collected in 2017 (before and after drilling)					
SE-01	03/08/2017	56 to 58	5000	2017_1	1
SE-01	03/08/2017	120	1820	2017_2	2
SE-01	03/08/2017	mix (~120)	980	2017_3	3
SE-01	03/08/2017	mix (~120)	805	2017_4	4
SE-01	03/08/2017	150	5000	2017_5	/
SE-01	08/08/2017	160	5000	2017_6	/
SE-01	08/08/2017	mix	850	2017_7	/
SE-03	06/09/2017	140 +/- 10	500	2017_11	11
SE-03	06/09/2017	200 +/- 10	500	2017_12	12
SE-03	06/09/2017	280 +/- 10	500	2017_13	13
SE-03	06/09/2017	mix	1000	2017_14	14
SE-03	06/09/2017	75 +/- 10	500	2017_15	15
SE-02B	05/09/2017	60	500	2017_16	16
SE-02B	05/09/2017	80	500	2017_17	17
SE-02B	05/09/2017	90	500	2017_18	18
SE-02B	05/09/2017	100	500	2017_19	19
SE-02B	05/09/2017	140	500	2017_20	20
SE-02B	05/09/2017	150	500	2017_21	21
SE-02B	05/09/2017	160	500	2017_22	22
SE-02B	05/09/2017	mix (~170)	500	2017_23	23
SE-02B	05/09/2017	mix (~0-160)	500		

Borehole fluids collected in 2018					
SE-02a	19/07/2018	60 to 100	800	2018_1	1
SE-02a	19/07/2018	110 to bottom	800	2018_2	2
SE-03	19/07/2018	mix	20000	2018_3	3
SE-02a	19/07/2018	mix (~120)	135	2018_4	/
SE-02a	19/07/2018	135	1000	2018_5	/

For cultivation, 50 ml of the sample were reduced by Na_2S solution (0.05% *w/v* final concentration) and stored at 4°C. The rest of the sample was filtrated through 0.22 µm Sterivex™ filters (Millipore) for future molecular analyses. Filters were closed at both ends and immediately frozen at −80 °C (Figure 14).

Drill core samples

A total of 56 core samples were collected from the drill holes (SE-02A, SE-02B and SE-03) for microbial detection and analysis. Seventeen drill core samples were collected from SE-02A, the first vertical hole, cored using filtered, doubly sterilized seawater drilling fluid; 17 from SE-02B, the second vertical hole (Table 3), cored using filtered, doubly sterilized seawater drilling fluid and occasional attapulgite mud; and 22 from SE-03, the inclined drill core, cored with untreated seawater and occasional attapulgite mud (Jackson *et al.*, 2019b; Weisenberger *et al.*, 2019).

Table 3. Drill core samples were collected from the Surtsey SE-02B borehole in 2017. ICDP labeling, core logs, temperatures, depths, and rock description.

ICDP labelling	Core run no.	Sampling date	Depth (m)		Temperature (°C)	Core description
			From	To		
5059-1C-4Z-4	4	22/08/2017	20.84	23.89	20	Armored Lapilli Tuff
5059-1C-9Z-3	9	22/08/2017	33.04	36.09	36	Armored Lapilli Tuff
5059-1C-13Z-3	13	22/08/2017	42.19	45.24	59	Armored Lapilli Tuff
5059-1C-17Z-3	17	23/08/2017	54.06	55.68	82.5	Armored Lapilli Tuff
5059-1C-22Z-3	22	23/08/2017	63.54	66.49	101.5	Armored Lapilli Tuff
5059-1C-27Z-3	27	23/08/2017	74.74	78.79	114	Lapilli Tuff
5059-1C-33Z-3	33	24/08/2017	90.99	94.04	123	Lapilli Tuff

5059-1C-36Z-3	36	24/08/2017	100.14	103.19	124	Lapilli Tuff
5059-1C-39Z-3	39	24/08/2017	109.29	112.34	121.5	Lapilli Tuff
5059-1C-42Z-3	42	24/08/2017	118.44	121.49	116	Lapilli Tuff
5059-1C-45Z-1	45	24/08/2017	127.49	130.64	107	Lapilli Tuff
5059-1C-49Z-4	49	25/08/2017	138.09	139.29	97	Lapilli Tuff
5059-1C-52Z-3	52	25/08/2017	145.89	148.94	84	Lapilli Tuff
5059-1C-55Z-3	55	25/08/2017	155.04	158.09	64	Lapilli Tuff
5059-1C-59Z-3	59	25/08/2017	164.19	167.24	55	Lapilli Tuff
5059-1C-62Z-3	62	25/08/2017	173.34	176.14	44.5	Lapilli Tuff
5059-1C-65Z-2	65	25/08/2017	179.44	182.49	37	Lapilli Tuff

Fifty-six core samples were immediately fixed and frozen on-site for microbial identification and analysis. Every third 3 m core run at the drill site had a 30-cm portion cut out of the middle, which was separated into three subsections: 10 cm for molecular analyses (MA), 8 cm for culture (Cu), and 2 cm for microscopic examination (M) (Figure 14). The MA sections were retained in the plastic core liner, wrapped in plastic, and stored in liquid nitrogen (196°C) until they were delivered to long-term laboratory storage at 80°C. Cu pieces were withdrawn from the liner as soon as possible, placed in a sterile plastic bag, oxygen removed by applying GasPak^{TM} (BD), and stored at 4°C. M sections were incubated in a solution of 1X phosphate buffer solution (PBS) and 2% formaldehyde, washed twice with PBS 1X, transferred to a solution of PBS 1X and 96% ethanol, and stored at −80°C. To detect microbial contaminants introduced during drilling, circulating fluid was collected at each core sample depth and passed through a Sterivex filter to retain the microbial fraction. Samples were stored in liquid nitrogen at −196°C on-site and then transferred to laboratory storage at −80°C for future molecular analyses (Jackson *et al.*, 2019b).

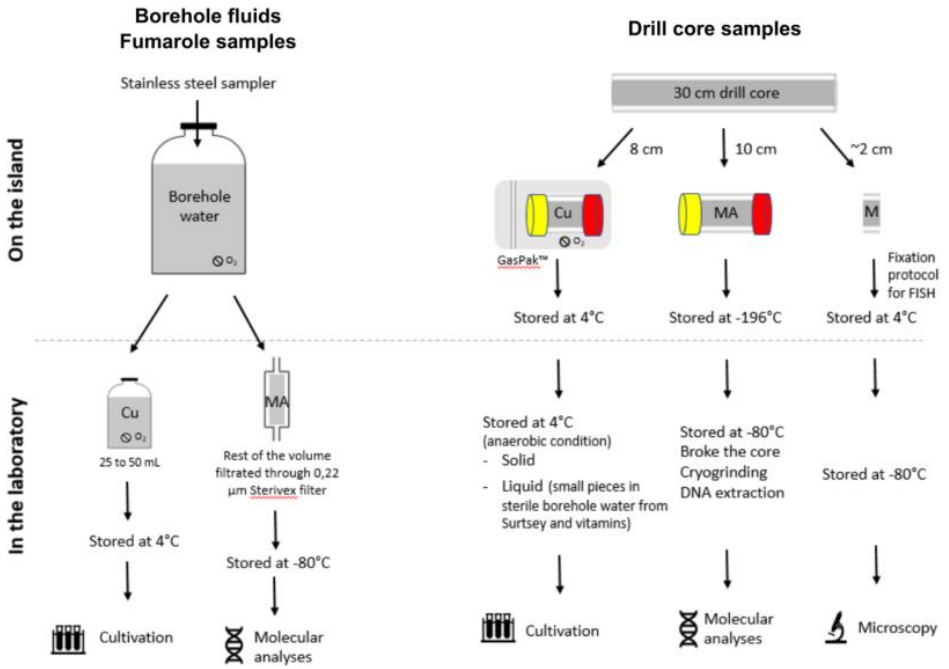

Figure 13. Sampling procedure and storage for borehole fluid and drill core samples.

Fumarole samples

Fumarole samples were collected on the top of Surtsey (N-63°18.249'; W020°36.085') for cultivation purposes and molecular analyses. On the 4th of August 2017, sterile tubes connected to 5 L sterile plastic containers were introduced into the fumarole SF-1. The next morning, 370 ml and 5 L of steam were collected in the bottles (Table 4).

For cultivation, 50 ml of the sample were reduced by Na_2S solution (0.05% *w/v* final concentration) and stored at 4°C. The rest of the sample was filtrated through 0.22 µm Sterivex™ filters (Millipore) for future molecular analyses. Filters were closed at both ends and immediately frozen at −196°C on the island before being transported to the laboratory and stored at -80 °C.

Table 4. Fumarole samples were collected in 2017.

Fumarole	Sampling date	GPS coordinates	Sample type and temperature	Volume (ml)	Sample ID	Cultivation ID
SF-1	05/08/2017	N-63°18.249'; W020°36.085'	Steam water (64.2 to 82.3°C)	370	2017_8	5
SF-1	05/08/2017	N-63°18.249'; W020°36.085'	Steam water (85.6°C)	5000	2017_9	7

Seawater samples

Seawater samples were collected as part of the Microbes in the Icelandic Marine Environment (MIME) project (Icelandic Research Fund - 163266-051), on August 18, during the annual oceanographic surveys from the Marine and Freshwater Research Institute of Iceland (https://www.hafogvatn.is/). Using a CTD rosette sampler, one liter of seawater was sampled at the station Selvogsbanki 2, a few kilometers from the northwest coast of Surtsey (63°28'58.8"N; 20°54'7.2"W) at 10, 20, 30 and 50 m below sea level. The temperatures of the samples were 12°C and 9.9°C at 10 and 50 m b.s.l., respectively.

ISCaR collection

The Icelandic Strain Collection and Records (ISCaR) database - a national database for microbial cultures and samples from Icelandic environments - monitored by the company Matís Ltd. includes 74 environmental samples from Surtsey. Biological material documented in ISCaR contains raw samples, DNA extracted from these samples (e.g., borehole fluid samples, drill core samples, steam and mud collected from fumaroles) and isolated strains. Each sample entered in the database is followed by information on the sampling's date, GPS coordinates, environmental parameters such as temperature and depth, and storage conditions. The consultation of the public part of these data is available online on the ISCaR website (http://iscar.matis.is/sampling-site/surts).

3.2 Microbial Diversity Analysis of the Surtsey Subsurface

3.2.1 DNA Extraction from Rock Samples

Different DNA extraction methods were tested before proceeding to the DNA extraction of the drill core samples extracted from the subsurface of Surtsey island in 2017. DNA extraction from fluid samples is also described in **Paper I**.

The rock samples used for the experimentations were collected on the surface of the island and at 170 m depth. Two negative controls were carried out in parallel throughout all extractions. One involved sterile water instead of the rock powder. The second control involved sterilized rock powder that has been sprayed with 70% ethanol, autoclaved and incubated in a hot air oven for 24 hours at 180°C. *Rhodothermus marinus* strain DSM 4252^T (Alfredsson *et al.*, 1988) was used as seed organism for positive control. The bacterium was grown to a late exponential phase (2 days) at 65°C in medium 166 supplemented with 1% NaCl (Hjorleifsdottir *et al.*, 2001). Optical density was measured at 580 nm using a Novaspec III Spectrophotometer (Biochrom) and the cell concentration was estimated at ~ 1.0×10^6 cells per ml (~ 100 cells/medium square; objective 40X) using Thoma cell counting chamber before the cell suspension was combined with sterilized rock sample at an equal volume of 1 ml.

Home-made pestle and mortar were used to break the rocks into small pieces. The equipment was autoclaved beforehand and was decontaminated by washing with ethanol (70%) and flaming between each use. Only pieces from the exterior of the core sample were selected for extraction. All the rock samples were weighted at ~500 mg.

The first method consisted of a custom-made protocol based on recommendations for the lysis protocol on basaltic samples (LP II) and the choice of an effective buffer to promote DNA desorption from minerals surfaces (Direito *et al.*, 2012; Lever *et al.*, 2015b). Samples supplemented with P/EtOH solution (1 M phosphate buffer from NaH_2PO_4/Na_2HPO_4, 15% ethanol, pH 8.0), Proteinase K (20 mg/ml), and lysis solution I (30 mM Tris-HCl, 30 mM EDTA, 800 mM guanidium hydrochloride, and 0.5% Triton X-100, pH 10) were homogenized by vortexing for 10 s, and incubated for 1 h at 55°C using a hybridization incubator with a rotisserie assembly. Bead-beating (autoclaved zirconia/silica beads, 0.2 g, 0,1 mm; BioSpec) for twice 30 s at 30 Hz with cooling on ice 1min in between. Two additional freeze-thaw+heat cycles (30min at -80°C/30min at 50°C) followed by centrifugation (1min; 10.000 × g; 4°C) and phenol/chloroform/isoamyl alcohol (PCI) extraction (25:24:1 *v/v*) on supernatant, purification using chloroform and DNA precipitation using isopropanol. The second method followed the PowerLyzer® PowerSoil® DNA Isolation Kit protocol according to the manufacturer´s instructions (MO BIO Laboratories, Inc.). Cell lysis occurs by mechanical (vortex) and chemical (Solution C1; SDS) interaction. The kit contained a patented humic substance/brown color removal procedure and therefore, is effective at removing PCR inhibitors (Solutions C2 and C3; Inhibitor Removal Technology®). Total genomic DNA is captured on a silica membrane in a spin column format and an ethanol-based wash solution is used for DNA elution. The third method consisted of a modified version of the PowerLyzer® kit following recommendations for low biomass samples (Direito *et al.*, 2012). The modification of the protocol consisted of adding a solution of P/EtOH at pH8 to the solution C1, two steps of bead-beating using autoclaved silica zirconium beads (0.5g, 1 mm) and Mixer Mill Grinder MM 400 (Retsch) for 30s at 30 Hz with cooling on ice 1min in between and incubation at 80°C for 40min before proceeding according to the PowerLyzer® PowerSoil® DNA Isolation Kit protocol.

DNA was quantified and quality assessed using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) and Qubit fluorometer (Invitrogen, Quant-iT™ dsDNA HS) and 16S rRNA gene amplifications were performed to test PCR inhibition. Visualization was performed on 1% (*w/v*) agarose gel.

3.2.2 16S rRNA Gene Amplicon Sequencing

Partial 16S rRNA gene amplification was tested using Q5® High-Fidelity polymerase (New England Biolabs, MA, United States) and the Earth Microbiome Project universal primers 515f (5'-GTG CCA GCM GCC GCG GTA A-3') and 806r (5'-GGA CTA CHV GGG TWT CTA AT-3') were used to amplify the variable region V4 of the bacterial and archaeal 16S rRNA genes (Caporaso *et al.*, 2012). Due to the lack of good coverage for archaea with this primer pair (Parada *et al.*, 2016), specific archaeal primer sets were utilized with a nested PCR technique as followed: first-round PCR (V3-V5 region) using Parch340F (5'- CCC TAY GGG GYG CAS CAG -3') and Arch958VR (5'- YCC GGC GTT GAV TCC AAT T -3') (Øvreås *et al.*, 1997; Klindworth *et al.*, 2013), and second round (V3 region) using Arch349F (5'- GYGCASCAGKCGMGAAW -3') and Parch519R (5'- TTACCGCGGCKGCTG -3') (Øvreås *et al.*, 1997; Takai and Horikoshi, 2000).

All PCR reactions were set up following the manufacturer's amplification protocol of Q5® High-Fidelity PCR Master Mix and 5µl of DNA at 10ng/µl (NanoDrop quantification) was used as template. Nuclease-free water was used to reach a total reaction volume of 25 µl.

The second round of the nested PCR used 5µl of amplified DNA from the first round. For rock samples that could not be amplified, bovine serum albumin was added to the master mix at a final concentration of 0.5 ng/µl. Conditions of the thermocycler (Bio-Rad) were set to: initial denaturation for 30s at 98°C followed by 30 cycles of denaturation for 10s at 98°C, annealing for 30s at 52°C (bacterial primer set) or 55°C (archaeal primer sets) and extension for 60s at 72°C. The final elongation was set for 2min at 72°C. Amplification products were visualized on 1% (*w/v*) agarose gels.

All PCR amplicons were multiplexed using Nextera®XT barcodes (8 cycles for index PCR) and were sequenced on a MiSeq sequencer (Illumina) with v3 chemistry and 2×300 cycles across sequencing runs. Raw sequences were demultiplexed and processed using the R Package DADA2 (Callahan *et al.*, 2016) version 1.4 or newer versions using the same parameters for the different runs. Unique Amplicon Sequence Variants (ASV) table was obtained after merging the different run outputs and taxonomic assignment of ASVs was conducted with the SILVA SSU database release 138 (Quast *et al.*, 2013). Statistical analysis and visual presentation of the data was conducted with RStudio running R version 4.0.2 (R Core Team, 2019; Rstudio, 2020) using packages Phyloseq (McMurdie and Holmes, 2013), ggplot2 (Wickham, 2011), vegan (Oksanen et al., 2007) and other packages detailed in **Paper I**.

3.2.3 Shotgun Metagenomics

Three metagenomes were obtained from DNA extracted from the subsurface of Surtsey. DNA was pooled from different samples to get enough DNA for the shotgun metagenome sequencing.

The sample SurW corresponds to pooled DNA extracted from borehole fluid samples collected from the 1979 SE-01 borehole at different depths (Sur161 to Sur16mix). Samples SurC1 and SurC2 correspond to pooled DNA extracted from drill core samples from the subaerial deposits (C4 to C22) and the submarine deposits (C27 to C65), respectively. DNA was quantified using high-sensitivity dsDNA reagents and Qubit fluorometer (Invitrogen™) and stored at −20°C before being sent on dry ice to the Marine Biological Laboratory (MBL), Woods Hole Oceanographic Institution, MA, USA, as a part of the Phase 14 DNA sequencing initiative of the Deep Carbon Observatory's Census of Deep Life. Based on Qubit ds HS quantification, DNA quantities sent were 10, 12 and 14 ng for SurW, SurC1 and SurC2, respectively. The DNA received was cleaned up using the DNEasy PowerClean Pro Cleanup kit to remove co-precipitates and concentrate the final product in SpeedVac. Purified DNA was then sheared to 400 bp on the Covaris S220 series and concentrated using AmPure XP beads to 10 µl for the library kit preparation. Due to the expected low DNA input, the samples were concentrated with a 2:1 ratio of beads (regular practice is 1:1) and cleaned with 75% ethanol. Libraries were prepared using the Ovation Ultralow V2 DNA-Seq library preparation kit (NuGEN, San Carlos, CA, USA), with the following modifications: 80% ethanol washed during concentration/cleanups of AMPure and elution volumes lower than recommended in the kit protocol for increased concentration. The input DNA was 0.4396, 0.4637 and 0.7085 ng for SurW, SurC1 and SurC2, respectively and 15 cycles and Illumina adapter-specific primers amplified the DNA fragments. A very small amount of primer dimers was obtained and removed using Sage Science Blue Pippin. The indexed libraries were pooled in equimolar concentration at 26 ng each and KAPA SYBR® FAST Universal qPCR kit (Kapa Biosystems, Boston,

MA, USA) was used to quantify the metagenome library DNA input concentrations. Shotgun sequencing was performed using a HiSeq system (Illumina). The run came back with overall high-quality values (90.9% Passing Filter, 85.8% Q30). Library preparation, multiplexing, and sequencing were performed at MBL (Woods Hole Oceanographic Institution, MA, USA). Samples were demultiplexed by MBL.

3.3 Isolation, Cultivation and Characterization of Bacterial Strains

3.3.1 Enrichment and Cultivation

All samples used for cultivation experiments were collected on the island between 2016 and 2018 and were immediately processed aseptically on-site and kept at 4°C. This included three sample types of drill cores, borehole fluids and condensed steam and biomass from fumarole outlets. Seven different culture media were tested, non-selective and selective (e.g., media for methanogens, iron, sulfate and sulfur reducers), at various temperatures (i.e., 22°C, 40°C, 60°C and 80°C) and under both aerobic and anaerobic conditions. Two solidifying agents were used: agar (14 g/L, Sigma-Aldrich, Deissenhofen, Germany) for incubation temperature below 60°C or Phytagel™ (8 g/L, Sigma-Aldrich, Deissenhofen, Germany) for incubation temperature above 60°C. Media and conditions used for enrichments are thoroughly described in **Paper II**. Direct plating, dilution-to-extinction and Hungate tube-roll techniques were tested (Hungate, 1950; Clark, 2019). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, MALDI-TOF MS (Microflex LT, Bruker Daltonics, Bremen, Germany), was used for differentiating the isolates in order to select colonies to be sequenced. Selected isolates were identified by partial 16S rRNA gene sequencing with an ABI 377 DNA sequencer. The short fragment was amplified using the primer set F9 (“5- GAGTTTGATCCTGGCTCAG-3”) and R805 (“5-GACTACCCGGGTATCTAATCC-3”). PCR and thermocycling was performed according to the recommendation of the manufacturers of OneTaq® Hot Start DNA Polymerase (New England BioLabs). The annealing temperature was set at 52°C. After purification by using Exonuclease I (ExoI, New England BioLabs) and Shrimp Alka-line Phosphatase (SAP, New England BioLabs), BigDye Terminator Cycle Sequencing Ready Reaction kit was used. The quality of the sequences was assessed using the software Sequencher 5.2.4 (Gene Codes Corporation). The sequences were taxonomically classified using BLASTn (Basic Local Alignment Search Tool) from NCBI (National Center for Biotechnology Information) and the 16S ribosomal RNA (Bacteria and Archaea type strains) database.

3.3.2 Phenotypic Analysis

Fifty-five 16S rRNA gene sequences were selected to represent each taxonomic group. The online portal of the SILVA Incremental Aligner (SINA 1. 2. 11) tool of the ARB-Silva database (http://www.arb-silva.de/aligner/) was used to classify and align sequences. RAxML BlackBox (https://raxml-ng.vital-it.ch/#/) (Kozlov *et al.*, 2019) implemented on the CIPRES webserver (Miller *et al.*, 2010) was used to perform maximum likelihood analyses using the GTR GAMMA model. The tree in NEWICK format was imported in Interactive Tree Of Life (iTOL) v6.4 (Letunic and Bork, 2021). In addition, representative

sequences of the novel strains isolated in **Paper II** were aligned against 16S rRNA gene sequences of the closest cultured type strains using the clustal_w program (Thompson *et al.*, 1994) and a neighbor-joining tree was built in MEGA7 (Kumar *et al.*, 2016).

3.3.3 Characterization of the Strain ISCAR-7401^T

The strain ISCAR-7401^T was characterized in **Paper III** according to the guidelines outlined in Tindall *et al.* (2010). Eight isolates were chosen for characterization in the context of Paper I and showed low 16S rRNA gene sequence similarity (96%) to the closest relative species belonging to the genus *Rhodothermus*. The phenotypic traits of the eight strains were similar. Strains ISCAR-7403 and 7404 were isolated from a tephra drill core collected at 70 m depth in the volcano (*in situ* temperature: ~100°C), while the others were isolated from borehole fluid samples collected at different depths: 75 m for strains ISCAR-7397 and 7398, 80 m for strains ISCAR-7399 and 7400, and 160 m for strains ISCAR-7401^T and 7402. All strains were routinely grown on medium 166 supplemented with 1% NaCl at 70°C, with or without agar (Hjorleifsdottir *et al.*, 2001). The cell size and morphology of strain ISCAR-7401^T were observed using a polarized light microscope Olympus BX51 (100x/1.30 Oil pH3) and a scanning electron microscope Supra25 Gemini FE-SEM (ZEISS). The Gram reaction was performed on fresh cells using the BBL™ Gram Stain Kit (BD) following the manufacturer's instructions. The growth range was determined in liquid medium 166 with 1% NaCl for 2-7 days. Optimal growth temperature, NaCl concentration from 0 to 7% and pH range from pH 5 to 10 were tested in liquid medium 166 at 70°C. *Rhodothermus marinus* DSM 4252^T was cultivated under the same conditions and served as a reference strain. Samples were collected at 1 or 2-hour intervals over 30 hours, and optical density was measured at 580 nm with a Novaspec III Spectrophotometer (Biochrom). The growth rates of ISCAR-7401^T at each condition were calculated using growth curves. Anaerobic growth was investigated for one week on agar plate medium 166, pH 7.5, and 1% NaCl at 70°C using a GasPak^{TM} (BD). The activities of catalase and oxidase were measured in triplicate using a 3% *(v/v)* H_2O_2 solution and an oxidase reagent kit (Difco BBL), respectively. Susceptibility to 4 antibiotics was also tested in triplicate. Analysis of cellular fatty acids, quinones and polar lipids for strain 7401^T were carried out on freeze dried cells from actively growing liquid cultures (medium 166, 1% NaCl at 70°C) by the Identification Service of Leibniz-institute DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

The DNA G+C content of the strain ISCAR-7401^T and the Average Nucleotide Identity (ANI) values were obtained by using the ANI Calculator from EZBioCloud (Yoon *et al.*, 2017) and the digital DNA–DNA hybridization (dDDH) values were obtained by using the Genome-to-Genome Distance Calculator 2.1 (GGDC) online tool from the DSMZ (https://ggdc.dsmz.de/) (Meier-Kolthoff *et al.*, 2013). A complete 16S rRNA gene sequence was extracted from the genome assembly of strain ISCAR-7401^T using RNAmmer (Lagesen *et al.*, 2007). The full 16S rRNA gene sequences of the closest relative species were downloaded from NCBI (e.g., *Rhodothermus profundi* PRI 2902^T, NR_116762, *Rhodothermus marinus* DSM 4252^T, NR_029282.2; and *Roseithermus sacchariphilus* MEBiC 09517^T, MG603595) and were aligned with the 16S rRNA gene sequence of the strain ISCAR-7401^T using the clustal_w program (Thompson *et al.*, 1994). Phylogenetic trees were reconstructed using neighbor-joining (Saitou and Nei, 1987), maximum likelihood and maximum parsimony tree algorithms in MEGA7 (Kumar *et al.*, 2016), based on 1000 bootstrap replications. *Balneola* species were always used as the outgroup.

3.4 Microscopic Investigations

Paper IV focuses on drill core samples that were stored under anaerobic conditions using gas-packs on site right after the drilling operation in 2017. Small fragments of samples were fixed using an overnight incubation with ethanol (50%). Fluorescent dyes, SYTO 9 and DAPI at a final concentration of 10 µM were used to stain cells and visualization was performed using an epifluorescence microscope. Different protocols were tested for the visualization of cells using Confocal laser scanning microscopy (CLSM - FluoView™ FV1000). Briefly, fixed rock fragments were ground into powder or smaller fragments and transferred on a glass slide. Solution of melted agarose 2% and SYTO 9 were added to the powder, antifading (citifluor) was also added and a cover slide was put on the top after the agarose solidified. For Scanning Electron Microscopy (SEM - Zeiss Auriga 40 Focused Ion Beam Field Emission Scanning Electron Microscope coupled with an Energy Dispersive X-ray analyzer) visualization, small fragments of drill core samples that were not previously fixed were dehydrated by four washes of 10 min in increasing concentrations of ethanol solutions (30, 50, 80, and 100%). After drying, samples were placed on carbon conductive tabs (PELCO Tabs™, 9mm) and were gold-coated for SEM visualization.

4 Results and Discussion

4.1 Contamination Assessments

The deep subsurface environments are estimated to contain around 70% of all prokaryotic cells on Earth (Magnabosco *et al.*, 2018). Yet, this large microbial habitat is still relatively unexplored today because it is very difficult and costly to obtain samples. In the past, the only way scientists had to access and study subsurface microorganisms was through "open windows" to subsurface ecosystems such as oil wells, mines, caves, and geothermal and hydrothermal environments (Deming and Baross, 1993; Marteinsson *et al.*, 2001; Huber *et al.*, 2006; Wanger *et al.*, 2008). Although pre-existing natural systems and infrastructure may be used for the collection of samples, recovering material from the subsurface generally requires drilling technologies to reach suitable depths. Over the years, drilling methods progressively became more efficient and allowed straight access to both deep terrestrial and marine subsurface environments. However, drilling is often inappropriate for microbiological sampling because it requires specific equipment and methods that were initially developed without consideration for the microorganisms inhabiting the subsurface. These drilling operations can extract cores at great depth but generate very high risks of introducing non-indigenous microorganisms into the samples (Smith *et al.*, 2000a; b; Smith and D'Hondt, 2006). Indeed, during a drilling operation, the corehole is constantly flushed with fluids that are not sterile, making the core samples contaminated. Injection of fluids is necessary to cool down the head bit and to equilibrate the pressure down in the hole. In addition, subsurface environments are low in biomass making the impact of external contamination higher. Injecting only a few non-indigenous cells into the samples can have a dramatic influence on the results of the analyses performed on these samples. For instance, cell numbers inside of the core can be overestimated because of contamination, and non-indigenous species can be cultivated and wrongly identified as endemic to deep subsurface environments. DNA extracted from contaminant species within the core samples is amplified, which can once again lead to misinterpretation and greatly bias the view of true microbial communities present in the subsurface (Smith and D'Hondt, 2006; Sheik *et al.*, 2018).

The risk of potential contamination when using standard techniques during drilling operations was recognized in the 1980s to interfere with the study of the deep biosphere. Over the years, numerous methods were tested to assess and evaluate the contamination from the surface during a drilling operation. After the "Subsurface Science Program" was formed in 1985, three drilling operations in South Carolina allowed the development of new techniques to facilitate the collection of sterile samples from deep cores (Fliermans and Balkwill, 1989; Lehman *et al.*, 1995). Many methods were tested including the separation of core and circulating fluids using sterilized plexiglas insert tubes (Fliermans and Balkwill, 1989) and the cutting of the outer layer to only keep the "uncontaminated" inner part of the core (Fliermans and Balkwill, 1989; Phelps *et al.*, 1989). However, the latter can be exceedingly challenging for crust samples. Lever et al. (2006) investigated a surface cleaning approach that involved washing samples with sterile seawater and burning the sample's outside until dry. Although sterilization of the material and removing the outer

layer of the core can reduce the risk of contamination (Smith *et al.*, 2000a), it cannot assure the integrity of the sample.

It has been shown that microbial contamination can easily infiltrate the inside of the core through cracks and fissures (Smith *et al.*, 2000a; b). Also, some drilling techniques make this problem worse by flushing the corehole with high-pressure drilling fluids that circulate up and down the hole. Those fluids transport microbial cells that can infiltrate straight inside of the core through the cracks and fissures. Therefore, the recovery of undisturbed rock samples is virtually impossible (Friese *et al.*, 2017; Kallmeyer, 2017), which makes it challenging to study indigenous microorganisms from the subsurface. In this matter, different methods were developed over the year to assess this problem. To follow fluid infiltration into the core, numerous physical and chemical tracers were tested (Diehl and Horchak-Morris, 1987; Chapelle and Lovley, 1990; Smith *et al.*, 2000b; D'Hondt *et al.*, 2002; Kallmeyer *et al.*, 2006; Friese *et al.*, 2017), including fluorescent dyes (Phelps *et al.*, 1989; Russell *et al.*, 1992; Pellizzari *et al.*, 2013), perfluorocarbon tracers (PFTs) (Senum, G. and Dietz, 1991; Colwell *et al.*, 1992; Russell *et al.*, 1992; Smith *et al.*, 2000b; House *et al.*, 2003; Lever *et al.*, 2006; Orcutt *et al.*, 2011a, 2017; Inagaki *et al.*, 2016) and microsphere tracers (Colwell *et al.*, 1992; Smith *et al.*, 2000b; Kallmeyer *et al.*, 2006; Yanagawa *et al.*, 2013).

Fluorescent dyes like fluorescein or rhodamine allowed the visualization of the microbial contaminants from the drilling fluids into the cores (Pedersen and Ekendahl, 1990; Russell *et al.*, 1992). One of the first fluorescent dyes used to calculate the contamination effect during drilling operations was fluorescein, which was first synthesized in 1871 (Duan *et al.*, 2009) and is among the most-commonly used tracer dyes today (Pellizzari *et al.*, 2013). Although fluorescent dyes have the advantages of high sensitivity for detection, low cost and are easy to use (Russell *et al.*, 1992), they are unstable at low pH values (Zhu *et al.*, 2005) and are sensitive to oxidizing agents and light degradation (Smart and Laidlaw, 1977; Diehl and Horchak-Morris, 1987; Zhu *et al.*, 2005). Also, fluorescence signal of the dye can greatly decrease due to the presence of humic substances and sorption on clay minerals limiting their applicability during drilling operations (Magal *et al.*, 2008; Hafuka *et al.*, 2015). Humic substances and clay minerals are commonly found in subsurface samples. Furthermore, fluorescent dyes can be toxic to the environment, which might cause problems with the disposal of the mud or fluids after drilling.

To identify the infiltration of drilling fluids into the core, perfluorocarbon tracers (PFTs) are also used (House *et al.*, 2003; Lehman *et al.*, 2004). They are chemically inert hydrophobic compounds and have a few advantages such as their non-toxicity to the environment and they can be detected at very low concentrations using gas chromatography (Smith *et al.*, 2000a). However, the detection of PFTs must be immediately performed on fresh cores because of their high volatility and requires elaborate equipment (Lever *et al.*, 2006). Standard contamination control methods during IODP drilling expeditions mainly use PFTs.

Today, one of the most used methods to assess microbial contamination during drilling is the utilization of microsphere tracers. They are very small (0.2–0.5 µm in diameter) fluorescent particles, used in both terrestrial and ocean drilling campaigns (Colwell *et al.*, 1992; Smith *et al.*, 2000a; Kallmeyer *et al.*, 2006; Yanagawa *et al.*, 2013). Since the microspheres have a similar size to indigenous microorganisms present in the subsurface, they mimic their infiltration into the core through cracks and fissures. They can be mixed

into the drilling fluids or locally released at a specific sampling depth. Then, they can be extracted from the core by density centrifugation (Kallmeyer *et al.*, 2006), transferred onto a filter membrane and counted by epifluorescence microscopy (Colwell *et al.*, 1992). However, microsphere tracers are very expensive, costing tens of dollars per milliliter, and they decompose under high-temperature conditions (Yanagawa *et al.*, 2013).

After their analysis for tracers' detection, the drill cores are analyzed for the presence of microorganisms, in parallel with the subsamples that would serve to identify the potential contamination. To thoroughly examine possible contamination, it is critical to incorporate sub-sampling of the material utilized during the drilling operation, core retrieval, and sample processing. Samples collected by swabbing surfaces of drilling materials, core liners, instruments used to recover the core such as used gloves and circular saws, and samples of drilling mud and fluids should all be included in subsampling for contamination analysis (Pfiffner *et al.*, 2008; Kieft, 2010). Negative controls, or "blanks," should be included in every phase of the procedure to identify laboratory or environmental contaminants from indigenous microorganisms. Gloves before use, laboratory regents, and so forth are examples of these. All the subsamples would be used to identify any possible contamination.

One of the most important aspects of sampling is to preserve the samples as fast as possible and use proper procedures for further microbiological analysis. The sample conservation varies depending on the analysis goal. Rapid freezing in liquid nitrogen or -80°C is generally appropriate for capturing microbial community composition and structure from nucleic acids when culture-independent techniques are applied. In low biomass subsurface environments, RNA-based microbial activity analyses are difficult. Messenger RNA signatures may change from *in situ* to surface conditions due to the time it takes to recover the samples. If samples were frozen throughout the coring process, those signatures that would normally alter fast would be preserved. If culture methods are utilized, samples should be stored anaerobically at 4°C and used as quickly as possible to avoid microbial overgrowth. However, long-term storage has been shown to affect core integrity (Lin *et al.*, 2010). During the storage of core material, changes in the microbial community structure and function occurred, leading to wrong conclusions about *in situ* microbial diversity (Mills *et al.*, 2012).

During the ICDP SUSTAIN drilling operation at Surtsey, no tracer compounds (e.g., fluorescent dyes (Pellizzari *et al.*, 2013), perfluorocarbon tracers (Lever *et al.*, 2006; Inagaki *et al.*, 2016; Orcutt *et al.*, 2017), and microsphere tracers (Kallmeyer *et al.*, 2006; Yanagawa *et al.*, 2013) were used due to the subsurface physico-chemical conditions, which prevented their utilization (pH, temperature, light, etc.). They were also prohibited on Surtsey due to the protected environmental status of the island (Baldursson, S. & Ingadóttir, 2007). Therefore, core samples were supposed to be drilled using doubly filtered and UV sterilized seawater, as the drilling fluid, to avoid any injection of microbial contaminants into the boreholes. However, we suspected that the drilling fluid was not sterile and samples were collected and analyzed. Biomass from the filters was extracted and the V4 region of bacterial and archaeal 16S rRNA genes was amplified and sequenced. The DADA2 pipeline (Callahan *et al.*, 2016) was used to analyze the microbial diversity in the drilling fluid samples. Samples 1B0ZC, 1B3ZC and 1B25ZC presented only two to five ASVs that belonged to the classes *Gammaproteobacteria*, *Cyanobacteria* and *Alphaproteobacteria* (Figure 13). This abundance was low in comparison to the rest of the samples. Figure 13 demonstrates that the sterilization system was efficient during the

drilling of the first half of SE-02A and failed sometime after 70 m depth (1B25ZC corresponding to the 25th core run). The depths of 58-70 m b. s. coincide with the coastal seawater level. Variation of pressure by crossing the sea level barrier could have changed the water flow rate needed to drill. The volume of fluids needed during the drilling process could have become too high in comparison to the lower flow rate provided by the sterilization system. It could have rushed the filtration (filters constantly clogged, Figure 12b) and reduced the time required under the UV to sterilized the seawater.

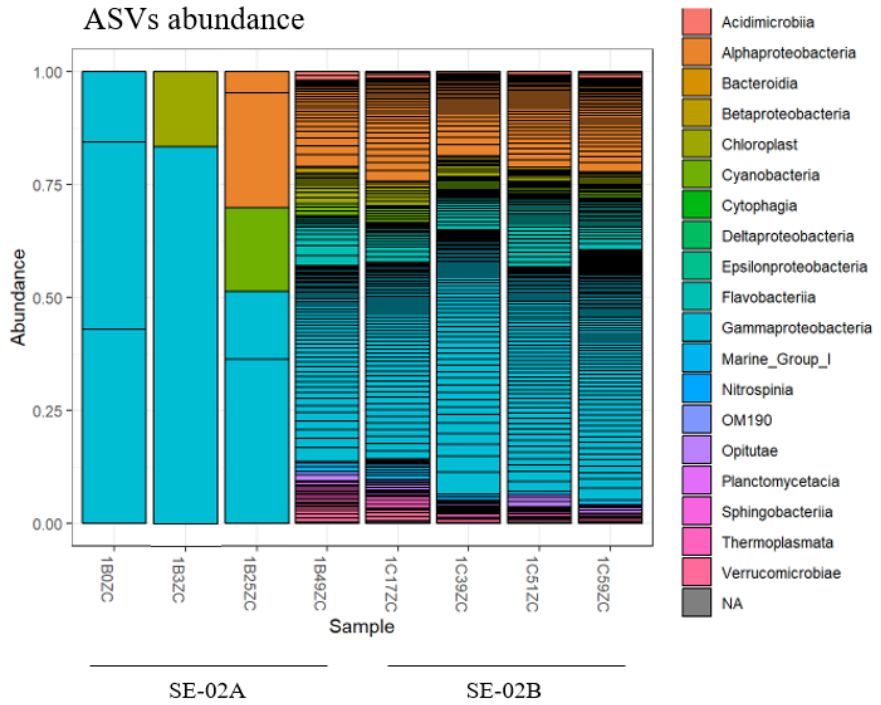

Figure 14. Amplicon Sequence Variant (ASV) abundance from drilling fluids, sampled during coring of SE-02A and SE-02B with the assigned taxonomy at the class level (SILVA SSU 132 database).

Many microbial species were expected to be injected from the drilling fluids – or non-sterile seawater – into the drill cores. However, we did not reject the hypothesis that marine bacteria and archaea colonized the subsurface of the island by seawater infiltration. While the three samples were removed from the dataset, the five remaining drilling fluids were retained to compare microbial communities between the drilling fluid and the drill core samples. **Paper I** develops in detail our strategies to distinguish indigenous microbial taxa of subsurface rocks from potential marine contaminants present in the drilling fluid based on a simple overlap approach at the ASV level.

4.2 DNA Extraction from Low Biomass and Mineralized Samples

Culture-independent studies, such as amplicon-based and "meta-omic" methods, provide by far a better representation of environmental microbial communities than culture-based methods. However, the origin of the samples and the choice of methods influence the efficiency of DNA extraction, microbial cell quantification, and the interpretation of the microbial community composition, which could lead to misinterpretations. Therefore, all the steps of DNA extraction and purification should be carefully considered.

Extracting DNA from all microorganisms present in one studied environment can be challenging, due to the nature of the sample. Indeed, mineralized environments, such as basaltic rock samples, contain chemical and physical factors that interfere with DNA extraction and amplification (Herrera and Cockell, 2007). For example, microbial cells can be encased in a physical matrix such as minerals, making cells inaccessible to the lysis. Also, certain spores, microbial cells or biofilms can be resistant to lysis because of their cell-wall composition, especially in extreme environments (Alain et al., 2011). Those two factors greatly decrease the lysis efficiency during DNA extraction and therefore the DNA yield. The absorption or binding of DNA to minerals or other elements can also limit the ability to extract DNA from basaltic rock samples, decreasing the DNA yield and affecting its purity (Barton et al., 2006; Herrera and Cockell, 2007; Direito et al., 2012; Yu et al., 2013). For instance, the absorption of DNA on clay minerals, that are abundant in such samples, has a strong impact on the efficiency of DNA extraction. In addition, the presence of enzymatic inhibitors in the rock (e.g., humic substances, metallic ions or some cations) affects enzymatic reactions. These can inhibit polymerase activity during DNA amplification (Dong et al., 2006; Kallmeyer and Smith, 2009). The cell density in a sample can also be challenging, being very low in subsurface environments, because contaminant DNA from the laboratory or from elsewhere can easily be amplified in a similar quantity to the DNA endemic to the environment, having a greater impact on the results (Barton et al., 2004).

Thus, a method to extract DNA from such samples must be optimized to improve the yield of DNA extraction, limit the biases mentioned above and obtain a correct representation of whole microbial communities in the environment (Cruaud et al., 2014). Nowadays, no standardized method is established for extracting DNA from a mineralized environment and multiple methods, including the use of commercial DNA extraction kits, were tested and compared over the years (Barton et al., 2006; Herrera and Cockell, 2007; Direito et al., 2012). To minimize the impact of minerals and inhibitors during DNA extraction, a strategy consists of separating the microbial cells from the mineral matrix before the cell lysis and DNA purification. However, this method takes time, increases the chance of contamination and often leads to low extraction efficiency (Kallmeyer et al., 2008; Morono et al., 2013). Direct DNA extraction on the rock samples, on the other hand, yields more DNA and is more representative of the environment, compared to the indirect method (Luna et al., 2006; Alain et al., 2011). This method is quicker and allows higher sample throughput. Different approaches of direct DNA extraction have been described in the literature (Webster et al., 2003; Luna et al., 2006; Direito et al., 2012; Lever, Torti, et al., 2015; Natarajan et al., 2016). Usually, DNA is directly extracted from the rock samples using one or different types of lysis methods combined with typical extraction methods using either commercial kits (e.g., DNeasy® UltraClean® DNA Isolation kit, DNeasy®

PowerSoil® DNA Isolation kit, DNeasy® PowerMax® Soil DNA Isolation kit, Qiagen) or optimized phenol-chloroform methods (Boivin-Jahns et al., 1996; Direito et al., 2012; Hurt et al., 2014). Lysis methods can be based on mechanical disruption (freeze/thaw cycles, bead-mill homogenization and/or sonication), chemical (detergents such as guanidium hydrochloride, sodium dodecyl sulfate (SDS) or Triton-X100 at different concentrations) or enzymatic approach (lysozyme, proteinase K).

To access microbial cells that are encased in minerals, different methods were tested such as the grinding samples with a sterile mortar and pestle (Barton et al., 2006; Postec et al., 2015) or using a cryogenic mill (Lipp et al., 2008; Alain et al., 2011), which chill samples in liquid nitrogen and pulverize them with a magnetically driven impactor. Absorption problems have been addressed by using competitor molecules to saturate adsorption sites in clay-rich samples. Indeed, it has been suggested that the addition of a solution of phosphate buffer/15% ethanol (pH8) helps to overcome the problem related to the absorption of DNA on minerals (Direito et al., 2012; Lever, Torti, et al., 2015). Phosphate groups bind competitively with DNA to clay and successfully decrease the amount of nucleic acid adsorbed on clay minerals (Direito et al., 2012; Lever, Torti, et al., 2015). Also, using ethanol at high concentrations affects the physical properties of the DNA, which changes its conformation (Fang et al., 1999). Linear DNA adsorbs more on minerals than when it is forming complex structures such as supercoiled plasmids. This is due to the availability of free phosphate groups that is higher in linearized DNA, binding more strongly than supercoiled DNA (Melzak et al., 1996; Poly et al., 2000). In addition, the pH can change the attraction between positively charged DNA and negatively charged clay minerals (Cai et al., 2006) and the adsorption of DNA to clay minerals increases with decreasing pH (Greaves and Wilson, 1969). Thus, at pH 8, adsorption to clay is reduced (Saeki et al., 2010). Likewise, the utilization of binding agents or cation chelators (e.g., EDTA) before the cell lysis could also prevent the adsorption of DNA to the mineral matrix. In addition to the DNA extraction, 16S rRNA gene amplicon sequencing results can be greatly biased by sequencing-preparation steps, primers and PCR amplification, sequencing errors, sequencing length and assignment errors (Poretsky et al., 2014).

Since extracting and sequencing environmental DNA from rock samples is a real challenge due to the low biomass, the adsorption of cells to minerals and the frequent co-extraction of enzymatic inhibitors (Herrera and Cockell, 2007; Direito *et al.*, 2012; Lever *et al.*, 2015b), different DNA extraction methods were tested before proceeding to the DNA extraction of the drill core samples extracted from the subsurface of Surtsey island in 2017. The method that achieved the highest DNA yield was then optimized.

While no statistical approach was performed to support the results, the first method seemed to give a better DNA yield than the others (see section 3.2.1). However, oxidation of the phenol was observed with the first DNA extraction method, which produced a pink/brown compound. As it can cause the degradation of the DNA, the use of reductants such as β-mercaptoethanol that could prevent phenol oxidation was tested but was unsuccessful (Lever *et al.*, 2015b). In addition, white and viscous pellets were observed after DNA precipitation, indicating that the purity of the samples had been compromised (e.g., contaminating humic acids, metal ions). No PCR amplification could be obtained from the DNA samples using the first method, reinforcing the previous observation. Therefore, the method using PCI was abandoned. The second and third methods seemed to give equivalent results. More tests were performed to optimize the method, including the addition of reductants, polyethylene glycol ([30% PEG 6000 and 1.6 M of NaCl] for better

DNA precipitation, the utilization of Freezer/Mill to obtain thin powder and the addition of BSA (Bovine serum albumin) to the PCR master mix to get better amplification.

The final method selected to extract the SE-02B drill cores is described in detail in the **Paper I**. It considered challenges related to the encasement of the microbial cells in a physical matrix (Freezer/Mill cryogenic grinder), cell resistance to lysis (bead-beating in addition to the lysis solution provided by the kit), DNA absorption on clay minerals (Phosphate-ethanol solution at pH 8) and PCR inhibition (addition of BSA to master mix). A low concentration of DNA was obtained for all the samples with a ratio of absorbance at 260 nm and 280 nm below 1.8, indicating the presence of impurities (Table 5). Nonetheless, amplifications of the 16S rRNA genes were successful.

Table 5. DNA concentration obtained after extracting 15g of each SE-02B drill core (Bergsten *et al.*, 2021).

Sample	Sampling date	Collection Depth (m b.s.)	Collection Temp (°C)	DNA concentration Nanodrop (ng/µl)	260/280	260/230	DNA concentration Qubit (ds DNA HS) (ng/µl)
C4	8/22/2017	23	20	181,61	1,42	1,24	0.172
C9	8/22/2017	35	36	196,47	1,44	1,15	0.143
C13	8/22/2017	44	59	202,15	1,33	1,56	0.16
C17	8/23/2017	55	82.5	137,89	1,43	1,51	0.105
C22	8/23/2017	65	101.5	115,60	1,46	1,59	0.408
C27	8/23/2017	78	114	204,83	1,42	1,25	0.11
C33	8/24/2017	93	123	202,74	1,38	1,16	0.089
C36	8/24/2017	102	124	202,32	1,41	1,21	0.157
C39	8/24/2017	111	121.5	197,72	1,35	1,35	0.149
C42	8/24/2017	120	116	57,15	1,43	-5,06	0.17
C45	8/24/2017	130	107	171,05	1,40	1,24	0.15
C49	8/25/2017	139	97	168,91	1,41	1,21	0.109
C52	8/25/2017	148	84	180,71	1,44	1,21	0.104
C55	8/25/2017	157	64	97,89	1,44	2,21	0.06
C59	8/25/2017	166	55	148,14	1,43	1,40	0.095
C62	8/25/2017	175	44.5	178,08	1,42	1,22	0.129
C65	8/25/2017	181	37	203,00	1,40	1,19	0.13

m b. s.: meters below surface

4.3 Microbial Diversity Analysis

Since the early days of deep biosphere research, studying life in the subsurface has been challenging, mainly due to the relative difficulties in obtaining samples. Therefore, initial investigations into the subsurface consisted of the collection of fluid samples from caves, mines, and oil wells or geothermal and hydrothermal places that can be viewed as “open windows” to the subsurface such as hot springs or hydrothermal vents. The early investigations focused on microscopic investigation and cultivation approaches (Zobell, 1947; Stetter *et al.*, 1993). For 30 years of research in deep life science, many drilling

projects have increased access to subsurface environments and have allowed the collection of solid samples in terrestrial and marine environments. Besides advances in sample collection, the study of microbial communities in their environment has progressed due to the development of new instruments and methodologies in several domains, such as cultivation, microscopy, and, most notably, molecular analysis. The rapid progress in next-generation sequencing technology with the simultaneous development of bioinformatic tools has allowed the discovery of a high number of previously unknown prokaryotic lineages (e.g., *Candidatus* Desulforudis and *Altiarchaeales*) and has provided critical information to start to unravel the ecological interactions and metabolic networks of the subsurface microbial communities. Indeed, subsurface biosphere research is closely linked with geomicrobiology and environmental microbiology, which aim to study the diversity, structure, functions, and interactions of microbial communities with their environment. Microbial communities are defined as multi-species assemblages of microorganisms coexisting and interacting with each other and their environment (Konopka, 2009). In the subsurface, microorganisms play a significant role in the geological and geochemical processes that shape the environment, for example, through processes such as bioweathering of rocks and minerals, biodegradation of organic substrates and biomineralization (D'Hondt *et al.*, 2019b). Understanding the role of subsurface microorganisms in their environment, such as how they affect global biogeochemical cycles, requires quantifying the extent, diversity, and activity of subsurface microbial communities. Different methods are used to study the subsurface biosphere, including microscopic investigations, culture-dependent and -independent methods. However, these methods have limitations and thus it is of crucial importance to use them all together to obtain a better representation of the studied microbiome.

4.3.1 Culture-Independent Studies

Due to developments in NGS technologies associated with bioinformatics tools, the use of culture-independent approaches has expanded significantly in the last 15 years to research unculturable microorganisms without isolating them and to study microbial communities in their habitats. These have allowed researchers to approach important questions in microbial ecology, in particular, "How to assess species diversity?", "What species are present in a particular environment?" and "What are the functions that each species performs?". The first step to answering these questions is to obtain DNA for sequencing, a process that can be challenging in low biomass and mineralized environments. After this crucial step, the microbial community diversity, composition and function can be assessed using two different methods: (1) Amplicon sequencing or (2) Shotgun metagenomics. In the first approach, primers target and amplify specific regions of interest of the DNA from microbial communities such as, for example, the 16S rRNA gene of prokaryotes. Shotgun metagenomics, the second approach, involves the sequencing of the total DNA from microbial communities, allowing the reconstruction of large fragments of DNA or even complete genomes.

Microbial Diversity and Species Richness

Species richness refers to the number of species in a microbial community and is the most basic way to characterize and measure a microbial community. However, species richness is not the sole factor that determines the diversity. To measure the unequal representation of species in communities, the concept of evenness or differential abundance is required. It is the description of a community's abundance distribution across species (Simpson, 1949).

When comparing two communities with the same number of species (same species richness) but different abundances, the more diverse community is the one with the same abundance of all species. As a result, species diversity is defined as the number of different species (richness) in a particular ecosystem, combined with the relative abundance of individuals within each of those species (Figure 15).

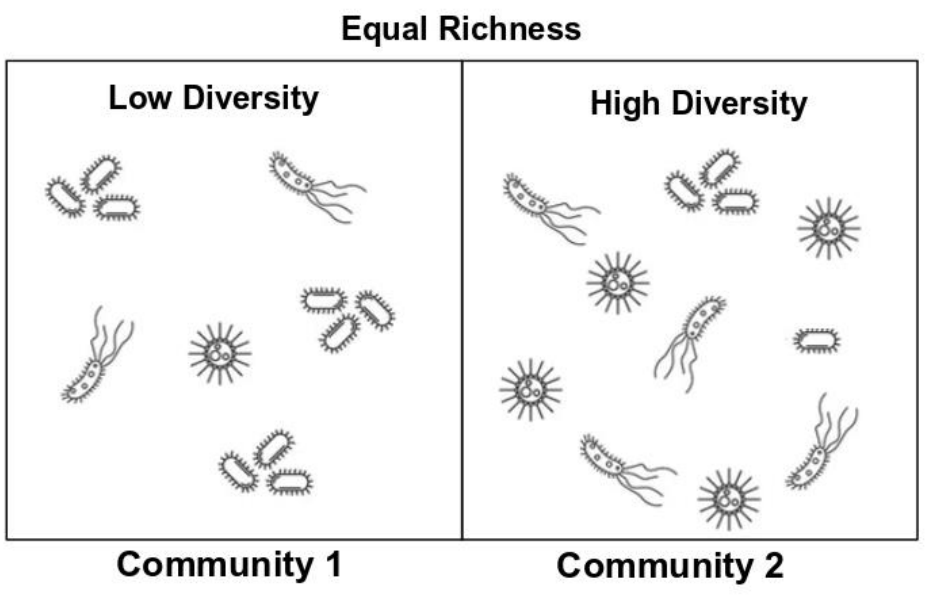

Figure 15. Microbial diversity, richness and evenness.

More metrics have been adapted to analyze the genetic information recovered directly from environmental samples in order to better describe and compare microbial communities. It includes the alpha (α), beta (β), and gamma (γ) metrics (Krebs, 2014). Alpha diversity measures a community's diversity in a sample (observed richness or evenness), beta diversity measures the variability in community composition (the identity of taxa observed) among samples, and gamma diversity measures the habitat's entire diversity, which comprises many different communities (total observed richness of all samples within in a habitat).

Nevertheless, processing, analyzing and statistical interpreting data from 16S rRNA gene amplicon sequencing is challenging (Weiss *et al.*, 2017). One concern is related to the library sizes. The microbial community of each sample is represented by the number of reads after sequencing, which can vary dramatically between samples. For this reason, the data cannot be directly compared because it reflects the differences in sequencing efficiency, even within a single sequencing run, rather than genuine variations in microbial communities (McMurdie and Holmes, 2014). Due to the variation in library size, two replicates with different numbers of reads are likely to have different read counts for certain sequence variants. These differences in library size could be caused by different amounts of loaded DNA for sequencing, DNA extraction or amplification biases. While parametric tools can provide a statistically complete framework for differential abundance analysis, normalizing library sizes is often required to compare amplicon sequencing data

and acquire meaningful results from the analysis. Rarefaction is a normalization tool that allows sample comparison without the bias caused by differences in library size by correcting it across samples and excluding sequences from larger libraries, resulting in all samples having the same, smallest size. However, rarefaction is statistically criticized because it gives only a snapshot of the microbial community by randomly eliminating a part of the observed sequences, which results in a loss of information and precision (McMurdie and Holmes, 2014).

In the alpha diversity assessment, rarefaction curves are used as a statistical approach to estimate the maximum species (or ASVs) number observed in a sample to determine the adequacy of the sequencing depth and to compare the samples with different sizes (Heck *et al.*, 1975). Other statistical estimators can be used to measure diversity, such as the Simpson index (Simpson, 1949) and the Shannon index (Shannon, 1948). The taxa abundance for the final richness estimation differs between the Simpson, giving more weight to species with more frequency in a sample, and the Shannon indices, giving more weight to rare species, respectively (Krebs, 2014).

A general mistake in microbiome analysis approaches is to presume that sequencing data is equal to ecological data. In a high-throughput sequencing run, however, the total read count is fixed, representing a random sample of the relative abundance of DNA in the ecosystem, and is not the absolute abundance of DNA in the input sample. When microbiome datasets are transformed to relative abundance values, normalized counts, or rarefied before analysis (McMurdie and Holmes, 2014; Weiss et al., 2017), this is implicitly admitted but there is no expected link between absolute abundance in the environment and relative abundance after sequencing. Thus, the compositional nature of the data needs to be addressed adequately when analyzing data from 16S rRNA gene amplicon sequencing using compositionally-appropriate tools (Gloor *et al.*, 2017).

The data set described in **Paper I** was obtained by 16S rRNA gene amplicon sequencing, therefore it is compositional. As a result, the analytical approaches (e.g., rarefaction, normalization) and statistical methods (e.g., ANOVA) employed to investigate microbiome data can possibly influence the results and lead to false discovery rates (Gloor *et al.*, 2017). This can result in a lack of consistency in microbiome research as well as misinterpretations of microbial community structures (e.g., alpha diversity). Nonetheless, variations in alpha diversity values among sample types (fumarole, borehole fluid, drill core and seawater samples) were evaluated using ANOVA and Tukey's HSD (Honestly Significant Difference) test. Before performing a non-metric multidimensional scaling (NMDS) ordination on Bray-Curtis dissimilarities for beta diversity analysis, the data were normalized using the "rarefy even depth" function. Permutational multivariate analysis of variance (PERMANOVA) using distance matrices and multilevel pairwise comparison was used to examine the importance of the sample type variable (Martinez Arbizu, 2020).

Amplicon Sequence Analysis

Amplicon sequence analysis, “metabarcoding” or “metaprofiling” are three terms that refer to the analysis of all organisms in a microbial community based on one gene or marker. Historically, ribosomal RNA genes were chosen as molecular markers for taxonomy or phylogenetic purposes because it is found in all organisms (Woese and Fox, 1977). One type of amplicon sequencing, the 16S rRNA gene sequencing targets a region of the small ribosomal subunit rRNA gene which is highly conserved between different species of

bacteria and archaea, implying that this type of sequencing can only identify these types of microorganisms.

The process of 16S rRNA gene amplicon sequencing, which involved platforms such as the Illumina MiSeq, includes a few steps: DNA extraction from the samples, PCR amplification of targeted hypervariable regions of the 16S rRNA gene, addition of molecular 'barcodes' to each DNA sample, clean-up of the amplicon to remove impurities, normalization to pool the samples together in equal proportions, quantification of the library and sequencing. The output of 16S rRNA sequencing provides sequence reads that can be analyzed using several basic bioinformatic steps, which when combined together are known as 'pipelines'. These bioinformatic pipelines remove sequencing errors and uncertain reads to 'clean' the data. Then, the good quality reads are aligned to microbial genomic databases to identify bacteria and archaea that are present in the samples. Several 16S sequencing pipelines are available, such as QIIME2, MOTHUR, USEARCH-UPARSE, DADA2 (Figure 16) (Schloss *et al.*, 2009; Caporaso *et al.*, 2010; Edgar, 2013; Callahan *et al.*, 2016).

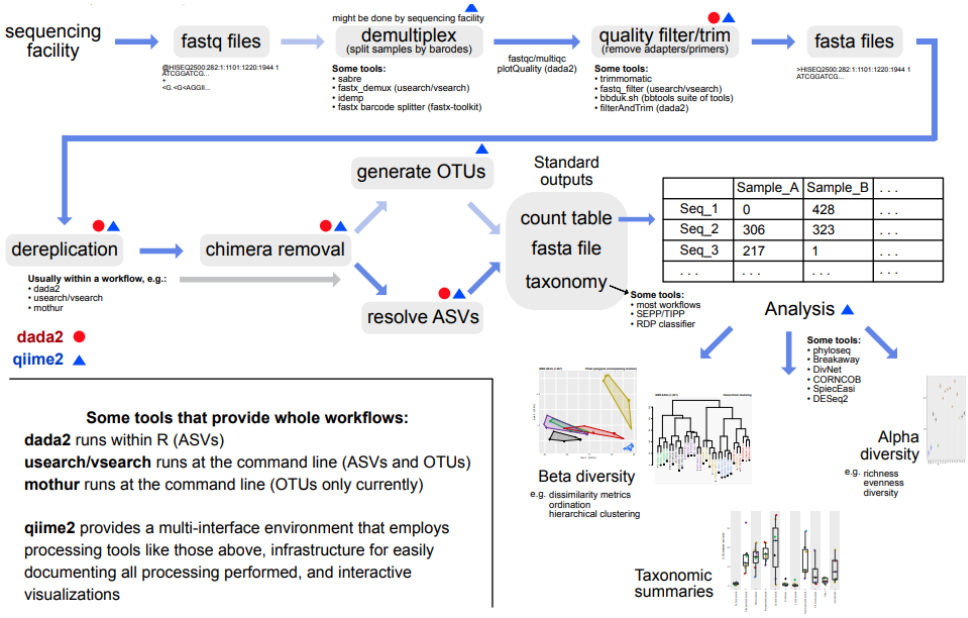

Figure 16. Overview of a standard amplicon workflow (from https://astrobiomike.github.io/amplicon/).

Recently, metabarcoding has shifted from Operating Taxonomic Units (OTUs) clustering to Amplicon Sequence Variant (ASV) approach (Callahan *et al.*, 2017). OTUs are defined as a cluster of sequences with sequence identity greater than a certain threshold, which is commonly set at 97%. On the other hand, ASV workflows divide the reads based on error models to correct sequencing errors while also accounting for abundance and sequence similarity (Callahan *et al.*, 2017). ASV calling increases the taxonomical resolution of the results by detecting the small biological sequence variants. It discards technical errors that are introduced by the library preparation or sequencing technology.

The 16S rRNA gene is widely represented in databases since it has been used as a phylogenetic marker for a long time, thus, it allows the taxonomic annotation of almost any microorganisms present in a sample to some extent. Some database examples are Greengenes (DeSantis *et al.*, 2006), and Silva (Quast *et al.*, 2013).

After the species level taxonomic annotation, functional information can be predicted. Taxonomical information is retrieved by extrapolating the functional annotation of related reference genomes (De Filippo *et al.*, 2012). For example, the software PICRUSt (Langille *et al.*, 2013) connects the taxonomic classification from metaprofiling results with metabolic information and gives 'predicted' functional profiles of the samples.

Paper I examined the diversity and distribution of microorganisms in subaerial and submarine basaltic rocks and fluids from Surtsey using 16S rRNA gene amplicon sequencing. To study the true residents of the subsurface, we first reported our strategy to access microbial contamination from the drilling fluids to the subsurface. In addition, DNA extraction controls were also carefully performed as the risk of introducing contaminating DNA from reagents is high in a low biomass environment and can critically interfere with downstream analyses. Both methodological and computational strategies were developed during the analyses of the data to distinguish endemic microbial taxa of subsurface rocks from potential contaminants from the laboratory and the drilling fluids. Since contaminating DNA was present in the drill cores, no significant differences were observed in the DNA concentration function of the depth, which could have been informative about the cell concentration.

Nonetheless, our study revealed diverse microbial communities in the drill core, associated fluids and fumarole samples, revealing rapid microbial colonization in the newly formed oceanic crust. Many lineages detected in this study were unknown. The microbial communities from those habitats (basaltic rock vs fluids) were distinct from each other, as well as different from those detected in seawater samples collected at the time of drilling 25 km offshore of the island. While various taxa detected in the SE-01 borehole fluids collected one year before the drilling operation were closely related to lineages previously reported in hydrothermal and deep subsurface environments, the presence of marine microorganisms (e.g., *Halomonas*, *Pseudoalteromonas*, *Sulfitobacter*, *Shewanella*) demonstrates that the infiltration of seawater transports marine microorganisms into the subsurface of Surtsey and its hydrothermal system. Microbial dispersion and adaptation to the harsh conditions occurring in the subsurface have probably driven its colonization. In addition, a distinction was observed in microbial communities from the subaerial and submarine deposits, where the environmental conditions are different, with vapor dominating above the coastal sea level and seawater dominating below (~58 m b. s.). The subaerial tuff is saturated with sea salt, which induces a rise in pH due to basaltic glass dissolution. At that alkaline pH and high NaCl concentration, taxa that are usually reported in alkaline and saline habitats were detected. This includes the genera *Thioalkalimicrobium*, *Ectothiorhodospira*, and *Salinarimonas*. The transfer of live cells from the zone of tidal flux and upper submarine deposits to the deeper submarine deposits is hardly possible because of the high temperature, acting as a dispersal barrier (>120°C; 110 m b. s.). In the deeper submarine deposits, microbial communities were similar to the ones detected in the seawater samples, where marine organisms are dominant (~50°C; 190 m b. s.). Although our study supports the dominance of bacterial lineages in marine basalts and associated fluids, many archaeal clades were detected (e.g., *Thermococcus*).

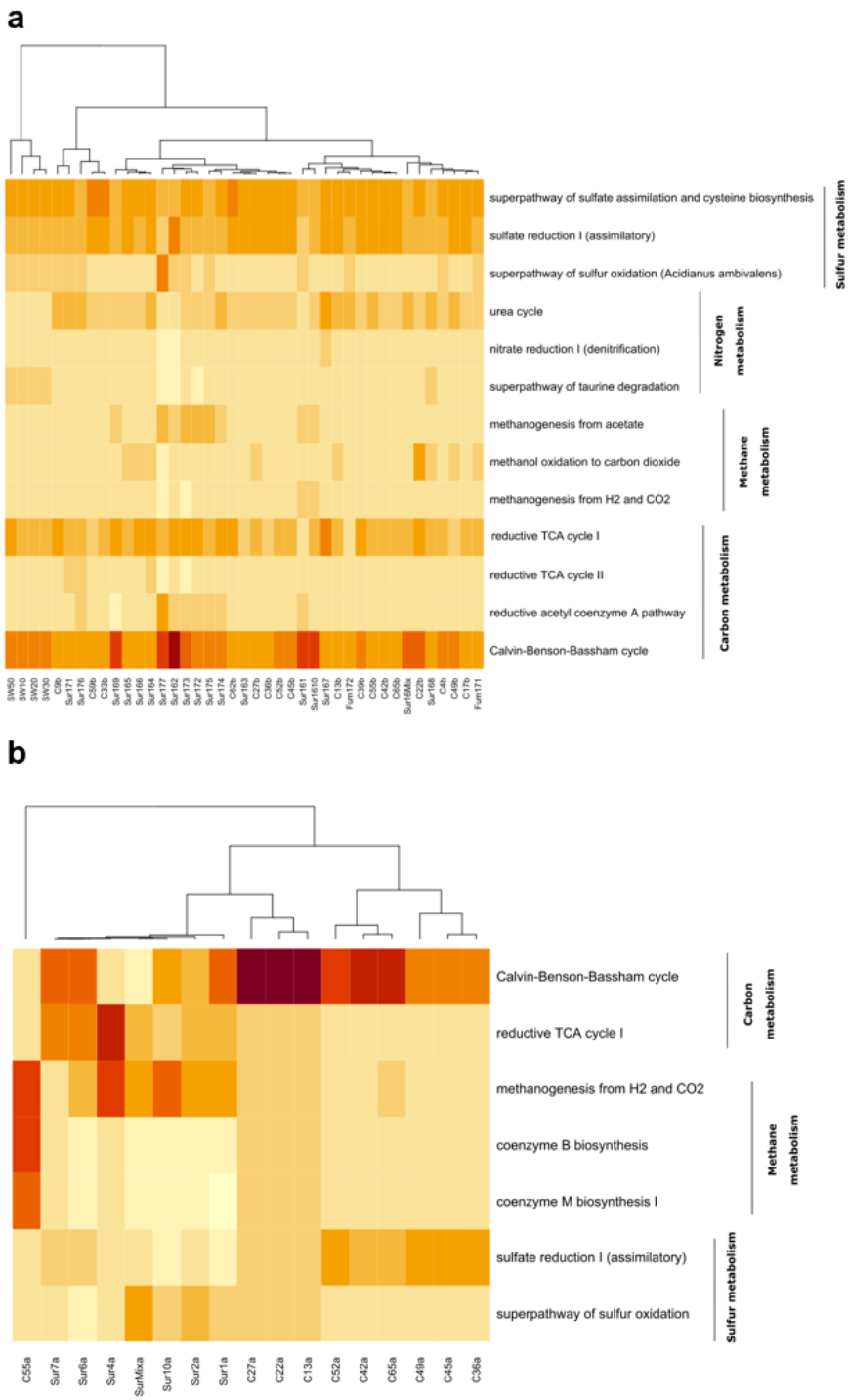

Figure 17. Predicted functional profiles (PICRUSt2) from the 16S rRNA gene amplicon sequencing datasets using the universal primer set (a) and the archaeal primer sets (b) (Bergsten *et al.*, 2021).

Furthermore, our predictive functional analyses based on taxonomic identifications detected by 16S rRNA gene sequence analysis suggest that the Surtsey subsurface microbiome is composed of heterotrophs, using organic carbon transported by the surface or the seawater inflows, and chemolithoautotrophs that can use dissolved inorganic carbon (DIC), such as CO_2, and inorganic compounds for their energy (e.g., sulfate reduction, sulfur oxidation) (Figure 17). At 157 m b. s., the sample C55a was dominated by the archaeal genus *Methanobacterium* from the order *Methanobacteriales* and genes involved in the methanogenesis from H_2 and CO_2, biosynthesis of coenzymes B and M suggest an active methane cycle at that depth (Figure 17b). The microbial communities from the subsurface of Surtsey might, to a certain degree, also participate in the depletion of some elemental concentrations (e.g., DIC, SO_4) previously reported in the borehole fluids in comparison with the seawater concentration (Kleine *et al.*, 2020), and could have a significant impact on the global biogeochemical cycles.

Shotgun Metagenomics

Shotgun metagenome sequencing, unlike 16S sequencing, which exclusively targets 16S rRNA genes, sequences genomic DNA randomly from a sample. During sequencing, the DNA is broken into many short fragments. The sequences of these fragments of DNA are then assembled back together using bioinformatic tools to determine both the taxa and genes present in the sample. It gives more information than 16S sequencing, as it allows a better taxonomic resolution (16S is generally limited at the genus level) and coverage (all taxa, including eukaryotes and viruses). It allows the reconstruction of genomes and the identification of microbial genes that are present in the sample, which provides additional information about the functional potential of the microbiome.

The process for metagenome sequencing is somewhat different from 16S rRNA sequencing. After the DNA is extracted from the sample, it is cleaved and tagged by a process called tagmentation. Fragmented and tagmented DNA is, then, cleaned to remove impurities. After tagmentation, PCR is performed, with index primers that uniquely index the samples, and amplifies the final library. Then, the unwanted large and small fragments are removed from the library that is subsequently purified, quantified and sequenced.

Shotgun sequencing reads require more complex bioinformatic methods than 16S rRNA sequencing outputs in order to analyze the results (Figure 18). The first step usually consists of removing human-related DNA sequence reads using tools such as Bowtie2 and SAMtools (Li *et al.*, 2009; Langmead and Salzberg, 2012). Shotgun metagenome bioinformatic pipelines perform quality filtering steps after which the cleaned sequence data can either be assembled or not. Read-based analyses consist of aligning the quality reads to databases of microbial marker genes, which provide taxonomic (using pipelines such as MetaPhlAn) and functional (HUMAnN) profiles (Beghini *et al.*, 2021). On the other hand, reads can be assembled to contigs to reconstruct full microbial genomes or Metagenome-Assembled Genomes (MAGs) using metagenomic assemblers, such as Megahit (Li *et al.*, 2015, 2016) or SPAdes (Bankevich *et al.*, 2012; Nurk *et al.*, 2013) and metagenome binning tools, such as CONCOCT (Alneberg *et al.*, 2014) or MetaBAT2 (Kang *et al.*, 2019). MAGs refer to a group of scaffolds with similar characteristics from a metagenome assembly that represent the microbial genome (Yang *et al.*, 2021). Many tools and pipelines exist to do downstream analyses to annotate MAGs. For example, tools involved in gene identification and annotation (e.g., Prodigal (Hyatt *et al.*, 2010)), functional annotation (e.g., MG-RAST (Keegan *et al.*, 2016)), taxonomic classification

(e.g., HMMER (Eddy, 2011) with GTDB-Tk (Chaumeil *et al.*, 2020)), and profiling MAG abundance (e.g., Kaiju, Kraken2, Bracken (Menzel *et al.*, 2016; Lu *et al.*, 2017; Wood *et al.*, 2019)).

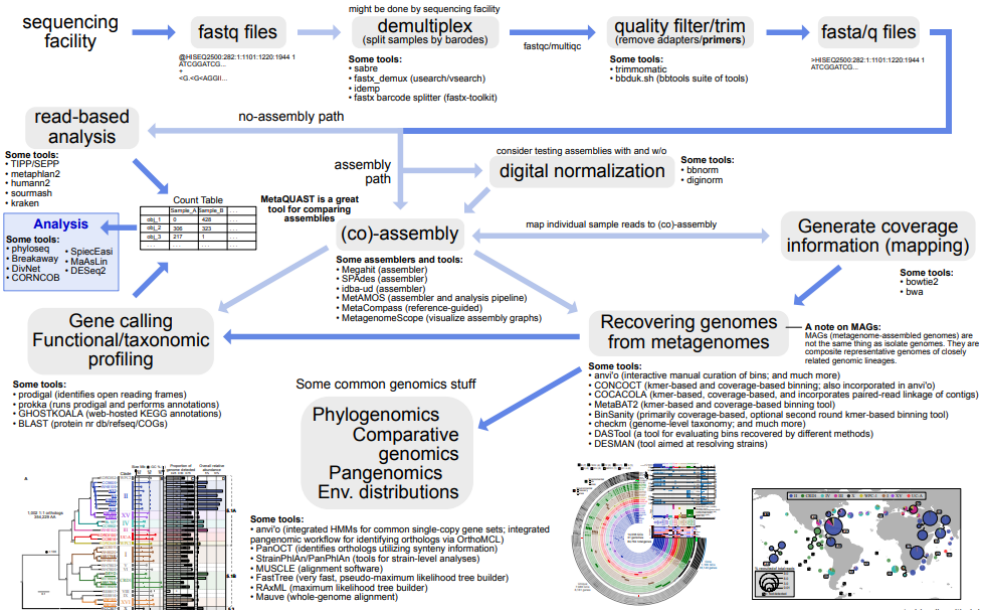

Figure 18. Overview of a standard metagenomic workflow (from https://astrobiomike.github.io/genomics/).

Metagenomics enable the discovery of new genes and proteins, as well as the entire genomes of non-cultivable organisms, in less time and with greater precision than traditional microbiological and molecular approaches. However, in the field of metagenomics, there are no universal methodologies or instruments and the lack of standards makes metagenomics a case-by-case research, limiting both reproducibility and comparison between similar projects. Census of Deep Life (CoDL) du Deep Carbon Observatory (DCO; https://deepcarbon.net/tag/census- deep-life) aims to finance the sequencing of genomic DNA of microbial communities from deep or extreme environments. The implementation of standard methods of metagenomic analyses would greatly help the comparison of subsurface microbial communities.

Shotgun metagenomes obtained from the subsurface of Surtsey were analysed. The quality of the reads was assessed using FastQC (Andrews, 2010). Reads were quality-filtered using iu-filter-quality-minoche (Minoche *et al.*, 2011). The number of read pairs obtained after being quality-filtered was 6867738, 15189727 and 16234791 for SurW, SurC1 and SurC2, respectively (Table 5). Reads mapping on the human genome GRCh38 (hg38) were removed using bowtie2 v 2.4.5 (Langmead and Salzberg, 2012). Percentage of read pairs kept after human contamination removal was 22.95%, 98.14% and 94.35% for SurW, SurC1 and SurC2, respectively (Table 6).

Table 6. Quality assessment of the reads obtained by shotgun metagenome sequencing.

SampleID	SurW	SurC1	SurC2
number of pairs analyzed	7,439,428	16,506,789	17,566,354
number of pairs passed after quality-filter	6,867,738 (%92.32 of all pairs)	15,189,727 (%92.02 of all pairs)	16,234,791 (%92.42 of all pairs)
number of pairs unmapped with hg (bowties2)	1,576,696 (22.95%)	14,907,485 (98.14%)	15,318,703 (94.35%)
Sequence length	27-151	27-151	27-151
%GC	43	52	54
Sequences classified with kraken2 on unassembled reads	769,250 (48.79%)	3,377,025 (22.65%)	6,048,382 (39.48%)

For classifying reads from the metagenome samples, the Kraken2 software (v2.1.2) was employed (Wood *et al.*, 2019). The database used was minikraken2_v2_8GB_201904_UPDATE, which has been built from the Refseq bacteria, archaea, and viral libraries and the GRCh38 human genome. All classifications were performed using default settings in Kraken2, -paired option being performed directly from the R1 and R2 quality-filtered and human-sequences-removed reads. The Bracken (Bayesian Reestimation of Abundance with KrakEN) software (Lu *et al.*, 2017) was used to statistically re-estimate the abundances at the species level of the taxonomic assignments made by Kraken2. KrakenTools was used to merge the kraken2/bracken profiles (Lu, 2021) and the auxiliary visualization tool hclust2 was used to generate the heatmap (parameters: --f_dist_f correlation and --slinkage complete) available at https://bitbucket.org/nsegata/hclust2.

SurW was dominated by reads mapping with *Streptococcus mitis* (18.42%), *Streptococcus pneumoniae* (13.7%), *Haemophilus parainfluenzae* (8.79*%), Streptococcus sanguinis* (8.7%) and *Neisseria subflava* (5.9%). Reads from SurC1 were mapping with *Escherichia coli* (27.95%), *Ralstonia pickettii* (14.67%), *Marinobacter* sp. LQ44 (7.565), *Alteromonas stellipolaris* (4.75%) and *Delftia acidovorans* (2.8%). SurC2 was dominated by reads mapping with *Escherichia coli* (29.23%), *Ralstonia pickettii* (15.88%), *Delftia acidovorans* (9.06%), *Delftia* sp. Cs1-4 (6.25%), *Delftia* sp. HK171 (5.70%), *Bradyrhizobium* sp. SK17 (4.49%) and *Cutibacterium acnes* (2.87%) (Figure 19). Since most of these taxa are related to the human microbiome such as found in the oral cavity, the gut or on the skin, we concluded that the samples were contaminated. Very few reads were assigned to archaea (Figure 20). MetaPhlAn3.0 was used as a complementary method to confirm the taxonomic profiles (Beghini et al., 2021).

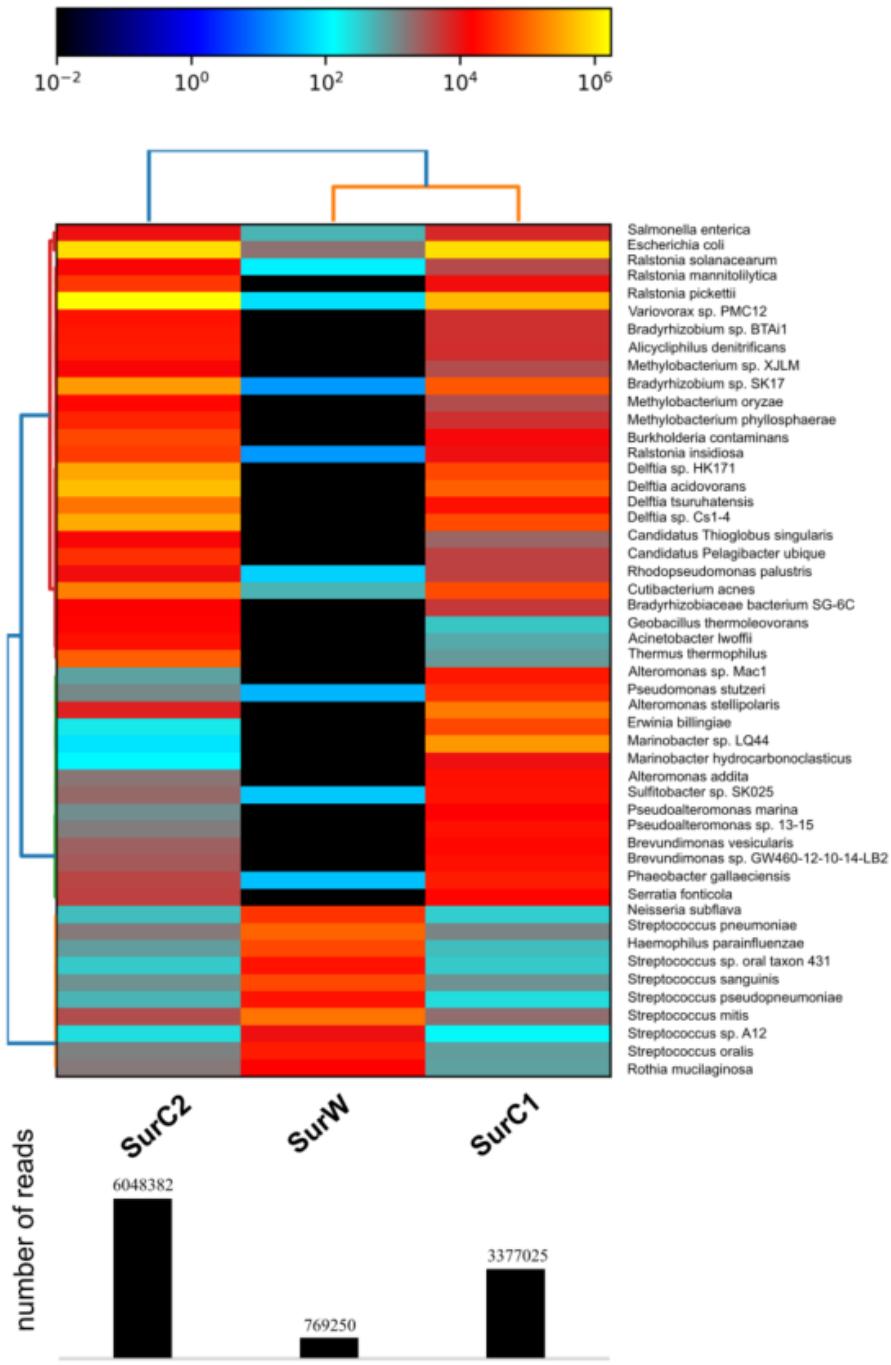

Figure 19. Heatmap showing the overall taxonomic profiles of the three metagenomes obtained using Kraken2/Bracken. The gradient of color indicates the number of reads that are mapped at the species level.

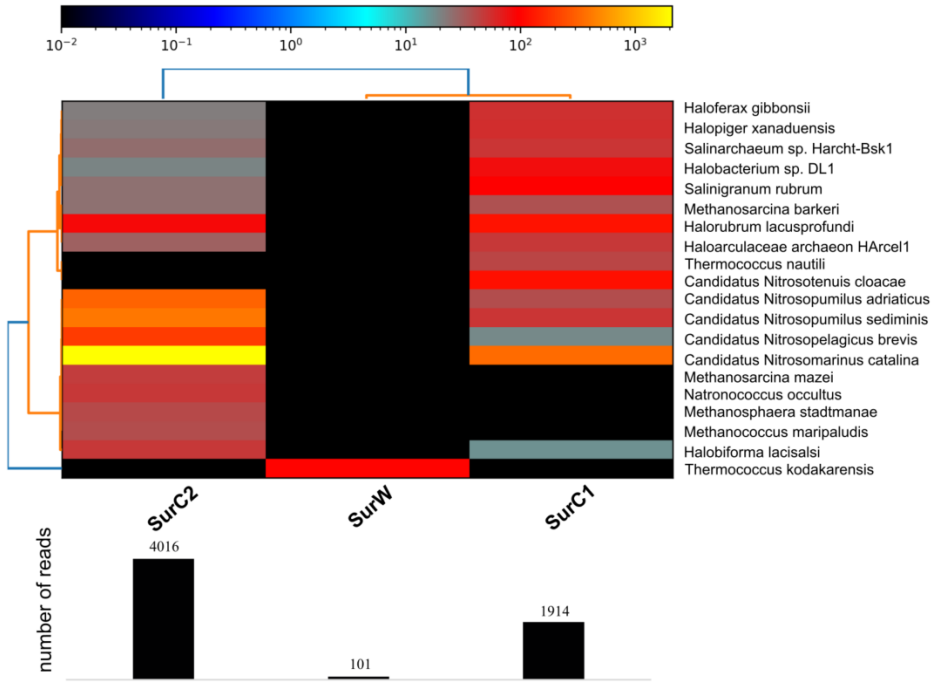

Figure 20. Heatmap showing the archaeal taxonomic profiles of the three metagenomes obtained using Kraken2/Bracken. The gradient of color indicates the number of reads that are mapped at the species level.

MetaPhlAn taxonomic profiles confirmed that the metagenomes were mainly composed of contaminants and no archaea were detected. Therefore, no functional profiles were performed on the reads. Quality-controlled reads were assembled individually using MegaHIT (Li *et al.*, 2015, 2016). Only contigs with a minimal length of 1000 bp were kept for analyses. Reads were mapped against filtered contigs using Bowtie2 (Langmead and Salzberg, 2012) and contigs binning was performed using MetaBAT2 (Kang *et al.*, 2019). CheckM was used to assess the quality of the bin (Parks *et al.*, 2015). However, no MAGs passing the MIMAG standard threshold (MQ: completeness >= 50% and contamination < 10%, HQ: comp. >90% and conta. <5%) were obtained from non-human-related bacteria.

As previously discussed, introducing a small amount of contaminating DNA in a very low biomass sample has a high impact on the sequence output. The many steps during the DNA extraction, as well as the library preparation, increase the chance of introducing contaminated DNA into the samples.

4.3.2 Culture-Dependent Studies

A recent global study on bacterial and archaeal diversity based on the OTUs approach estimated that the number of prokaryotic species on Earth is around 2.2–4.3 million (Louca et al., 2019). Yet, using standard cultivation techniques or common media and culture conditions, the vast majority of prokaryotes remain uncultivable or "yet to be cultured" (Amann *et al.*, 1995; Hugenholtz *et al.*, 1998; Rappé and Giovannoni, 2003; Steen *et al.*, 2019). The current number of prokaryotic species with validly published names is around

21 thousand (Hugenholtz *et al.*, 2021). The cultivated microbes, which account for less than 2% of the estimated total on Earth (Steen *et al.*, 2019; Martiny, 2020), are frequently found in low abundance in the environment and hence do not represent key players in the global metabolism of an ecosystem (Pace *et al.*, 1986). More than 70% of cells in the subsurface belong to uncultured clades, therefore their physiologies and ecological consequences are mostly unknown (Lloyd *et al.*, 2018; Wu *et al.*, 2020). Microbiologists have been unable to adequately examine the roles and functions of microorganisms in subsurface ecosystems due to difficulties in microbial cultivation in the laboratory. Although some studies have been successful in cultivating and isolating microbial taxa that are abundant in their ecosystem (Giovannoni *et al.*, 2005a; Giovannoni *et al.*, 2005b; Hahn *et al.*, 2012), diversity studies based on culture-dependent methods drastically underestimate the actual diversity of bacteria present in an ecosystem. Archaea are far less cultivated in the lab than bacteria. This limits research since the cultivation and isolation of bacteria and archaea are essential experimental tools for studying physiology, genetics, metabolism, and ecology. In addition, bacteria generate a significant number of novel molecules and thus unculturable subsurface microorganisms represent a massive unexplored source of compounds (e.g., primary metabolites, enzymes, antibiotics) and their biotechnological potential can only be fully accessed through isolation and characterization. Therefore, the development of more effective cultivation methods is critical.

The standard method for cultivation and isolation of "readily" cultivable microorganisms (Kell *et al.*, 1998) involves direct plating of the environmental samples on agar plates (Stewart, 2012). These samples can be liquid (e.g., freshwater, seawater) or solid (e.g., soil, rocks). Standard media commonly used to cultivate marine and freshwater/soil microorganisms include Marine Agar (ZoBell, 1941) and R2A Medium (Reasoner and Geldreich, 1985), respectively. After a few days of incubation, colonies may be growing on the plate, with distinct colors or morphologies, which can indicate the presence of different taxa. The next step of the general workflow is isolation, which involves the physical separation of a microorganism from the rest by, for example, transferring a single colony to a different plate (Figure 21). However, this method favors fast-growing microorganisms while neglecting the slow-growers that may be more ecologically relevant.

Other approaches for cultivating and isolating specific microorganisms have been developed and were proved to be successful. These include new media formulations and their optimization, enrichment for specific groups of microorganisms using oligotrophic or selective media, specific incubation conditions (e.g., temperature, pH, presence or absence of oxygen), and pre-treatment of the inoculum (e.g., antibiotics). Over the years, many media compositions have been designed to target various microorganisms based on their physiological characteristics (Atlas, 2010). Enrichment cultures are frequently used to increase the number of cells of specific types of microorganisms present in low abundance in the original samples. Those enrichments are normally carried out in liquid media that are more or less specific, and then an aliquot is employed as inoculum on agar plates that are suited for the microorganisms being studied.

Various procedures, such as transferring a colony to liquid media and plating different concentrations of dilution on agar, can be used. The dilution-to-extinction method performs several dilutions in liquid media until the last dilution generates no growth, allowing the most abundant organisms in a sample to be cultured (Button *et al.*, 1993). Although this method does not work for the less abundant microorganisms in a sample,

when applied as a high-throughput culturing procedure, it has been successful in isolating a few bacterial lineages that were previously uncultured and only detected by sequencing (Connon and Giovannoni, 2002). A good example is the successful isolation of SAR11 clade bacteria from saltwater, such as *Pelagibacter ubique* (Rappé *et al.*, 2002). The filtration-acclimatization method (FAM), microscale manipulation systems, and fluorescence-activated cell sorting (FACS) are some of the other isolation techniques. While the FAM physically separates cells by using a filter with different pore sizes to exclude the fast-growing cells (Hahn *et al.*, 2004), manipulation systems and FACS identify a single prokaryotic cell from a mixed population, based on distinct characteristics and isolate it from the rest. The FACS sorts cells based on morphological (shape, size, granulometry) or fluorescence characteristics (Akselband *et al.*, 2006; Cellamare *et al.*, 2010; Poniedziałek *et al.*, 2017). Single cells can be isolated from the microbial community using micromanipulator techniques such as optical tweezers (Zhang and Liu, 2008) or laser microdissection (Fröhlich and König, 2000). These are based on morphological characteristics of the cells as well, but they are under direct visual control.

Other methods, or a combination of different methods, can be used to obtain pure cultures of unknown species that contain specific 16S rRNA gene sequences previously detected in the environment. Fluorescence *in situ* hybridization (FISH) is a technique that uses labeled DNA probes to for example target the rRNA of specific taxonomic groups (Pernthaler *et al.*, 2001; Pernthaler and Pernthaler, 2007). Previously uncultivated archaea were successfully isolated using a combination of optical tweezers and visual recognition of single-cell staining after *in situ* hybridizations of an enrichment culture with fluorescently labeled DNA probes, which were targeting an archaeal-specific region within the 16S rRNA gene sequence (Huber *et al.*, 1995). The live-FISH approach combines FACS and FISH to select and cultivate specific taxonomic groups of bacteria from a community, necessitating protocol improvement, such as omitting fixation, centrifugation, and using buffers optimized for cell survival (Batani *et al.*, 2019). However, those techniques necessitated the use of sophisticated equipment.

The next step of cultivation and isolation is the maintenance of the culture (Figure 21). Indeed, subcultivation and optimal culture maintenance are important aspects of effective microorganism cultivation. In many circumstances, the first subcultivation fails, and the strain can be lost. For example, trace micronutrient substances or nutrients originally present in the samples that are essential for growth can vanish after the first transfer to a new medium. There is a difference between temporary cultivation and a successful subcultivation with the maintenance of the strain. These are crucial for the investigations of cultivated microorganisms for their physiological and taxonomic characterization, especially for the description of novel species and the deposition of strains in public culture collections. To validly name a new species of prokaryote, it is nowadays a mandatory requirement to proceed to the deposition of a culture of the type strain in two different public collections in at least two different countries (Tindall *et al.*, 2010). If not, some journals such as the International Journal of Systematic and Evolutionary Microbiology (IJSEM) do not accept publishing the taxonomic description and the requirements such as for the phenotypic characterization of the proposed species are very strict.

Even with the advancement of new technology, most environmental microorganisms remain refractory to laboratory cultivation. Various factors can make cultivation attempts unsuccessful such as insufficient care during sampling, transportation, and wrong storage of the samples used as inoculum. Also, inadequate cultivation conditions can be due to a

lack of consideration of the physicochemical conditions of the sampling sites and the absence of design of new media formulations that would best mimic the ecological habitat (Alain and Querellou, 2009; Overmann, 2013). Growth inhibition or even death of the cells due to nutritional shock can occur when microorganisms from oligotrophic habitats are transferred into nutrient-rich media. The presence of fast-growers and short incubation time are also factors influencing the success of the culture. Subsurface microorganisms can be slow growers, making their study in the laboratory fastidious, yet they are metabolically active and therefore relevant to the environment (Lok, 2015). In addition, the culture and isolation of certain taxa can also be inhibited by syntrophic relationships, competition, specific phages, or inhibition amongst microorganisms (Muniesa *et al.*, 2005; Alain and Querellou, 2009). Often the subsurface microorganisms that live in extreme and oligotrophic environments depend on each other to survive or need specific conditions to grow that are difficult to reproduce in the laboratory, making the cultivation, isolation and subculture of these microorganisms even more challenging. Yet, many subsurface microorganisms have been successfully cultured, but there are still far too few, and global efforts should be made to continue in this direction with the application of innovative methods, such as *in situ* cultivation that incubate the microorganisms in their natural environment (Silver *et al.*, 2010; Berdy *et al.*, 2017; Jung *et al.*, 2021a; b). The *Candidatus* Desulforudis audaxviator, for example, was successfully cultivated in the laboratory around 10 years after its genome was retrieved from a deep gold mine in Africa (Chivian *et al.*, 2008), even though it was thought that the species had not divided for hundreds to thousands of years (Karnachuk *et al.*, 2019).

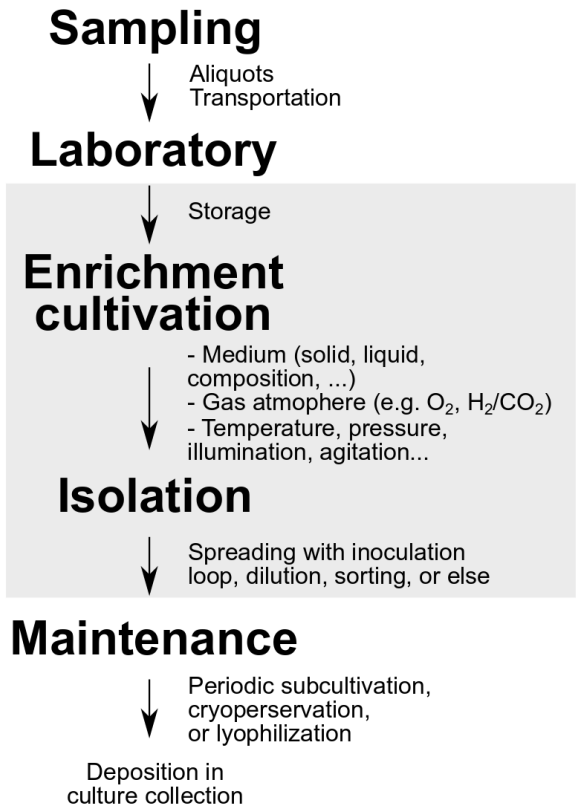

Figure 21. General workflow of isolation and cultivation. The steps outlined in grey comprise what is commonly referred to as a cultivation process (modified from Hahn *et al.*, 2019).

Culturable diversity from the subsurface of Surtsey

In **Paper II**, we evaluated the culturable diversity from the subsurface of Surtsey island. Different conditions, selective and non-selective media and temperatures (22-80°C) were used to enrich subsurface microbial communities from borehole fluid, drill core and fumarole samples. A total of 270 enrichments were tested and 195 bacterial isolates were successfully cultivated, purified and identified based on 16S rRNA gene sequencing. Most of the isolated strains were grown under aerobic conditions and in media for heterotrophic growth. This indicates that the media and conditions tested were ineffective for isolating archaea and chemolithoautotrophs, although those taxa were detected in the subsurface of the Surtsey volcano (**Paper I**). All isolates belonged to the domain Bacteria and were grouped within the clades *Firmicutes*, *Gammaproteobacteria*, *Actinobacteriota*, *Bacteroidota*, *Alphaproteobacteria* and *Deinococcota*. While most of the isolated strains were found to be closely related to species previously detected or isolated from soil, seawater, and hydrothermally active habitats, according to phylogenetic analysis, some potentially represented new species. These included new species of *Rubrobacter*, *Planococcus*, *Halomonas*, *Microbacterium*, *Polaromonas* and one was characterized and described as a new species, *Rhodothermus bifroesti* (**Paper III**). Comparative analyses performed between the isolated strains and 16S rRNA gene amplicon sequence datasets from **Paper I** revealed that 2.15% of the ASVs detected in the amplicon dataset were

cultivated. Some of the cultivated genera were not detected with a culture-independent approach, indicating that they could represent members of the rare biosphere and thus, cultivation-dependent method could increase the total diversity present in a sample. We thus emphasized the importance of applying both culture-dependent and -independent approaches to investigate the microbial diversity of a microbiome. We also discussed possible improvements to the cultivation methods used, even though it is well known that recovering and cultivating diverse environmentally relevant microorganisms from the subsurface remains extremely difficult.

Novel species characterization

Paper III was initiated with the isolation of the strain 7401^T by A. Mougeolle in the context of **Paper II**, which aimed to study the culturable part of the subsurface microbial diversity of Surtsey and isolate new species. Of many bacteria isolated, strain 7401^T was selected for characterization because of its low 16S rRNA gene sequence similarity (~96%) to any other species. Its closest relatives were bacterial species belonging to the genus *Rhodothermus*. The only two cultivated species of this genus were *Rhodothermus marinus* and *Rhodothermus profundi*. While *R. marinus* was isolated in 1988 from submarine alkaline hot springs in Iceland by Alfredsson and collaborators, *R. profundi* was isolated in 2010 from a deep-sea hydrothermal vent in the Pacific Ocean by Marteinsson and collaborators. The description of this taxa is adding a novel species to the family of *Rhodothermaceae*, enabling a better taxonomic classification and comparison within the order *Rhodothermales* that contained only ten members. In **Paper III** we proposed the name *Rhodothermus bifroesti* of which strain 7401 is the type strain and a viable culture has been deposited in two publicly accessible service collections in different countries: The Leibniz Institute German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) in Germany and the Pasteur Institutes Collection (CIP) in France (strain 7401^T =DSM 112103^T = CIP 111906^T).

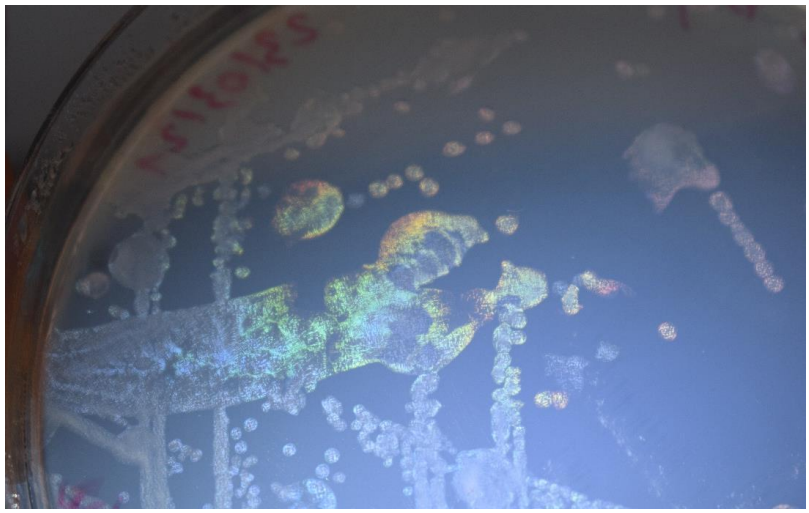

Figure 22. *Rhodothermus bifroesti* (bi.froes'ti. N.L. gen. n. *bifroesti*, named after the burning rainbow bridge Bifröst in Norse mythology). The species name refers to its thermophilic and iridescent characteristic on agar plate medium 166, as well as to its origin, the subsurface of Surtsey island.

The Gram-negative bacteria are rod-shaped, aerobic, non-sporulating, non-motile and of 0.3–0.4 m width and 1.5–7 m length. Colonies of the eight strains appeared circular with entire margins, 2.5-3.5 µm in diameter, flat, translucent and pinkish-pigmented after 3 days of incubation at 70°C on agar plate medium 166, pH 7.5 and 1% NaCl. Furthermore, they displayed an iridescent characteristic on agar plate medium 166, exhibiting all spectral colors ranging from red to blue under the condition of oblique illumination (Figure 22). At 70°C, pH 7-7.5, and 1% NaCl, optimal growth was observed. Based on 16S rRNA gene sequence similarity (~96%), genomic ANI (~72-73%), and digital DNA–DNA hybridization (~17%), the type strain, ISCAR-7401^T, was genetically distinct from its closest relatives *R. marinus* DSM 4252^T and *R. profundi* PRI 2902^T. We also provided a high-quality draft genome for *Rhodothermus bifroesti* type strain 7401^T (DDBJ/ENA/GenBank accession number: JAGKTL000000000).

More effort should be given to isolating species from subsurface habitats in order to test their limits and adaptive capabilities. In addition, research on microbial life in extreme environments is of high relevance due to the biotechnological potential of extremophiles, for example being a source of thermostable enzymes such as amylases, cellulases, chitinases, pectinases, xylanases, proteases, lipase, and DNA polymerases.

4.4 Biomass Estimation and Microscopic Investigations

The detection and enumeration of microbial cells are the first steps toward a better understanding of the extent of the subsurface biosphere. It usually involves fluorescent dyes such 4′,6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980), allowing direct cell counts via microscopic fluorescence observations. A standard procedure to enumerate microorganisms in a liquid environmental sample consists of filtration through filters, staining using fluorescent dye and visualization under an epifluorescence microscope (Lunau *et al.*, 2005). However, cell counting from turbid environments and rocky samples can be challenging and cell separation methods are needed to isolate the cells from the mineral matrix. To overcome these challenges, new methods were developed. The separation methods include, for example, gentle centrifugation and bilayer density separation, followed by filtration to separate cells from sediment particles (Kallmeyer et al., 2008) or using flow cytometry to separate the cells from mineral matrices (Patel *et al.*, 2007) coupled with the utilization of a higher sensitivity DNA dye called SYBR-Green I (Noble and Fuhrman, 1998) (Morono *et al.*, 2013). Another example is the preparation of a thin section of the rock sample that is infiltrated by a resin after being dehydrated. The cells in the rock interior are stained with SYBR-Green I that diffuses inside the resin and are visualized by fluorescence microscopy (Sueoka *et al.*, 2019).

Using thin sections of rock, spectrometric analyses are also used to characterize microbial cells and the composition of minerals in the near environment (Yamashita *et al.*, 2019; Suzuki *et al.*, 2020), for example, using scanning electron microscopy coupled with energy-dispersive spectroscopy (SEM-EDS) or confocal laser scanning microscopy (CLSM) coupled with Raman spectroscopy at the micrometric scale (Gérard *et al.*, 2013; Thomazo *et al.*, 2017). This allows the study of interactions between microbial cells and the mineral matrix. Nonetheless, it is crucial to evaluate microbial contamination within

the rock samples before microscopic investigations and make sure that the SYBR-Green I signals actually originate from microorganisms.

The main limitation in microscopic cell counting methods from mineralized environments is due to the difficulty to distinguish the cell-derived fluorescent signals from inorganic material. The choice of the dye is very important since fluorescent molecules can bind to DNA, RNA and EPS, not specifically staining cells and inducing non-biological background noise and false-positive signals (Gérard *et al.*, 2013; Escudero *et al.*, 2018). The fluorescence characteristic of SYBR Green I at different wavelengths allow the distinction between cell-derived fluorescence signals and nonspecific background fluorescence signals from minerals (Morono *et al.*, 2009). In addition, some minerals are auto-fluorescent. It is therefore important to carefully choose the right fluorescent dye and always have a control sample without any fluorescent dye.

Paper IV originated in the context of the program Jules Verne. The work was performed in Paris at the "Institute de Physique du Globe de Paris" (IPGP, Paris, France). The project focuses on microscopic investigations of the SE-02B drill cores through microscopic methods such as Epifluorescence, Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM). Challenges related to cell concentration estimation and the use of fluorescent dyes in a mineralized environment are discussed. The microbial cell numbers present in the drill cores were estimated to range from 5×10^4 to 1.36×10^6 cells per gram of rock based on DNA content. This study revealed possible biotic structures whose size, morphology, and fabrics are consistent with extracellular polymeric substances or EPS-microcolony complexes that are attached to the basaltic substrate. This cell cluster organization could be an adaptation strategy for microbial survival and protection from the extreme environmental conditions occurring in the subsurface of the Surtsey volcano.

5 Conclusions and Perspectives

This thesis aimed at broadening the understanding of the subsurface biosphere hosted in the basaltic oceanic crust through the study of the Surtsey volcano geothermal system. Surtsey is one of the world's newest islands, making it an ideal place to study the subsurface biosphere associated with newly formed basaltic tuff deposits in a still active seawater-hydrothermal system.

With the coastal sea level located at around 58 m depth, the Surtsey volcano geothermal system is composed of subaerial and submarine deposits with gradients of temperature varying from 40°C to 124.6°C in 2017. Its maximum temperature at around 100 m depth was higher than the presumed thermal limit for microbial growth of 122°C and thus prevents the transfer of living microorganisms from the upper deposits to the deeper submarine deposits. In terms of seawater-oceanic crust interactions, the Surtsey submarine basaltic deposits behave similarly to seamounts in ridge flank systems, where the chemical composition of rocks and fluids changes and seawater inflows introduce organic matter and oxygen into the system. Seawater and meteoric water passes through the porous and permeable crust's cracks and fissures, where it reacts with minerals to modify the chemical content of the fluid over time and create chemical fluxes that microorganisms can use for metabolic energy and biomass production. A highly porous zone situated between 143-150 m depth possibly channels cool seawater into the geothermal system, which causes the temperature of the hydrothermal system to decrease over the years. The Surtsey subsurface ecosystem is unique due to a singular combination of physico-chemical conditions, which include the exceptionally young crustal age, temperature gradients, geological settings, redox conditions, and rapid temporal evolution. These characteristics undoubtedly have a significant impact on the Surtsey subsurface biosphere.

Surtsey is a protected area and thus permission is needed to collect samples on the island, which occurs only once per year making the samples rare. Through a collection of diverse subsurface samples, obtained during the ICDP SUSTAIN drilling operation at Surtsey in 2017, we had the opportunity to explore for the first time the Surtsey subsurface biosphere and bring evidence on the processes that sustain it. A combination of methods was applied, providing complementary insights to the study, which included 16S rRNA gene amplicon sequence analyses, bacterial cultivation and microscopic investigations. Nonetheless, many challenges arose during this work such as issues related to low biomass that increased the risk of external contamination, the difficulties in handling the inevitable microbial contamination from the surface during the drilling operation, the presence of minerals that generate DNA binding and inhibitions, and issues related to the application of fluorescence techniques. Indeed, due to the strict environmental protection of the island and the extreme physico-chemical conditions occurring in the subsurface, no tracer compounds could be used to track microbial contamination and the cores were drilled using non-sterile seawater as drilling fluid. Drilling fluid samples were sequenced simultaneously with subsurface samples, and 16S rRNA gene amplicon sequence analyses allowed the development of computational strategies to distinguish indigenous microbial taxa of subsurface rocks from potential marine contaminants present in the drilling fluid.

Nevertheless, only 50 years after the end of the eruptions, diverse and active microbial communities were detected in basalt and fluids. The microbial cell numbers present in the drill cores were estimated to range from 5×10^4 to 1×10^6 cells per gram of sample based on DNA yield. A range that is consistent with analog but far older crustal environments. Microbial communities were detected in the subsurface zones crossing the hydrothermal temperature maximum. Using culture-dependent and -independent approaches, many taxa inhabiting the subsurface of Surtsey were found to be closely related to species that have been previously detected in environments such as soil, hot springs, hydrothermal vents and seawater, suggesting that fluid infiltration (meteorite water and seawater) bring microorganisms into the subsurface from the surrounding environment. Despite that drill cores, borehole fluids, steam from fumarole, and seawater samples all hosted significantly distinct microbial communities, several of the same species were discovered in numerous samples, suggesting the possible transfer of microorganisms across those ecosystems. The ability of those microorganisms to possibly adapt to the physico-chemical conditions occurring in the Surtsey volcano geothermal system and disseminate between ecosystems could have driven the colonization of the young basalt. Based on taxonomic identifications, predictive functional analyses suggested that the Surtsey subsurface biosphere is mostly composed of aerobic and heterotrophic microorganisms that use organic carbon transported by the surface and the oxygenated seawater inflows. Nevertheless, chemoautotrophic microorganisms were also detected, which indicated that a portion of the microbial communities can use dissolved inorganic carbon (DIC), such as CO_2, and inorganic compounds for their energy. Those taxa were involved in the sulfur, nitrogen and methane cycles. However, the metagenome analyses were unsuccessful and could not confirm the latter predictions. The microbial communities from the subsurface of Surtsey might, to a certain degree, participate in the depletion of some elemental concentrations (e.g., DIC, SO_4) previously reported in the borehole fluids in comparison with the seawater concentration (Kleine et al., 2020), and could have a significant impact on the biogeochemical cycles.

A variety of enrichment cultures were grown under different conditions, and approximately 200 bacterial strains were isolated, including several novel species. *Rhodothermus bifroesti*, a novel thermophilic bacterial species, was thoroughly characterized and described in this context. Comparative investigations found that strains cultivated using standard methods constituted 2.15% of the amplicon sequence variants from the 16S rRNA gene amplicon sequence datasets. In addition, putative microbial structures, or EPS-microcolony complexes, were observed attached to the basalt inside spherical cavities (vesicles) within basalt glass, at the boundary between the clay and volcanic glass of the vesicles. All the evidence collected during this work point to active microbial colonization of the Surtsey deposits and associated fluids, with possible sources of colonization coming from nearby ecosystems via microbial dissemination and possible adaptions. Microbial communities inhabiting the subsurface depend on the composition of the rock, availability of carbon and energy sources, temperature gradients, active circulation of fluids, and limited available space within the rock. Those constraints might play a major role in the colonization of rocks and the succession of the microbial communities. We expect that when the Surtsey hydrothermal system cools, its subsurface microbial communities will be comparable to those found in cold and oxic oceanic crust. While the thesis provides the foundation for future research of the Surtsey subsurface microbial communities, as well as their temporal succession in the context of a changing hydrothermal environment (e.g., temperature and basaltic tuff consolidation), many questions remain unanswered.

Being the first extensive investigation of the Surtsey subsurface biosphere, the experiments presented in this thesis need to be repeated. More metadata should be collected, including, for instance, the concentrations of O_2, CO_2 and CH_4 in the subsurface fluids in order to perform correlation analyses and determine the factors that structure the microbial communities. In addition, new methods should be tested. This includes DNA extractions from larger sample volumes to allow replicate analyses and shotgun metagenome sequencing from all the samples, especially from the drill core samples collected at each depth. Indeed, further efforts should be given to recovering higher DNA yield with better quality to perform metagenomic analyses. The reconstruction of Metagenome-Assembled Genomes (MAGs) would bring better evidence of the metabolic potential of the Surtsey subsurface biosphere through the study of specific genes. Furthermore, the data could reveal potential phenotypic variability in genomic populations, which could testify to adaptation processes that enable microbial communities to survive in this extreme environment. The metagenomic data would also better inform the connection between the ecological functions and taxonomic groups, and thus provide evidence on the impact of living microorganisms on the biogeochemical cycles. Additionally, metatranscriptomic approaches could be considered, however, RNA extraction is more challenging than DNA extraction, and because messenger RNA has such a short half-life, its quantity and quality are likely to fluctuate significantly during sampling. New cultivation methods should also be tested as the basis of media, which could involve the utilization of sterilized natural fluid from the subsurface. Ideally, *in situ* cultivation experiments should be tested. In this context, a subsurface observatory was deployed in September 2017 in the borehole SE-02B. It involved incubation chamber experiments with basaltic glass and olivine and this approach could lead to extensive future research on microbial-mineral interactions (Türke et al., 2019). Besides, in-depth microscopic investigations on drill core samples should be conducted, including more advanced methods such as laser microdissection combined with whole-genome amplification (Gérard *et al.*, 2018). This would allow the characterization of microbial communities on a smaller scale and a better understanding of local ecosystems. Overall, the importance of using a multi-scale approach is emphasized for future research on the characterization of microbial ecosystems from mineralized environments.

References

Abe, F. (2007) Exploration of the effects of high hydrostatic pressure on microbial growth, physiology and survival: Perspectives from piezophysiology. *Bioscience, Biotechnology and Biochemistry*. doi: 10.1271/bbb.70015.

Akselband, Y., Cabral, C., Castor, T.P., Chikarmane, H.M., and McGrath, P. (2006) Enrichment of slow-growing marine microorganisms from mixed cultures using gel microdrop (GMD) growth assay and fluorescence-activated cell sorting. *Journal of Experimental Marine Biology and Ecology*. doi: 10.1016/j.jembe.2005.08.018.

Alain, K. and Querellou, J. (2009) Cultivating the uncultured: Limits, advances and future challenges. *Extremophiles*. doi: 10.1007/s00792-009-0261-3.

Alfredsson, G.A., Kristjansson, J.K., Hjorleifsdottir, S., and Stetter, K.O. (1988) *Rhodothermus marinus*, gen. nov., sp. nov., a Thermophilic, Halophilic Bacterium from Submarine Hot Springs in Iceland. *Microbiology* 134: 299–306. doi: 10.1099/00221287-134-2-299.

Alneberg, J., Bjarnason, B.S., De Bruijn, I., Schirmer, M., Quick, J., Ijaz, U.Z., et al. (2014) Binning metagenomic contigs by coverage and composition. *Nature Methods*. doi: 10.1038/nmeth.3103.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*. doi: 10.1128/mr.59.1.143-169.1995.

Amend, J.P. and Shock, E.L. (2001) Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. *FEMS Microbiology Reviews*. doi: 10.1016/S0168-6445(00)00062-0.

Amend, J.P. and Teske, A. (2005) Expanding frontiers in deep subsurface microbiology. *Palaeogeography, Palaeoclimatology, Palaeoecology*. doi: 10.1016/j.palaeo.2004.10.018.

Anantharaman, K., Brown, C.T., Hug, L.A., Sharon, I., Castelle, C.J., Probst, A.J., et al. (2016) Thousands of microbial genomes shed light on interconnected biogeochemical processes in an aquifer system. *Nature Communications*. doi: 10.1038/ncomms13219.

Andreani, M. and Ménez, B. (2019) New Perspectives on Abiotic Organic Synthesis and Processing during Hydrothermal Alteration of the Oceanic Lithosphere. In *Deep Carbon*. doi: 10.1017/9781108677950.015.

Andrews, S. (2010) FastQC - A quality control tool for high throughput sequence data. *Babraham Bioinformatics*. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Antunes, A., Taborda, M., Huber, R., Moissl, C., Nobre, M.F., and da Costa, M.S. (2008) *Halorhabdus tiamatea* sp. nov., a non-pigmented extremely halophilic archaeon from a deep-sea hypersaline anoxic basin of the Red Sea, and emended description of the genus Halorhabdus. *International Journal of Systematic and Evolutionary Microbiology*. doi: 10.1099/ijs.0.65316-0.

Atlas, R.M. (2010) Handbook of Microbiological Media doi: 10.1201/ebk1439804063.

Baas-Becking, L.G.M. (1934) Baas Becking's: Geobiology translated from Geobiologie of inleiding tot de milieukunde. doi: 10.1002/9781118295472.

Bach, W. and Edwards, K.J. (2003) Iron and sulfide oxidation within the basaltic ocean crust: Implications for chemolithoautotrophic microbial biomass production. *Geochimica et Cosmochimica Acta*. doi: 10.1016/S0016-7037(03)00304-1.

Baldursson, S. & Ingadóttir, Á. (2007) Nomination of Surtsey for the UNESCO World Heritage List. *Icelandic Institute of Natural History, Reykjavik*. Available online at: https://whc.unesco.org/uploads/nominations/1267.pdf

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., et al. (2012) SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*. doi: 10.1089/cmb.2012.0021.

Baquiran, J.P.M., Ramírez, G.A., Haddad, A.G., Toner, B.M., Hulme, S., Wheat, C.G., et al. (2016) Temperature and redox effect on mineral colonization in Juan de Fuca Ridge Flank subsurface crustal fluids. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2016.00396.

Bar-On, Y.M., Phillips, R., and Milo, R. (2018) The biomass distribution on Earth. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1711842115.

Bartlett, D.H. (2002) Pressure effects on in vivo microbial processes. *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*. doi: 10.1016/S0167-4838(01)00357-0.

Bastin, E.S. and Greer, F.E. (1930) Additional Data on Sulphate-Reducing Bacteria in Soils and Waters of Illinois Oil Fields. *AAPG Bulletin* 14: 153–159. doi: 10.1306/3D9328AA-16B1-11D7-8645000102C1865D.

Bastin, E.S., Greer, F.E., Merritt, C.A., and Moulton, G. (1926) The presence of sulphate reducing bacteria in oil field waters. *Science*. doi: 10.1126/science.63.1618.21.

Batani, G., Bayer, K., Böge, J., Hentschel, U., and Thomas, T. (2019) Fluorescence in situ hybridization (FISH) and cell sorting of living bacteria. *Scientific Reports*. doi: 10.1038/s41598-019-55049-2.

Becker, K. and Davis, E.E. (2005) A review of CORK designs and operations during the Ocean Drilling Program. In *Proceedings of the IODP*, *301*. doi: 10.2204/iodp.proc.301.104.2005.

Beghini, F., McIver, L.J., Blanco-Míguez, A., Dubois, L., Asnicar, F., Maharjan, S., et al. (2021) Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *eLife*. doi: 10.7554/eLife.65088.

Berdy, B., Spoering, A.L., Ling, L.L., and Epstein, S.S. (2017) In situ cultivation of previously uncultivable microorganisms using the ichip. *Nature Protocols*. doi: 10.1038/nprot.2017.074.

Bergsten, P., Vannier, P., Klonowski, A.M., Knobloch, S., Gudmundsson, M.T., Jackson, M.D., and Marteinsson, V.T. (2021) Basalt-Hosted Microbial Communities in the Subsurface of the Young Volcanic Island of Surtsey, Iceland. *Frontiers in Microbiology* 12:. doi: 10.3389/fmicb.2021.728977.

Biddle, J.F., Sylvan, J.B., Brazelton, W.J., Tully, B.J., Edwards, K.J., Moyer, C.L., et al. (2012) Prospects for the study of evolution in the deep biosphere. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2011.00285.

Biebl, H. and Pfennig, N. (1978) Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. *Archives of Microbiology*. doi: 10.1007/BF00689344.

Blank, J.G., Green, S.J., Blake, D., Valley, J.W., Kita, N.T., Treiman, A., and Dobson, P.F. (2009) An alkaline spring system within the Del Puerto Ophiolite (California, USA): A Mars analog site. *Planetary and Space Science*. doi: 10.1016/j.pss.2008.11.018.

Boetius, A. and Joye, S. (2009) Thriving in Salt. *Science*. doi: 10.1126/science.1172979.

Bomberg, M., Nyyssönen, M., Nousiainen, A., Hultman, J., Paulin, L., Auvinen, P., and Itävaara, M. (2014) Evaluation of Molecular Techniques in Characterization of Deep Terrestrial Biosphere. *Open Journal of Ecology*. doi: 10.4236/oje.2014.48040.

Braun, S., Mhatre, S.S., Jaussi, M., Røy, H., Kjeldsen, K.U., Pearce, C., et al. (2017) Microbial turnover times in the deep seabed studied by amino acid racemization modeling. *Scientific Reports*. doi: 10.1038/s41598-017-05972-z.

Brazelton, W.J., Ludwig, K.A., Sogin, M.L., Andreishcheva, E.N., Kelley, D.S., Shen, C.C., et al. (2010) Archaea and bacteria with surprising microdiversity show shifts in dominance over 1,000-year time scales in hydrothermal chimneys. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0905369107.

Brazelton, W.J., Schrenk, M.O., Kelley, D.S., and Baross, J.A. (2006) Methane- and sulfur-metabolizing microbial communities dominate the lost city hydrothermal field ecosystem. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.00574-06.

Brock, T. D. (1966) Microbial Life on Surtsey. Surtsey Research Progress Report 11, p. 9-13. Available online at: https://surtsey.is/wp-content/uploads/2019/08/1966_II_2_01.pdf.

Burra, P. V., Kalmar, L., and Tompa, P. (2010) Reduction in structural disorder and functional complexity in the thermal adaptation of prokaryotes. *PLoS ONE*. doi: 10.1371/journal.pone.0012069.

Butterfield, D.A., Roe, K.K., Lilley, M.D., Huber, J.A., Baross, J.A., Embley, R.W., and Massoth, G.J. (2004) Mixing, reaction and microbial activity in the sub-seafloor revealed by temporal and spatial variation in diffuse flow vents at axial volcano. In *Geophysical Monograph Series*. doi: 10.1029/144GM17.

Button, D.K., Schut, F., Quang, P., Martin, R., and Robertson, B.R. (1993) Viability and isolation of marine bacteria by dilution culture: Theory, procedures, and initial results. *Applied and Environmental Microbiology*. doi: 10.1128/aem.59.3.881-891.1993.

Cacciapuoti, G., Moretti, M.A., Forte, S., Brio, A., Camardella, L., Zappia, V., and Porcelli, M. (2004) Methylthioadenosine phosphorylase from the archaeon *Pyrococcus furiosus*: Mechanism of the reaction and assignment of disulfide bonds. *European Journal of Biochemistry*. doi: 10.1111/j.1432-1033.2004.04449.x.

Callahan, B.J., McMurdie, P.J., and Holmes, S.P. (2017) Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME Journal*. doi: 10.1038/ismej.2017.119.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016) DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*. doi: 10.1038/nmeth.3869.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*. doi: 10.1038/nmeth.f.303.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME Journal*. doi: 10.1038/ismej.2012.8.

Cario, A., Grossi, V., Schaeffer, P., and Oger, P.M. (2015) Membrane homeoviscous adaptation in the piezo-hyperthermophilic archaeon *Thermococcus barophilus*. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2015.01152.

Cario, A., Oliver, G.C., and Rogers, K.L. (2019) Exploring the Deep Marine Biosphere: Challenges, Innovations, and Opportunities. *Frontiers in Earth Science*. doi: 10.3389/feart.2019.00225.

Cellamare, M., Rolland, A., and Jacquet, S. (2010) Flow cytometry sorting of freshwater phytoplankton. *Journal of Applied Phycology*. doi: 10.1007/s10811-009-9439-4.

Chapelle, F.H. and Lovley, D.R. (1990) Rates of microbial metabolism in deep subsurface environments. *Applied and Environmental Microbiology*. doi: https://doi.org/10.1128/aem.56.6.1865-1874.1990.

Chapelle, F.H., O'Neill, K., Bradley, P.M., Methá, B.A., Ciufo, S.A., Knobel, L.R.L., and Lovley, D.R. (2002) A hydrogen-based subsurface microbial community dominated by methanogens. *Nature*. doi: 10.1038/415312a.

Charlou, J.L., Donval, J.P., Fouquet, Y., Jean-Baptiste, P., and Holm, N. (2002) Geochemistry of high H2 and CH4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36°14′N,MAR). *Chemical Geology*. doi: 10.1016/S0009-2541(02)00134-1.

Chaumeil, P.A., Mussig, A.J., Hugenholtz, P., and Parks, D.H. (2020) GTDB-Tk: A toolkit to classify genomes with the genome taxonomy database. *Bioinformatics*. doi: 10.1093/bioinformatics/btz848.

Chivian, D., Brodie, E.L., Alm, E.J., Culley, D.E., Dehal, P.S., DeSantis, T.Z., et al. (2008) Environmental genomics reveals a single-species ecosystem deep within earth. *Science*. doi: 10.1126/science.1155495.

Ciobanu, M.C., Burgaud, G., Dufresne, A., Breuker, A., Rédou, V., Ben Maamar, S., et al. (2014) Microorganisms persist at record depths in the subseafloor of the Canterbury Basin. *ISME Journal*. doi: 10.1038/ismej.2013.250.

Clark, H. (2019) Culturing anaerobes. *Nature Milestones*. S5. Available online at: https://media.nature.com/original/magazine-assets/d42859-019-00007-1/d42859-019-00007-1.pdf

Cockell, C.S., Voytek, M.A., Gronstal, A.L., Finster, K., Kirshtein, J.D., Howard, K., et al. (2012) Impact disruption and recovery of the deep subsurface biosphere. *Astrobiology*. doi: 10.1089/ast.2011.0722.

Colwell, F.S. and Smith, R.P. (2004) Unifying principles of the deep terrestrial and deep marine biospheres. In *Geophysical Monograph Series*. doi: 10.1029/144GM22.

Colwell, F.S., Stormberg, G.J., Phelps, T.J., Birnbaum, S.A., McKinley, J., Rawson, S.A., et al. (1992) Innovative techniques for collection of saturated and unsaturated subsurface basalts and sediments for microbiological characterization. *Journal of Microbiological Methods*. doi: 10.1016/0167-7012(92)90047-8.

Connon, S.A. and Giovannoni, S.J. (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.68.8.3878-3885.2002.

Corliss, J.B., Dymond, J., Gordon, L.I., Edmond, J.M., Von Herzen, R.P., Ballard, R.D., et al. (1979) Submarine thermal springs on the Galápagos Rift. *Science*. doi: 10.1126/science.203.4385.1073.

Cowen, J.P., Giovannoni, S.J., Kenig, F., Johnson, H.P., Butterfield, D., Rappé, M.S., et al. (2003) Fluids from aging ocean crust that support microbial life. *Science*. doi: 10.1126/science.1075653.

Cragg, B.A. and Parkes, R.J. (1994) Bacterial profiles in hydrothermally active deep sediment layers from Middle Valley (NE Pacific), Sites 857 and 858. *Proc, scientific results, ODP Leg 139, Middle Valley, Juan de Fuca Ridge*. doi: 10.2973/odp.proc.sr.139.236.1994.

D'Hondt, S., Inagaki, F., Orcutt, B.N., and Hinrichs, K.U. (2019a) Iodp advances in understanding of subseafloor life. *Oceanography* **32**: 198–207. doi: 10.5670/OCEANOG.2019.146.

D'Hondt, S., Inagaki, F., Zarikian, C.A., Abrams, L.J., Dubois, N., Engelhardt, T., et al. (2015) Presence of oxygen and aerobic communities from sea floor to basement in deep-sea sediments. *Nature Geoscience*. doi: 10.1038/ngeo2387.

D'Hondt, S., Jørgensen, B.B., Miller, D.J., Batzke, A., Blake, R., Cragg, B.A., et al. (2004) Distributions of microbial activities in deep subseafloor sediments. *Science*. doi: 10.1126/science.1101155.

D'Hondt, S., Pockalny, R., Fulfer, V.M., and Spivack, A.J. (2019b) Subseafloor life and its biogeochemical impacts. *Nature Communications*. doi: 10.1038/s41467-019-11450-z.

D'Hondt, S., Rutherford, S., and Spivack, A.J. (2002) Metabolic activity of subsurface life in deep-sea sediments. *Science*. doi: 10.1126/science.1064878.

Davis, E.E., Becker, K., Pettigrew, T., Carson, B., and Macdonald, R. (1992) Cork: a hydrologic seal and downhole observatory for deep-ocean boreholes. *Proc, initial reports, ODP, Leg 139, Middle Valley, Juan de Fuca Ridge*. doi: 10.2973/odp.proc.ir.139.103.1992.

Deming, J.W. and Baross, J.A. (1993) Deep-sea smokers: Windows to a subsurface biosphere? *Geochimica et Cosmochimica Acta*. doi: 10.1016/0016-7037(93)90535-5.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., et al. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.03006-05.

Diehl, H. and Horchak-Morris, N. (1987) Studies on fluorescein-V The absorbance of fluorescein in the ultraviolet, as a function of pH. *Talanta*. doi: 10.1016/0039-9140(87)80232-1.

Direito, S.O.L., Marees, A., and Röling, W.F.M. (2012) Sensitive life detection strategies for low-biomass environments: Optimizing extraction of nucleic acids adsorbing to terrestrial and Mars analogue minerals. *FEMS Microbiology Ecology*. doi: 10.1111/j.1574-6941.2012.01325.x.

Dong, Y., Kumar, C.G., Chia, N., Kim, P.J., Miller, P.A., Price, N.D., et al. (2014) *Halomonas sulfidaeris*-dominated microbial community inhabits a 1.8 km-deep subsurface Cambrian sandstone reservoir. *Environmental Microbiology*. doi: 10.1111/1462-2920.12325.

Duan, Y., Liu, M., Sun, W., Wang, M., Liu, S., and Li, Q. (2009) Recent Progress on Synthesis of Fluorescein Probes. *Mini-Reviews in Organic Chemistry*. doi: 10.2174/157019309787316111.

Dutta, A., Sar, P., Sarkar, J., Dutta Gupta, S., Gupta, A., Bose, H., et al. (2019) Archaeal Communities in Deep Terrestrial Subsurface Underneath the Deccan Traps, India. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2019.01362.

Eddy, S.R. (2011) Accelerated profile HMM searches. *PLoS Computational Biology*. doi: 10.1371/journal.pcbi.1002195.

Edgar, R.C. (2013) UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*. doi: 10.1038/nmeth.2604.

Edwards, K.J., Bach, W., and Kalus, A. (2012a) Expedition 336 Scientists. *Expedition 336 Summary College Station, TX: Ocean Drilling Program*. doi: doi:10.2204/iodp.proc.336.101.2012.

Edwards, K.J., Bach, W., and McCollom, T.M. (2005) Geomicrobiology in oceanography: Microbe-mineral interactions at and below the seafloor. *Trends in Microbiology*. doi: 10.1016/j.tim.2005.07.005.

Edwards, K.J., Becker, K., and Colwell, F. (2012b) The deep, dark energy biosphere: Intraterrestrial life on earth. *Annual Review of Earth and Planetary Sciences*. doi: 10.1146/annurev-earth-042711-105500.

Edwards, K.J., Fisher, A.T., and Wheat, C.G. (2012c) The deep subsurface biosphere in igneous ocean crust: Frontier habitats for microbiological exploration. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2012.00008.

Emerson, D., Rentz, J.A., Lilburn, T.G., Davis, R.E., Aldrich, H., Chan, C., and Moyer, C.L. (2007) A novel lineage of proteobacteria involved in formation of marine Fe-oxidizing microbial mat communities. *PLoS ONE*. doi: 10.1371/journal.pone.0000667.

Escudero, C., Vera, M., Oggerin, M., and Amils, R. (2018) Active microbial biofilms in deep poor porous continental subsurface rocks. *Scientific Reports*. doi: 10.1038/s41598-018-19903-z.

Fernández-Remolar, D.C., Prieto-Ballesteros, O., Rodríguez, N., Gómez, F., Amils, R., Gómez-Elvira, J., and Stoker, C.R. (2008) Underground habitats in the Río tinto basin: A model for subsurface life habitats on mars. *Astrobiology*. doi: 10.1089/ast.2006.0104.

De Filippo, C., Ramazzotti, M., Fontana, P., and Cavalieri, D. (2012) Bioinformatic approaches for functional annotation and pathway inference in metagenomics data. *Briefings in Bioinformatics*. doi: 10.1093/bib/bbs070.

Fisher, C.R., Takai, K., and Le Bris, N. (2007) Hydrothermal Vent Ecosystems. *Oceanography* 20: 14–23.

Fisk, M.R., Storrie-Lombardi, M.C., Douglas, S., Popa, R., McDonald, G., and Di Meo-Savoie, C. (2003) Evidence of biological activity in Hawaiian subsurface basalts. *Geochemistry, Geophysics, Geosystems*. doi: 10.1029/2002GC000387.

Flemming, H.C. and Wuertz, S. (2019) Bacteria and archaea on Earth and their abundance in biofilms. *Nature Reviews Microbiology*. doi: 10.1038/s41579-019-0158-9.

Fliermans, C.B. and Balkwill, D.L. (1989) Microbial Life in Deep Terrestrial Subsurfaces. *BioScience*. doi: 10.2307/1311066.

Fones, E.M., Colman, D.R., Kraus, E.A., Nothaft, D.B., Poudel, S., Rempfert, K.R., et al. (2019) Physiological adaptations to serpentinization in the Samail Ophiolite, Oman. *ISME Journal*. doi: 10.1038/s41396-019-0391-2.

Fredrickson, J.K. and Balkwill, D.L. (2006) Geomicrobial processes and biodiversity in the deep terrestrial subsurface. *Geomicrobiology Journal*. doi: 10.1080/01490450600875571.

Fredrickson, J.K., McKinley, J.P., Bjornstad, B.N., Long, P.E., Ringelberg, D.B., White, D.C., et al. (1997) Pore-size constraints on the activity and survival of subsurface bacteria in a late cretaceous shale-sandstone sequence, northwestern new mexico. *Geomicrobiology Journal*. doi: 10.1080/01490459709378043.

Fredrickson, J.K. and Onstott, T.C. (1996) Microbes deep inside the earth. *Scientific American*. doi: 10.1038/scientificamerican1096-68.

Friese, A., Kallmeyer, J., Kitte, J.A., Martínez, I.M., Bijaksana, S., and Wagner, D. (2017) A simple and inexpensive technique for assessing contamination during drilling operations. *Limnology and Oceanography: Methods*. doi: 10.1002/lom3.10159.

Fröhlich, J. and König, H. (2000) New techniques for isolation of single prokaryotic cells. *FEMS Microbiology Reviews*. doi: 10.1016/S0168-6445(00)00045-0.

Furnes, H. and Staudigel, H. (1999) Biological mediation in ocean crust alteration: How deep is the deep biosphere? *Earth and Planetary Science Letters*. doi: 10.1016/S0012-821X(99)00005-9.

Gérard, E., De Goeyse, S.D., Hugoni, M., Agogué, H., Richard, L., Milesi, V., et al. (2018) Key role of Alphaproteobacteria and Cyanobacteria in the formation of stromatolites of Lake Dziani Dzaha (Mayotte, Western Indian Ocean). *Frontiers in Microbiology*. doi: 10.3389/fmicb.2018.00796.

Gérard, E., Ménez, B., Couradeau, E., Moreira, D., Benzerara, K., Tavera, R., and López-García, P. (2013) Specific carbonate-microbe interactions in the modern microbialites of Lake Alchichica (Mexico). *ISME Journal*. doi: 10.1038/ismej.2013.81.

Ghiorse, W.C. and Wilson, J.T. (1988) Microbial Ecology of the Terrestrial Subsurface. *Advances in Applied Microbiology*. doi: 10.1016/S0065-2164(08)70206-5.

Gihring, T.M., Moser, D.P., Lin, L.H., Davidson, M., Onstott, T.C., Morgan, L., et al. (2006) The distribution of microbial taxa in the subsurface water of the kalahari shield, south africa. *Geomicrobiology Journal*. doi: 10.1080/01490450600875696.

Giovannoni, S.J., Bibbs, L., Cho, J.C., Stapels, M.D., Desiderio, R., Vergin, K.L., et al. (2005a) Proteorhodopsin in the ubiquitous marine bacterium SAR11. *Nature*. doi: 10.1038/nature04032.

Giovannoni, S.J., Tripp, H.J., Givan, S., Podar, M., Vergin, K.L., Baptista, D., et al. (2005b) Genome Streamlining in a Cosmopolitan Oceanic Bacterium (supporting material). *Science*. doi: 10.1126/science.1114057.

Gloor, G.B., Macklaim, J.M., Pawlowsky-Glahn, V., and Egozcue, J.J. (2017) Microbiome datasets are compositional: And this is not optional. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2017.02224.

Gold, T. (1992) The deep, hot biosphere. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.89.13.6045.

Hafuka, A., Ding, Q., Yamamura, H., Yamada, K., and Satoh, H. (2015) Interactions of dissolved humic substances with oppositely charged fluorescent dyes for tracer techniques. *Water Research*. doi: 10.1016/j.watres.2015.08.039.

Hahn, M.W., Koll, U., and Schmidt, J. (2019) Isolation and Cultivation of Bacteria. doi: 10.1007/978-3-030-16775-2_10.

Hahn, M.W., Scheuerl, T., Jezberová, J., Koll, U., Jezbera, J., Šimek, K., et al. (2012) The passive yet successful way of planktonic life: Genomic and experimental analysis of the ecology of a free-living polynucleobacter population. *PLoS ONE*. doi: 10.1371/journal.pone.0032772.

Hahn, M.W., Stadler, P., Wu, Q.L., and Pöckl, M. (2004) The filtration-acclimatization method for isolation of an important fraction of the not readily cultivable bacteria. *Journal of Microbiological Methods*. doi: 10.1016/j.mimet.2004.02.004.

Hallsworth, J.E., Yakimov, M.M., Golyshin, P.N., Gillion, J.L.M., D'Auria, G., De Lima Alves, F., et al. (2007) Limits of life in MgCl2-containing environments: Chaotropicity defines the window. *Environmental Microbiology*. doi: 10.1111/j.1462-2920.2006.01212.x.

Heck, K.L., van Belle, G., and Simberloff, D. (1975) Explicit Calculation of the Rarefaction Diversity Measurement and the Determination of Sufficient Sample Size. *Ecology*. doi: 10.2307/1934716.

Heim, C., Quéric, N.V., Ionescu, D., Schäfer, N., and Reitner, J. (2017) Frutexites-like structures formed by iron oxidizing biofilms in the continental subsurface (Äspö Hard Rock Laboratory, Sweden). *PLoS ONE*. doi: 10.1371/journal.pone.0177542.

Henriksson, L. E. and Henriksson, E. (1974) Studies in the nitrogen cycles of Surtsey in 1972. Surtsey Reseach Progress Report 7: 36–44. Available online at: https://english.surtsey.is/wp-content/uploads/2019/08/1974_VII_2_07.pdf.

Henriksson, L. E. and Henriksson, E. (1982) Concerning the biological nitrogen fixation on Surtsey. Surtsey Research Progress Report IX: 9-12. Available online at: https://surtsey.is/wp-content/uploads/2019/08/1982_IX_2_01.pdf.

Herrera, A. and Cockell, C.S. (2007) Exploring microbial diversity in volcanic environments: A review of methods in DNA extraction. *Journal of Microbiological Methods*. doi: 10.1016/j.mimet.2007.04.005.

Higashi, Y., Sunamura, M., Kitamura, K., Nakamura, K.I., Kurusu, Y., Ishibashi, J.I., et al. (2004) Microbial diversity in hydrothermal surface to subsurface environments of Suiyo Seamount, Izu-Bonin Arc, using a catheter-type in situ growth chamber. *FEMS Microbiology Ecology*. doi: 10.1016/S0168-6496(04)00004-2.

Hjorleifsdottir, S., Skirnisdottir, S., Hreggvidsson, G.O., Holst, O., and Kristjansson, J.K. (2001) Species composition of cultivated and noncultivated bacteria from short filaments in an icelandic hot spring at 88°C. *Microbial Ecology* 42: 117–125. doi: 10.1007/s002480000110.

Hoehler, T.M. and Jørgensen, B.B. (2013) Microbial life under extreme energy limitation. *Nature Reviews Microbiology*. doi: 10.1038/nrmicro2939.

House, C.H., Cragg, B.A., and Teske, A. (2003) Drilling Contamination Tests during ODP Leg 201 Using Chemical and Particulate Tracers. In *Proceedings of the Ocean Drilling Program, 201 Initial Reports*. doi: 10.2973/odp.proc.ir.201.102.2003.

Huber, G.W., Iborra, S., and Corma, A. (2006) Synthesis of transportation fuels from biomass: Chemistry, catalysts, and engineering. *Chemical Reviews*. doi: 10.1021/cr068360d.

Huber, R., Burggraf, S., Mayer, T., Barns, S.M., Rossnagel, P., and Stetter, K.O. (1995) Isolation of a hyperthermophilic archaeum predicted by in situ RNA analysis. *Nature*. doi: 10.1038/376057a0.

Hugenholtz, P., Chuvochina, M., Oren, A., Parks, D.H., and Soo, R.M. (2021) Prokaryotic taxonomy and nomenclature in the age of big sequence data. *ISME Journal*. doi: 10.1038/s41396-021-00941-x.

Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology*. doi: 10.1128/jb.180.18.4765-4774.1998.

Hungate, R.E. (1950) The anaerobic mesophilic cellulolytic bacteria. *Bacteriological reviews*. doi: 10.1128/mmbr.14.1.1-49.1950.

Hyatt, D., Chen, G.L., LoCascio, P.F., Land, M.L., Larimer, F.W., and Hauser, L.J. (2010) Prodigal: Prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics*. doi: 10.1186/1471-2105-11-119.

Inagaki, F., Hinrichs, K.U., Kubo, Y., Bihan, M., Bowden, S.A., Bowles, M., et al. (2016) IODP Expedition 337: Deep Coalbed Biosphere off Shimokita - Microbial processes and hydrocarbon system associated with deeply buried coalbed in the ocean. *Scientific Drilling*. doi: 10.5194/sd-21-17-2016.

Inagaki, F., Hinrichs, K.U., Kubo, Y., Bowles, M.W., Heuer, V.B., Hong, W.L., et al. (2015) Exploring deep microbial life in coal-bearing sediment down to ~2.5 km below the ocean floor. *Science*. doi: 10.1126/science.aaa6882.

Ino, K., Konno, U., Kouduka, M., Hirota, A., Togo, Y.S., Fukuda, A., et al. (2016) Deep microbial life in high-quality granitic groundwater from geochemically and geographically distinct underground boreholes. *Environmental Microbiology Reports*. doi: 10.1111/1758-2229.12379.

Itävaara, M., Nyyssönen, M., Bomberg, M., Kapanen, A., Nousiainen, A., Ahonen, L., et al. (2011) Microbiological sampling and analysis of the Outokumpu Deep Drill Hole biosphere in 2007-2009. *Special Paper of the Geological Survey of Finland*.

Iversen, N. and Jorgensen, B.B. (1985) Anaerobic methane oxidation rates at the sulfate-methane transition in marine sediments from Kattegat and Skagerrak (Denmark). *Limnology and Oceanography*. doi: 10.4319/lo.1985.30.5.0944.

Jackson, M.D. (2020) Petrograpic and material observations of basaltic lapilli tuff, 1979 and 2017 Surtsey drill cores, Iceland. *Surtsey research*. doi: 10.33112/surtsey.14.4.

Jackson, M.D., Couper, S., Stan, C. V., Ivarsson, M., Czabaj, M.W., Tamura, N., et al. (2019a) Authigenic Mineral Texture in Submarine 1979 Basalt Drill Core, Surtsey Volcano, Iceland. *Geochemistry, Geophysics, Geosystems*. doi: 10.1029/2019GC008304.

Jackson, M.D., Gudmundsson, M.T., Bach, W., Cappelletti, P., Coleman, N.J., Ivarsson, M., et al. (2015) Time-lapse characterization of hydrothermal seawater and microbial interactions with basaltic tephra at Surtsey Volcano. *Scientific Drilling* **20**: 51–58. doi: 10.5194/sd-20-51-2015.

Jackson, M.D., Gudmundsson, M.T., Weisenberger, T.B., Rhodes, J.M., Stefánsson, A., Kleine, B.I., et al. (2019b) SUSTAIN drilling at Surtsey volcano, Iceland, tracks hydrothermal and microbiological interactions in basalt 50 years after eruption. *Scientific Drilling* 25: 35–46. doi: 10.5194/sd-25-35-2019.

Jacobson Meyers, M.E., Sylvan, J.B., and Edwards, K.J. (2014) Extracellular enzyme activity and microbial diversity measured on seafloor exposed basalts from Loihi seamount indicate the importance of basalts to global biogeochemical cycling. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01038-14.

Jakobsson, S.P. (1978) Environmental factors controlling the palagonitization of the Surtsey tephra, Iceland. *Bulletin of the Geological Society of Denmark*. doi: 10.37570/bgsd-1978-si-11.

Jakobsson, S.P. and Moore, J.G. (1986) Hydrothermal minerals and alteration rates at Surtsey volcano, Iceland. *Geological Society of America Bulletin* 97: 648–659. doi: 10.1130/0016-7606(1986)97<648:HMAARA>2.0.CO;2.

Jakobsson, S.P. and Moore, J.G. (1982) The Surtsey Research Drilling Project of 1979. *Surtsey Research Progress Report* 9: 76–93.

Jannasch, H.W., Eimhjellen, K., Wirsen, C.O., and Farmanfarmaian, A. (1971) Microbial degradation of organic matter in the deep sea. *Science* 171: 672–6.

Jin, Q. and Kirk, M.F. (2018) pH as a primary control in environmental microbiology: 1. thermodynamic perspective. *Frontiers in Environmental Science* 6: 21. doi: 10.3389/fenvs.2018.00021.

Jones, A.A. and Bennett, P.C. (2017) Mineral ecology: Surface specific colonization and geochemical drivers of biofilm accumulation, composition, and phylogeny. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2017.00491.

Jorgensen, B.B. and Marshall, I.P.G. (2016) Slow Microbial Life in the Seabed. *Annual Review of Marine Science*. doi: 10.1146/annurev-marine-010814-015535.

Jørgensen, S.L. and Zhao, R. (2016) Microbial inventory of deeply buried oceanic crust from a young ridge flank. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2016.00820.

Jung, D., Liu, L., and He, S. (2021a) Application of in situ cultivation in marine microbial resource mining. *Marine Life Science and Technology*. doi: 10.1007/s42995-020-00063-x.

Jung, D., Machida, K., Nakao, Y., Kindaichi, T., Ohashi, A., and Aoi, Y. (2021b) Triggering Growth via Growth Initiation Factors in Nature: A Putative Mechanism for in situ Cultivation of Previously Uncultivated Microorganisms. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2021.537194.

Jungbluth, S.P., Amend, J.P., and Rappé, M.S. (2017) Metagenome sequencing and 98 microbial genomes from Juan de Fuca Ridge flank subsurface fluids. *Scientific Data*. doi: 10.1038/sdata.2017.37.

Jungbluth, S.P., Grote, J., Lin, H.T., Cowen, J.P., and Rappé, M.S. (2013) Microbial diversity within basement fluids of the sediment-buried Juan de Fuca Ridge flank. *ISME Journal*. doi: 10.1038/ismej.2012.73.

Jungbluth, S.P., Lin, H.T., Cowen, J.P., Glazer, B.T., and Rappé, M.S. (2014) Phylogenetic diversity of microorganisms in subseafloor crustal fluids from Holes 1025C and 1026B along the Juan de Fuca Ridge flank. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2014.00119.

Kallmeyer, J. (2017) Contamination Control for Scientific Drilling Operations. *Advances in Applied Microbiology*. doi: 10.1016/bs.aambs.2016.09.003.

Kallmeyer, J., Mangelsdorf, K., Cragg, B., and Horsfield, B. (2006) Techniques for contamination assessment during drilling for terrestrial subsurface sediments. *Geomicrobiology Journal*. doi: 10.1080/01490450600724258.

Kallmeyer, J., Pockalny, R., Adhikari, R.R., Smith, D.C., and D'Hondt, S. (2012) Global distribution of microbial abundance and biomass in subseafloor sediment. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 16213–16216. doi: 10.1073/pnas.1203849109.

Kang, D.D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., and Wang, Z. (2019) MetaBAT 2: An adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ*. doi: 10.7717/peerj.7359.

Karnachuk, O. V., Frank, Y.A., Lukina, A.P., Kadnikov, V. V., Beletsky, A. V., Mardanov, A. V., and Ravin, N. V. (2019) Domestication of previously uncultivated Candidatus *Desulforudis audaxviator* from a deep aquifer in Siberia sheds light on its physiology and evolution. *ISME Journal*. doi: 10.1038/s41396-019-0402-3.

Keegan, K.P., Glass, E.M., and Meyer, F. (2016) MG-RAST, a metagenomics service for analysis of microbial community structure and function. In *Methods in Molecular Biology*. doi: 10.1007/978-1-4939-3369-3_13.

Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R., and Barer, M.R. (1998) Viability and activity in readily culturable bacteria: A review and discussion of the practical issues. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*. doi: 10.1023/A:1000664013047.

Kieft, T.L. (2016) Microbiology of the Deep Continental Biosphere. doi: 10.1007/978-3-319-28071-4_6.

Kieft, T.L. (2010) Sampling the Deep Sub-Surface Using Drilling and Coring Techniques. In *Handbook of Hydrocarbon and Lipid Microbiology*. doi: 10.1007/978-3-540-77587-4_267.

Kieft, T.L., Murphy, E.M., Haldeman, D.L., Amy, P.S., Bjornstad, B.N., McDonald, E. V., et al. (1998) Microbial transport, survival, and succession in a sequence of buried sediments. *Microbial Ecology*. doi: 10.1007/s002489900120.

Kleine, B.I., Stefánsson, A., Kjartansdóttir, R., Prause, S., Weisenberger, T.B., Reynolds, H.I., et al. (2020) The Surtsey volcano geothermal system: An analogue for seawater-oceanic crust interaction with implications for the elemental budget of the oceanic crust. *Chemical Geology*. doi: 10.1016/j.chemgeo.2020.119702.

Kleine, B.I., Stefánsson, A., Zierenberg, R.A., Jeon, H., Whitehouse, M.J., Jónasson, K., et al. (2022) Sulfate (re-)cycling in the oceanic crust: Effects of seawater-rock interaction, sulfur reduction and temperature on the abundance and isotope composition of anhydrite. *Geochimica et Cosmochimica Acta*. doi: 10.1016/j.gca.2021.10.016.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., and Glöckner, F.O. (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*. doi: 10.1093/nar/gks808.

Kokelaar, B.P. (1983) The mechanism of Surtseyan volcanism. *Journal of the Geological Society*. doi: 10.1144/gsjgs.140.6.0939.

Konopka, A. (2009) What is microbial community ecology. *ISME Journal*. doi: 10.1038/ismej.2009.88.

Kozlov, A.M., Darriba, D., Flouri, T., Morel, B., and Stamatakis, A. (2019) RAxML-NG: A fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics*. doi: 10.1093/bioinformatics/btz305.

Krebs, C.J. (2014) Species Diversity Measures. In *Ecological Methodology*. Available online at: https://www.zoology.ubc.ca/~krebs/downloads/krebs_chapter_13_2017.pdf.

Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular biology and evolution* 33: 1870–1874. doi: 10.1128/jcm.16.3.584-586.1982.

Kusube, M., Kyaw, T.S., Tanikawa, K., Chastain, R.A., Hardy, K.M., Cameron, J., and Bartlett, D.H. (2017) *Colwellia marinimaniae* sp. nov., a hyperpiezophilic species isolated from an amphipod within the challenger deep, Mariana Trench. *International Journal of Systematic and Evolutionary Microbiology*. doi: 10.1099/ijsem.0.001671.

Lagesen, K., Hallin, P., Rødland, E.A., Stærfeldt, H.H., Rognes, T., and Ussery, D.W. (2007) RNAmmer: Consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Research*. doi: 10.1093/nar/gkm160.

Lang, S.Q., Osburn, M.R., and Steen, A.D. (2019) Carbon in the Deep Biosphere. In *Deep Carbon: Past to Present*. pp. 480–523.

Langille, M.G.I., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., et al. (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology*. doi: 10.1038/nbt.2676.

Langmead, B. and Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nature Methods*. doi: 10.1038/nmeth.1923.

LaRowe, D.E. and Amend, J.P. (2015) Power limits for microbial life. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2015.00718.

Lau, M.C.Y., Kieft, T.L., Kuloyo, O., Linage-Alvarez, B., Van Heerden, E., Lindsay, M.R., et al. (2016) An oligotrophic deep-subsurface community dependent on syntrophy is dominated by sulfur-driven autotrophic denitrifiers. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1612244113.

Lecoeuvre, A., Ménez, B., Cannat, M., Chavagnac, V., and Gérard, E. (2021) Microbial ecology of the newly discovered serpentinite-hosted Old City hydrothermal field (southwest Indian ridge). *ISME Journal*. doi: 10.1038/s41396-020-00816-7.

Lee, M.D., Walworth, N.G., Sylvan, J.B., Edwards, K.J., and Orcutt, B.N. (2015) Microbial communities on seafloor basalts at Dorado Outcrop reflect level of alteration and highlight global lithic clades. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2015.01470.

Lehman, R.M., Colwell, F.S., Ringelberg, D.B., and White, D.C. (1995) Combined microbial community-level analyses for quality assurance of terrestrial subsurface cores. *Journal of Microbiological Methods*. doi: 10.1016/0167-7012(95)00012-A.

Lehman, R.M., O'Connell, S.P., Banta, A., Fredrickson, J.K., Reysenbach, A.L., Kieft, T.L., and Colwell, F.S. (2004) Microbiological comparison of core and groundwater samples collected from a fractured basalt aquifer with that of dialysis chambers incubated in Situ. *Geomicrobiology Journal*. doi: 10.1080/01490450490275848.

Letunic, I. and Bork, P. (2021) Interactive tree of life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Research*. doi: 10.1093/nar/gkab301.

Lever, M.A., Alperin, M., Engelen, B., Inagaki, F., Nakagawa, S., Steinsbu, B.O., and Teske, A. (2006) Trends in basalt and sediment core contamination during IODP expedition 301. *Geomicrobiology Journal*. doi: 10.1080/01490450600897245.

Lever, M.A., Rogers, K.L., Lloyd, K.G., Overmann, J., Schink, B., Thauer, R.K., et al. (2015a) Life under extreme energy limitation: A synthesis of laboratory- and field-based investigations. *FEMS Microbiology Reviews*. doi: 10.1093/femsre/fuv020.

Lever, M.A., Rouxel, O., Alt, J.C., Shimizu, N., Ono, S., Coggon, R.M., et al. (2013) Evidence for microbial carbon and sulfur cycling in deeply buried ridge flank basalt. *Science*. doi: 10.1126/science.1229240.

Lever, M.A., Torti, A., Eickenbusch, P., Michaud, A.B., Šantl-Temkiv, T., and Jørgensen, B.B. (2015b) A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2015.00476.

Li, D., Liu, C.M., Luo, R., Sadakane, K., and Lam, T.W. (2015) MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics*. doi: 10.1093/bioinformatics/btv033.

Li, D., Luo, R., Liu, C.M., Leung, C.M., Ting, H.F., Sadakane, K., et al. (2016) MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. *Methods*. doi: 10.1016/j.ymeth.2016.02.020.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. doi: 10.1093/bioinformatics/btp352.

Lin, L.-H., Wang, P.-L., Rumble, D., Lippmann-Pipke, J., Boice, E., Pratt, L.M., et al. (1961) Long-Term Sustainability of a High-Energy, Low-Diversity Crustal Biome. *Science*. doi: 10.1126/science.1127376.

Lin, L.H., Wang, P.L., Rumble, D., Lippmann-Pipke, J., Boice, E., Pratt, L.M., et al. (2006) Long-term sustainability of a high-energy, low-diweniff crystal-biome. *Science*. doi: 10.1126/science.1127376.

Lin, Y.S., Biddle, J.F., Lipp, J.S., Orcutt, B.N., Holler, T., Teske, A., and Hinrichs, K.U. (2010) Effect of storage conditions on archaeal and bacterial communities in subsurface marine sediments. *Geomicrobiology Journal*. doi: 10.1080/01490450903410423.

Lloyd, K.G., Steen, A.D., Ladau, J., Yin, J., and Crosby, L. (2018) Phylogenetically Novel Uncultured Microbial Cells Dominate Earth Microbiomes. *mSystems*. doi: 10.1128/msystems.00055-18.

Lok, C. (2015) Mining the microbial dark matter. *Nature*. doi: 10.1038/522270a.

Lu, J. (2021) KrakenTools. Available online at: https://github.com/jenniferlu717/KrakenTools.

Lu, J., Breitwieser, F.P., Thielen, P., and Salzberg, S.L. (2017) Bracken: Estimating species abundance in metagenomics data. *PeerJ Computer Science*. doi: 10.7717/peerj-cs.104.

Lunau, M., Lemke, A., Walther, K., Martens-Habbena, W., and Simon, M. (2005) An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Environmental Microbiology*. doi: 10.1111/j.1462-2920.2005.00767.x.

Lysnes, K., Torsvik, T., Thorseth, I.H., and Pedersen, R.B. (2004) Microbial Populations in Ocean Floor Basalt: Results from ODP Leg 187. In *Proceedings of the Ocean Drilling Program, 187 Scientific Results*. doi: 10.2973/odp.proc.sr.187.203.2004.

Magal, E., Weisbrod, N., Yakirevich, A., and Yechieli, Y. (2008) The use of fluorescent dyes as tracers in highly saline groundwater. *Journal of Hydrology*. doi: 10.1016/j.jhydrol.2008.05.035.

Magnabosco, C., Biddle, J.F., Cockell, C.S., Jungbluth, S.P., and Twing, K.I. (2019) Biogeography, Ecology, and Evolution of Deep Life. In *Deep Carbon: Past to Present*. pp. 524–555. doi: 10.1017/9781108677950.017.

Magnabosco, C., Lin, L.H., Dong, H., Bomberg, M., Ghiorse, W., Stan-Lotter, H., et al. (2018) The biomass and biodiversity of the continental subsurface. *Nature Geoscience* 1, 707–717. doi: 10.1038/s41561-018-0221-6.

Magnabosco, C., Ryan, K., Lau, M.C.Y., Kuloyo, O., Sherwood Lollar, B., Kieft, T.L., et al. (2016) A metagenomic window into carbon metabolism at 3 km depth in Precambrian continental crust. *ISME Journal*. doi: 10.1038/ismej.2015.150.

Magnabosco, C., Tekere, M., Lau, M.C.Y., Linage, B., Kuloyo, O., Erasmus, M., et al. (2014) Comparisons of the composition and biogeographic distribution of the bacterial communities occupying South African thermal springs with those inhabiting deep subsurface fracture water. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2014.00679.

Magnússon, B., Magnússon, S.H., Ólafsson, E., and Sigurdsson, B.D. (2014) Plant colonization, succession and ecosystem development on Surtsey with reference to neighbouring islands. *Biogeosciences*. doi: 10.5194/bg-11-5521-2014.

Marteinsson, V., Klonowski, A., Reynisson, E., Vannier, P., Sigurdsson, B.D., and Ólafsson, M. (2015) Microbial colonization in diverse surface soil types in Surtsey and diversity analysis of its subsurface microbiota. *Biogeosciences*. doi: 10.5194/bg-12-1191-2015.

Marteinsson, V.T., Hauksdóttir, S., Hobel, C.F.V., Kristmannsdóttir, H., Hreggvidsson, G.O., and Kristjánsson, J.K. (2001) Phylogenetic Diversity Analysis of Subterranean Hot Springs in Iceland. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.67.9.4242-4248.2001.

Martinez Arbizu, P. (2020) pairwiseAdonis: Pairwise multilevel comparison using adonis. *R package version 04*. Available online at: https://github.com/pmartinezarbizu/pairwiseAdonis.

Martiny, A.C. (2020) The ‘1% culturability paradigm’ needs to be carefully defined. *ISME Journal*. doi: 10.1038/s41396-019-0507-8.

Mason, O.U., Nakagawa, T., Rosner, M., van Nostrand, J.D., Zhou, J., Maruyama, A., et al. (2010) First investigation of the microbiology of the deepest layer of ocean crust. *PLoS ONE*. doi: 10.1371/journal.pone.0015399.

McCollom, T.M. and Amend, J.P. (2005) A thermodynamic assessment of energy requirements for biomass synthesis by chemolithoautotrophic micro-organisms in oxic and anoxic environments. *Geobiology*. doi: 10.1111/j.1472-4669.2005.00045.x.

McCollom, T.M. and Shock, E.L. (1997) Geochemical constraints on chemolithoautotrophic metabolism by microorganisms in seafloor hydrothermal systems. *Geochimica et Cosmochimica Acta*. doi: 10.1016/S0016-7037(97)00241-X.

Mcloughlin, N., Staudigel, H., Furnes, H., Eickmann, B., and Ivarsson, M. (2010) Mechanisms of microtunneling in rock substrates: Distinguishing endolithic biosignatures from abiotic microtunnels. *Geobiology*. doi: 10.1111/j.1472-4669.2010.00243.x.

Mcmahon, S. and Parnell, J. (2014) Weighing the deep continental biosphere. *FEMS Microbiology Ecology*. doi: 10.1111/1574-6941.12196.

McMurdie, P.J. and Holmes, S. (2013) Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*. doi: 10.1371/journal.pone.0061217.

McMurdie, P.J. and Holmes, S. (2014) Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. *PLoS Computational Biology*. doi: 10.1371/journal.pcbi.1003531.

McPhie, J., White, J.D.L., Gorny, C., Jackson, M.D., Gudmundsson, M.T., and Couper, S. (2020) Lithofacies from the 1963-1967 Surtsey eruption in SUSTAIN drill cores SE-2a, SE-2b and SE-03. *Surtsey research*. doi: 10.33112/surtsey.14.2.

Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.P., and Göker, M. (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 14:. doi: 10.1186/1471-2105-14-60.

Meier, D. V., Pjevac, P., Bach, W., Hourdez, S., Girguis, P.R., Vidoudez, C., et al. (2017) Niche partitioning of diverse sulfur-oxidizing bacteria at hydrothermal vents. *ISME Journal*. doi: 10.1038/ismej.2017.37.

Menzel, P., Ng, K.L., and Krogh, A. (2016) Fast and sensitive taxonomic classification for metagenomics with Kaiju. *Nature Communications*. doi: 10.1038/ncomms11257.

Meyer, J.L., Jaekel, U., Tully, B.J., Glazer, B.T., Wheat, C.G., Lin, H.T., et al. (2016) A distinct and active bacterial community in cold oxygenated fluids circulating beneath the western flank of the Mid-Atlantic ridge. *Scientific Reports*. doi: 10.1038/srep22541.

Michoud, G. and Jebbar, M. (2016) High hydrostatic pressure adaptive strategies in an obligate piezophile Pyrococcus yayanosii. *Scientific Reports*. doi: 10.1038/srep27289.

Miller, M.A., Pfeiffer, W., and Schwartz, T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *2010 Gateway Computing Environments Workshop, GCE 2010*. doi: 10.1109/GCE.2010.5676129.

Mills, H.J., Reese, B.K., and Peter, C.S. (2012) Characterization of microbial population shifts during sample storage. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2012.00049.

Minoche, A.E., Dohm, J.C., and Himmelbauer, H. (2011) Evaluation of genomic high-throughput sequencing data generated on Illumina HiSeq and Genome Analyzer systems. *Genome Biology*. doi: 10.1186/gb-2011-12-11-r112.

Momper, L., Jungbluth, S.P., Lee, M.D., and Amend, J.P. (2017a) Energy and carbon metabolisms in a deep terrestrial subsurface fluid microbial community. *ISME Journal*. doi: 10.1038/ismej.2017.94.

Momper, L., Kiel Reese, B., Zinke, L., Wanger, G., Osburn, M.R., Moser, D., and Amend, J.P. (2017b) Major phylum-level differences between porefluid and host rock bacterial communities in the terrestrial deep subsurface. *Environmental Microbiology Reports* 9: 501–511. doi: 10.1111/1758-2229.12563.

Moore, J.C. and Jackson, M.D. (2020) Observations on the structure of Surtsey. *Surtsey research*. doi: 10.33112/surtsey.14.3.

Morono, Y., Terada, T., Kallmeyer, J., and Inagaki, F. (2013) An improved cell separation technique for marine subsurface sediments: Applications for high-throughput analysis using flow cytometry and cell sorting. *Environmental Microbiology*. doi: 10.1111/1462-2920.12153.

Morono, Y., Terada, T., Masui, N., and Inagaki, F. (2009) Discriminative detection and enumeration of microbial life in marine subsurface sediments. *ISME Journal*. doi: 10.1038/ismej.2009.1.

Morono, Y., Terada, T., Nishizawa, M., Ito, M., Hillion, F., Takahata, N., et al. (2011) Carbon and nitrogen assimilation in deep subseafloor microbial cells. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1107763108.

Morris, B.E.L., Henneberger, R., Huber, H., and Moissl-Eichinger, C. (2013) Microbial syntrophy: Interaction for the common good. *FEMS Microbiology Reviews*. doi: 10.1111/1574-6976.12019.

Moser, D.P., Gihring, T.M., Brockman, F.J., Fredrickson, J.K., Balkwill, D.L., Dollhopf, M.E., et al. (2005) Desulfotomaculum and Methanobacterium spp. dominate a 4- to 5-kilometer-deep fault. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.71.12.8773-8783.2005.

Muniesa, M., Blanch, A.R., Lucena, F., and Jofre, J. (2005) Bacteriophages may bias outcome of bacterial enrichment cultures. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.71.8.4269-4275.2005.

Murakami, Y., Fujita, Y., Naganuma, T., and Iwatsuki, T. (2002) Abundance and Viability of the Groundwater Microbial Communities from a Borehole in the Tono Uranium Deposit Area, Central Japan. *Microbes and Environments*. doi: 10.1264/jsme2.2002.63.

Nigro, L.M., Harris, K., Orcutt, B.N., Hyde, A., Clayton-Luce, S., Becker, K., and Teske, A. (2012) Microbial communities at the borehole observatory on the Costa Rica Rift flank (Ocean Drilling Program Hole 896A). *Frontiers in Microbiology*. doi: 10.3389/fmicb.2012.00232.

Noble, R.T. and Fuhrman, J.A. (1998) Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquatic Microbial Ecology*. doi: 10.3354/ame014113.

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., et al. (2013) Assembling genomes and mini-metagenomes from highly chimeric reads. In *Deng M., Jiang R., Sun F., Zhang X. (eds) Research in Computational Molecular Biology. Lecture Notes in Computer Science*. Springer, Berlin, Heidelberg, pp. 158–170. doi: 10.1007/978-3-642-37195-0_13.

Nyyssönen, M., Hultman, J., Ahonen, L., Kukkonen, I., Paulin, L., Laine, P., et al. (2014) Taxonomically and functionally diverse microbial communities in deep crystalline rocks of the Fennoscandian shield. *ISME Journal*. doi: 10.1038/ismej.2013.125.

Oger, P.M. and Jebbar, M. (2010) The many ways of coping with pressure. *Research in Microbiology*. doi: 10.1016/j.resmic.2010.09.017.

Ólafsson, M. and Jakobsson, S.P. (2009) Chemical composition of hydrothermal water and water-rock interactions on Surtsey volcanic island: A preliminary report. *Surtsey Research* 29–38.

Onstott, T.C., Moser, D.P., Pfiffner, S.M., Fredrickson, J.K., Brockman, F.J., Phelps, T.J., et al. (2003) Indigenous and contaminant microbes in ultradeep mines. *Environmental Microbiology*. doi: 10.1046/j.1462-2920.2003.00512.x.

Onstott, T.C., Phelps, T.J., Kieft, T., Colwell, F.S., Balkwill, D.L., Fredrickson, J.K., and Brockman, F.J. (1999) A Global Perspective on the Microbial Abundance and Activity in the Deep Subsurface. In *Enigmatic Microorganisms and Life in Extreme Environments*. doi: 10.1007/978-94-011-4838-2_38.

Opatkiewicz, A.D., Butterfield, D.A., and Baross, J.A. (2009) Individual hydrothermal vents at Axial Seamount harbor distinct subseafloor microbial communities. *FEMS Microbiology Ecology*. doi: 10.1111/j.1574-6941.2009.00747.x.

Orcutt, B.N., Bach, W., Becker, K., Fisher, A.T., Hentscher, M., Toner, B.M., et al. (2011a) Colonization of subsurface microbial observatories deployed in young ocean crust. *ISME Journal* **5**: 692–703. doi: 10.1038/ismej.2010.157.

Orcutt, B.N., Bergenthal, M., Freudenthal, T., Smith, D., Lilley, M.D., Schnieders, L., et al. (2017) Contamination tracer testing with seabed drills: IODP Expedition 357. *Scientific Drilling*. doi: 10.5194/sd-23-39-2017.

Orcutt, B.N., D'Angelo, T., Wheat, C.G., and Trembath-Reichert, E. (2020) Microbe-mineral biogeography from multi-year incubations in oceanic crust at North Pond, Mid-Atlantic Ridge. *Environmental Microbiology*. doi: 10.1111/1462-2920.15366.

Orcutt, B.N., LaRowe, D.E., Biddle, J.F., Colwell, F.S., Glazer, B.T., Reese, B.K., et al. (2013a) Microbial activity in the marine deep biosphere: Progress and prospects. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2013.00189.

Orcutt, B.N., Sylvan, J.B., Knab, N.J., and Edwards, K.J. (2011b) Microbial Ecology of the Dark Ocean above, at, and below the Seafloor. *Microbiology and Molecular Biology Reviews*. doi: 10.1128/mmbr.00039-10.

Orcutt, B.N., Wheat, C.G., Rouxel, O., Hulme, S., Edwards, K.J., and Bach, W. (2013b) Oxygen consumption rates in subseafloor basaltic crust derived from a reaction transport model. *Nature Communications*. doi: 10.1038/ncomms3539.

Oremland, R.S. and Polcin, S. (1982) Methanogenesis and Sulfate Reduction: Competitive and Noncompetitive Substrates in Estuarine Sediments. *Applied and Environmental Microbiology* 44: 1270–1276. doi: 10.1128/aem.44.6.1270-1276.1982.

Orsi, W.D., Edgcomb, V.P., Christman, G.D., and Biddle, J.F. (2013) Gene expression in the deep biosphere. *Nature*. doi: 10.1038/nature12230.

Osburn, M.R., LaRowe, D.E., Momper, L.M., and Amend, J.P. (2014) Chemolithotrophy in the continental deep subsurface: Sanford underground research facility (SURF), USA. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2014.00610.

Óskarsson, B.V., Jónasson, K., Valsson, G., and Belart, J.M.C. (2020) Erosion and sedimentation in Surtsey island quantified from new DEMs. *Surtsey research*. doi: 10.33112/surtsey.14.5.

Overmann, J. (2013) Principles of enrichment, isolation, cultivation, and preservation of prokaryotes. In *The Prokaryotes: Prokaryotic Biology and Symbiotic Associations*. doi: 10.1007/978-3-642-30194-0_7.

Øvreås, L., Forney, L., Daae, F.L., and Torsvik, V. (1997) Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology*. doi: 10.1128/aem.63.9.3367-3373.1997.

Pace, N.R., Stahl, D.A., Lane, D.J., and Olsen, G.J. (1986) The Analysis of Natural Microbial Populations by Ribosomal RNA Sequences. doi: 10.1007/978-1-4757-0611-6_1.

Parada, A.E., Needham, D.M., and Fuhrman, J.A. (2016) Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology*. doi: 10.1111/1462-2920.13023.

Parkes, R.J., Cragg, B.A., and Wellsbury, P. (2000) Recent studies on bacterial populations and processes in subseafloor sediments: A review. *Hydrogeology Journal* 8: 11–28.

Parkes, R.J., Webster, G., Cragg, B.A., Weightman, A.J., Newberry, C.J., Ferdelman, T.G., et al. (2005) Deep sub-seafloor prokaryotes stimulated at interfaces over geological time. *Nature*. doi: 10.1038/nature03796.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., and Tyson, G.W. (2015) CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research*. doi: 10.1101/gr.186072.114.

Patel, A., Noble, R.T., Steele, J.A., Schwalbach, M.S., Hewson, I., and Fuhrman, J.A. (2007) Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR Green I. *Nature Protocols*. doi: 10.1038/nprot.2007.6.

Payler, S.J., Biddle, J.F., Lollar, B.S., Fox-Powell, M.G., Edwards, T., Ngwenya, B.T., et al. (2019) An ionic limit to life in the deep subsurface. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2019.00426.

Pedersen, K. (2000) Exploration of deep intraterrestrial microbial life: Current perspectives. *FEMS Microbiology Letters*. doi: 10.1016/S0378-1097(00)00061-6.

Pedersen, K. and Ekendahl, S. (1990) Distribution and activity of bacteria in deep granitic groundwaters of southeastern sweden. *Microbial Ecology*. doi: 10.1007/BF02543865.

Pellizzari, L., Neumann, D., Alawi, M., Voigt, D., Norden, B., and Würdemann, H. (2013) The use of tracers to assess drill-mud penetration depth into sandstone cores during deep drilling: Method development and application. *Environmental Earth Sciences*. doi: 10.1007/s12665-013-2715-2.

Pernthaler, A. and Pernthaler, J. (2007) Fluorescence in situ hybridization for the identification of environmental microbes. *Methods in Molecular Biology*. doi: 10.1385/1-59745-229-7:153.

Pernthaler, J., Glöckner, F.O., Schönhuber, W., and Amann, R. (2001) Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes. *Methods in Microbiology*. doi: 10.1016/s0580-9517(01)30046-6.

Pfiffner, S.M., Onstott, T.C., Ruskeeniemi, T., Talikka, M., Bakermans, C., McGown, D., et al. (2008) Challenges for coring deep permafrost on earth and mars. In *Astrobiology*. doi: 10.1089/ast.2007.0159.

Phelps, T.J., Fliermans, C.B., Garland, T.R., Pfiffner, S.M., and White, D.C. (1989) Methods for recovery of deep terrestrial subsurface sediments for microbiological studies. *Journal of Microbiological Methods*. doi: 10.1016/0167-7012(89)90069-9.

Poniedziałek, B., Falfushynska, H.I., and Rzymski, P. (2017) Flow cytometry as a valuable tool to study cyanobacteria:A mini-review. *Limnological Review*. doi: 10.1515/limre-2017-0009.

Porter, K.G. and Feig, Y.S. (1980) The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography*. doi: 10.4319/lo.1980.25.5.0943.

Postec, A., Quéméneur, M., Bes, M., Mei, N., Benaïssa, F., Payri, C., et al. (2015) Microbial diversity in a submarine carbonate edifice from the serpentinizing hydrothermal system of the Prony Bay (New Caledonia) over a 6-year period. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2015.00857.

Prause, S., Weisenberger, T.B., Cappelletti, P., Grimaldi, C., Rispoli, C., Jónasson, K., et al. (2020) Alteration progress within the Surtsey hydrothermal system, SW Iceland – A time-lapse petrographic study of cores drilled in 1979and 2017. *Journal of Volcanology and Geothermal Research*. doi: 10.1016/j.jvolgeores.2019.106754.

Probst, A.J., Weinmaier, T., Raymann, K., Perras, A., Emerson, J.B., Rattei, T., et al. (2014) Biology of a widespread uncultivated archaeon that contributes to carbon fixation in the subsurface. *Nature Communications*. doi: 10.1038/ncomms6497.

Proskurowski, G., Lilley, M.D., Seewald, J.S., Früh-Green, G.L., Olson, E.J., Lupton, J.E., et al. (2008) Abiogenic hydrocarbon production at lost city hydrothermal field. *Science*. doi: 10.1126/science.1151194.

Purkamo, L., Bomberg, M., Kietäväinen, R., Salavirta, H., Nyyssönen, M., Nuppunen-Puputti, M., et al. (2016) Microbial co-occurrence patterns in deep Precambrian bedrock fracture fluids. *Biogeosciences*. doi: 10.5194/bg-13-3091-2016.

Purkamo, L., Bomberg, M., Nyyssönen, M., Kukkonen, I., Ahonen, L., and Itävaara, M. (2014) Heterotrophic Communities Supplied by Ancient Organic Carbon Predominate in Deep Fennoscandian Bedrock Fluids. *Microbial Ecology*. doi: 10.1007/s00248-014-0490-6.

Purkamo, L., Kietäväinen, R., Nuppunen-Puputti, M., Bomberg, M., and Cousins, C. (2020) Ultradeep microbial communities at 4.4 km within crystalline bedrock: Implications for habitability in a planetary context. *Life*. doi: 10.3390/life10010002.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013) The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*. doi: 10.1093/nar/gks1219.

Quéméneur, M., Bes, M., Postec, A., Mei, N., Hamelin, J., Monnin, C., et al. (2014) Spatial distribution of microbial communities in the shallow submarine alkaline hydrothermal field of the Prony Bay, New Caledonia. *Environmental Microbiology Reports*. doi: 10.1111/1758-2229.12184.

R Core Team (2019) R: A language and environment for statistical computing. *R Foundation for Statistical Computing*. Available online at: https://www.R-project.org/.

Ramírez-Flandes, S., González, B., and Ulloa, O. (2019) Redox traits characterize the organization of global microbial communities. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1817554116.

Ramírez, G.A., Garber, A.I., Lecoeuvre, A., D'angelo, T., Wheat, C.G., and Orcutt, B.N. (2019) Ecology of subseafloor crustal biofilms. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2019.01983.

Rappé, M.S., Connon, S.A., Vergin, K.L., and Giovannoni, S.J. (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature*. doi: 10.1038/nature00917.

Rappé, M.S. and Giovannoni, S.J. (2003) The Uncultured Microbial Majority. *Annual Review of Microbiology*. doi: 10.1146/annurev.micro.57.030502.090759.

Rathsack, K., Stackebrandt, E., Reitner, J., and Schumann, G. (2009) Microorganisms isolated from deep sea low-temperature influenced oceanic crust basalts and sediment samples collected along the Mid-Atlantic Ridge. *Geomicrobiology Journal*. doi: 10.1080/01490450902892456.

Reasoner, D.J. and Geldreich, E.E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology*. doi: 10.1128/aem.49.1.1-7.1985.

Rebata-Landa, V. and Santamarina, J.C. (2006) Mechanical limits to microbial activity in deep sediments. *Geochemistry, Geophysics, Geosystems*. doi: 10.1029/2006GC001355.

Reese, B.K., Zinke, L.A., Sobol, M.S., LaRowe, D.E., Orcutt, B.N., Zhang, X., et al. (2018) Nitrogen Cycling of Active Bacteria within Oligotrophic Sediment of the Mid-Atlantic Ridge Flank. *Geomicrobiology Journal*. doi: 10.1080/01490451.2017.1392649.

Rempfert, K.R., Miller, H.M., Bompard, N., Nothaft, D., Matter, J.M., Kelemen, P., et al. (2017) Geological and geochemical controls on subsurface microbial life in the Samail Ophiolite, Oman. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2017.00056.

Reysenbach, A.L., Liu, Y., Banta, A.B., Beveridge, T.J., Kirshtein, J.D., Schouten, S., et al. (2006) A ubiquitous thermoacidophilic archaeon from deep-sea hydrothermal vents. *Nature*. doi: 10.1038/nature04921.

Robador, A., Jungbluth, S.P., LaRowe, D.E., Bowers, R.M., Rappé, M.S., Amend, J.P., and Cowen, J.P. (2015) Activity and phylogenetic diversity of sulfate-reducing microorganisms in low-temperature subsurface fluids within the upper oceanic crust. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2014.00748.

Røy, H., Kallmeyer, J., Adhikari, R.R., Pockalny, R., Jørgensen, B.B., and D'Hondt, S. (2012) Aerobic microbial respiration in 86-million-year-old deep-sea red clay. *Science*. doi: 10.1126/science.1219424.

Rstudio, T. (2020) RStudio: Integrated Development for R. *Rstudio Team, PBC, Boston, MA URL http://www.rstudio.com/*. doi: 10.1145/3132847.3132886.

Russell, B.F., Phelps, T.J., Griffin, W.T., and Sargent, K.A. (1992) Procedures for Sampling Deep Subsurface Microbial Communities in Unconsolidated Sediments. *Groundwater Monitoring & Remediation*. doi: 10.1111/j.1745-6592.1992.tb00414.x.

Sahl, J.W., Schmidt, R., Swanner, E.D., Mandernack, K.W., Templeton, A.S., Kieft, T.L., et al. (2008) Subsurface microbial diversity in deep-granitic-fracture water in Colorado. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01133-07.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution* 4: 406–425. doi: 10.1093/oxfordjournals.molbev.a040454.

Santelli, C.M., Edgcomb, V.P., Bach, W., and Edwards, K.J. (2009) The diversity and abundance of bacteria inhabiting seafloor lavas positively correlate with rock alteration. *Environmental Microbiology*. doi: 10.1111/j.1462-2920.2008.01743.x.

Santelli, C.M., Orcutt, B.N., Banning, E., Bach, W., Moyer, C.L., Sogin, M.L., et al. (2008) Abundance and diversity of microbial life in ocean crust. *Nature*. doi: 10.1038/nature06899.

Schipper, C.I., Jakobsson, S.P., White, J.D.L., Michael Palin, J., and Bush-Marcinowski, T. (2015) The surtsey magma series. *Scientific Reports*. doi: 10.1038/srep11498.

Schipper, C.I., Le Voyer, M., Moussallam, Y., White, J.D.L., Thordarson, T., Kimura, J.I., and Chang, Q. (2016) Degassing and magma mixing during the eruption of Surtsey Volcano (Iceland, 1963–1967): the signatures of a dynamic and discrete rift propagation event. *Bulletin of Volcanology*. doi: 10.1007/s00445-016-1025-4.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01541-09.

Schrenk, M.O., Brazelton, W.J., and Lang, S.Q. (2013) Serpentinization, carbon, and deep life. *Reviews in Mineralogy and Geochemistry*. doi: 10.2138/rmg.2013.75.18.

Schrenk, M.O., Kelley, D.S., Bolton, S.A., and Baross, J.A. (2004) Low archaeal diversity linked to subseafloor geochemical processes at the Lost City Hydrothermal Field, Mid-Atlantic Ridge. *Environmental Microbiology*. doi: 10.1111/j.1462-2920.2004.00650.x.

Schwartz, W. and Schwartz, A. (1972) Microbial activity in Surtsey. Surtsey Research Progress Report VI: 90. Available online at: https://surtsey.is/wp-content/uploads/2019/08/1972_VI_2_22.pdf.

Senum, G. and Dietz, R. (1991) Perfluorocarbon tracer tagging of drilling muds for the assessment of sample contamination. *Brookhaven National Lab*. Available online at: https://www.osti.gov/servlets/purl/7267282.

Shannon, C.E. (1948) A Mathematical Theory of Communication. *Bell System Technical Journal*. doi: 10.1002/j.1538-7305.1948.tb01338.x.

Sharma, A., Scott, J.H., Cody, G.D., Fogel, M.L., Hazen, R.M., Hemley, R.J., and Huntress, W.T. (2002) Microbial activity at gigapascal pressures. *Science*. doi: 10.1126/science.1068018.

Sheik, C.S., Reese, B.K., Twing, K.I., Sylvan, J.B., Grim, S.L., Schrenk, M.O., et al. (2018) Identification and removal of contaminant sequences from ribosomal gene databases: Lessons from the Census of Deep Life. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2018.00840.

Shelobolina, E., Xu, H., Konishi, H., Kukkadapu, R., Wu, T., Blöthe, M., and Roden, E. (2012) Microbial lithotrophic oxidation of structural Fe(II) in biotite. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01034-12.

Silver, B.J., Onstott, T.C., Rose, G., Lin, L.-H., Ralston, C., Sherwood-Lollar, B., et al. (2010) In Situ Cultivation of Subsurface Microorganisms in a Deep Mafic Sill: Implications for SLiMEs. *Geomicrobiology Journal* 27: 329–348. doi: 10.1080/01490451003705183.

Simpson, E.H. (1949) Measurement of diversity [16]. *Nature*. doi: 10.1038/163688a0.

Skennerton, C.T., Chourey, K., Iyer, R., Hettich, R.L., Tyson, G.W., and Orphan, V.J. (2017) Methane-fueled syntrophy through extracellular electron transfer: Uncovering the genomic traits conserved within diverse bacterial partners of anaerobic methanotrophic archaea. *mBio*. doi: 10.1128/mBio.00530-17.

Smart, P.L. and Laidlaw, I.M.S. (1977) An evaluation of some fluorescent dyes for water tracing. *Water Resources Research*. doi: 10.1029/WR013i001p00015.

Smith, A., Popa, R., Fisk, M., Nielsen, M., Wheat, C.G., Jannasch, H.W., et al. (2011) In situ enrichment of Ocean crust microbes on igneous minerals and glasses using an osmotic flow-through device. *Geochemistry, Geophysics, Geosystems*. doi: 10.1029/2010GC003424.

Smith, A.R., Fisk, M.R., Thurber, A.R., Flores, G.E., Mason, O.U., Popa, R., and Colwell, F.S. (2017) Deep Crustal Communities of the Juan de Fuca Ridge Are Governed by Mineralogy. *Geomicrobiology Journal*. doi: 10.1080/01490451.2016.1155001.

Smith, D.C. and D'Hondt, S. (2006) Exploration of life in deep subseafloor sediments. *Oceanography*. doi: 10.5670/oceanog.2006.05.

Smith, D.C., Spivack, A.J., Fisk, M.R., Haveman, S.A., and Staudigel, H. (2000a) Methods for Quantifying Potential Microbial Contamination during Deep Ocean Coring doi: 10.2973/odp.tn.28.2000.

Smith, D.C., Spivack, A.J., Fisk, M.R., Haveman, S.A., and Staudigel, H. (2000b) Tracer-based estimates of drilling-induced microbial contamination of deep sea crust. *Geomicrobiology Journal*. doi: 10.1080/01490450050121170.

Starnawski, P., Bataillon, T., Ettema, T.J.G., Jochum, L.M., Schreiber, L., Chen, X., et al. (2017) Microbial community assembly and evolution in subseafloor sediment. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1614190114.

Staudigel, H., Furnes, H., McLoughlin, N., Banerjee, N.R., Connell, L.B., and Templeton, A. (2008) 3.5 billion years of glass bioalteration: Volcanic rocks as a basis for microbial life? *Earth-Science Reviews*. doi: 10.1016/j.earscirev.2008.04.005.

Steen, A.D., Crits-Christoph, A., Carini, P., DeAngelis, K.M., Fierer, N., Lloyd, K.G., and Cameron Thrash, J. (2019) High proportions of bacteria and archaea across most biomes remain uncultured. *ISME Journal*. doi: 10.1038/s41396-019-0484-y.

Stetter, K.O., Huber, R., Blöchl, E., Kurr, M., Eden, R.D., Fielder, M., et al. (1993) Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. *Nature*. doi: 10.1038/365743a0.

Stevens, T. (1997) Lithoautotrophy in the subsurface. In *FEMS Microbiology Reviews*. doi: 10.1016/S0168-6445(97)00015-6.

Stevens, T.O. and McKinley, J.P. (1995) Lithoautotrophic microbial ecosystems in deep basalt aquifers. *Science*. doi: 10.1126/science.270.5235.450.

Stevenson, A., Burkhardt, J., Cockell, C.S., Cray, J.A., Dijksterhuis, J., Fox-Powell, M., et al. (2015) Multiplication of microbes below 0.690 water activity: Implications for terrestrial and extraterrestrial life. *Environmental Microbiology*. doi: 10.1111/1462-2920.12598.

Stewart, E.J. (2012) Growing unculturable bacteria. *Journal of Bacteriology*. doi: 10.1128/JB.00345-12.

Stokke, R., Dahle, H., Roalkvam, I., Wissuwa, J., Daae, F.L., Tooming-Klunderud, A., et al. (2015) Functional interactions among filamentous Epsilonproteobacteria and Bacteroidetes in a deep-sea hydrothermal vent biofilm. *Environmental Microbiology*. doi: 10.1111/1462-2920.12970.

Sueoka, Y., Yamashita, S., Kouduka, M., and Suzuki, Y. (2019) Deep Microbial Colonization in Saponite-Bearing Fractures in Aged Basaltic Crust: Implications for Subsurface Life on Mars. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2019.02793.

Suzuki, S., Ishii, S., Wu, A., Cheung, A., Tenney, A., Wanger, G., et al. (2013) Microbial diversity in the Cedars, an ultrabasic, ultrareducing, and low salinity serpentinizing ecosystem. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1302426110.

Suzuki, S., Kuenen, J.G., Schipper, K., Van Der Velde, S., Ishii, S., Wu, A., et al. (2014) Physiological and genomic features of highly alkaliphilic hydrogen-utilizing Betaproteobacteria from a continental serpentinizing site. *Nature Communications* 5:. doi: 10.1038/ncomms4900.

Suzuki, Y., Yamashita, S., Kouduka, M., Ao, Y., Mukai, H., Mitsunobu, S., et al. (2020) Deep microbial proliferation at the basalt interface in 33.5–104 million-year-old oceanic crust. *Communications Biology*. doi: 10.1038/s42003-020-0860-1.

Swanner, E.D. and Templeton, A.S. (2011) Potential for nitrogen fixation and nitrification in the granite-hosted subsurface at Henderson Mine, CO. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2011.00254.

Takai, K. and Horikoshi, K. (2000) Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Applied and Environmental Microbiology*. doi: 10.1128/AEM.66.11.5066-5072.2000.

Takai, K., Komatsu, T., and Horikoshi, K. (2001) *Hydrogenobacter subterraneus* sp. nov., an extremely thermophilic, heterotrophic bacterium unable to grow on hydrogen gas, from deep subsurface geothermal water. *International Journal of Systematic and Evolutionary Microbiology*. doi: 10.1099/00207713-51-4-1425.

Takai, K., Moyer, C.L., Miyazaki, M., Nogi, Y., Hirayama, H., Nealson, K.H., and Horikoshi, K. (2005) *Marinobacter alkaliphilus* sp. nov., a novel alkaliphilic bacterium isolated from subseafloor alkaline serpentine mud from Ocean Drilling Program Site 1200 at South Chamorro Seamount, Mariana Forearc. *Extremophiles*. doi: 10.1007/s00792-004-0416-1.

Takai, K., Nakamura, K., Toki, T., Tsunogai, U., Miyazaki, M., Miyazaki, J., et al. (2008) Cell proliferation at 122°C and isotopically heavy CH4 production by a hyperthermophilic methanogen under high-pressure cultivation. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0712334105.

Templeton, A.S., Staudigel, H., and Tebo, B.M. (2005) Diverse Mn(II)-oxidizing bacteria isolated from submarine basalts at Loihi seamount. *Geomicrobiology Journal*. doi: 10.1080/01490450590945951.

Thomazo, C., Buoncristiani, J.F., Vennin, E., Pellenard, P., Cocquerez, T., Mugnier, J.L., and Gérard, E. (2017) Geochemical processes leading to the precipitation of subglacial carbonate crusts at bossons glacier, mont blanc massif (French alps). *Frontiers in Earth Science*. doi: 10.3389/feart.2017.00070.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*. doi: 10.1093/nar/22.22.4673.

Thorarinsson, S., Einarsson, T., Sigvaldason, G., and Elisson, G. (1964) The submarine eruption off the Vestmann islands 1963–64. *Bulletin Volcanologique* 27: 435–445. doi: 10.1007/BF02597544.

Thorarinsson, S. and Þórarinsson, S. (1965) The Surtsey eruption: Course of events and the development of the new island. *Surtsey Research Progress Report* 1: 51–55.

Tindall, B.J., Rosselló-Móra, R., Busse, H.J., Ludwig, W., and Kämpfer, P. (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. *International Journal of Systematic and Evolutionary Microbiology*. doi: 10.1099/ijs.0.016949-0.

Toner, B.M., Lesniewski, R.A., Marlow, J.J., Briscoe, L.J., Santelli, C.M., Bach, W., et al. (2013) Mineralogy Drives Bacterial Biogeography of Hydrothermally Inactive Seafloor Sulfide Deposits. *Geomicrobiology Journal*. doi: 10.1080/01490451.2012.688925.

Tully, B.J., Wheat, C.G., Glazer, B.T., and Huber, J.A. (2018) A dynamic microbial community with high functional redundancy inhabits the cold, oxic subseafloor aquifer. *ISME Journal*. doi: 10.1038/ismej.2017.187.

Vannier, P., Michoud, G., Oger, P., Marteinsson, V.P., and Jebbar, M. (2015) Genome expression of *Thermococcus barophilus* and *Thermococcus kodakarensis* in response to different hydrostatic pressure conditions. *Research in Microbiology*. doi: 10.1016/j.resmic.2015.07.006.

Volpi, M., Lomstein, B.A., Sichert, A., Røy, H., Jørgensen, B.B., and Kjeldsen, K.U. (2017) Identity, abundance, and reactivation kinetics of thermophilic fermentative endospores in cold marine sediment and seawater. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2017.00131.

Walker, G.P.L. (1973) Explosive volcanic eruptions - a new classification scheme. *Geologische Rundschau*. doi: 10.1007/BF01840108.

Walton, A.W. (2008) Microtubules in basalt glass from Hawaii Scientific Driling Project #2 phase 1 core and Hilina slope, Hawaii: Evidence of the occurrence and behavior of endolithic microorganisms. *Geobiology*. doi: 10.1111/j.1472-4669.2008.00149.x.

Wanger, G., Onstott, T.C., and Southam, G. (2008) Stars of the terrestrial deep subsurface: A novel "star-shaped" bacterial morphotype from a South African platinum mine. *Geobiology*. doi: 10.1111/j.1472-4669.2008.00163.x.

Weisenberger, T.B., Gudmundsson, M.T., Jackson, M.D., Gorny, C.F., Türke, A., Kleine, B.I., et al. (2019) Operational Report for the 2017 Surtsey Underwater volcanic System for Thermophiles, Alteration processes and INnovative Concretes (SUSTAIN) drilling project at Surtsey Volcano, Iceland. *Potsdam: GFZ German Research Centre for Geosciences* 240. doi: 10.2312/ICDP.5059.001.

Weiss, S., Xu, Z.Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., et al. (2017) Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*. doi: 10.1186/s40168-017-0237-y.

Wheat, C.G., Jannasch, H.W., Kastner, M., Hulme, S., Cowen, J., Edwards, K.J., et al. (2011) Fluid sampling from oceanic borehole observatories: design and methods for CORK activities (1990–2010). doi: 10.2204/iodp.proc.327.109.2011.

Whelan, J., Oremland, R., Tarafa, M., Smith, R., Howarth, R., and Lee, C. (1986) Evidence for Sulfate-Reducing and Methane-Producing Microorganisms in Sediments from Sites 618, 619, and 622. doi: 10.2973/dsdp.proc.96.147.1986.

Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998) Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.95.12.6578.

Wickham, H. (2011) ggplot2. *Wiley Interdisciplinary Reviews: Computational Statistics*. doi: 10.1002/wics.147.

Winogradsky, S. (1887) Über Schwefelbacterien. *Botanische Zeitung* 40: 489–507.

Woese, C.R. and Fox, G.E. (1977) Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.74.11.5088.

Wood, D.E., Lu, J., and Langmead, B. (2019) Improved metagenomic analysis with Kraken 2. *Genome Biology*. doi: 10.1186/s13059-019-1891-0.

Wörmer, L., Hoshino, T., Bowles, M.W., Viehweger, B., Adhikari, R.R., Xiao, N., et al. (2019) Microbial dormancy in the marine subsurface: Global endospore abundance and response to burial. *Science Advances*. doi: 10.1126/sciadv.aav1024.

Wu, X., Spencer, S., Gushgari-Doyle, S., Yee, M.O., Voriskova, J., Li, Y., et al. (2020) Culturing of "Unculturable" Subsurface Microbes: Natural Organic Carbon Source Fuels the Growth of Diverse and Distinct Bacteria From Groundwater. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2020.610001.

Yamashita, S., Mukai, H., Tomioka, N., Kagi, H., and Suzuki, Y. (2019) Iron-rich Smectite Formation in Subseafloor Basaltic Lava in Aged Oceanic Crust. *Scientific Reports*. doi: 10.1038/s41598-019-47887-x.

Yanagawa, K., Nunoura, T., McAllister, S.M., Hirai, M., Breuker, A., Brandt, L., et al. (2013) The first microbiological contamination assessment by deep-sea drilling and coring by the D/V chikyu at the iheya north hydrothermal field in the mid-okinawa trough (IODP Expedition 331). *Frontiers in Microbiology*. doi: 10.3389/fmicb.2013.00327.

Yang, C., Chowdhury, D., Zhang, Z., Cheung, W.K., Lu, A., Bian, Z., and Zhang, L. (2021) A review of computational tools for generating metagenome-assembled genomes from metagenomic sequencing data. *Computational and Structural Biotechnology Journal*. doi: 10.1016/j.csbj.2021.11.028.

Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H., and Chun, J. (2017) Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *International Journal of Systematic and Evolutionary Microbiology* 67: 613–1617. doi: 10.1099/ijsem.0.001755.

Zhang, C., Palumbo, A. V., Phelps, T.J., Beauchamp, J.J., Brockman, F.J., Murray, C.J., et al. (1998) Grain size and depth constraints on microbial variability in coastal plain subsurface sediments. *Geomicrobiology Journal*. doi: 10.1080/01490459809378074.

Zhang, H. and Liu, K.K. (2008) Optical tweezers for single cells. *Journal of the Royal Society Interface*. doi: 10.1098/rsif.2008.0052.

Zhang, Z.M., Rumble, D., Liou, J.G., Xiao, Y.L., and Gao, Y.J. (2005) Oxygen isotope geochemistry of rocks from the Pre-Pilot Hole of the Chinese Continental Scientific Drilling Project (CCSD-PPH1). *American Mineralogist*. doi: 10.2138/am.2005.1650.

Zhao, R., Hannisdal, B., Mogollon, J.M., and Jørgensen, S.L. (2019) Nitrifier abundance and diversity peak at deep redox transition zones. *Scientific Reports*. doi: 10.1038/s41598-019-44585-6.

Zhao, R., Mogollon, J.M., Abby, S.S., Schleper, C., Biddle, J.F., Roerdink, D.L., et al. (2020) Geochemical transition zone powering microbial growth in subsurface sediments. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.2005917117.

Zhao, W., Weber, C., Zhang, C.L., Romanek, C.S., King, G.M., Mills, G., et al. (2006) *Thermalkalibacillus uzonensis* gen. nov. sp. nov, a novel aerobic alkali-tolerant thermophilic bacterium isolated from a hot spring in Uzon Caldera, Kamchatka. *Extremophiles*. doi: 10.1007/s00792-006-0511-6.

Zhu, H., Derksen, R.C., Krause, C.R., Fox, R.D., Brazee, R.D., and Ozkan, H.E. (2005) Fluorescent intensity of dye solutions under different pH conditions. *Journal of ASTM International*. doi: 10.1520/jai12926.

Ziebis, W., McManus, J., Ferdelman, T., Schmidt-Schierhorn, F., Bach, W., Muratli, J., et al. (2012) Interstitial fluid chemistry of sediments underlying the North Atlantic gyre and the influence of subsurface fluid flow. *Earth and Planetary Science Letters*. doi: 10.1016/j.epsl.2012.01.018.

Zobell, C.E. (1947) Microbial Transformation of Molecular Hydrogen in Marine Sediments, with Particular Reference to Petroleum. *AAPG Bulletin*. doi: 10.1306/3d933a4c-16b1-11d7-8645000102c1865d.

Zobell, C.E. (1938) Studies on the Bacterial Flora of Marine Bottom Sediments. *SEPM Journal of Sedimentary Research* 8: 10–18. doi: 10.1306/d4268fd6-2b26-11d7-8648000102c1865d.

ZoBell, C.E. (1941) Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *Journal of Marine Research* 4: 41–75.

ZoBell, C.E.D., Anderson, Q., and Claude E. ZoBell, D.Q.A. (1936) Vertical Distribution of Bacteria in Marine Sediments. *AAPG Bulletin* 20: 258–269. doi: 10.1306/3d932db2-16b1-11d7-8645000102c1865d.

ZoBell, C.E.D. and Morita, R.Y. (1957) Barophilic bacteria in some deep sea sediments. *Journal of bacteriology* 73: 563–568. doi: ZOBELL, C E, and R Y MORITA. “Barophilic bacteria in some deep sea sediments.” Journal of bacteriology vol. 73,4 (1957): 563-8. doi:10.1128/jb.73.4.563-568.1957.

Original papers

Paper I Bergsten, P., Vannier, P., Klonowski, A.M., Knobloch, S., Gudmundsson, M.T., Jackson, M.D., and Marteinsson, V.T. (2021) **Basalt-Hosted Microbial Communities in the Subsurface of the Young Volcanic Island of Surtsey, Iceland.** *Frontiers in Microbiology* 12. doi: 10.3389/fmicb.2021.728977.

Paper II Bergsten, P., Vannier, P., Frion, J., Mougeolle, A., and Marteinsson, V.T. (2022) **Culturable Bacterial Diversity from the Basaltic Subsurface of the Young Volcanic Island of Surtsey, Iceland**. *Microorganisms.* doi: 10.3390/microorganisms10061177.

Paper III Bergsten, P., Vannier, P., Mougeolle, A., Rigaud, L., and Marteinsson, V.T. (2022) ***Rhodothermus bifroesti* sp. nov., a thermophilic bacterium isolated from the basaltic subsurface of the volcanic island Surtsey.** *International Journal of Systematic and Evolutionary Microbiology*. doi: 10.1099/ijsem.0.005214.

Paper IV Bergsten, P., and Marteinsson, V.T. **Microscopic investigations revealed putative biotic structures attached to basaltic drill cores collected from the subsurface of the Surtsey volcano.** Unpublished manuscript.

Other paper relevant to the thesis manuscript

Jackson, M.D., Gudmundsson, M.T., Weisenberger, T.B., Rhodes, J.M., Stefánsson, A., Kleine, B.I., Lippert, P.C., Marquardt, J.M., Reynolds, H.I., Kück, J., Marteinsson, V.T., Vannier, P., Bach, W., Barich, A., Bergsten, P., Bryce, J.G., Cappelletti, P., Couper, S., Fahnestock, M.F., Gorny, C., Grimaldi, C., Groh, M., Gudmundsson, A., Gunnlaugsson, A., Hamlin, C., Högnadóttir., T., Jónasson, K., Jónsson, S.S., Jørgensen, S.L., Klonowski., A.M., Marshall, B., Massey, E., McPhie, J., Moore, J.G., Ólafsson, E.S., Onstad, S.L., Perez, V., Prause, S., Snorrason, S.P., Türke, A., White, J.D.L., and Zimanowski, B. (2019) **SUSTAIN drilling at Surtsey volcano, Iceland, tracks hydrothermal and microbiological interactions in basalt 50 years after eruption.** *Scientific Drilling* 25: 35–46. doi: 10.5194/sd-25-35-2019.

Paper I

Basalt-Hosted Microbial Communities in the Subsurface of the Young Volcanic Island of Surtsey, Iceland.

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Published in *Frontiers in Microbiology*, section *Extreme Microbiology* (2021) *Volume 12*.

Candidate contribution:
Conceived and designed the study, performed fieldwork, performed laboratory work, analyzed data, wrote the manuscript, reviewed and edited the manuscript and corresponded during the publication process.

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.728977/full#supplementary-material

frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 September 2021
doi: 10.3389/fmicb.2021.728977

Basalt-Hosted Microbial Communities in the Subsurface of the Young Volcanic Island of Surtsey, Iceland

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OPEN ACCESS

Edited by:
Mark Alexander Lever, ETH Zürich, Switzerland

Reviewed by:
Gustavo Antonio Ramírez, University of North Carolina at Chapel Hill, United States
Florentin Constancias, ETH Zürich, Switzerland

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

Received: *22 June 2021*
Accepted: *30 August 2021*
Published: *29 September 2021*

Citation:
Bergsten P, Vannier P, Klonowski AM, Knobloch S, Gudmundsson MT, Jackson MD and Marteinsson VT (2021) Basalt-Hosted Microbial Communities in the Subsurface of the Young Volcanic Island of Surtsey, Iceland. Front. Microbiol. 12:728977. doi: 10.3389/fmicb.2021.728977

The island of Surtsey was formed in 1963–1967 on the offshore Icelandic volcanic rift zone. It offers a unique opportunity to study the subsurface biosphere in newly formed oceanic crust and an associated hydrothermal-seawater system, whose maximum temperature is currently above 120°C at about 100 m below surface. Here, we present new insights into the diversity, distribution, and abundance of microorganisms in the subsurface of the island, 50 years after its creation. Samples, including basaltic tuff drill cores and associated fluids acquired at successive depths as well as surface fumes from fumaroles, were collected during expedition 5059 of the International Continental Scientific Drilling Program specifically designed to collect microbiological samples. Results of this microbial survey are investigated with 16S rRNA gene amplicon sequencing and scanning electron microscopy. To distinguish endemic microbial taxa of subsurface rocks from potential contaminants present in the drilling fluid, we use both methodological and computational strategies. Our 16S rRNA gene analysis results expose diverse and distinct microbial communities in the drill cores and the borehole fluid samples, which harbor thermophiles in high abundance. Whereas some taxonomic lineages detected across these habitats remain uncharacterized (e.g., Acetothermiia, Ammonifexales), our results highlight potential residents of the subsurface that could be identified at lower taxonomic rank such as *Thermaerobacter*, BRH-c8a (Desulfallas-Sporotomaculum), *Thioalkalimicrobium,* and *Sulfurospirillum*. Microscopy images reveal possible biotic structures attached to the basaltic substrate. Finally, microbial colonization of the newly formed basaltic crust and the metabolic potential are discussed on the basis of the data.

Keywords: 16S rRNA gene amplicon sequencing, bacterial and archaeal communities, microbial diversity, extreme environment, subsurface, oceanic basaltic crust, Iceland

INTRODUCTION

The subsurface biosphere, defined as an ecosystem encompassing regions beneath soils and sediments, occupies roughly twice the volume of the oceans and holds about 15% of the total biomass on Earth (Pedersen, 2000; Heberling et al., 2010; Bar-On et al., 2018; Orcutt et al., 2019). Recent estimates suggest that these zones (i.e., continental subsurface, subseafloor sediments, and oceanic crust) contain ~70% of all prokaryotic cells and possibly more than 80% of all bacterial and archaeal species (Magnabosco et al., 2018). This large biome has only recently become the focus of research studies (Gold, 1992), and most microbial surveys have, to date, focused on subseafloor sediments (Parkes et al., 2000; D'Hondt et al., 2004; Inagaki et al., 2015). The deep biosphere hosted in the basaltic ocean crust, on the contrary, has been understudied because of various challenges: the relative inaccessibility of the samples (drilling operation often required), the low biomass (high risk of external contamination), the presence of minerals which generates DNA binding and inhibitions (Direito et al., 2012; Lever et al., 2015), and the difficulties in handling the inevitable microbial contamination from the surface (Baross et al., 2004; Sheik et al., 2018; Ramírez et al., 2019).

Over the past decades, many microorganisms have been discovered within Earth's igneous oceanic crust despite extreme conditions, such as oligotrophy, temperature gradients, active circulation, and limited available space within the rock, which were once thought unsuitable to supporting life (Cowen et al., 2003; Nakagawa et al., 2006; Orcutt et al., 2011b; Jungbluth et al., 2013, 2014; Lever et al., 2013; Baquiran et al., 2016). Studies of ridge-flank systems have demonstrated that crustal aquifers harbor aerobic mesophiles and anaerobic thermophiles, involved in hydrogen, nitrogen, carbon, and sulfur cycling (Cowen et al., 2003; Nakagawa et al., 2006; Smith et al., 2011; Lever et al., 2013; Orcutt et al., 2013; Robador et al., 2015; Jungbluth et al., 2016). The comparison of biomes in very young (<3 Ma) and old (80 Ma) oceanic crust indicates that microbial diversity increases with the age of the basalt and the community compositions converge toward similar profiles over time (Lee et al., 2015). The colonization of rocks and the succession of microbial communities mainly depend on the temperature and reduction-oxidation conditions (Baquiran et al., 2016; Ramírez et al., 2019). While microbial communities hosted by young basalt (<10 Ma) have been detected (Konhauser et al., 2002; Templeton et al., 2005), our knowledge of the pioneering communities inhabiting newly erupted oceanic basalt (<100 years) is extremely limited.

A recent drilling operation has addressed this gap in knowledge through the study of the volcanic island, Surtsey, located on the southern offshore extension of the Icelandic volcanic rift zone (**Figure 1**). The island is the visible part of a volcano formed by underwater and basaltic eruptions from the seafloor between 1963 and 1967 (Þórarinsson, 1965; Jakobsson and Moore, 1982; Jakobsson et al., 2009). Since the initiation of eruptive activity, the entire area has been accessible only to scientific investigations. Surtsey is the site of long-term longitudinal studies, which have provided a unique record of pioneering species of plants and animals colonizing the surface of the basaltic deposits (Baldursson and Ingadóttir, 2007; Magnússon et al., 2014). In 1979, a 181 m core (SE-01) drilled through the eastern sector of the Surtur vent (**Figure 1**), probed the hydrothermal system in the subaerial and the submarine deposits – above and below coastal sea level, respectively (Jakobsson and Moore, 1982, 1986; Jackson et al., 2019a). In 2017, three new cored boreholes (SE-02a, SE-02b and SE-03) were acquired through the International Continental Scientific Drilling Program (ICDP) 5,059 expedition, SUSTAIN drilling operation (Jackson et al., 2015, 2019b; Weisenberger et al., 2019). Annual monitoring of temperatures in the 1979 borehole, SE-01, indicates that the hydrothermal system has cooled down over the years. The maximal temperature in 1980 was 141.3°C at 100 m depth, and it has decreased gradually to 124.6°C in 2017 (**Figure 1**; Jakobsson and Moore, 1986; Ólafsson and Jakobsson, 2009; Marteinsson et al., 2015; Jackson et al., 2019b). The current temperatures exceed a presumed upper limit for functional microbial life (122°C; Prieur and Marteinsson, 1998; Kashefi and Lovley, 2003; Takai et al., 2008). The highly porous subaerial and submarine tephra deposits were largely transformed to palagonitized lapilli tuff by 1979, described in studies of the SE-01 drill core (Jakobsson and Moore, 1982; Jakobsson and Moore, 1986). Progressive alteration was recorded in samples from a parallel, time-lapse drill core acquired in 2017, SE-02b (**Figure 1**; Prause et al., 2020). A recent study of geothermal water chemistries in the 1979 and 2017 Surtsey boreholes indicates depletion in boron, magnesium, iron, carbon dioxide, and sulfate concentrations, suggesting that the fluid compositions in the subsurface deposits are controlled by seawater-basalt interactions and temperature (Kleine et al., 2020). This further suggests that fluid-rock interactions in the submarine Surtsey basaltic deposits behave similarly to those interactions in basaltic oceanic crust, where the chemical composition of rocks and fluids changes and introduces organic matter and oxygen into the system (Furnes and Staudigel, 1999; Edwards et al., 2005; Orcutt et al., 2011a). The Surtsey volcano geothermal system represents an exceptional natural laboratory for studying fluid-rock-microbe interactions at temperatures approaching the presumed thermal limit for functional life on Earth. Its boreholes can be viewed as windows opened from the land surface that allow the study of subsurface processes at high temperature associated with the basaltic oceanic crust.

Several studies have described the volcanic structure of Surtsey and the geochemical, chemical, and mineralogical changes in the altered basaltic deposits (Jakobsson and Moore, 1982; Jakobsson and Moore, 1986; Schipper et al., 2016; McPhie et al., 2020; Moore and Jackson, 2020; Prause et al., 2020). Little is known, however, about the microorganisms inhabiting the subsurface and their metabolic potential. A pioneering study by Marteinsson et al. (2015) detected archaea of the *Archaeoglobus* genus at 145 m depth (80°C) and one taxon from the Methanobacteriales order in the SE-01 borehole at depths to 170 m (55°C; Marteinsson et al., 2015). Furthermore, recent studies of 1979 and 2017 drill core samples show abundant microtubules in basaltic glass that resemble endolithic microborings (Jackson et al., 2019a; Jackson, 2020); these are

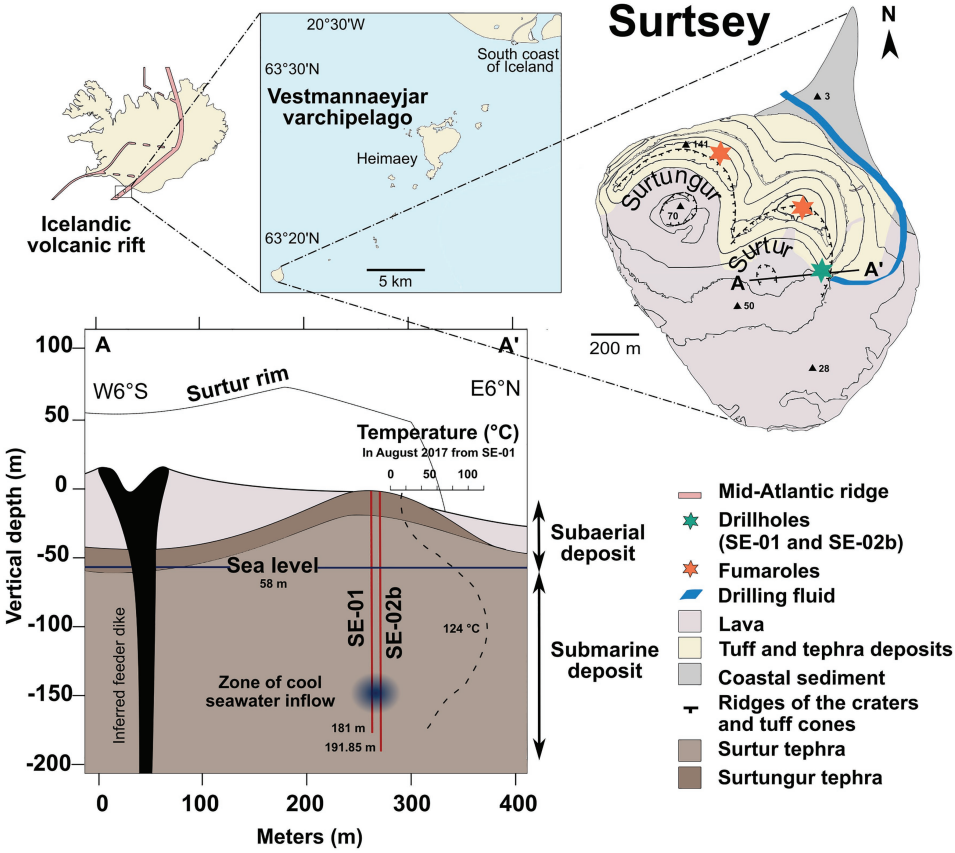

FIGURE 1 | Location of the study area of the south coast of Iceland. Map of the Surtsey volcanic island within the Westman Islands (Vestmannaeyjar) archipelago and location of sampling sites: 1979 borehole SE-01 and 2017 borehole SE-02b (green star), drilling fluid (blue line), and fumaroles (orange stars). Schematic cross section of Surtur crater showing the geological setting of the subsurface deposits and the location of the time-lapse cored boreholes (see Jackson et al., 2019b) and a zone of seawater inflow.

thought to indicate microbial microboring into glass (Fisk et al., 2003; Staudigel et al., 2008; Walton, 2008; Mcloughlin et al., 2010). These investigations suggest that Surtsey's basalt and fluids could reveal new information about the microbial communities associated with the young hydrothermal-seawater system and the newly formed oceanic basaltic crust.

This work focuses on the following questions: (i) how can we distinguish the true residents of the subsurface from the marine microbial taxa derived from the drilling fluid? (ii) which microorganisms inhabit Surtsey's basalt and fluids? (iii) does life persist in the subsurface zones crossing the presumed thermal limit for life? and (iv) can we detect actual microbial structures or biotic signatures in the basaltic cores only 50 years after eruptions terminated? We used high-throughput sequencing (16S rRNA gene amplicon sequence analysis) and scanning electron microscopy (SEM) to address these questions and enable an in-depth characterization of the basalt-hosted microbial communities within the volcanic system. Microbial communities from drill core samples collected at successive depths from the subaerial and submarine basaltic tephra deposits were compared to the communities in borehole fluid samples associated with the active hydrothermal system and in fumarole samples from the surface of the island and seawater samples collected several kilometers offshore. In this study, it was of crucial importance to collect drilling fluids, sequence control samples representing potential sources of contamination, and implement computational methods to distinguish the endemic microbial taxa of the subsurface rocks from the microorganisms found in the drilling fluid and other potential contaminants. Here, we report our strategy to identify and minimize contaminants in the data sets; we describe the archaeal and bacterial taxa that are candidate residents of the oceanic crust; we discuss the microbial colonization and dissemination from the surrounding ecosystems, and we explore microbial metabolic

potentials. The results provide unique data on subsurface microbial life in one of the newest and most pristine oceanic basalt environments on Earth.

MATERIALS AND METHODS

Site Description

The volcanic island of Surtsey is located in the Vestmannaeyjar archipelago, approximately 35 km from the south coast of Iceland (63°18'10.8'N; 20°36'16.9'W; **Figure 1**) within the southern offshore extension of Iceland's Eastern Volcanic Zone. The ocean depth was 130 m below sea level prior to eruption. After 3.5 years of submarine, explosive, and effusive lava-producing eruptions of basalts from the seafloor in 1963–1967, Surtsey had a subaerial area of 2.6 km^2, and the highest point on the island was 174 m above sea level. The island has now eroded to less than 1.2 km^2 and a height of 150 m above sea level (Baldursson and Ingadóttir, 2007; Óskarsson et al., 2020).

The cores extracted from the SE-01 borehole in 1979 and the SE-02b borehole in 2017 indicate that the subsurface of Surtsey consists mainly of lithified tephra, mainly lapilli tuff, and minor amounts of weakly consolidated tephra and alkali olivine basalt intrusions (Jakobsson and Moore, 1982; McPhie et al., 2020; **Figure 1**). The principal authigenic minerals in the lapilli tuff are smectitic clay mineral (nontronite and clinochlore), analcite, phillipsite, Al-tobermorite, and anhydrite (Jakobsson and Moore, 1986; Jackson et al., 2019a; Prause et al., 2020). Alteration through palagonitization and production of authigenic cementitious minerals has progressed during the past 38 years, from 1979 to 2017 (Jackson, 2020; Prause et al., 2020). *In situ* subsurface fluid temperatures have been measured annually since 1980 in SE-01, usually at 1-m intervals from the surface to the bottom of the borehole (e.g., Jakobsson and Moore, 1986; Marteinsson et al., 2015). Geochemical analyses and pH measurements of borehole fluids and pore water extracted from the 2017 drill cores are described by Kleine et al. (2020).

Drilling Operation and Drilling Fluid

One of the principal objectives of the 2017 Surtsey Underwater volcanic System for Thermophiles, Alteration processes and Innovative concretes (SUSTAIN) drilling project at Surtsey volcano, sponsored in part by the ICDP, was to investigate microbial diversity within the basaltic tephra. All possible precautions were made to avoid microbial contamination (Jackson et al., 2019b). Water from the sea was pumped to the drill site and used as drilling fluid since no fresh water is available on the island (**Figure 1**). The conventional methods used to track contamination during drilling operations, such as the addition of tracer compounds in the circulating fluid, could not be applied during drilling at Surtsey due to the strict environmental protection of the island (see discussion). To avoid contamination, drilling fluid was sterilized using two filtration units with a pore size of 30 µm (Pentek Big Blue, R30-BB 30 Micron cartridge filter, Lenntech, The Netherlands) and two ultraviolet sterilization devices (AQUA4ALT from WEDECO, Aquaculture systems, Xylem Water Solutions Herford GmbH, Germany) with a maximum flow rate of 1.58 ls^{-1}. The decontaminated drilling fluid was stored in 1000 L containers before pumping into the borehole. To track potential problems with the sterilization system, 1 l of drilling fluid was collected regularly at different drilling depths to assess potential microbial contaminants (**Figure 1**).

Sample Collection

Four types of samples were collected for molecular investigation and comparison of microbial diversity among these sample types: drill cores from the 2017 SE-02b borehole, borehole fluids from the 1979 SE-01 borehole, steam from surface cracks of fumaroles, and seawater samples collected few kilometers from the northwest coast of Surtsey (**Figure 1**). A description of the samples is shown in **Table 1**.

Seventeen drill core samples were collected for microbial analyses from the vertical SE-02b cored borehole that extends to 192 m below surface and terminates in poorly consolidated tephra a few meters above the presumed depth of the pre-eruption seafloor (Jackson et al., 2019b). At the drill site, drill core samples were collected from every third 3-m core run for molecular analyses by cutting a 10-cm section at 70 cm from the top of the core run. Immediately after sampling, each section was kept in the plastic core liner, taped at both ends, wrapped in a plastic bag, kept in liquid nitrogen on site, and at −80°C for long-term laboratory storage. Fluid samples from SE-01 borehole were collected in 2016 and in 2017, before drilling operations started, using a custom sampler made of stainless steel, as described in Marteinsson et al. (2015). The sampler was rinsed with 70% ethanol before each sampling. Eighteen fluid samples were collected from the SE-01 borehole: 18 in 2016 and seven in 2017 (**Table 1**). Steam from fumaroles, located on the summit of Surtur, the eastern tephra cone (~150 m above sea level; 63°18'15.4'N 20°36'07.7'W) and Surtlungur, the western tephra cone (63°18'19.9'N 20°36'24.7'W) were collected in 2017 by introducing a sterile rubber hose into the outlet of the fumarole, with the other end connected to a sterile plastic container. This generated 5,350 ml of condensed water from the fumaroles over 12 h of continuous sampling. Four liters of seawater samples were collected 25 km offshore during the drilling operation (63°28'58.8'N; 20°54'7.2'W). All water samples (drilling fluid, borehole fluid, fumarole, and seawater samples) were immediately filtrated through 0.22-µm Sterivex™ filters (Merck Millipore). The filters were stored in liquid nitrogen on site and at −80°C for long-term storage in the laboratory.

DNA Extraction

DNA Extraction From Rock Samples

A modified PowerMax® Soil DNA Isolation Kit protocol (MO BIO Laboratories, Inc.) was applied to extract the DNA from the drill core samples. Small fragments of tuff from the interior of the 17 frozen core samples from SE-02b were broken aseptically. After 2 min pre-cooling on ice,

TABLE 1 | Sample description table: locations, sampling date, depth, temperature, and DNA concentration.

Sample ID	Sample types and categories	Sample site	Sampling date	Collection depth (m b.s.)	Collection Temp (°C)	Sample amount	DNA concentration NanoDrop (ng/μl)	260/280	DNA concentration QuBit dsDNA (ng/μl)
Sur161	Borehole fluid	SE-01	6/9/2016	164	56.56	500 ml	16.14	1.41	0.16
Sur162	Borehole fluid	SE-01	6/9/2016	164	56.56	300 ml	15.48	1.35	0.30
Sur163	Borehole fluid	SE-01	6/9/2016	166	54.24	500 ml	26.65	1.43	Too low
Sur164	Borehole fluid	SE-01	6/9/2016	162	58.88	400 ml	8.33	1.29	Too low
Sur165	Borehole fluid	SE-01	6/9/2016	70	109.17	250 ml	56.12	1.56	0.09
Sur166	Borehole fluid	SE-01	6/9/2016	100	124.62	500 ml	34.07	1.58	0.15
Sur167	Borehole fluid	SE-01	6/9/2016	100	124.62	250 ml	38.24	1.50	0.81
Sur168	Borehole fluid	SE-01	6/9/2016	120	115.78	550 ml	97.37	1.61	0.56
Sur169	Borehole fluid	SE-01	6/9/2016	160	61.27	500 ml	17.59	1.05	0.13
Sur1610	Borehole fluid	SE-01	6/9/2016	160	61.27	150 ml	12.31	1.21	0.11
Sur16Mix	Borehole fluid	SE-01	6/9/2016	mix	n.a.	1,400 ml	52.23	1.14	0.15
Sur171	Borehole fluid	SE-01	8/3/2017	58	87.75	5,000 ml	48.80	1.49	n.a.
Sur172	Borehole fluid	SE-01	8/3/2017	120	115.78	1,820 ml	38.25	1.46	n.a.
Sur173	Borehole fluid	SE-01	8/3/2017	mix	n.a.	980 ml	35.38	1.49	n.a.
Sur174	Borehole fluid	SE-01	8/3/2017	mix	n.a.	805 ml	23.07	1.53	n.a.
Sur175	Borehole fluid	SE-01	8/3/2017	150	74.22	5,000 ml	52.80	1.45	n.a.
Sur176	Borehole fluid	SE-01	8/8/2017	160	61.27	5,000 ml	35.95	1.49	n.a.
Sur177	Borehole fluid	SE-01	8/8/2017	mix	n.a.	850 ml	16.59	1.58	n.a.
C4	Drill core (DC_1)	SE-02b	8/22/2017	23*	20.00	15 g	181.61	1.42	0.17
C9	Drill core (DC_1)	SE-02b	8/22/2017	35*	36.00	15 g	196.47	1.44	0.14
C13	Drill core (DC_2)	SE-02b	8/22/2017	44*	59.00	15 g	202.15	1.33	0.16
C17	Drill core (DC_2)	SE-02b	8/23/2017	55*	82.50	15 g	137.89	1.43	0.11
C22	Drill core (DC_2)	SE-02b	8/23/2017	65	101.50	15 g	115.60	1.46	0.41
C27	Drill core (DC_3)	SE-02b	8/23/2017	78	114.00	15 g	204.83	1.42	0.11
C33	Drill core (DC_3)	SE-02b	8/24/2017	93	123.00	15 g	202.74	1.38	0.09
C36	Drill core (DC_3)	SE-02b	8/24/2017	102	124.00	15 g	202.32	1.41	0.16
C39	Drill core (DC_3)	SE-02b	8/24/2017	111	121.50	15 g	197.72	1.35	0.15
C42	Drill core (DC_3)	SE-02b	8/24/2017	120	116.00	15 g	57.15	1.43	0.17
C45	Drill core (DC_3)	SE-02b	8/24/2017	130	107.00	15 g	171.05	1.40	0.15
C49	Drill core (DC_3)	SE-02b	8/25/2017	139	97.00	15 g	168.91	1.41	0.11
C52	Drill core (DC_4)	SE-02b	8/25/2017	148	84.00	15 g	180.71	1.44	0.10
C55	Drill core (DC_4)	SE-02b	8/25/2017	157	64.00	15 g	97.89	1.44	0.06
C59	Drill core (DC_4)	SE-02b	8/25/2017	166	55.00	15 g	148.14	1.43	0.10
C62	Drill core (DC_4)	SE-02b	8/25/2017	175	44.50	15 g	178.08	1.42	0.13
C65	Drill core (DC_4)	SE-02b	8/25/2017	181	37.00	15 g	203.00	1.40	0.13
Fum_1	Fumarole	63°18'19.9'N 20°36'24.7'W	8/4/2017	0	82.30	350 ml	19.05	1.88	Too low
Fum_2	Fumarole	63°18'15.4'N 20°36'07.7'W	8/4/2017	0	85.60	5,000 ml	8.39	2.33	Too low
SW_10	Seawater	63°28'58.8'N; 20°54'7.2'W	8/18/2017	10 (m b.s.l.)	12	1,000 ml	330.37	2.00	n.a.
SW_20	Seawater	63°28'58.8'N; 20°54'7.2'W	8/18/2017	20 (m b.s.l.)	11.97	1,000 ml	607.05	1.58	n.a.
SW_30	Seawater	63°28'58.8'N; 20°54'7.2'W	8/18/2017	30 (m b.s.l.)	10.81	1,000 ml	449.85	1.85	n.a.
SW_50	Seawater	63°28'58.8'N; 20°54'7.2'W	8/18/2017	50 (m b.s.l.)	9.9	1,000 ml	175.98	1.81	n.a.
1B0ZC	Drilling fluid	63°18'30.7'N 20°36'21.0'W	8/9/2017	1 (m b.s.l.)	10.00	1,000 ml	220.69	1.98	n.a.
1B3ZC	Drilling fluid	63°18'30.7'N 20°36'21.0'W	8/10/2017	1 (m b.s.l.)	10.00	1,000 ml	92.74	1.91	n.a.
1B25ZC	Drilling fluid	63°18'30.7'N 20°36'21.0'W	8/12/2017	1 (m b.s.l.)	10.00	1,000 ml	103.01	2.03	n.a.
149ZC	Drilling fluid	63°18'30.7'N 20°36'21.0'W	8/16/2017	1 (m b.s.l.)	10.00	1,000 ml	129.22	1.90	n.a.
1C17ZC	Drilling fluid	63°18'30.7'N 20°36'21.0'W	8/23/2017	1 (m b.s.l.)	10.00	1,000 ml	118.26	1.97	n.a.
1C39ZC	Drilling fluid	63°18'30.7'N 20°36'21.0'W	8/24/2017	1 (m b.s.l.)	10.00	1,000 ml	96.39	1.91	n.a.

(Continued)

TABLE 1 | Continued

Sample ID	Sample types and categories	Sample site	Sampling date	Collection depth (m b.s.)	Collection Temp (°C)	Sample amount	DNA concentration NanoDrop (ng/μl)	260/280	DNA concentration QuBit dsDNA (ng/μl)
1C51ZC	Drilling fluid	63°18'30.7'N 20°36'21.0'W	8/25/2017	1 (m b.s.l.)	10.00	1,000 ml	252.97	2.05	n.a.
1C59ZC	Drilling fluid	63°18'30.7'N 20°36'21.0'W	8/24/2017	1 (m b.s.l.)	10.00	1,000 ml	152.45	2.04	n.a.
Cw	Control	n.a.	n.a.	n.a.	n.a.	n.a.	27.11	1.74	Too low
Cr	Control	n.a.	n.a.	n.a.	n.a.	15 g	25.05	1.69	Too low

*Drill core samples from the subaerial tuff cone, located above the sea level. Too low: for detection with Qubit fluorometer and high-sensitivity dsDNA reagents, <0.5 ng/ml. n.a.: not available. m b.s.l.: meter below sea level. m b.s.: meter below surface.

15 g were cryo-ground at an impact rate of 8 cycles per second for 1 min using a 6,700 Freezer/Mill cryogenic grinder (SPEX). Phosphate-ethanol solution (1 M phosphate buffer, 15% ethanol, pH 8.0; Direito et al., 2012) and proteinase K (20 mg/ml) were added to the lysis solution provided by the kit. Samples were vortexed twice for 30 s with a 1 min cooling step in between instead of the bead-beating step, followed by incubations at 55°C for 1 h and at 80°C for 40 min. Subsequent DNA isolation was carried out according to the standard PowerMax® Soil DNA isolation protocol. DNA precipitation was done overnight with isopropanol (0.7 volume) and glycogen (20 mg/ml). After a washing step with 70% ethanol, DNA was resuspended in Tris buffer (10 mM, pH 8) and quantified using both NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) and a Qubit fluorometer and high-sensitivity dsDNA reagents (Invitrogen™) before being stored at −20°C. Attempts to estimate the microbial biomass within the drill core samples using fluorescence microscopy were unsuccessful because of the non-biological background signals that occurred during recognition of cells for counting. Two controls were carried out to test for contamination during DNA extraction of the core samples. One used sterile water (instead of the basalt powder) to test for contaminants derived from the kit and reagents. The other used 15 g of basalt powder from a drill core that had been treated with 70% ethanol and heated at 180°C for 24 h. Both controls resulted in amplifications with primers targeting bacterial and archaeal 16S rRNA genes, which were then sequenced.

DNA Extraction From Fluid Samples

DNA was extracted from Sterivex™ filters containing biomass from borehole water, condensed fumarole water and drilling fluid samples following a modified protocol by Neufeld et al. (2007). Sucrose EDTA Tris buffer (SET buffer: 40 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl pH 9, and 0.75 M sucrose) and 20 mg/ml lysozyme solution were added to the Sterivex™ filters. Filters were incubated at 37°C for 30 min. After the addition of 10% (w/v) sodium dodecyl sulfate (SDS) and proteinase K (20 mg/ml), the filters were incubated with rotation at 55°C for 2 h. Lysates were collected into syringes, while the filters were rinsed twice with SET buffer and the rinsed buffer was combined to the lysate. One volume of phenol:chloroform:isoamyl alcohol (PCI: 25:24:1, pH 8) was added, and the aqueous phase was transferred to a new tube after a 15-min centrifugation at 10,000 × *g* at 4°C. The phenol was removed from the aqueous phase by adding 1 volume of chloroform. The cleaned aqueous phase was transferred to a new tube after a 5-min centrifugation at 10,000 × *g* at 4°C, and 0.7 volume of cold isopropanol was added. After inverting the tube several times, the sample was incubated for 15 min at room temperature and then overnight at −20°C. After a 20-min centrifugation at 16,000 × *g* at 4°C, the DNA pellet was washed twice with 75% (v/v) ethanol, dried for 5 min using a SpeedVac™ and 15 min at room temperature, and finally resuspended in sterile Tris-HCl buffer (10 mM, pH 8). DNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) and Qubit fluorometer (Invitrogen, Quant-iT™ dsDNA HS) and stored at −20°C. One negative extraction control was carried out for each round of extractions by rinsing a sterile Sterivex™ filter with sterile laboratory-grade water.

Partial 16S rRNA Gene Amplification and Tag Sequencing

Illumina MiSeq paired-end (2 × 300 base pair) tag sequencing was carried out using the Earth Microbiome Project universal primers 515f (5'-GTG CCA GCM GCC GCG GTA A-3') and 806r (5'-GGA CTA CHV GGG TWT CTA AT-3'), which amplify the V4 region of the bacterial and archaeal 16S rRNA genes (Caporaso et al., 2012). Since this primer pair does not show a high coverage for Archaea (Parada et al., 2016), specific archaeal primer sets were used with a nested PCR approach. The first-round PCR was performed to amplify the V3-V5 region of archaeal 16S rRNA gene with the primer set Parch340F (5'- CCC TAY GGG GYG CAS CAG -3'; Øvreås et al., 1997) and Arch958VR (5'- YCC GGC GTT GAV TCC AAT T -3'; Klindworth et al., 2013). Then, a second round was performed on the first PCR product to amplify the V3 region of archaeal 16S rRNA gene with the primer set Arch349F (5'-GYGCASCAGKCGMGAAW -3'; Takai and Horikoshi, 2000) and Parch519R (5'- TTACCGCGGCKGCTG -3'; Øvreås et al., 1997).

All PCR reactions were carried out in 25 µl reactions with 20 µl of Q5® High-Fidelity PCR Master Mix (New England Biolabs, MA, United States) following the manufacturer's amplification protocol and 5 µl of DNA at 10 ng/µl (NanoDrop quantification). The second round of the nested PCR used 5 µl of amplified DNA from the first round. Bovine serum albumin was added to the master mix at a final concentration of 0.5 ng/µl for rock samples that could not be amplified. Thermal cycling consisted of an initial denaturation step at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 52°C (using the bacterial primer set) or at 55°C (using the archaeal primer sets) for 30 s, and elongation at 72°C for 60 s. Final elongation was set at 72°C for 2 min. Amplification products were visualized on 1% (w/v) agarose gels.

Sequencing libraries were generated using the "16S Metagenomic Sequencing Library Preparation guide" from Illumina and barcodes from the Illumina Nextera®XT DNA Sample Preparation Kit (8 cycles for index PCR). The libraries were assessed on a Qubit Fluorometer (Invitrogen, Quant-iT™ dsDNA HS) and a Bioanalyzer system (Agilent Technologies). After normalization and quantification, the final pooled library was loaded on a MiSeq Desktop sequencer (Illumina) and sequenced with V3 chemistry and 2 × 300 cycles across two sequencing runs. Raw sequences have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number ERP126178.

16S rRNA Gene Amplicon Sequence Analysis

Bioinformatic analysis was conducted in RStudio running R version 4.0.2 (R Core Team, 2019; Rstudio, 2020). Sequence variants were inferred using the R Package DADA2 (Callahan et al., 2016) version 1.4, available at https://benjjneb.github.io/dada2/tutorial.html. The following trimming parameters were used: trimLeft = 10, maxN = 0, maxEE = c(2,5), truncQ = 2, and truncLen = c(230,215) for the universal primer set and truncLen = c(115,110) for the archaeal primer sets. After quality screening and trimming, forward and reverse reads were merged to remove chimeric variants and singletons and to identify amplicon sequence variants (ASV). Non-target-length sequences were removed, and only amplicons of 270–275 bp length were kept with the universal primer set and amplicons of 130–165 bp for the archaeal primer sets. Data sets from three MiSeq sequencing runs (universal primer set) were processed separately using the same pipeline and same parameters and merged into a unique ASV table using the function "mergeSequenceTables." The taxonomy was assigned using the function "assignTaxonomy" (minimum bootstrap confidence at 50) and the SILVA SSU database release 138 (Quast et al., 2013).

In total, all 51 samples were successfully sequenced using the universal primer set, including extraction controls and drilling fluid samples. The R package Decontam (version 1.10.0; Davis et al., 2018[1]) was used to identify contaminant ASVs in the universal primer data set. We identified 160 contaminant ASVs (**Supplementary Table S1**) using the Decontam prevalence method (threshold value of 0.5), based on the prevalence comparison of each sequence in true samples and negative controls (**Supplementary Figure S1**). In addition, sequences that were identified as chloroplast at the order level, mitochondria at the family level, Eukaryote at the kingdom level, and those that could not be identified at the kingdom level were subtracted from the data set prior to analysis, as well as putative contaminants identified by taxonomic affiliation at the genus level by a study on common contaminants from the Census of Deep Life data set (Sheik et al., 2018). **Supplementary Table S2** gives a list of 95 potentially contaminant genera removed from the analyses. A total of 588,510 sequences were removed from the samples by the abovementioned procedures (Details are available in **Supplementary Table S3**).

From the archaeal nested PCR, 17 samples were analyzed. Sequences identified at the kingdom level as Eukaryote, Bacteria, or not assigned were removed from libraries prior to analysis, as well as sequences detected in the DNA extraction blanks (**Supplementary Table S4**, 26 ASVs). Using the archaeal primer sets, the number of sequences removed from the samples was 74,315 (Details available in **Supplementary Table S3**).

Microbial Community Analysis

Microbial community analysis (α and β diversity, community composition, and statistical analysis) was conducted in R (R Core Team, 2019) with the Phyloseq (McMurdie and Holmes, 2013) and Vegan (Oksanen et al., 2007) packages. ANOVA and Tukey's HSD (Honestly Significant Difference) test were conducted to evaluate the differences in α diversity values. For β diversity assessment, the data were normalized using "rarefy_even_depth" function prior to performing a non-metric multidimensional scaling (NMDS) ordination on Bray-Curtis dissimilarities. The significance of sample type variable was assess using permutational multivariate analysis of variance (PERMANOVA) using distance matrices and multilevel pairwise comparison (Martinez Arbizu, 2020). The command envfit was used to investigate the correlation between the community structure and environmental variables (depth and temperature). Finally, DESeq2 was used to identify ASVs significantly different among sample types (F, fumarole; BF, borehole fluid; DC, drill core; SW, seawater samples) and categories of drill cores (DC_1, DC_2, DC_3 and DC_4; Love et al., 2014). Details of the data analyses can be found in the Supplementary Material. Predictive functional analyses of the prokaryotic communities were performed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) on both data sets, obtained with the universal primer set and the archaeal primer sets, separately (Douglas et al., 2020). From the universal data set, a total of 2,333 PICRUSt2 predicted KEGG orthologs (enzymes) were collapsed into 425 MetaCyc pathways, while 828 predicted KEGG orthologs and 124 MetaCyc pathways were obtained from the archaeal data set. Only few MetaCyc pathways were selected to represent sulfur, nitrogen, methane, and carbon metabolism (**Supplementary Figures S8, S9**).

[1]https://github.com/benjjneb/decontam

Microscopy

For SEM images, drill core samples were crushed in a sterile mortar to obtain rock grains <0.5 mm diameter, which were dehydrated by four 10-min wash steps in increasing concentrations of ethanol (30, 50, 80, and 100%). After drying, samples were placed on carbon conductive tabs (PELCO Tabs™, 9 mm) and gold-coated. SEM used a Zeiss Auriga 40 Focused Ion Beam Field Emission Scanning Electron Microscope coupled with an energy dispersive X-ray spectroscopy analyzer (EDX) at the Institute de Physique du Globe de Paris (University Sorbonne Paris Cité, Paris, France) using two types of secondary electron detectors: In-Lens and SESI, and a backscattered electron detector: EsB. The acceleration voltage (EHT) ranged from 5 to 15 kV.

RESULTS

DNA Yields From Rocks

Based on Qubit quantification, DNA concentration extracted from 15 g of each drill core sample yields from 0.2 to 1.36 ng. g^{-1} of rocks with an average of 0.48 ng.g^{-1}. However, no significant correlations in DNA yield were apparent with sampling depth or *in situ* temperature (**Supplementary Figure S2**).

Special Considerations for the Drilling Operation and the Drilling Fluid

The island of Surtsey is subjected to strict environmental protection and is a UNESCO World Heritage site (Baldursson and Ingadóttir, 2007). For this reason (and other reasons mentioned later in the study), the use of tracer compounds to track contamination could not be considered during the design of the 2017 SUSTAIN drilling operation. Instead, a different approach was used to assess contamination. Filtered and UV-sterilized water pumped from the sea was used as drilling fluid (see above). Aliquots of those fluids were then collected to track microbial DNA potentially contaminating the core. DNA extracted from 1 l of each drilling fluid sample yields ~96 to 252 ng/μL of DNA (**Table 1**).

The 16s rRNA gene sequencing of the drilling fluid samples thus showed, unfortunately, that the sterilization system failed (**Supplementary Table S3**, **Supplementary Figure S3**). Consequently, marine microbial taxa from the drilling fluid were introduced into the subsurface. The failure can possibly be explained by the clogging of filters and an overly high flow rate for the UV system.

As the contamination of the samples was evidently pervasive, the data were evaluated with caution. A NMDS ordination plot conducted using Bray-Curtis dissimilarity metrics (stress value = 0.124) revealed that some of the drill core samples (e.g., C45, C52, C65) showed a microbial community composition similar to the drilling fluid and seawater samples (**Supplementary Figure S4**). Whereas differences in microbial community structure between drill core and drilling fluid and between drill core and seawater samples proved to be significant by a Tukey HSD test (p adjusted values of 0.01 and 0.0008, respectively), a strategy was adopted to distinguish endemic microbial taxa of Surtsey subsurface rocks from the marine residents introduced to the subsurface by the drilling fluid. The distributions of individual ASVs were evaluated by a simple overlap approach using a Venn diagram to compare ASVs shared among the seawater samples, drilling fluid, drill core from the subaerial deposits, and from the submarine deposits (**Figure 2**).

In the subaerial tephra deposits, located above coastal sea level, meteoric water was presented before drilling. In the zone of tidal flux at ~58 m b.s., temperatures up to 100°C could operate as a natural autoclave or biological barrier, preventing transfer of live microorganisms between the subaerial and submarine basaltic deposits (Ólafsson and Jakobsson, 2009; Marteinsson et al., 2015). Hence considering that no marine taxa should be detected in the subaerial deposit, all ASVs shared between the subaerial samples (C4, C9, C13, and C17; 23–55 m b.s.; 20–82.5°C), the drilling fluid, and the seawater samples (**Figure 2**: 93 + 84 + 14 + 11 + 16 + 14 ASVs, **Supplementary Table S6**) were considered as potential marine contaminants; these sequences were removed from the data set. In a second iteration, ASVs shared only between the drill core samples located in the submarine deposits and the drilling fluid (**Figure 2, Supplementary Table S6**, 128 ASVs) were also considered as marine contaminants and were removed from the data set. Natural infiltration of cool seawater occurs in the subsurface of Surtsey at 144–155 m b.s (Jakobsson and Moore, 1986; Jackson et al., 2019b; Kleine et al., 2020). This suggests that marine taxa detected in the submarine deposit could derive from the inflow of seawater that infiltrate the subsurface. Therefore, ASVs shared between the submarine drill core, seawater samples, and the drilling fluid were retained (**Figure 2**, 61 ASVs). The total number of sequences removed from the drill core samples by the abovementioned procedures was 138,913 (**Supplementary Table S3**), which represents on average 47% of the reads per drill core samples. The subsequent analyses were performed on the decontaminated data set, excluding the drilling fluid samples.

Microbial Community Structure Among the Sample Types

Sequencing provided enough reads to capture the total richness of the samples as all libraries reached saturation in rarefaction curves (**Supplementary Figure S5**). The 41 samples were catalogued by sample type: 18 samples from borehole fluids, 17 samples from drill cores, 2 samples from fumaroles, and 4 seawater samples (**Table 1, Supplementary Table S3**). Amplification of the partial 16S rRNA gene using the universal primer set was successful for all samples, while only 17 samples could be amplified using the archaeal primer sets.

We obtained a total of 455,545 and 848,053 high-quality sequences using universal and archaeal 16S rRNA primer sets, respectively (**Supplementary Table S3**). A total of 4,222 ASVs ranging from 41 (Sur168) to 317 (SW50) ASVs per sample

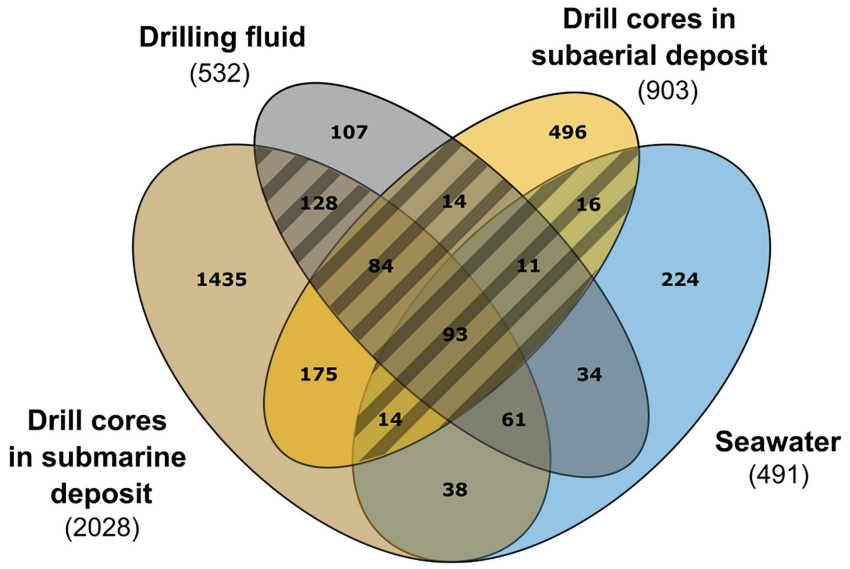

FIGURE 2 | Contamination assessment by an overlap approach between drill cores from the subaerial and submarine deposits, drilling fluid, and seawater samples. Five drilling fluid samples (in grey): 1B49ZC, 1C17ZC, 1C39ZC, 1C51ZC, 1C59ZC, 4 sea samples (in blue): SW_10, SW_20, SW_30, SW_50, 4 samples from the subaerial deposit (in yellow): C4, C9, C13 and C17, and 13 samples from the submarine deposit (in light brown): C22, C27, C33, C36, C39, C42, C45, C49, C52, C55, C59, C62, C65. Hatched area: 360 ASVs considered as contaminants.

were obtained using the universal primer set, whereas a total of 157 ASVs ranging between 3 (Sur4a) and 41 (Sur1a) ASVs per sample were obtained using the archaeal primer sets.

α Diversity

Analysis of variance on species richness showed significant differences between sample types (ANOVA, F value = 9.082, Pr(>F) = 0.000123). A Tukey HSD test highlighted significant differences among them between seawater samples and borehole fluid (<0.001), seawater samples and drill core (0.0339), and between drill core and borehole fluid (0.0184). Shannon diversity also differed significantly among them (ANOVA, F value = 5.998, Pr(>F) = 0.00195), and Tukey's HSD test revealed significant differences between seawater samples and borehole fluid (0.00323) and between seawater samples and drill core (0.02570; **Figure 3A, Supplementary Table S7**). The SE-01 borehole fluids displayed a significantly lower observed diversity than the drill cores, but Shannon diversity was not significantly different, indicating that the evenness of the species present in these sample types was comparable. Observed diversity and evenness were significantly higher in the seawater samples than in drill core and borehole fluid samples.

β Diversity

The resulting NMDS ordination conducted using Bray-Curtis dissimilarity metrics revealed distinct microbial communities specific to each sample types, which clustered individually (**Figure 3B**). The stress value equaled 0.18. This was further confirmed by a PERMANOVA analysis showing that sample types differed significantly [Pr(>F) = 0.001, r2 = 274], and a multilevel pairwise comparison showed significant differences among drill cores and borehole fluids (adjusted p value = 0.0184), seawater samples and borehole fluids (<0.001), and between seawater samples and drill cores (0.0338). This indicates that the samples within a given sample type show microbial communities that are more similar to one another than to samples from a different sample type. The cluster of SE-01 borehole fluid samples appears wider than the drill core cluster, indicating more differences in microbial community (**Figure 3B**).

Community Composition

At the phylum level, some taxa were common across the four sample types (**Figure 3C**). The phylum Proteobacteria dominated all sample types, for example, whereas its relative proportion varied greatly among them. Within Proteobacteria, Alphaproteobacteria was the most abundant class followed by Gammaproteobacteria. Fumarole samples showed a higher relative proportion of Cyanobacteria (15.3%), Deinococcota (12.6%), Acidobacteriota (7.84%), and Chloroflexi (4.52%) than borehole fluids and drill cores. Borehole fluids displayed the highest occurrence of the bacterial phyla Acetothermia (5.56%) and Patescibacteria (2.26%), as well as the archaeal phyla Thermoplasmatota (5.60%) and Euryarchaeota (3.13%), in comparison with the other sample types. The class

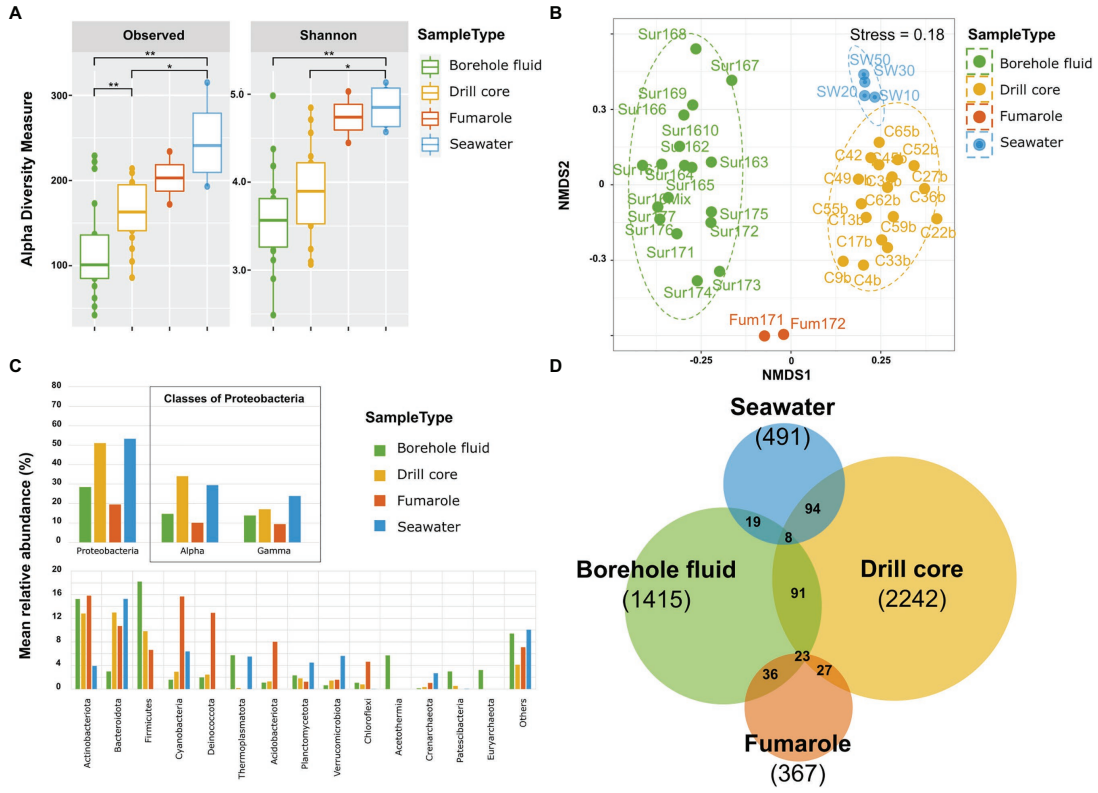

FIGURE 3 | Overview of the microbial community diversity, structure, and composition of the samples types: borehole fluid, drill core, fumarole, and seawater samples. **(A)** α diversity indices: observed diversity and Shannon's diversity indexes. Signif. codes: 0.001 "***"0.01 "*". **(B)** β diversity assessed with non-metric multidimensional scaling ordination using Bray-Curtis dissimilarity metrics of microbial community within sample types. The stress value equaled 0.18. **(C)** Mean relative abundance of the 14 most abundant phyla. Proteobacteria are divided into classes. **(D)** Venn diagram showing the number of shared and unique ASVs among the sample types.

Desulfotomaculia represented more than 5% of the borehole fluid community (data not shown). Nevertheless, due to the compositional nature of the data, lineage-specific enrichments necessitate further investigation, using for example a qPCR approach (Jian et al., 2020). The drill core samples show the highest sequence variants (2,242 ASVs), followed by the borehole fluids (1,415 ASVs), seawater samples (494 ASVs), and fumarole samples (367 ASVs). Most ASVs were not shared between the sample types (**Figure 3D**).

Microbial Community Composition and Structure of the Rock Samples and Influence of Depth and Temperature

The bacterial sequences from the drill core samples are classified into 35 phyla, with the ten phyla Proteobacteria, Bacteroidota, Actinobacteriota, Firmicutes, Cyanobacteria, Deinococcota, Planctomycetota, Verrucomicrobiota, Acidobacteriota, and Chloroflexi, comprising more than 95% of the sequences (**Figure 4**). The 29 ASVs classified as Archaea using the universal primer set fall into the phyla Thermoplasmatota, Crenarchaeota, Nanoarchaeota, and Iainarchaeota.

Archaeal sequences obtained by nested PCR from 10 of the drill cores were more diverse. The 88 unique ASVs include the phyla Crenarchaeota (mainly Nitrososphaeria), Thermoplasmatota (mainly Thermoplasmata), Euryarchaeota, Halobacterota, Hydrothermarchaeota, Nanoarchaeota, and unassigned Archaea (**Figure 4**). Remarkably, the sample C55 (157 m b.s.; 64°C) below the submarine inflow zone is dominated by the archaeal genus *Methanobacterium,* while the sample C65 (181 m b.s.; 37°C) in weakly consolidated tephra near the pre-eruption seafloor shows the highest abundance of the genus *Thermococcus* (**Figure 4**).

Canonical correspondence analysis (CCA) ordination (ANOVA, $p = 0.018$) and envfit analyses demonstrate that both depth and *in situ* temperature are significantly correlated with microbial community structure of the drill core samples (**Supplementary Figure S6**). However, it is unclear which

variable has the most influence on the microbial community structure, since both are linked. Observed and Shannon diversity indices showed no significant difference between the four categories of drill core samples (data not shown).

To identify the ASVs causing the dissimilarity of community structure in the CCA ordination plot, the drill core samples are grouped in four categories as follows (**Supplementary Figure S6**): (DC_1) drill core samples from the subaerial deposits (23–35 m b.s.; 20–36°C in 2017; $n = 2$, C4, C9) (DC_2) drill core samples from the zone of daily intertidal fluctuations at coastal sea level (44–65 m b.s.; 59–101.5°C; $n = 3$, C13, C17, C22), (DC_3) drill core samples from the submarine deposits near the hydrothermal temperature maximum (78–139 m b.s.; 97–124°C in 2017; $n = 7$, C27, C33, C36, C39, C42, C45, C49), and (DC_4) drill core samples from the submarine deposits, below the zone of seawater inflow (157–181 m b.s.; 37–84°C in 2017; $n = 5$, C52, C55, C65, C62, C59).

Differential Abundance Analyses

To identify differences in ASVs abundance between the four sample types (F, fumarole; BF, borehole fluid; DC, drill core; SW, seawater samples), we performed differential abundance analyses (two-by-two comparisons) using separately the universal primer and the archaeal primer data sets (**Supplementary Table S5**). Additionally, the same analyses were performed for the four categories of drill core samples to highlight the ASVs contributing to the dissimilarity in the CCA plot (**Supplementary Figure S6**). Due to the compositional features of the data, the identification of differentially abundant taxa between the different groups of samples must be assessed carefully, since it is based on a count-based method. Being aware of the limitations of this approach, a total of 95 and 26 unique ASVs were proved to be significantly represented using the universal primer data set (grouping into 59 taxonomic bins) and the archaeal primer sets (7 taxonomic bins), respectively (**Figure 5**), in accordance with the log fold change of the mean normalized read counts ($p < 0.01$).

Compared to the seawater samples, the drill cores showed overrepresented ASVs assigned to the taxa *Caldisericum*, *Galbitalea*, *Geobacillus*, *Glaciecola*, *Oceaniserpentilla*, *Oleispira*, *Piscinibacter*, *Pseudoarthrobacter*, *Psychromonas*, *Reyranella*, *Sulfurospirillum*, *Thermaerobacter*, *Thermus*, unassigned bacteria from the Sphingomonadaceae family, and unassigned Thermoplasmata. ASVs assigned to *Aliivibrio* and *Pseudoalteromonas* were significantly underrepresented in the drill cores compared to the seawater samples. Although the latter genera are generally detected in seawater, they were

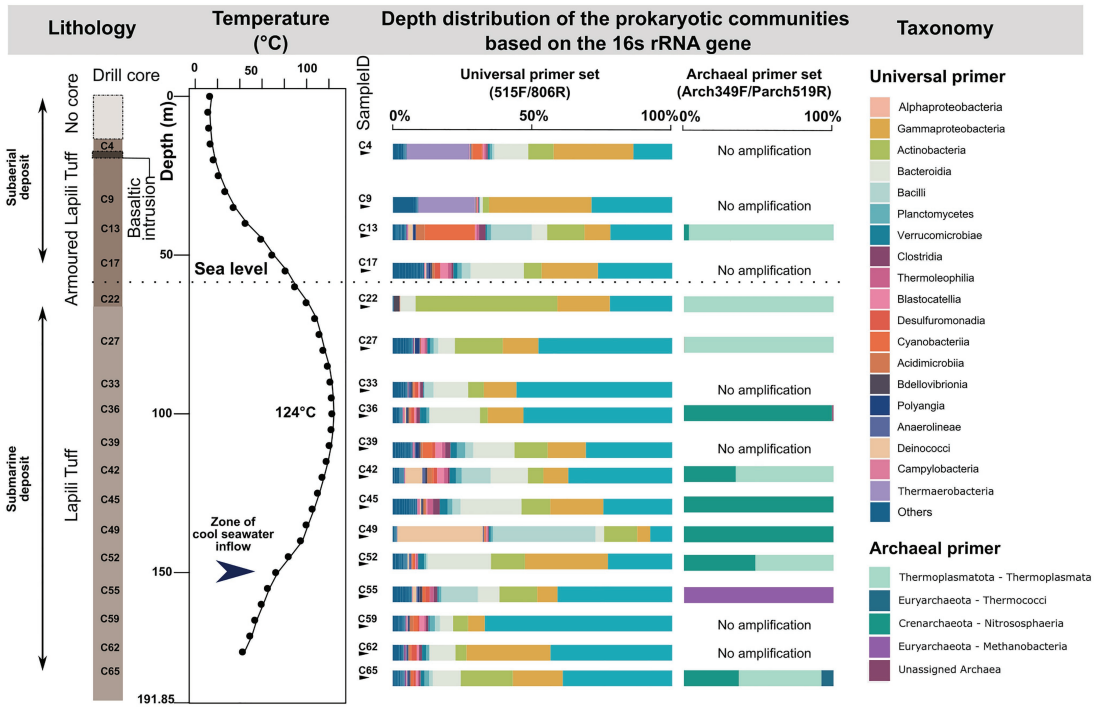

FIGURE 4 | Vertical distribution of the microbial communities at the phylum or class level detected in the 17 drill core samples from the subsurface of the Surtsey volcano using universal primer set and archaeal primer sets.

significantly overrepresented in the borehole fluids collected before drilling, indicating that marine microorganisms (or their DNA) naturally infiltrate the subsurface basaltic deposits. Other overrepresented ASVs in the borehole fluids, as compared to the drill core samples, included unassigned Acetothermiia, BRH-c8a (Desulfallas-Sporotomaculum), *Ruegeria*, *Rhodobaca*, unassigned RBG-16-55-12 (Actinobacteriota), *Conexibacter*, *Desulfosporosinus*, *Desulfatiglans*, unassigned Ammonifexales, and Ammonificaceae, among others (**Supplementary Table S5**). Archaeal taxa that proved to be overrepresented in the borehole fluids included unassigned Thermoplasmata, Hydrothermarchaeales, Syntrophoarchaeaceae, and the genus *Thermococcus*. Using the archaeal primer sets, unassigned Thermoplasmatota and Bathyarchaeia were added to the list of overrepresented archaeal taxa detected using the universal primer set (**Figure 5, Supplementary Table S5**).

Comparing the four categories of drill cores, ASVs overrepresented in the drill core samples from the subaerial tuff cone (DC_1; 23–35 m b.s.; 20–36°C in 2017) were assigned to the genera *Thermaerobacter*, *Thioalkalimicrobium*, *Salinarimonas*, *Marinobacter*, *Nioella*, *Ectothiorhodospira*, *Polaribacter,* and unassigned Limnochordaceae (**Figure 5**). No ASVs were significantly overrepresented in drill core samples from the zone of daily intertidal fluctuations (DC_2; 44–65 m b.s.; 59–101.5°C in 2017) compared with the other categories of drill core samples. Although one ASV assigned to *Geobacillus* is significantly overrepresented in the drill core samples from the hydrothermal temperature maximum (DC_3; 78–139 m b.s.; 97–124°C in 2017), this is similar to the drill core samples located below the zone of seawater inflow (DC_4; 157–181 m b.s.; 37–84°C in 2017). These samples shared significant ASVs with the seawater samples, including *Flavicella*, NS4 marine group from the Flavobacteriaceae family, *Synechococcus* CC9902, *Planktomarina,* and *Sulfitobacter*. The presence of these taxa further suggests an infiltration of marine microorganisms associated with the infiltration of cool seawater at 145–155 m depth, if we presume that no drilling fluid contamination remains.

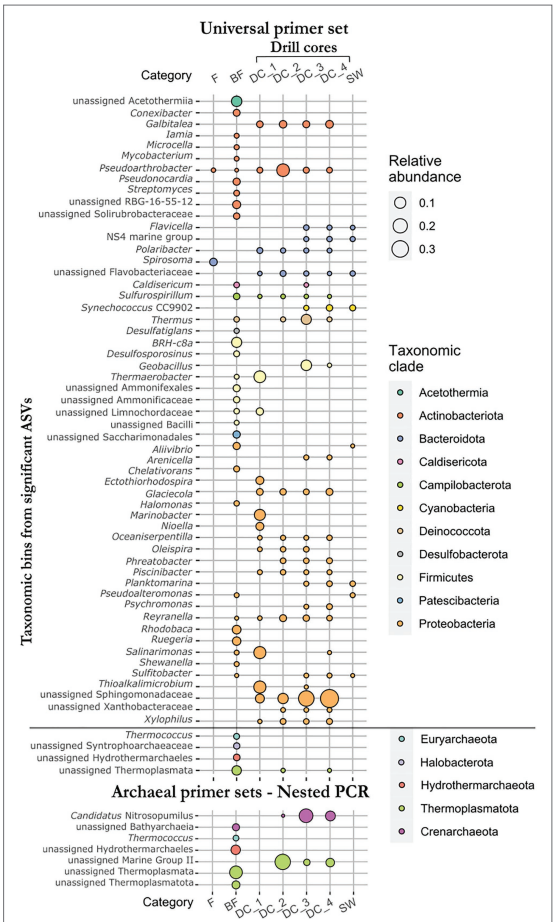

FIGURE 5 | Relative abundance of taxonomic bins from all significant ASVs identified by differential abundance analyses using both universal and archaeal primer data sets. Sample types: F, fumarole ($n = 2$); BF, borehole fluid ($n = 18$); DC, drill core ($n = 17$); SW, seawater samples ($n = 4$). Categories of drill cores: DC_1, drill cores from the subaerial deposit ($n = 2$); DC_2, just above and at a zone of daily intertidal fluctuations at coastal sea level ($n = 3$); DC_3, in the submarine deposit at the hydrothermal temperature maximum ($n = 7$); DC_4, in the submarine deposit below the zone of seawater inflow ($n = 5$).

Possible Biotic Structures Revealed by SEM Images

To determine whether the detected DNA sequences could be derived from planktonic cells in interstitial pore fluids or, alternatively, attached to surfaces of the lapilli tuff and to investigate their organization on the basaltic substrate, SEM studies were undertaken on six drill core samples, differing in depth (C4, C9, C22, C49, C55, and C65).

All six lapilli tuff samples are porous with high water absorption (Jackson et al., 2019b). Vesicles with spherical shapes 10–80 µm in diameter occur in all samples, and platy clay mineral structures, with flower petal morphologies, indicate alteration of the original volcanic glass (Jakobsson and Moore, 1986; **Figure 6B**). The distribution of microstructures varies with sampling depth; here, we focus on instructive samples at 32 m b.s (C9) and 65 m b.s (C22). Some vesicle surfaces are covered by a net of thin filaments with an approximate diameter of 10 nm. These features are closely intertwined with one other and could possibly correspond to extracellular polymeric substances-like (EPS) structures (Thorseth et al., 2001, 2003; Sudek et al., 2017), perhaps representing a "relic" of past biofilm activity (**Figures 6A**,**B**). In other vesicles, spheroidal elements correspond to putative microbial cells 2 µm in diameter (**Figure 6C**) as previously described (Thorseth et al., 2001, 2003). Furthermore, these vesicles are covered by fibrillar-like microstructures (**Figure 6D**), that appear texturally different from the EPS-like structures (**Figure 6B**). They occur as nets of oriented fibers with ramifications; some seem to be attached

to the spheroidal structures. Energy dispersive X-ray analysis on the spheroidal elements does not detect carbon or phosphate but, instead, detects magnesium, aluminum, and iron. They therefore seem to have an inorganic origin.

Wrinkled dome structures are observed in many vesicles at 65 m b.s (**Figure 6E**) with a diameter size of 10–20 µm. A network of intercrossed filaments covers the surface of the dome or mound (**Figure 6F**). Their wrinkled appearance could be explained by the sample preparation for SEM images, which includes gradual dehydration. EDX analyses investigate the chemical signature of the wrinkled dome structures (**Supplementary Figure S7**). The superposition of the spectra corresponding to the adjacent clay mineral(s) (**Supplementary Figure S7**, red area) and the wrinkled dome structures (**Supplementary Figure S7**, blue area) shows enrichment in carbon, oxygen, sodium, phosphate, and calcium in the crinkled ridge of the mound. These could possibly indicate elements associated with the cells and biomass of a biofilm.

DISCUSSION

Microbial colonization by pioneer communities can occur within months on freshly deposited erupted volcanic rocks in geothermally active environments (e.g., Konhauser et al., 2002; Kelly et al., 2014). The Surtsey geothermal system offers a highly unusual site to study rapid microbial colonization in newly formed oceanic crust at <100 years and > 50°C. Results of the analysis described in **Figures 3, 4**, and **6** suggest that diverse microbial communities have developed in the Surtsey deposits only 50 years after the eruptions ended. Communities attached to the lapilli tuff of drill core samples are significantly different from communities in associated fluids (**Figures 3A**,**B**). Similar observations have previously been reported in crustal environments (Ramírez et al., 2019). The Surtsey data suggest that fumaroles, borehole fluids, drill cores, and seawater samples harbor significantly distinct microbial communities (**Figures 3B**,**C**). Yet, taxa are shared between these sample types (**Figure 3D**), suggesting possible transfer of microorganisms among these habitats. The hot fumarole samples from the surface of Surtsey at 82.3 and 85.6°C harbor microbial communities that are more similar to those of the drill core samples located above sea level in the subaerial deposits (e.g., samples C4, C9, 23–35 m b.s.; **Figure 2B**). This suggests that some species could disperse through the subaerial tuff cone above sea level up to the surface through fumarole activity (Tin et al., 2011). Excluding all the drilling fluid ASVs contaminants, microbial communities detected in the seawater samples are closer in community structure to the drill core samples from the zone of seawater inflow (sample C52, 148 m b.s., **Figure 2B**) and to the samples in proximity to the seafloor (sample C65, 181 m b.s., **Figure 2B**). This suggests that microorganisms from the seawater surrounding the island (i) infiltrate the submarine tephra deposits, following the seawater inflow at about 148 m b.s (Jakobsson and Moore, 1986; Jackson et al., 2019b; Kleine et al., 2020), and (ii) occupy the deeper zone of poorly consolidated tuff through indirect infiltration of the seawater and its circulation *via* the seafloor. The ability of some species to expand across ecosystems and adapt to new environmental conditions (Sriswasdi et al., 2017) has possibly driven the subsurface colonization of the Surtsey volcano. Species that cannot survive might serve as supply of fermentable organic molecules for the subsurface heterotrophic microorganisms (Li et al., 2020).

Assessment of contamination by Drilling Fluid

The problem of subsurface sample contamination during drilling operations is well-known (Barton et al., 2006; Lever et al., 2006; Kieft, 2010; Santelli et al., 2010; Sheik et al., 2018) since drilling operations require the use of circulating fluid that inevitably infiltrates into the drill core. As a result, the recovery of uncontaminated rock is nearly impossible (Friese et al., 2017; Kallmeyer, 2017). Conventional methods to assess contamination include the addition of tracer compounds to the drilling fluid. These include fluorescent dyes (Pellizzari et al., 2013), perfluorocarbon tracers (PFT; Lever et al., 2006; Inagaki et al., 2016; Orcutt et al., 2017), and microsphere tracers (Kallmeyer et al., 2006; Yanagawa et al., 2013). The use of these tracer compounds is prohibited on Surtsey due to the protected environmental status of the island (Baldursson and Ingadóttir, 2007). Furthermore, their performance in the Surtsey system would have been quite problematic. Although fluorescent dyes have the advantages of high sensitivity for detection, low cost, and ease of use (Pellizzari et al., 2013), they are unstable at low pH (Zhu et al., 2005) and are also susceptible to degradation in the presence of light (Diehl and Horchak-Morris, 1987). Measurements of Surtsey subsurface borehole fluids extracted from SE-01 in 2016 showed pH values decreasing to 5 at some depths (Kleine et al., 2020). Water tanks containing the drilling fluid were exposed to sunlight during the long Icelandic summer days. The detection of PFTs must be performed immediately on fresh cores because of the high volatility of the PFTs and requires elaborate equipment (Lever et al., 2006) whose transport by helicopter to Surtsey would have been very difficult. Finally, microsphere tracers decompose under high-temperature conditions (Yanagawa et al., 2013) and would not have performed well in the Surtsey hydrothermal system, which currently exceeds 120°C at some depths (Marteinsson et al., 2015; Jackson et al., 2019b). Because of the unique circumstances of drilling on Surtsey, the filtration and UV sterilization of the drilling fluid was the most effective strategy to manage contamination. We then mitigated this strategy with a simple overlap approach that is commonly used in environmental microbiology studies to identify and remove contaminants (Sheik et al., 2018). At the ASVs level, this approach enabled the distinction between marine contaminants from the drilling fluid and possible endemic residents, providing a firm basis for the exploration of microbial life in this extreme and unusual habitat (**Figure 2**).

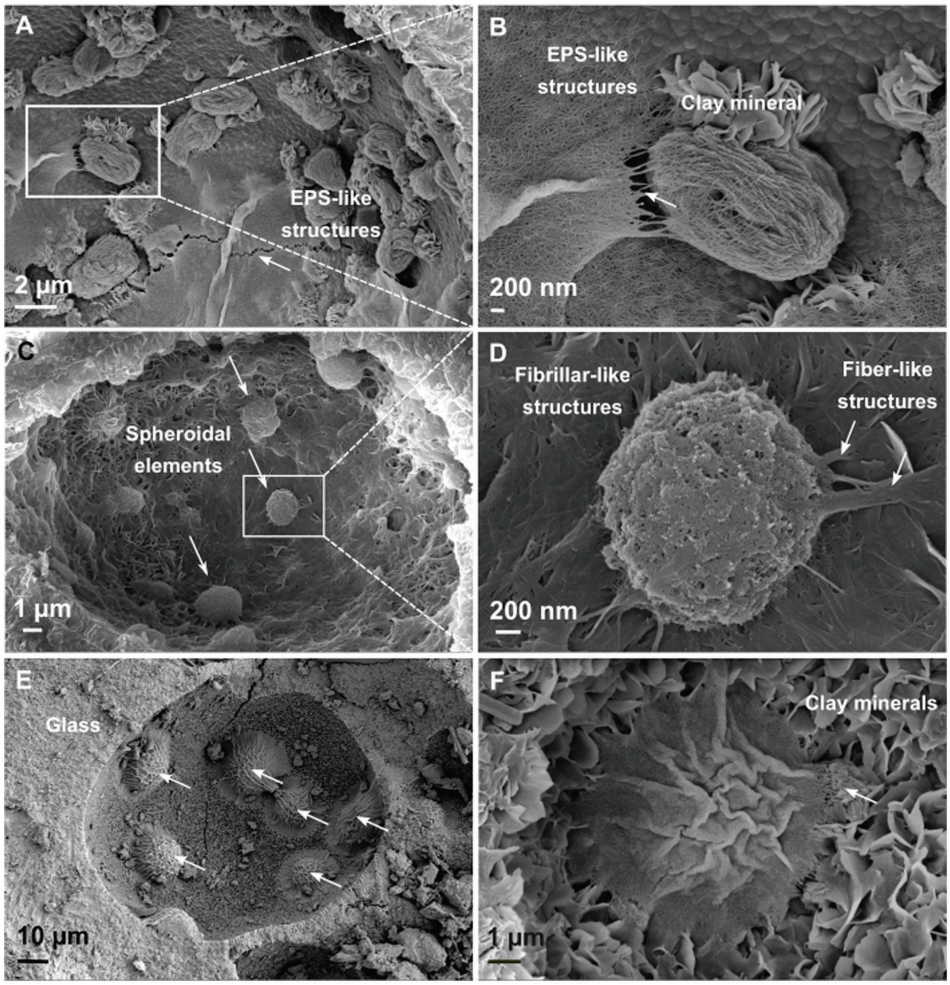

FIGURE 6 | Scanning electron micrographs of Surtsey tephra surfaces in drill core samples. **(A,B)** Platey clay mineral and EPS-like structures, 65 m b.s., sample C22. **(C,D)** Vesicle surfaces in altered basaltic glass are covered by a net of fibrillar-like structures that appears to be attached to spheroidal elements, 32 m b.s, sample C9. **(E,F)** Wrinkled dome structures, or mounds, are covered by a network of filaments, 65 m b.s, sample C22.

The DNA concentration in the drill core samples, measured using a QuBit fluorometer, ranged from 0.2–1.36 ng.g^{-1} of basalt, which is in good correlation with other basaltic environments (Fisk et al., 2003; Herrera and Cockell, 2007). Surprisingly, DNA concentration did not correlate with variations in temperature (**Supplementary Figure S2a**). It does decrease with depth (**Supplementary Figure S2b**), as previously observed in terrestrial and marine subsurface habitats (Cockell et al., 2012; Ciobanu et al., 2014; Mcmahon and Parnell, 2014), but not significantly. The presence of contaminant DNA in the low-biomass basaltic core samples quite possibly distorts these results. Indeed, low DNA concentration exacerbates issues of external contamination (Sheik et al., 2018). When using 16S rRNA gene sequencing to analyze low-biomass samples, the amplification and sequencing of contaminant DNA are introduced during extraction and library preparation steps, and the risk of well-to-well contamination is high (Minich et al., 2019). The low biomass and the presence of external contamination also influence the sequencing results by affecting the magnitude and biological provenance of analyzed sequences. Therefore, it is of crucial importance to use negative controls and optimal decontamination approaches. The inclusion of bacterial mock communities as extraction and sequencing controls would have been beneficial for the study (Pollock et al., 2018). We emphasize

that all samples were handled with extreme care during all steps of the project to minimize, identify and remove contaminants using a combination of experimental and computational methods. The lists of contaminants that have been identified based on the presence/absence of ASVs across samples, their relative abundances, and their taxonomic assignment (**Supplementary Tables S1, S2 and S4**) should be valuable for future microbial explorations of the oceanic crust. In addition to the low biomass and the presence of external contamination affecting the results, we should keep in mind that the compositional nature of the data has limits. Indeed, the data set reported in this study was obtained by 16S rRNA gene amplicon sequencing; thus, the data are compositional being based on relative abundances, which sum to a constant. Therefore, the analytical approaches (e.g., rarefaction, normalization) and statistical methods (e.g., ANOVA) used to study microbiome data influence the results and can potentially be are subjected to inflated false discovery rates (Gloor et al., 2017). This can lead to the lack of reproducibility among microbiome studies and misinterpretations of microbial community structures (e.g., alpha diversity).

Putative Inhabitants of the Subsurface

Many of the taxa detected in the SE-01 borehole fluid samples match DNA sequences previously identified in hot springs, hydrothermal vents, and subsurface environments (**Figure 5**). For example, sequences assigned to the class Acetothermiia closely match with DNA sequences previously found in hydrothermal sediments (GenBank: FM868292). Members of this class were previously detected in anaerobic digesters, hot springs, and other deep biosphere studies (Takami et al., 2012; Jungbluth et al., 2017; Zaitseva et al., 2017; Hao et al., 2018; Korzhenkov et al., 2018). Sequences assigned to the genus BRH-c8a from the family Desulfallas-Sporotomaculum match closely with sequences retrieved from deep groundwater (LC179584) and group with the genus *Desulfotomaculum* that can be found in deep subsurface environments (Sousa et al., 2018; Watanabe et al., 2018). Other sequences belonging to the class Desulfotomaculia are assigned to Ammonifexales and Ammonificaceae; these are related to DNA sequences retrieved from petroleum reservoirs (MF470409). In addition, sequences assigned to *Desulfosporosinus* closely match sequences retrieved from coal formation waters (KC215435), while sequences assigned to *Desulfatiglans* match with sequences found in hydrothermal vents (AB294892). Among the abundant ASVs detected in the borehole fluids, an early-branching, uncultivated actinobacterial clade identified as RBG-16-55-12 in the SILVA database release 138 has been previously detected in serpentinite-hosted systems (Merino et al., 2020). Uncharacterized Thermoplasmata are also detected in serpentinite subsurface deposits (Motamedi et al., 2020), yet numerous sequences assigned to this class from the SE-01 borehole fluid samples showed less than 90% of sequence similarity with the first match on the NCBI Nucleotide collection database (AB327321). These results suggest that all the latter taxa could be endemic in the Surtsey subsurface deposits and perhaps could be common in other oceanic or continental subsurface habitats, as well. Other taxa detected in the SE-01 borehole fluid samples are usually found in seawater, including *Halomonas*, *Pseudoalteromonas*, *Sulfitobacter*, *Aliivibrio,* and *Shewanella* (**Figure 5**). The presence of marine microorganisms in the SE-01 borehole fluid before new drilling began in 2017 further demonstrates that the infiltration of seawater transports marine microorganisms into the subsurface and its hydrothermal system. These species are also frequently detected in cool basaltic oceanic crust (Templeton et al., 2005; Mason et al., 2007; Zhang et al., 2016), and the question of whether or not these species survive in the Surtsey hydrothermal system could depend on the influence of temperature (Edwards et al., 2012; Baquiran et al., 2016; Ramírez et al., 2019).

Scanning electron microscopy studies of instructive drill core samples reveal possible biotic structures whose size, morphology, and fabrics seem to be consistent with EPS-microcolony complexes that are attached to the basaltic substrate (**Figure 6, Supplementary Figure S7**; Thorseth et al., 2001, 2003; Nakagawa et al., 2006). Indeed, some taxa detected in the drill core samples could represent endemic inhabitant of the basaltic subsurface of the island, which can be divided into two distinct habitats: the subaerial tuff cone and the submarine tuff deposits. In the subaerial tuff cone (DC_1; 23–35 m b.s.; 20–36°C in 2017), the taxa include the genera *Thermaerobacter*, *Thioalkalimicrobium*, *Salinaromonas*, *Marinobacter,* and *Ectothiorhodospira* (**Figure 5**). *Thermaerobacter* are thermophilic to extremely thermophilic bacteria found in terrestrial and oceanic subsurface environments (Spanevello et al., 2002; Takai et al., 1999). *Thioalkalimicrobium*, *Ectothiorhodospira,* and *Salinarimonas* are detected in alkaline and saline habitats such as soda lakes (Sorokin et al., 2001), saline soil (Cai et al., 2011), or salt mines (Liu et al., 2010). The presence of *Marinobacter* only in the tuff deposits above sea level is curious since it is a common lineage found in marine basaltic habitats, including ridge-flank systems and seamount (Templeton et al., 2005; Zhang et al., 2016). Nevertheless, the presence of these taxa in the subaerial deposits suggests adaptation of the microbial communities to more extreme environmental conditions, such as high temperature, high salt concentration produced by NaCl saturation from seawater evaporation or from alkaline pH produced by basaltic glass dissolution at low fluid-rock ratios (Kleine et al., 2020). No hyperthermophilic species were significantly enriched in the drill core samples from the hydrothermal temperature maximum (DC_3; 78–139 m b.s.; 97–124°C in 2017 and 100–141°C in 1979), yet two thermophilic species *Thermus* and *Geobacillus* from the Thermoleovorans group were detected (**Figure 5**). Based on these results and the taxa detected in the SE-01 borehole fluids, it seems that microbial life may persist in subsurface deposits that have experienced temperatures >120°C, the presumed temperature for functional microbial life. The hydrothermal zone at about 100 m b.s. could act as a dispersal barrier that provides an obstacle to the transfer of live cells from the zone of tidal flux and

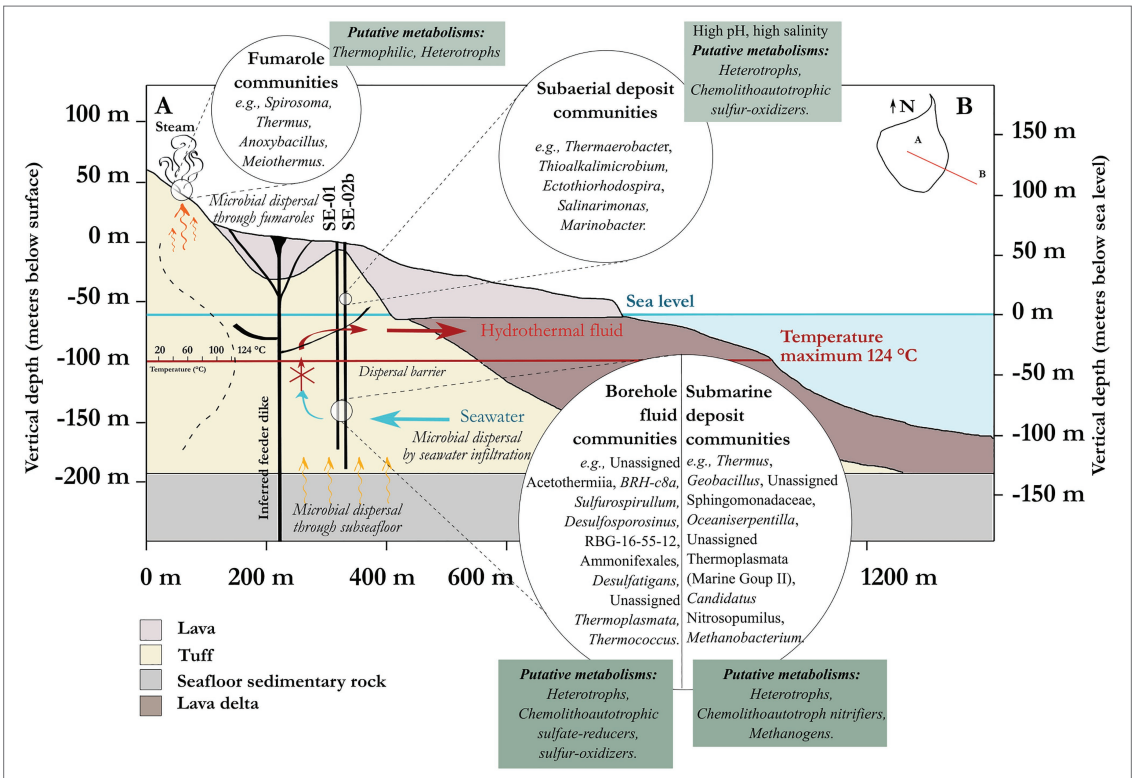

FIGURE 7 | Overview of the putative metabolisms and microbial dispersion scenario of the subsurface of Surtsey volcano.

upper submarine deposits to the deeper submarine deposits (e.g., samples C27; 78 m b.s. and C52; 148 m b.s.). Furthermore, no significant difference exists between the observed microbial communities from the lowermost submarine deposits below the zone of cool seawater inflow (DC_4; 157–181 m b.s.; 37–84°C in 2017) and the other submarine deposits (**Supplementary Figure S6**). Shared taxa mainly include mesophilic Proteobacteria such as unassigned Sphingomonadaceae, *Glaciecola*, *Arenicella*, *Oceaniserpentilla*, and *Piscinibacter* that are commonly found in marine habitats. In addition, *Candidatus* Nitrosopumilus and unassigned Thermoplasmata from the Marine Group II were detected in great abundance (**Figure 5**). Those taxa have been previously detected in marine basalts and deep seawater circulation through oceanic crust (Mason et al., 2007; Singer et al., 2015; Suzuki et al., 2020; Bergo et al., 2021). Although the deep ocean is typically enriched in archaeal cells, other marine environments usually show a dominance of bacterial lineages (Karner et al., 2001). This has been previously observed in the basaltic crust. For example, Gammaproteobacteria and Alphaproteobacteria are detected in great abundance in the seafloor basaltic glass of the East Pacific Rise (Santelli et al., 2008, 2009), the Arctic spreading ridges (Lysnes et al., 2004), altered basalts from the Hawaiian Loihi Seamount (Templeton et al., 2005; Santelli et al., 2008; Jacobson Meyers et al., 2014), and the Mid-Atlantic Ridge (Rathsack et al., 2009; Mason et al., 2010). Deltaproteobacteria, Firmicutes, Gammaproteobacteria, and Bacteroidetes are also detected in great abundance in the Juan de Fuca Ridge flank and the Costa Rica Rift (Nigro et al., 2012; Jungbluth et al., 2013, 2014). Our results support the dominance of bacterial lineages in marine basalts.

While most taxa could be identified at a low taxonomic rank, many others were not assigned to a known genus, family, or even order. For example, many sequences detected in this study fell into unknown clades, including Acetothermiia, Ammonifexales, Bacilli, RBG-16-55-12 from the phylum Actinobacteriota, Sphingomonadaceae, Limnochordaceae, and Saccharimonadales, among others. Likewise, many archaeal sequences could not be identified further than the phylum level, including unassigned Thermoplasmatota and Halobacterota, or the class level, including unassigned Thermoplasmata and Bathyarchaeia. Hence, the subsurface biosphere of Surtsey lapilli tuff and tephra, as well as fluids, could have high potential for discoveries of new microbial clades.

Metabolic Potential

The metabolic potential of the endemic subsurface microbial communities can be discussed with some degree of certainty, while remaining mindful of the difficulties inherent to identifications based on 16S rRNA gene sequence analysis. To support our hypotheses, predictive functional analyses were performed using PICRUSt2. A few MetaCyc pathways were selected to represent the functional potential of the bacterial and archaeal communities for carbon, sulfur, nitrogen, and methane metabolism (**Supplementary Figures S8, S9**). Many genera reported in this study belong to taxonomic clades with known metabolisms that are involved in both heterotrophy and chemolithoautotrophy (**Figure 7**). The presence of putative sulfate-reducing bacteria strongly suggests a potential for active sulfate reduction, including *Desulfosporosinus*, *Desulfatiglans,* and the *Desulfotomaculia* class, among others. In addition, sulfur oxidizers were detected, such as the genera *Thioalkalimicrobium*, *Sulfurospirillum*, *Sulfurimonas*, *Ectothiorhodospira,* and *Sulfurihydrogenibium*. The observation of these bacterial taxa possibly involved in sulfate reduction and sulfur oxidation suggests an active sulfur cycle in the subsurface of Surtsey (**Supplementary Figure S8**), as has previously been reported in similar ecosystems (Bach and Edwards, 2003; Lever et al., 2013; Suzuki et al., 2020). This is further reinforced by the detection of archaea possibly involved in the sulfur cycle, such as *Thermococcus*, *Pyrococcus*, and *Archaeoglobus*. Also, characterized members of the Thermoplasmatales are typically involved in sulfur cycling (Barton et al., 2014; Arce-Rodríguez et al., 2019). These taxa coincide with deposits that contain sulfate minerals, principally anhydrite and gypsum (Jakobsson and Moore, 1986; Kleine et al., 2020; Prause et al., 2020).

In addition, the genus *Methanobacterium* from the order Methanobacteriales dominates the archaeal sequences of drill core sample C55, at 157 m depth (**Figure 3**). The MetaCyc pathways detected in this sample using the archaeal data set were mainly involved in methane metabolism (e.g., methanogenesis from H_2 and CO_2, coenzyme B biosynthesis, coenzyme M biosynthesis I; **Supplementary Figure S9**). One taxon from the same order of Methanobacteriales was previously reported to dominate the SE-01 borehole fluid microbial communities sampled in 2009 at similar depth (Marteinsson et al., 2015). *Methanobacterium* spp. grows by reducing carbon dioxide to methane and uses molecular hydrogen as the electron donor (Kern et al., 2015). Hence, these taxa could play an important role as primary producers in this ecosystem, at least at certain depths. The possible occurrence of an active methane cycle is supported by the presence of other methanogens (e.g., Methanosarcinia (Syntrophoarchaeaceae), Methanomassiliicoccales (*Methanothermus*, *Methanoregula*), as well as methanotrophic (e.g., *Methylocella*, Methylacidiphilaceae), and methylotrophic (e.g., *Hansschlegelia*, *Methylopila*, Methylophilaceae (OM43 clade), *Methylophaga*) bacteria, despite their relatively low abundances. The observation of genera such as *Geobacter*, *Rhodoferax*, *Marinobacter*, *Shewanella,* and *Ferruginibacter*, as well as *Hydrogenophilus* and *Hydrogenophaga,* could indicate that iron and hydrogen are electron donors within the ecosystem, as previously reported for other subsurface habitats (Bach and Edwards, 2003; Bach, 2016; Zhang et al., 2016). In addition, putative ammonium-oxidizing archaea belonging to the Marine Group II and *Candidatus* Nitrosopumilus suggest an ability to transform nitrogen compounds as previously reported in the oceanic crust (Daae et al., 2013; Orcutt et al., 2015; Jørgensen and Zhao, 2016; Zhao et al., 2020).

CONCLUSIONS

The 1979 and 2017 drilling projects at Surtsey volcano provide a rare opportunity to explore the subsurface microbial diversity of a very young basaltic island associated with an active hydrothermal-seawater system in newly formed oceanic crust. A cored borehole dedicated to microbiology research in the 2017 drilling operation probes a low biomass but highly diverse habitat that hosts bacterial and archaeal clades, including extremophiles, that have been previously detected in other terrestrial and marine environments. Many clades, however, fall into as-yet-unknown lineages. The 16 s rRNA gene amplicon sequencing data provide insights into diverse sources of microbial colonization in newly formed oceanic crust, with potential dissemination from the deep subsurface, surrounding seawater, and surface ecosystems. The island of Surtsey may thus be regarded as a porous, sponge-like basaltic structure that absorbs cells from the surrounding environments and selects microorganisms that can adapt to the extreme environmental conditions that exist within the volcano. The data also provide a baseline for long-term observations of the microbial communities inhabiting the subsurface of Surtsey and their temporal succession amid a changing hydrothermal environment, in which poorly consolidated tephra lithify to form well-consolidated lapilli tuff. Further research, including metagenomic sequencing, will increase our knowledge of the metabolism and function of the microbiome in this very young basaltic environment.

DATA AVAILABILITY STATEMENT

The data sets (accession number ERP126178) for this study can be found in the European Nucleotide Archive (ENA) at EMBL-EBI (https://www.ebi.ac.uk/ena/browser/view/PRJEB42339).

AUTHOR CONTRIBUTIONS

VM (PI of the microbiology part of drilling operation), MJ, and MG conceived the study. PB, AK, VM, and members of the SUSTAIN onsite, science teams conducted field operations and sampling. PB and PV processed the samples and conducted the molecular biology experiments. PB, PV, and SK performed the data analysis. PB wrote the original draft. All authors took part in writing the manuscript.

FUNDING

Sample collection and sample processing were funded with a grant of excellence from the Icelandic Science fund, ICF-RANNÍS IceSUSTAIN (163083-051). The drilling operation was funded with International Continental Scientific Drilling Program (ICDP) through the SUSTAIN project and the IceSUSTAIN grant. A doctoral student grant from RANNÍS (206582-051) was attributed to PB.

CODE AVAILABILITY STATEMENT

The pipeline used to analyze the data set reported in this manuscript can be found at https://benjjneb.github.io/dada2/tutorial.html. The code used to identify contaminants can be found at https://benjjneb.github.io/decontam/vignettes/decontam_intro.html, and the code used to build the figures can be found in the supplementary material under the section code.

ACKNOWLEDGMENTS

The authors would like to thank all members of the SUSTAIN onsite and science teams, and the Surtsey Research Society for their contributions to the drilling project and sampling, especially B. Kleine, T. Weisenberger, S. Prause, C.F. Gorny, and S. Couper. The Icelandic Science fund, ICF-RANNÍS Jules Verne fund (scientific and technical cooperation between Iceland and France) contributed additional funds. The authors thank E. Gérard, A. Lecoeuvre, B. Ménez, and S. Borensztajn for their contributions to sample preparation and SEM images.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.728977/full#supplementary-material

REFERENCES

Arce-Rodríguez, A., Puente-Sánchez, F., Avendaño, R., Martínez-Cruz, M., de Moor, J. M., Pieper, D. H., et al. (2019). Thermoplasmatales and sulfur-oxidizing bacteria dominate the microbial community at the surface water of a CO_2-rich hydrothermal spring located in Tenorio volcano National Park, Costa Rica. *Extremophiles* 23, 177–187. doi: 10.1007/s00792-018-01072-6

Bach, W. (2016). Some compositional and kinetic controls on the bioenergetic landscapes in oceanic basement. *Front. Microbiol.* 7:107. doi: 10.3389/fmicb.2016.00107

Bach, W., and Edwards, K. J. (2003). Iron and sulfide oxidation within the basaltic ocean crust: implications for chemolithoautotrophic microbial biomass production. *Geochim. Cosmochim. Acta* 67, 3871–3887. doi: 10.1016/S0016-7037(03)00304-1

Baldursson, S., and Ingadóttir, Á. (2007). Nomination of Surtsey for the UNESCO world heritage list. *Icelandic Inst. Nat. Hist. Reykjavik* Available at: https://whc.unesco.org/uploads/nominations/1267.pdf

Baquiran, J. P. M., Ramírez, G. A., Haddad, A. G., Toner, B. M., Hulme, S., Wheat, C. G., et al. (2016). Temperature and redox effect on mineral colonization in Juan de Fuca Ridge Flank subsurface crustal fluids. *Front. Microbiol.* 7:396. doi: 10.3389/fmicb.2016.00396

Bar-On, Y. M., Phillips, R., and Milo, R. (2018). The biomass distribution on earth. *Proc. Natl. Acad. Sci. U. S. A.* 115, 6506–6511. doi: 10.1073/pnas.1711842115

Baross, J. A., Wilcock, W. S. D., Kelley, D. S., Delong, E. F., and Cary, S. C. (2004). The subsurface biosphere at mid-ocean ridges: issues and challenges. *Geophys. Monogr. Ser.* doi: 10.1029/GM144

Barton, L. L., Fardeau, M. L., and Fauque, G. D. (2014). Hydrogen sulfide: A toxic gas produced by dissimilatory sulfate and sulfur reduction and consumed by microbial oxidation. *Met. Ions Life Sci.* 14, 237–277. doi: 10.1007/978-94-017-9269-1_10

Barton, H. A., Taylor, N. M., Lubbers, B. R., and Pemberton, A. C. (2006). DNA extraction from low-biomass carbonate rock: An improved method with reduced contamination and the low-biomass contaminant database. *J. Microbiol. Methods* 66, 21–31. doi: 10.1016/j.mimet.2005.10.005

Bergo, N. M., Bendia, A. G., Ferreira, J. C. N., Murton, B. J., Brandini, F. P., and Pellizari, V. H. (2021). Microbial diversity of deep-sea ferromanganese crust field in the Rio Grande Rise, Southwestern Atlantic Ocean. *Microb. Ecol.* 82, 344–355. doi: 10.1007/s00248-020-01670-y

Cai, M., Wang, L., Cai, H., Li, Y., Wang, Y. N., Tang, Y. Q., et al. (2011). Salinarimonas ramus sp. nov. and Tessaracoccus oleiagri sp. nov., isolated from a crude oil-contaminated saline soil. *Int. J. Syst. Evol. Microbiol.* 61, 1767–1775. doi: 10.1099/ijs.0.025932-0

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Ciobanu, M. C., Burgaud, G., Dufresne, A., Breuker, A., Rédou, V., Maamar, S. B., et al. (2014). Microorganisms persist at record depths in the subseafloor of the Canterbury Basin. *ISME J.* 8, 1370–1380. doi: 10.1038/ismej.2013.250

Cockell, C. S., Voytek, M. A., Gronstal, A. L., Finster, K., Kirshtein, J. D., Howard, K., et al. (2012). Impact disruption and recovery of the deep subsurface biosphere. *Astrobiology* 12, 231–246. doi: 10.1089/ast.2011.0722

Cowen, J. P., Giovannoni, S. J., Kenig, F., Johnson, H. P., Butterfield, D., Rappé, M. S., et al. (2003). Fluids from aging ocean crust that support microbial life. *Science* 299, 120–123. doi: 10.1126/science.1075653

D'Hondt, S., Jørgensen, B. B., Miller, D. J., Batzke, A., Blake, R., Cragg, B. A., et al. (2004). Distributions of microbial activities in deep subseafloor sediments. *Science* 306, 2216–2221. doi: 10.1126/science.1101155

Daae, F. L., Økland, I., Dahle, H., Jørgensen, S. L., Thorseth, I. H., and Pedersen, R. B. (2013). Microbial life associated with low-temperature alteration of ultramafic rocks in the Leka ophiolite complex. *Geobiology* 11, 318–339. doi: 10.1111/gbi.12035

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., and Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 6:226. doi: 10.1186/s40168-018-0605-2

Diehl, H., and Horchak-Morris, N. (1987). Studies on fluorescein-V The absorbance of fluorescein in the ultraviolet, as a function of pH. *Talanta* 34, 739–741. doi: 10.1016/0039-9140(87)80232-1

Direito, S. O. L., Marees, A., and Röling, W. F. M. (2012). Sensitive life detection strategies for low-biomass environments: optimizing extraction of nucleic acids adsorbing to terrestrial and Mars analogue minerals. *FEMS Microbiol. Ecol.* 81, 111–123. doi: 10.1111/j.1574-6941.2012.01325.x

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6

Edwards, K. J., Bach, W., and McCollom, T. M. (2005). Geomicrobiology in oceanography: microbe-mineral interactions at and below the seafloor. *Trends Microbiol.* 13, 449–456. doi: 10.1016/j.tim.2005.07.005

Edwards, K. J., Fisher, A. T., and Wheat, C. G. (2012). The deep subsurface biosphere in igneous ocean crust: frontier habitats for microbiological exploration. *Front. Microbiol.* 3:8. doi: 10.3389/fmicb.2012.00008

Fisk, M. R., Storrie-Lombardi, M. C., Douglas, S., Popa, R., McDonald, G., and Di Meo-Savoie, C. (2003). Evidence of biological activity in Hawaiian subsurface basalts. *Geochem. Geophys. Geosyst*, 12:1103. doi: 10.1029/2002GC000387

Friese, A., Kallmeyer, J., Kitte, J. A., Martínez, I. M., Bijaksana, S., and Wagner, D. (2017). A simple and inexpensive technique for assessing contamination during drilling operations. *Limnol. Oceanogr. Methods* 15, 200–211. doi: 10.1002/lom3.10159

Furnes, H., and Staudigel, H. (1999). Biological mediation in ocean crust alteration: how deep is the deep biosphere? *Earth Planet. Sci. Lett.* 166, 97–103. doi: 10.1016/S0012-821X(99)00005-9

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., and Egozcue, J. J. (2017). Microbiome datasets are compositional: And this is not optional. *Front. Microbiol.* 8:2224. doi: 10.3389/fmicb.2017.02224

Gold, T. (1992). The deep, hot biosphere. *Proc. Natl. Acad. Sci. U. S. A.* 89, 6045–6049. doi: 10.1073/pnas.89.13.6045

Hao, L., McIlroy, S. J., Kirkegaard, R. H., Karst, S. M., Fernando, W. E. Y., Aslan, H., et al. (2018). Novel prosthecate bacteria from the candidate phylum Acetothermia. *ISME J.* 12, 2225–2237. doi: 10.1038/s41396-018-0187-9

Heberling, C., Lowell, R. P., Liu, L., and Fisk, M. R. (2010). Extent of the microbial biosphere in the oceanic crust. *Geochem. Geophys. Geosyst.* 8, 1525–2027. doi: 10.1029/2009GC002968

Herrera, A., and Cockell, C. (2007). Exploring microbial diversity in volcanic environments: A review of methods in DNA extraction. *J. Microbiol. Method.* 70, 1–12. doi: 10.1016/j.mimet.2007.04.005

Inagaki, F., Hinrichs, K. U., Kubo, Y., Bihan, M., Bowden, S. A., Bowles, M., et al. (2016). IODP expedition 337: deep coalbed biosphere off Shimokita – microbial processes and hydrocarbon system associated with deeply buried coalbed in the ocean. *Sci. Drill.* 21, 17–28. doi: 10.5194/sd-21-17-2016

Inagaki, F., Hinrichs, K. U., Kubo, Y., Bowles, M. W., Heuer, V. B., Hong, W. L., et al. (2015). Exploring deep microbial life in coal-bearing sediment down to ~2.5 km below the ocean floor. *Science* 349, 420–424. doi: 10.1126/science.aaa6882

Jackson, M. D. (2020). Petrograpic and material observations of basaltic lapilli tuff, 1979 and 2017 Surtsey drill cores, Iceland. *Surtsey Res.* 14, 47–62. doi: 10.33112/surtsey.14.4

Jackson, M. D., Couper, S., Stan, C. V., Ivarsson, M., Czabaj, M. W., Tamura, N., et al. (2019a). Authigenic mineral texture in submarine 1979 basalt drill Core, Surtsey volcano, Iceland. *Geochem. Geophys. Geosyst.* 20, 3751–3773. doi: 10.1029/2019GC008304

Jackson, M. D., Gudmundsson, M. T., Bach, W., Cappelletti, P., Coleman, N. J., Ivarsson, M., et al. (2015). Time-lapse characterization of hydrothermal seawater and microbial interactions with basaltic tephra at Surtsey volcano. *Sci. Drill.* 20, 51–58. doi: 10.5194/sd-20-51-2015

Jackson, M. D., Gudmundsson, M. T., Weisenberger, T. B., Michael Rhodes, J., Stefánsson, A., Kleine, B. I., et al. (2019b). SUSTAIN drilling at Surtsey volcano, Iceland, tracks hydrothermal and microbiological interactions in basalt 50 years after eruption. *Sci. Drill.* 25, 35–46. doi: 10.5194/sd-25-35-2019

Jacobson Meyers, M. E., Sylvan, J. B., and Edwards, K. J. (2014). Extracellular enzyme activity and microbial diversity measured on seafloor exposed basalts from Loihi seamount indicate the importance of basalts to global biogeochemical cycling. *Appl. Environ. Microbiol.* 80, 4854–4864. doi: 10.1128/AEM.01038-14

Jakobsson, S. P., and Moore, J. G. (1982). The Surtsey research drilling project of 1979. *Surtsey Res.* 9, 76–93.

Jakobsson, S. P., and Moore, J. G. (1986). Hydrothermal minerals and alteration rates at Surtsey volcano, Iceland. *Geol. Soc. Am. Bull.* 97, 648–659. doi: 10.1130/0016-7606(1986)97<648:HMAARA>2.0.CO;2

Jakobsson, S. P., Thors, K., Vésteinsson, Á. T., and Ásbjörnsdóttir, L. (2009). Some aspects of the seafloor morphology at Surtsey volcano: The new multibeam bathymetric survey of 2007. *Surtsey Res.* 12,9–20.

Jian, C., Luukkonen, P., Yki-Järvinen, H., Salonen, A., and Korpela, K. (2020). Quantitative PCR provides a simple and accessible method for quantitative microbiota profiling. *PLoS One* 15:e0227285. doi: 10.1371/journal.pone.0227285

Jørgensen, S. L., and Zhao, R. (2016). Microbial inventory of deeply buried oceanic crust from a young ridge flank. *Front. Microbiol.* 7:820. doi: 10.3389/fmicb.2016.00820

Jungbluth, S. P., Bowers, R. M., Lin, H. T., Cowen, J. P., and Rappé, M. S. (2016). Novel microbial assemblages inhabiting crustal fluids within mid-ocean ridge flank subsurface basalt. *ISME J.* 10, 2033–2047. doi: 10.1038/ismej.2015.248

Jungbluth, S. P., del Rio, T. G., Tringe, S. G., Stepanauskas, R., and Rappé, M. S. (2017). Genomic comparisons of a bacterial lineage that inhabits both marine and terrestrial deep subsurface systems. *PeerJ* 5:e3134. doi: 10.7717/peerj.3134

Jungbluth, S. P., Grote, J., Lin, H. T., Cowen, J. P., and Rappé, M. S. (2013). Microbial diversity within basement fluids of the sediment-buried Juan de Fuca Ridge flank. *ISME J.* 7, 161–172. doi: 10.1038/ismej.2012.73

Jungbluth, S. P., Lin, H. T., Cowen, J. P., Glazer, B. T., and Rappé, M. S. (2014). Phylogenetic diversity of microorganisms in subseafloor crustal fluids from holes 1025C and 1026B along the Juan de Fuca Ridge flank. *Front. Microbiol.* 5:119. doi: 10.3389/fmicb.2014.00119

Kallmeyer, J. (2017). Contamination control for scientific drilling operations. *Adv. Appl. Microbiol.* 98, 61–91. doi: 10.1016/bs.aambs.2016.09.003

Kallmeyer, J., Mangelsdorf, K., Cragg, B., and Horsfield, B. (2006). Techniques for contamination assessment during drilling for terrestrial subsurface sediments. *Geomicrobiol J.* 23, 227–239. doi: 10.1080/01490450600724258

Karner, M. B., Delong, E. F., and Karl, D. M. (2001). Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* 409, 507–510. doi: 10.1038/35054051

Kashefi, K., and Lovley, D. R. (2003). Extending the upper temperature limit for life. *Science* 301:934. doi: 10.1126/science.1086823

Kelly, L. C., Cockell, C. S., Thorsteinsson, T., Marteinsson, V., and Stevenson, J. (2014). Pioneer microbial communities of the Fimmvörðuháls lava flow, Eyjafjallajökull, Iceland. *Microb. Ecol.* 68, 504–518. doi: 10.1007/s00248-014-0432-3

Kern, T., Linge, M., and Rother, M. (2015). Methanobacterium Aggregans sp. nov., a hydrogenotrophic methanogenic archaeon isolated from an anaerobic digester. *Int. J. Syst. Evol. Microbiol.* 65, 1975–1980. doi: 10.1099/ijs.0.000210

Kieft, T. L. (2010). "Sampling the deep sub-surface using drilling and coring techniques," in *Handbook of Hydrocarbon and Lipid Microbiology.* ed. K. N. Timmis (Berlin/Heidelberg: Springer).

Kleine, B. I., Stefánsson, A., Kjartansdóttir, R., Prause, S., Weisenberger, T. B., Reynolds, H. I., et al. (2020). The Surtsey volcano geothermal system: An analogue for seawater-oceanic crust interaction with implications for the elemental budget of the oceanic crust. *Chem. Geol.* 550:119702. doi: 10.1016/j.chemgeo.2020.119702

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808

Konhauser, K. O., Schiffman, P., and Fisher, Q. J. (2002). Microbial mediation of authigenic clays during hydrothermal alteration of basaltic tephra, Kilauea volcano. *Geochem. Geophys. Geosyst.* 3, 1–13. doi: 10.1029/2002GC000317

Korzhenkov, A. A., Teplyuk, A. V., Lebedinsky, A. V., Khvashchevskaya, A. A., Kopylova, Y. G., Arakchaa, K. D., et al. (2018). Members of the uncultured taxon OP1 ("Acetothermia") predominate in the microbial community of an alkaline hot spring at East-Tuvinian upland. *Microbiol.* 87, 783–795. doi: 10.1134/S0026261718060115

Lee, M. D., Walworth, N. G., Sylvan, J. B., Edwards, K. J., and Orcutt, B. N. (2015). Microbial communities on seafloor basalts at Dorado Outcrop reflect level of alteration and highlight global lithic clades. *Front. Microbiol.* 6:1470. doi: 10.3389/fmicb.2015.01470

Lever, M. A., Alperin, M., Engelen, B., Inagaki, F., Nakagawa, S., Steinsbu, B. O., et al. (2006). Trends in basalt and sediment core contamination during IODP expedition 301. *Geomicrobiol J.* 23, 517–530. doi: 10.1080/01490450600897245

Lever, M. A., Rogers, K. L., Lloyd, K. G., Overmann, J., Schink, B., Thauer, R. K., et al. (2015). Life under extreme energy limitation: A synthesis of laboratory- and field-based investigations. *FEMS Microbiol. Rev.* 39, 688–728. doi: 10.1093/femsre/fuv020

Lever, M. A., Rouxel, O., Alt, J. C., Shimizu, N., Ono, S., Coggon, R. M., et al. (2013). Evidence for microbial carbon and sulfur cycling in deeply buried ridge flank basalt. *Science* 339, 1305–1308. doi: 10.1126/science.1229240

Li, J., Mara, P., Schubotz, F., Sylvan, J. B., Burgaud, G., Klein, F., et al. (2020). Recycling and metabolic flexibility dictate life in the lower oceanic crust. *Nature* 579, 250–255. doi: 10.1038/s41586-020-2075-5

Liu, J. H., Wang, Y. X., Zhang, X. X., Wang, Z. G., Chen, Y. G., Wen, M. L., et al. (2010). Salinarimonas rosea gen. nov., sp. nov., a new member of

the α-2 subgroup of the proteobacteria. *Int. J. Syst. Evol. Microbiol.* 60, 55–60. doi: 10.1099/ijs.0.006981-0

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Lysnes, K., Torsvik, T., Thorseth, I.H., and Pedersen, R.B. (2004). "Microbial populations in ocean floor basalt: Results from ODP leg 187." in *Proc. ODP, Sci. Results. eds*. R. B. Pedersen, D. M. Christie and D. J. Miller, 187.

Magnabosco, C., Lin, L. H., Dong, H., Bomberg, M., Ghiorse, W., Stan-Lotter, H., et al. (2018). The biomass and biodiversity of the continental subsurface. *Nat. Geosci.* 11, 707–717. doi: 10.1038/s41561-018-0221-6

Magnússon, B., Magnússon, S. H., Ólafsson, E., and Sigurdsson, B. D. (2014). Plant colonization, succession and ecosystem development on Surtsey with reference to neighbouring islands. *Biogeosciences* 11, 5521–5537. doi: 10.5194/bg-11-5521-2014

Marteinsson, V., Klonowski, A., Reynisson, E., Vannier, P., Sigurdsson, B. D., and Ólafsson, M. (2015). Microbial colonization in diverse surface soil types in Surtsey and diversity analysis of its subsurface microbiota. *Biogeosciences* 12, 1191–1203. doi: 10.5194/bg-12-1191-2015

Martinez Arbizu, P. (2020). pairwiseAdonis: pairwise multilevel comparison using Adonis. *R Packag version 04.* Available at: https://github.com/pmartinezarbizu/pairwiseAdonis

Mason, O. U., Nakagawa, T., Rosner, M., van Nostrand, J. D., Zhou, J., Maruyama, A., et al. (2010). First investigation of the microbiology of the deepest layer of ocean crust. *PLoS One* 5:e15399. doi: 10.1371/journal.pone.0015399

Mason, O. U., Stingl, U., Wilhelm, L. J., Moeseneder, M. M., Di Meo-Savoie, C. A., Fisk, M. R., et al. (2007). The phylogeny of endolithic microbes associated with marine basalts. *Environ. Microbiol.* 9, 2539–2550. doi: 10.1111/j.1462-2920.2007.01372.x

Mcloughlin, N., Staudigel, H., Furnes, H., Eickmann, B., and Ivarsson, M. (2010). Mechanisms of microtunneling in rock substrates: distinguishing endolithic biosignatures from abiotic microtunnels. *Geobiology* 8, 245–255. doi: 10.1111/j.1472-4669.2010.00243.x

Mcmahon, S., and Parnell, J. (2014). Weighing the deep continental biosphere. *FEMS Microbiol. Ecol.* 87, 113–120. doi: 10.1111/1574-6941.12196

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

McPhie, J., White, J. D. L., Gorny, C., Jackson, M. D., Gudmundsson, M. T., and Couper, S. (2020). Lithofacies from the 1963-1967 Surtsey eruption in SUSTAIN drill cores SE-2a, SE-2b and SE-03. *Surtsey Res.* 14, 19–32. doi: 10.33112/surtsey.14.2

Merino, N., Kawai, M., Boyd, E. S., Colman, D. R., McGlynn, S. E., Nealson, K. H., et al. (2020). Single-cell genomics of novel actinobacteria with the Wood–Ljungdahl pathway discovered in a serpentinizing system. *Front. Microbiol.* 11:1031. doi: 10.3389/fmicb.2020.01031

Minich, J. J., Sanders, J. G., Amir, A., Humphrey, G., Gilbert, J. A., and Knight, R. (2019). Quantifying and understanding well-to-well contamination in microbiome research. *mSystems.* 4:e00186-19. doi: 10.1128/mSystems.00186-19

Moore, J. C., and Jackson, M. D. (2020). Observations on the structure of Surtsey. *Surtsey Res.* 14, 33–45. doi: 10.33112/surtsey.14.3

Motamedi, S., Orcutt, B. N., Früh-Green, G. L., Twing, K. I., Pendleton, H. L., and Brazelton, W. J. (2020). Microbial residents of the Atlantis massif's shallow serpentinite subsurface. *Appl. Environ. Microbiol.* 86:e00356-20. doi: 10.1128/AEM.00356-20

Nakagawa, S., Inagaki, F., Suzuki, Y., Steinsbu, B. O., Lever, M. A., Takai, K., et al. (2006). Microbial community in black rust exposed to hot ridge flank crustal fluids. *Appl. Environ. Microbiol.* 72, 6789–6799. doi: 10.1128/AEM.01238-06

Neufeld, J. D., Schäfer, H., Cox, M. J., Boden, R., McDonald, I. R., and Murrell, J. C. (2007). Stable-isotope probing implicates Methylophaga spp and novel Gammaproteobacteria in marine methanol and methylamine metabolism. *ISME J.* 1, 480–491. doi: 10.1038/ismej.2007.65

Nigro, L. M., Harris, K., Orcutt, B. N., Hyde, A., Clayton-Luce, S., Becker, K., et al. (2012). Microbial communities at the borehole observatory on the Costa Rica Rift flank (ocean drilling program hole 896A). *Front. Microbiol.* 3:232. doi: 10.3389/fmicb.2012.00232

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2007). The vegan package. *Commun. Ecol. Packag* 10, 631–637.

Ólafsson, M., and Jakobsson, S. P. (2009). Chemical composition of hydrothermal water and water-rock interactions on Surtsey volcanic island: A preliminary report. *Surtsey Res*, 12, 29–38.

Orcutt, B. N., Bach, W., Becker, K., Fisher, A. T., Hentscher, M., Toner, B. M., et al. (2011b). Colonization of subsurface microbial observatories deployed in young ocean crust. *ISME J.* 5, 692–703. doi: 10.1038/ismej.2010.157

Orcutt, B. N., Bergenthal, M., Freudenthal, T., Smith, D., Lilley, M. D., Schnieders, L., et al. (2017). Contamination tracer testing with seabed drills: IODP expedition 357. *Sci. Drill.* 23, 39–46. doi: 10.5194/sd-23-39-2017

Orcutt, B.N., Daniel, I., and Dasgupta, R. (2019). Book deep carbon: past to present. Cambridge: Cambridge University Press.

Orcutt, B. N., Sylvan, J. B., Knab, N. J., and Edwards, K. J. (2011a). Microbial ecology of the Dark Ocean above, at, and below the seafloor. *Microbiol. Mol. Biol. Rev.* 75, 361–422. doi: 10.1128/MMBR.00039-10

Orcutt, B. N., Sylvan, J. B., Rogers, D. R., Delaney, J., Lee, R. W., and Girguis, P. R. (2015). Carbon fixation by basalt-hosted microbial communities. *Front. Microbiol.* 6:904. doi: 10.3389/fmicb.2015.00904

Orcutt, B. N., Wheat, C. G., Rouxel, O., Hulme, S., Edwards, K. J., and Bach, W. (2013). Oxygen consumption rates in subseafloor basaltic crust derived from a reaction transport model. *Nat. Commun.* 4:2539. doi: 10.1038/ncomms3539

Óskarsson, B. V., Jónasson, K., Valsson, G., and Belart, J. M. C. (2020). Erosion and sedimentation in Surtsey island quantified from new DEMs. *Surtsey Res.* 4, 63–77. doi: 10.33112/surtsey.14.5

Øvreås, L., Forney, L., Daae, F. L., and Torsvik, V. (1997). Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* 63, 3367–3373. doi: 10.1128/aem.63.9.3367-3373.1997

Parada, A. E., Needham, D. M., and Fuhrman, J. A. (2016). Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ. Microbiol.* 18, 1403–1414. doi: 10.1111/1462-2920.13023

Parkes, J. R., Cragg, B. A., and Wellsbury, P. (2000). Recent studies on bacterial populations and processes in subseafloor sediments. *A review. Hydrogeol. J.* 8, 11–28. doi: 10.1007/PL00010971

Pedersen, K. (2000). Exploration of deep intraterrestrial microbial life: current perspectives. *FEMS Microbiol. Lett.* 185, 9–16. doi: 10.1111/j.1574-6968.2000.tb09033.x

Pellizzari, L., Neumann, D., Alawi, M., Voigt, D., Norden, B., and Würdemann, H. (2013). The use of tracers to assess drill-mud penetration depth into sandstone cores during deep drilling: method development and application. *Environ. Earth Sci.* 70, 3727–3738. doi: 10.1007/s12665-013-2715-2

Pollock, J., Glendinning, L., Wisedchanwet, T., and Watson, M. (2018). The madness of microbiome: attempting to find consensus "best practice" for 16S microbiome studies. *Appl. Environ. Microbiol.* 84:e02627-17. doi: 10.1128/AEM.02627-17

Prause, S., Weisenberger, T. B., Cappelletti, P., Grimaldi, C., Rispoli, C., Jónasson, K., et al. (2020). Alteration progress within the Surtsey hydrothermal system, SW Iceland – A time-lapse petrographic study of cores drilled in 1979and 2017. *J. Volcanol. Geotherm. Res.* 392:106754. doi: 10.1016/j.jvolgeores.2019.106754

Prieur, D., and Marteinsson, V.T. (1998) "Prokaryotes living under elevated hydrostatic pressure," in *Biotechnology of Extremophiles. Advances in Biochemical Engineering/Biotechnology. Vol 61*. ed. G. Antranikian (Berlin, Heidelberg: Springer).

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

R Core Team (2019). R: A language and environment for statistical computing. *R Found Stat Comput*. Vienna. Available at: https://www.R-project.org/

Ramírez, G. A., Garber, A. I., Lecoeuvre, A., D'angelo, T., Wheat, C. G., and Orcutt, B. N. (2019). Ecology of subseafloor crustal biofilms. *Front. Microbiol.* 10:1983. doi: 10.3389/fmicb.2019.01983

Rathsack, K., Stackebrandt, E., Reitner, J., and Schumann, G. (2009). Microorganisms isolated from deep sea low-temperature influenced oceanic crust basalts and sediment samples collected along the Mid-Atlantic Ridge. *Geomicrobiol J.* 26, 264–274. doi: 10.1080/01490450902892456

Robador, A., Jungbluth, S. P., LaRowe, D. E., Bowers, R. M., Rappé, M. S., Amend, J. P., et al. (2015). Activity and phylogenetic diversity of sulfate-

reducing microorganisms in low-temperature subsurface fluids within the upper oceanic crust. *Front. Microbiol.* 5:748. doi: 10.3389/fmicb.2014.00748

Rstudio, T. (2020) *RStudio: Integrated Development for R*. Rstudio Team, PBC, Boston, MA. Available at: Available at: http://www.rstudio.com/

Santelli, C. M., Banerjee, N., Bach, W., and Edwards, K. J. (2010). Tapping the subsurface ocean crust biosphere: low biomass and drilling-related contamination calls for improved quality controls. *Geomicrobiol J.* 27, 158–169. doi: 10.1080/01490450903456780

Santelli, C. M., Edgcomb, V. P., Bach, W., and Edwards, K. J. (2009). The diversity and abundance of bacteria inhabiting seafloor lavas positively correlate with rock alteration. *Environ. Microbiol.* 11, 86–98. doi: 10.1111/j.1462-2920.2008.01743.x

Santelli, C. M., Orcutt, B. N., Banning, E., Bach, W., Moyer, C. L., Sogin, M. L., et al. (2008). Abundance and diversity of microbial life in ocean crust. *Nature* 453, 653–656. doi: 10.1038/nature06899

Schipper, C. I., Le Voyer, M., Moussallam, Y., White, J. D. L., Thordarson, T., Kimura, J. I., et al. (2016). Degassing and magma mixing during the eruption of Surtsey volcano (Iceland, 1963–1967): the signatures of a dynamic and discrete rift propagation event. *Bull. Volcanol.* 78:33. doi: 10.1007/s00445-016-1025-4

Sheik, C. S., Reese, B. K., Twing, K. I., Sylvan, J. B., Grim, S. L., Schrenk, M. O., et al. (2018). Identification and removal of contaminant sequences from ribosomal gene databases: lessons from the census of deep life. *Front. Microbiol.* 9:840. doi: 10.3389/fmicb.2018.00840

Singer, E., Chong, L. S., Heidelberg, J. F., and Edwards, K. J. (2015). Similar microbial communities found on two distant seafloor basalts. *Front. Microbiol.* 6:1409. doi: 10.3389/fmicb.2015.01409

Smith, A., Popa, R., Fisk, M., Nielsen, M., Wheat, C. G., Jannasch, H. W., et al. (2011). In situ enrichment of ocean crust microbes on igneous minerals and glasses using an osmotic flow-through device. *Geochem. Geophys. Geosyst.* 6, 1525–2027. doi: 10.1029/2010GC003424

Sorokin, D. Y., Lysenko, A. M., Mityushina, L. L., Tourova, T. P., Jones, B. E., Rainey, F. A., et al. (2001). Thioalkalimicrobium aerophilum gen. nov., sp. nov. and Thioalkalimicrobium sibericum sp. nov., and Thioalkalivibrio versutus gen. nov., sp. nov., Thioalkalivibrio nitratis sp. nov. and Thioalkalivibrio denitrificans sp. nov., novel obligately alkaliphilic. *Int. J. Syst. Evol. Microbiol.* 51, 565–580. doi: 10.1099/00207713-51-2-565

Sousa, D. Z., Visser, M., Van Gelder, A. H., Boeren, S., Pieterse, M. M., Pinkse, M. W. H., et al. (2018). The deep-subsurface sulfate reducer Desulfotomaculum kuznetsovii employs two methanol-degrading pathways. *Nat. Commun.* 9:239. doi: 10.1038/s41467-017-02518-9

Spanevello, M. D., Yamamoto, H., and Patel, B. K. C. (2002). Thermaerobacter subterraneus sp. nov., a novel aerobic bacterium from the Great Artesian Basin of Australia, and emendation of the genus Thermaerobacter. *Int. J. Syst. Evol. Microbiol.* doi: 10.1099/ijs.0.01959-0

Sriswasdi, S., Yang, C. C., and Iwasaki, W. (2017). Generalist species drive microbial dispersion and evolution. *Nat. Commun.* 8:1162. doi: 10.1038/s41467-017-01265-1

Staudigel, H., Furnes, H., McLoughlin, N., Banerjee, N. R., Connell, L. B., and Templeton, A. (2008). 3.5 billion years of glass bioalteration: volcanic rocks as a basis for microbial life? *Earth Sci. Rev.* 89, 156–176. doi: 10.1016/j.earscirev.2008.04.005

Sudek, L. A., Wanger, G., Templeton, A. S., Staudigel, H., and Tebo, B. M. (2017). Submarine basaltic glass colonization by the heterotrophic Fe(II)-oxidizing and siderophore-producing deep-sea bacterium pseudomonas stutzeri VS-10: The potential role of basalt in enhancing growth. *Front. Microbiol.* 8:363. doi: 10.3389/fmicb.2017.00363

Suzuki, Y., Yamashita, S., Kouduka, M., Ao, Y., Mukai, H., Mitsunobu, S., et al. (2020). Deep microbial proliferation at the basalt interface in 33.5–104 million-year-old oceanic crust. *Commun. Biol.* 3:136. doi: 10.1038/s42003-020-0860-1

Takai, K., and Horikoshi, K. (2000). Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Appl. Environ. Microbiol.* 66, 5066–5072. doi: 10.1128/AEM.66.11.5066-5072.2000

Takai, K., Inoue, A., and Horikoshi, K. (1999). Thermaerobacter marianensis gen. nov., sp. nov., an aerobic extremely thermophilic marine bacterium from the 11 000 m deep Mariana Trench. *Int. J. Syst. Bacteriol.* 2, 619–628. doi: 10.1099/00207713-49-2-619

Takai, K., Nakamura, K., Toki, T., Tsunogai, U., Miyazaki, M., Miyazaki, J., et al. (2008). Cell proliferation at 122°C and isotopically heavy CH4 production by a hyperthermophilic methanogen under high-pressure cultivation. *Proc. Natl. Acad. Sci. U. S. A.* 105, 10949–10954. doi: 10.1073/pnas.0712334105

Takami, H., Noguchi, H., Takaki, Y., Uchiyama, I., Toyoda, A., Nishi, S., et al. (2012). A deeply branching thermophilic bacterium with an ancient acetyl-CoA pathway dominates a subsurface ecosystem. *PLoS One* 7:e30559. doi: 10.1371/journal.pone.0030559

Templeton, A. S., Staudigel, H., and Tebo, B. M. (2005). Diverse Mn(II)-oxidizing bacteria isolated from submarine basalts at Loihi seamount. *Geomicrobiol J.* 22, 127–139. doi: 10.1080/01490450590945951

Þórarinsson, S. (1965). The Surtsey eruption: course of events and the development of the new island. *Surtsey Res* 1, 51–55.

Thorseth, I. H., Pedersen, R. B., and Christie, D. M. (2003). Microbial alteration of 0-30-Ma seafloor and sub-seafloor basaltic glasses from the Australian Antarctic Discordance. *Earth Planet. Sci. Lett.* 215, 237–247. doi: 10.1016/S0012-821X(03)00427-8

Thorseth, I. H., Torsvik, T., Torsvik, V., Daae, F. L., and Pedersen, R. B. (2001). Diversity of life in ocean floor basalt. *Earth Planet. Sci. Lett.* 194, 31–37. doi: 10.1016/S0012-821X(01)00537-4

Tin, S., Bizzoco, R. W., and Kelley, S. T. (2011). Role of the terrestrial subsurface in shaping geothermal spring microbial communities. *Environ. Microbiol. Rep.* 3, 491–499. doi: 10.1111/j.1758-2229.2011.00248.x

Walton, A. W. (2008). Microtubules in basalt glass from Hawaii Scientific Driling Project #2 phase 1 core and Hilina slope, Hawaii: evidence of the occurrence and behavior of endolithic microorganisms. *Geobiology* 6, 351–364. doi: 10.1111/j.1472-4669.2008.00149.x

Watanabe, M., Kojima, H., and Fukui, M. (2018). Review of Desulfotomaculum species and proposal of the genera Desulfallas gen. Nov., Desulfofundulus gen. nov., Desulfofarcimen gen. nov. and Desulfohalotomaculum gen. nov. *Int. J. Syst. Evol. Microbiol.* 68, 2891–2899. doi: 10.1099/ijsem.0.002915

Weisenberger, T. B., Gudmundsson, M. T., Jackson, M. D., Türke, A., Kleine, B. I., and Marshall, B. (2019). Operational report for the 2017 Surtsey underwater volcanic system for thermophiles, alteration processes and INnovative concretes (SUSTAIN) drilling project at Surtsey volcano, Iceland. *Geo. Forschungs Zent Rep.* Available at: https://gfzpublic.gfz-potsdam.de/rest/items/item_4342891_3/component/file_4342889/content

Yanagawa, K., Nunoura, T., McAllister, S. M., Hirai, M., Breuker, A., Brandt, L., et al. (2013). The first microbiological contamination assessment by deep-sea drilling and coring by the D/V chikyu at the Iheya North hydrothermal field in the Mid-Okinawa trough (IODP Expedition 331). *Front. Microbiol.* 4:327. doi: 10.3389/fmicb.2013.00327

Zaitseva, S., Lavrentieva, E., Radnagurueva, A., Baturina, O., Kabilov, M., and Barkhutova, D. (2017) Distribution of Acetothermia-dominated microbial communities in alkaline hot springs of Baikal Rift Zone. *Peer. J. Preprints.* 5:e3492v1. doi: 10.7287/peerj.preprints.3492v1

Zhang, X., Feng, X., and Wang, F. (2016). Diversity and metabolic potentials of subsurface crustal microorganisms from the western flank of the Mid-Atlantic Ridge. *Front. Microbiol.* 7:363. doi: 10.3389/fmicb.2016.02094

Zhao, R., Dahle, H., Ramírez, G. A., and Jørgensen, S. L. (2020). Indigenous ammonia-oxidizing archaea in oxic subseafloor oceanic crust. *mSystems.* 5:e00758-19. doi: 10.1128/mSystems.00758-19

Zhu, H., Derksen, R. C., Krause, C. R., Fox, R. D., Brazee, R. D., and Ozkan, H. E. (2005). Fluorescent intensity of dye solutions under different pH conditions. *J. ASTM Int.* 2:12926. doi: 10.1520/JAI12926

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Paper II

Culturable Bacterial Diversity from the Basaltic Subsurface of the Young Volcanic Island of Surtsey, Iceland.

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Accepted in *Microorganisms*, section *Environmental Microbiology*, special issue *Extremophilic Microorganisms and Their Communities* (2022) *Volume 10*.

Candidate contribution:
Conceived and designed the study, performed fieldwork, performed laboratory work, analyzed data, wrote the manuscript, reviewed and edited the manuscript and corresponded during the publication process.

microorganisms

MDPI

Article

Culturable Bacterial Diversity from the Basaltic Subsurface of the Young Volcanic Island of Surtsey, Iceland

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Abstract: The oceanic crust is the world's largest and least explored biosphere on Earth. The basaltic subsurface of Surtsey island in Iceland represents an analog of the warm and newly formed-oceanic crust and offers a great opportunity for discovering novel microorganisms. In this study, we collected borehole fluids, drill cores, and fumarole samples to evaluate the culturable bacterial diversity from the subsurface of the island. Enrichment cultures were performed using different conditions, media and temperatures. A total of 195 bacterial isolates were successfully cultivated, purified, and identified based on MALDI-TOF MS analysis and by 16S rRNA gene sequencing. Six different clades belonging to Firmicutes (40%), Gammaproteobacteria (28.7%), Actinobacteriota (22%), Bacteroidota (4.1%), Alphaproteobacteria (3%), and Deinococcota (2%) were identified. *Bacillus* (13.3%) was the major genus, followed by *Geobacillus* (12.33%), *Enterobacter* (9.23%), *Pseudomonas* (6.15%), and *Halomonas* (5.64%). More than 13% of the cultured strains potentially represent novel species based on partial 16S rRNA gene sequences. Phylogenetic analyses revealed that the isolated strains were closely related to species previously detected in soil, seawater, and hydrothermal active sites. The 16S rRNA gene sequences of the strains were aligned against Amplicon Sequence Variants (ASVs) from the previously published 16S rRNA gene amplicon sequence datasets obtained from the same samples. Compared with the culture-independent community composition, only 5 out of 49 phyla were cultivated. However, those five phyla accounted for more than 80% of the ASVs. Only 121 out of a total of 5642 distinct ASVs were culturable ($\geq$98.65% sequence similarity), representing less than 2.15% of the ASVs detected in the amplicon dataset. Here, we support that the subsurface of Surtsey volcano hosts diverse and active microbial communities and that both culture-dependent and -independent methods are essential to improving our insight into such an extreme and complex volcanic environment.

Keywords: oceanic subsurface; culturable microbial diversity; bacteria; extreme environment; Surtsey; Iceland

Citation: Bergsten, P.; Vannier, P.; Frion, J.; Mougeolle, A.; Marteinsson, V.Þ. Culturable Bacterial Diversity from the Basaltic Subsurface of the Young Volcanic Island of Surtsey, Iceland. *Microorganisms* **2022**, *10*, 1177. https://doi.org/10.3390/microorganisms10061177

Academic Editors: Olga V. Golyshina and Michail M. Yakimov

Received: 16 May 2022
Accepted: 6 June 2022
Published: 8 June 2022

1. Introduction

The oceanic crust represents the largest habitable environment on Earth and one of the least explored ecosystems on our planet [1]. Numerous extremophiles were previously reported in crustal fluids, including thermophilic fermenters, sulfate reducers, and methanogens [2–5]. Despite the undeniable existence of microbial activity in the oceanic crust [4–11], little is known about the diversity of microbial species from such environments. Advances in molecular techniques, high-throughput sequencing technologies, and bioinformatics have led to major improvements in exploring microbial communities in their environment and provided tools for studying the diversity, distributions, and activities of microorganisms without the need to cultivate them [12]. Such methods resulted in

the discovery and characterization of rare biospheres in the most extreme environments, giving precious clues to the metabolic potential and activity of many yet-to-be cultivated microorganisms [12–15]. Nevertheless, cultivation-dependent methods still remain the best tool for studying a microorganism's physiology, metabolism, and ecology in ways that cannot be achieved using culture-independent approaches (e.g., [16]). Furthermore, cultivation can offer complementary insights into an ecosystem in combination with molecular tools, although the culturable microorganisms represent a minor component of the actual microbial community [17–20]. This includes the ecological role of the culturable microorganisms in the environment, their physiological limits, and their adaptive capacities to extreme conditions.

The island Surtsey is a rift zone volcano located in the south of Iceland that emerged from the seafloor between 1963 and 1967 [21–23]. In 1979, a cored borehole (SE-01) was drilled through the young volcano for geological exploration [22,24]. The active hydrothermal-seawater system discovered passes through the subsurface deposits, in which the maximal temperature of 124.6 °C in 2017 exceeded the presumed upper limit for functional life [22,25–28]. Thirty-eight years later, three new cored boreholes (SE-02a, SE-02b, and SE-03) were drilled specifically for microbiological and geological analysis [29–31]. Over the years, many studies have explored the Surtsey edifice in terms of geology, mineral, and chemical composition, suggesting that fluid–rock interactions in the submarine Surtsey basaltic deposits behave similarly to basaltic oceanic crust [32–36]. The Surtsey volcano geothermal system can be considered as analog for seawater–oceanic crust interactions that occur at seamounts and in ridge flank systems without the presence of overlying sediments [33]. Surtsey thus serves as a unique natural laboratory for investigating fluid–rock–microbe interactions, and its boreholes can be thought of as opened windows from the surface, allowing for the examination of subsurface microbial processes at high temperatures associated with oceanic crust. Diverse bacterial and archaeal taxa were detected in the subsurface of Surtsey using high-throughput sequencing [27,37]. Although many of these taxa were previously reported in surface and subsurface habitats in both terrestrial and marine settings, many of the newly discovered clades belonged to previously unknown lineages [37].

In this study, we used both specific and non-specific culture media under various temperature conditions to enrich high diversity of microorganisms from the subsurface of Surtsey, which includes fumarole, borehole fluid, and drill core samples. The objectives were (i) to describe the subsurface culturable microbial diversity; (ii) to determine if the application of a diverse range of media, temperature, and cultivation methods can enable the isolation of subsurface microorganisms previously only detected through culture-independent analysis; and (iii) to contribute to the understanding of the cultivable diversity of extreme subsurface environments, especially in the warm and newly formed oceanic crust.

2. Materials and Methods

2.1. Study Site and Sample Collection

The sampling site, Surtsey island (63°18′10.8″ N; 20°36′16.9″ W), is located approximately 35 km from the south coast of Iceland, within the southern offshore extension of Iceland's Eastern Volcanic Zone. All samples were collected on the island between 2016 and 2018. The hydrothermal system was active with a maximal temperature of 124.6 °C at 100 m depth in 2017 [24,26–28]. Three sample types were collected for cultivation purposes (Table 1): (i) drill core samples collected at successive depths from SE-02a and SE-02b, (ii) borehole fluids from the four drill holes (SE-01, SE-02a, SE-02b, and SE-03), and (iii) condensed steam and biomass from fumarole outlets located on the two tephra cones (63°18′15.4″ N 20°36′07.7″ W and 63°18′19.9″ N 20°36′24.7″ W). All samples were immediately processed aseptically on-site and kept at 4 °C [30,37].

Drill cores were sampled during the ICDP SUSTAIN drilling operation at Surtsey in 2017 [30,37]. At the drill site, an 8 cm section was cut from every third 3 m core run at 70 cm from the top and was immediately removed from the liner, put into a sterile plastic bag,

oxygen-removed by GasPak™ (BD), and stored at 4 °C. In the laboratory, drill core samples were fragmented with a hammer into an anaerobic chamber (atmosphere: $N_2/CO_2/H_2$: 80/10/10, Coy Laboratory Inc., Grass Lake, MI, USA), and interior fractions were split into smaller pieces in a mortar. All tools for crushing were autoclaved, disinfected by ethanol, and flamed before and between each use. Two cultivation methods were tested with the drill core samples. Some basaltic samples were transferred into artificial seawater (ASW) supplemented by Wolin's vitamin solution (DSMZ, medium 141) (1X) [38]. After being shaken overnight and stored at 4 °C for sedimentation, the supernatant was used for cultivation. In addition, small pieces of basalt were directly transferred into media for enrichment. Borehole fluid samples were collected using a stainless-steel bailer, as previously described in [27]. Steam from fumaroles was collected over 12 h of continuous sampling by introducing a sterile rubber hose into the outlet of the fumarole with the other end connected to a sterile plastic container. Both borehole fluids and steam, with were condensed into water, from fumarole were aliquoted, reduced by Na_2S solution (0.05% *w*/*v* final concentration), and stored at 4 °C. In addition, biomass samples (mud and dead flies) were collected aseptically near the outlet fluxes and were stored at 4 °C in falcon tubes in anaerobic conditions using GasPak™ (BD).

Table 1. Sample collection.

Sample ID	Sample Type	Sampling Date	Collection Depth (m b.s.)	Collection Temperature (°C)
16.2	Borehole fluid	9 June 2016	166	54
16.7	Borehole fluid	9 June 2016	160	60
16.8	Borehole fluid	9 June 2016	mix	n.a.
17.1	Borehole fluid	3 August 2017	58	85
17.2	Borehole fluid	3 August 2017	120	116
17.3	Borehole fluid	3 August 2017	150	76
17.4	Borehole fluid	3 August 2017	160	52
17.5	Fumarole	5 August 2017	0	64.2–82.3
17.6	Fumarole	4 August 2017	0	40.8
17.8, 17.9, 17.F	Fumarole	4 August 2017	0	56.1–74.6
17.11	Borehole fluid	6 September 2017	140	116
17.13	Borehole fluid	6 September 2017	280	58
17.14	Borehole fluid	6 September 2017	mix	n.a.
17.15	Borehole fluid	6 September 2017	75	98
17.16	Borehole fluid	5 September 2017	60	90
17.17	Borehole fluid	5 September 2017	80	116
17.18	Borehole fluid	5 September 2017	90	122
17.19	Borehole fluid	5 September 2017	100	124
17.22	Borehole fluid	5 September 2017	160	61
17.23	Borehole fluid	5 September 2017	mix	n.a.
18.1	Borehole fluid	19 September 2018	mix	n.a.
18.2	Borehole fluid	19 September 2018	mix	n.a.
18.3	Borehole fluid	19 September 2018	mix	n.a.
B3	Drill core	10 August 2017	15	15.3
B9	Drill core	11 August 2017	32	30
B24	Drill core	12 August 2017	70	109
B30	Drill core	13 August 2017	87	121
B36	Drill core	14 August 2017	105	123
C55	Drill core	25 August 2017	156	64
C59	Drill core	25 August 2017	167	55
C62	Drill core	25 August 2017	176	44.5
C65	Drill core	25 August 2017	181	37

n.a.: not availble.

2.2. Media Preparation, Enrichment, and Strains Isolation

Seven different culture media were tested, non-selective and selective (e.g., media for methanogens, iron, sulfate, and sulfur reducers), at various temperatures (i.e., 22 °C, 40 °C, 60 °C, and 80 °C) and under both aerobic and anaerobic conditions.

Aerobic cultures were performed on plates and in liquid non-selective media for marine heterotrophs (MB: Marine Broth 514 medium, BD Difco™) at pH 7 and 9 and in medium 166, a standard medium for aerobic thermophiles [39], supplemented with 2% NaCl (Table S1). Two jellifying agents were used: agar (14 g/L, Sigma-Aldrich, Deissenhofen, Germany) for incubation temperature below 60 °C or Phytagel™ (8 g/L, Sigma-Aldrich, Deissenhofen, Germany) for incubation temperature above 60 °C.

Anaerobic cultures were prepared in liquid selective media containing modified ASW, with the addition of different substrates (solution A) (Tables S2–S5). Those media were tested with different salinities (1 to 3% (*w*/*v*) NaCl). The modified ASW basis contained per liter of distilled water: NaCl (10 to 30 g), NH_4Cl (0.5 g), $MgSO_4 \cdot 7H_2O$ (3.4 g), $MgCl_2 \cdot 6H_2O$ (4.18 g), KCl (0.33 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), $Na_2SeO_3 \cdot 5H_2O$ (1 mg), and PIPES buffer (3 g). Before being autoclaved, a few drops of resazurin (0.1% *w*/*v*) were added to the modified ASW as an indicator of O_2 variations and substrates were differentially added to the different media as follows (per liter): the medium for sulfur-reducing microorganisms (modified YPS, Table S2) contained a yeast extract (0.5 g) and peptone (0.5 g), and pH was adjusted to 7; the medium for sulfate-reducing microorganisms (SO, Table S3) was supplemented by Na_2SO_4 (0.2 g), a yeast extract (0.2 g), L-lactate (0.5 g), Na-pyruvate (0.5 g), and L-ascorbate (0.5 g), and pH was adjusted to 7.5; the medium for iron-reducing microorganisms (I, Table S4) contained Fe (III) citrate (10 g) and Na-acetate (0.1 g), and pH was adjusted to 8; and the medium for methanogens (M, Table S5) was supplemented by a yeast extract (1 g), and pH was adjusted to 7.5.

In parallel, solutions B (K_2HPO_4 at 2.8% *w*/*v*) and C ($CaCl_2 \cdot 2H_2O$ at 10% *w*/*v*) were prepared and autoclaved separately. A trace elements solution (DMSZ 141 medium) was prepared as well and was filtrated-sterilized (0.22 µm filter). After sterilization and cooling, the solutions were added to solution A as follows: 5 mL of solution B, 5 mL of solution C, and 10 mL of the trace element solution. In addition, 10 g of elemental sulfur and 10 mL of the filtrated-sterilized solution of vitamins (DMSZ 141 medium) were added into the medium YPS (Table S2); 20 mL of $NaHCO_3$ (0.1% *w*/*v*) was added into the medium SO (Table S3); 10 mL of a filtrated-sterilized solution of vitamins (DMSZ 141 medium), 20 mL of $NaHCO_3$ (0.2% *w*/*v*), and 0.25 mL of $Na_2WO_4 \cdot 2H_2O$ (0.1% *w*/*v*, N_2) were added into the medium for I (Table S4); and 10 mL of a vitamin solution (DMSZ 141 medium), 20 mL of $NaHCO_3$ (0.2% *w*/*v*), 0.5 g/L of coenzyme M (2-mercaptoethanesulfonic acid), and 5 mL of methanol were added into the medium M (Table S5).

All media were supplemented with a gas phase of H_2/CO_2 (80/20, 1.5 to 2 bars), except medium SO, which was supplemented with a gas phase of N_2 (100, 1.5 to 2 bars). All media were reduced with a sterile $Na_2S \cdot 9H_2O$ solution (0.05% *w*/*v*, pH 7, N_2) with the addition of a L-Cysteine-HCl·H_2O solution (0.05% *w*/*v*, pH 7, N_2) in the medium M.

In the dark, culture media were inoculated with 1 to 3% of the basaltic suspension, small bits of rock, fumarole biomass fragments, or 1% of fluid samples (borehole fluid and condensed water from fumarole). The uninoculated culture media were incubated for each medium and incubation temperature under the same conditions as the negative controls. After growth was observed in the enrichment cultures, the plates were directly inoculated with the enrichment culture as an inoculum. If no growth was observed on the plate after 5 days, the dilution-to-extinction technique was employed. Growth was monitored by colony observation on plates or under the microscope (Olympus BX51 at 100X/1.30 Oil pH3). Colonies with unique morphological features were selected and were streaked at least six times before being considered pure. Using the dilution-to-extinction technique for strain isolation in liquid cultures, pure cultures were obtained from the highest positive dilution tube [40].

In total, 195 isolates were added to the Icelandic Strain Collection and Records (ISCaR) and stored at −140 °C (Table 2). Glycerol (20% *v*/*v*) was used as cryoprotectant for aerobic isolates, and two different preservation methods were used for anaerobic isolates—glycerol (20% *v*/*v*) and dimethyl sulfoxide (DMSO) (0.025% *v*/*v*).

Table 2. Phylogenetic affiliations of the isolates based on a comparative analysis of their 16S rRNA gene sequences with the SILVA database.

Phylogenetic Phylum or Class	Family or Genus	Sample Origin	Culture Conditions	Number of Strains Isolated	Borehole Fluid	Fumarole	Drill Core
Actinobacteriota	*Arthrobacter*	17.9, 18.1	166, O_2, 22 °C	3	1	2	0
	Cryobacterium	16.8	M, O_2, 40 °C	1	1	0	0
	Frigoribacterium	16.8	M, O_2, 40 °C	1	1	0	0
	Microbacterium	B3, C59	166, MB, SO, O_2, 22 °C	4	0	0	4
	Dietzia	B9	MB, O_2, 22 °C	1	0	0	1
	Georgenia	B24	MB, O_2, 22 °C	1	0	0	1
	Glutamicibacter	17.16	166 and MB, O_2, 22 °C	4	4	0	0
	Janibacter	17.3, 16.7	166, O_2, 22 °C	4	4	0	0
	Leifsonia	16.8	M, O_2, 40 °C	1	1	0	0
	Intrasporangiaceae	17.4	166, O_2, 22 °C	2	2	0	0
	Kocuria	17.15, 17.16	166, O_2, 22 °C	2	2	0	0
	Micrococcus	16.8, B3	M and SO, O_2, 22 and 40 °C	2	1	0	1
	Paeniglutamicibacter	17.17, 17.19, 18.2, 18.3	166 and MB, O_2, 22 °C	7	7	0	0
	Rhodococcus group 1	17.17	MB, O_2, 22 °C	1	1	0	0
	Rhodococcus group 2	17.2, 17.17, 17.22, 18.1	166 and MB, O_2, 22 °C	5	5	0	0
	Rubrobacter	17.15, 17.2, 17.22, B24	166, O_2, 60 °C	4	3	0	1
				43	**33**	**2**	**8**
Bacteroidota	*Rhodothermus*	17.15, 17.2, 17.22, B24	166, O_2, 60 °C	8	6	0	2
				8	**6**	**0**	**2**
Deinococcota	*Thermus*	17.5, 17.8, 17.9	166, O_2, 80 °C	4	0	4	0
				4	**0**	**4**	**0**
Firmicutes	*Bacillus* group 1	17.11, 17.14, 17.15, B9	MB, O_2, 22 °C	5	5	0	0
	Bacillus group 2	17.1	166, O_2, 22 °C	1	0	0	1
	Bacillus (para)licheniformis	17.2, 17.5, 17.8, 17.9, 17.F	166, with and without O_2, 22, 50 and 60 °C	15	2	13	0
	Bacillus cereus group	17.8, 17.15, 17.F, C55, C65	166, with and without O_2, 22 and 37 °C	5	1	2	2
	Brevibacillus	17.8	166, O_2, 60 °C	2	0	2	0
	Brevibacillus thermoruber	17.1, 17.5, 17.9	166, O_2, 60 °C	4	1	3	0
	Caldalkalibacillus	17.1, 17.4	166, O_2, 60 °C	6	6	0	0
	Geobacillus	17.16	166, O_2, 22 and 60 °C	4	4	0	0
	Geobacillus thermoleovorans group	17.5, 17.8, 17.F	166, O_2, 60 and 65 °C	20	2	18	0
	Paenibacillus	B3, C55, C65	166, O_2, 22 °C	8	0	0	8
	Planifilum	18.3	166, O_2, 60 °C	1	1	0	0

Table 2. *Cont.*

Phylogenetic Phylum or Class	Family or Genus	Sample Origin	Culture Conditions	Number of Strains Isolated	Borehole Fluid	Fumarole	Drill Core
	Planococcaceae	18.3	MB, O_2, 22 °C	1	1	0	0
	Planomicrobium	18.3	166, O_2, 22 °C	2	2	0	0
	Ureibacillus	17.4, 17.6	166, O_2, 60 °C	4	1	3	0
				78	**26**	**41**	**11**
Alpha-proteobacteria	*Brevundimonas*	17.16	166, O_2, 22 °C	1	1	0	0
	Paracoccus	C65	MB, O_2, 22 °C	1	0	0	1
	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	B9	MB, O_2, 22 °C	2	0	0	2
	Sphingomonadaceae	B3	YPS, O_2, 22 °C	1	0	0	1
	Rhodobacteraceae	B3	SO, O_2, 22 °C	1	0	0	1
				6	**1**	**0**	**5**
Gamma-proteobacteria	*Acinetobacter*	16.8	M, O_2, 40 °C	1	1	0	0
	Halomonas	17.15, 17.23, 18.2, 18.3	166 and MB, O_2, 22 °C	11	11	0	0
	Marinomonas	17.13, 17.15	166 and MB, O_2, 22 °C	2	2	0	0
	Enterobacter	16.2, 16.7, 17.5, B3, C55, C65	166, M, I and SO, without O_2, 22 °C	18	5	1	12
	Enterobacteriaceae	17.5	166, O_2, 22 °C	1	0	1	0
	Pseudoalteromonas	17.23	166 and MB, O_2, 22 °C	7	7	0	0
	Pseudomonas group 1	B30, B36, B9, C62	166 and MB, O_2, 22 °C	8	0	0	8
	Pseudomonas group 2	18.2, 18.3	166, O_2, 22 °C	3	3	0	0
	Pseudomonas group 3	17.8	166, O_2, 22 °C	1	0	1	0
	Serratia	17.5	166, O2, 22 °C	1	0	1	0
	Shewanella	B9	MB, O_2, 22 °C	2	0	0	2
	Polaromonas	16.8	M, O_2, 40 °C	1	1	0	0
				56	**30**	**4**	**22**
Total number of isolated strains				**195**	**96**	**51**	**48**

2.3. Identification of Isolates by 16S rRNA Gene Sequencing

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, MALDI-TOF MS (Microflex LT, Bruker Daltonics, Bremen, Germany), was used for strain differentiation, allowing for the selection of colonies to be sequenced. Each colony was extracted using a standard formic acid/acetonitrile procedure. In brief, a full loop of fresh culture was diluted in 300 µL of autoclaved milliQ water and 900 µL of ethanol. After centrifugation and elution of ethanol, the pellet was dried. Then, depending on the size of the pellet, a volume between 5 and 30 µL of a 70% formic acid solution was added, followed by the same volume of acetonitrile. After centrifugation, 1 µL of the supernatant was spotted on the target plate using a saturated α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution. Measurements were consistently carried out using the same instrument parameters.

In parallel, single colony DNA was extracted from pure cultures and was carried out using a fresh 6% Chelex® 100 solution, as previously described [41]. DNA was quantified with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). From the selected isolates, partial sequences of the 16S rRNA

gene were amplified using forward primer F9 ("5-GAGTTTGATCCTGGCTCAG-3") and reverse primer R805 ("5-GACTACCCGGGTATCTAATCC-3") [42]. PCR was performed using OneTaq® Hot Start DNA Polymerase (New England BioLabs Inc. (NEB), Ipswich, MA, USA; #M0481L), according to the recommendation of the manufacturers. The PCR program started with an initial denaturation step at 94 °C for 30 s, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, and extension at 68 °C for 1 min. A final extension at 68 °C for 5 min was also included. The negative control was always used to exclude contamination. The amplification products were confirmed by agarose gel electrophoresis (1%) stained with SYBR® Safe DNA Gel Stain (Thermo Fischer Scientific, Waltham, MA, USA; #S33102) for 40 min at 100V and 400 mA in a 1X TAE buffer (2 M Tris, 1 M acetic acid, 50 mM EDTA disodium salt). PCR products were purified using Exonuclease I (ExoI, NEB, Ipswich, MA, USA; #M0293S), Shrimp Alkaline Phosphatase (SAP, NEB, Ipswich, MA, USA; #M0289S) and were sequenced with an ABI 377 DNA sequencer using a BigDye Terminator Cycle Sequencing Ready Reaction kit according to the manufacturer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The quality and analysis of partial 16S rRNA gene sequences obtained from the isolates were checked with the software Sequencher 5.2.4 software (Gene Codes Corp., Ann Arbor, MI, USA), and closely related sequences were identified using BLASTn (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information) and the 16S ribosomal RNA (Bacteria and Archaea type strains) database.

2.4. Construction of Phylogenetic Trees

One or two representative 16S rRNA gene sequences of each taxonomic group (55 sequences) were selected to build the phylogenetic tree. Sequences were classified and aligned using the online portal of the SILVA Incremental Aligner (SINA 1. 2. 11) tool of the ARB-Silva database (http://www.arb-silva.de/aligner/, accessed on 7 July 2021) [43]. The SILVA reference alignment searched the related sequences (two nearest neighbors per sequence) to 90% min identity of the 16S rRNA gene sequences from this study. Columns containing 10% or less rows of sequences were stripped of the full alignment, generating a final alignment with 158 taxa (55 from this study) and 1571 nucleotides. Maximum likelihood analyses were carried out using RAxML BlackBox (https://raxml-ng.vital-it.ch/#/, accessed on 13 September 2021) [44], as implemented on the CIPRES webserver [45] under the GTR GAMMA model. The tree in NEWICK format was imported into Interactive Tree Of Life (iTOL) v6.4 [46]. An additional tree that aligned all the sequences (151) with 442 sequences from the ARB-Silva database can be found in the Supplementary Materials (Figure S1).

Seven representative sequences of the novel strains (<98.65% 16S rRNA gene sequence similarity) isolated in this study were selected and aligned against 16S rRNA gene sequences of the closest cultured type strains using the clustal_w program [47]. Phylogenetic trees were reconstructed using the neighbour-joining tree algorithm [48] in MEGA7 [49], based on 1000 bootstrap replications and a total of 224 positions in the final dataset.

2.5. Bacterial Cultured Collection vs. 16S rRNA Amplicon Gene Sequencing

The 16S rRNA amplicon datasets used for the comparison with the sequences of the isolated strains were previously published in the European Nucleotide Archive (ENA) at EMBL-EBI (accession number ERP126178) [37]. Briefly, the datasets were obtained by Illumina MiSeq paired-end (2 × 300 base pair) tag sequencing using the universal primers 515f (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806r (5′-GGA CTA CHV GGG TWT CTA AT-3′) [50]. Sequence variants were inferred using the R Package DADA2 [51] version 1.4, as described elsewhere [37], and the SILVA SSU database release 138 was used for taxonomic assignation [43]. A phyloseq object [52] was constructed directly from the DADA2 outputs and the Amplicon Sequence Variant (ASV) table before any contaminant removal was used for the alignment. The comparisons between the ASVs from 16S rRNA amplicon gene sequencing datasets and partial 16S rRNA gene sequences obtained by Sanger sequencing

were performed using BLASTn optimized for highly similar sequences (megablast). All ASVs were basted against the 55 representative sequences of the isolated strains (Figure S2).

3. Results

3.1. Cultivated Bacterial Diversity

Under the microscope, 151 of the 270 enrichment cultures in the tested media and temperatures indicated growth. Positive growing enrichments were used as an inoculum in a subsequent medium. The isolates were obtained after either randomly selecting colonies that appeared on plates or after using the dilution-to-extinction method [40]. We obtained 195 isolates of bacteria from fumarole samples, borehole fluids, and drill cores collected from Surtsey island (Table 1). Most of the isolates were grown on the culture media 166 (101 strains), at 22 °C (120 strains), and in aerobic conditions (148 strains). The highest number of isolated strains came from borehole fluid samples (49.23%), followed by fumarole (26.15%) and drill cores (24.62%) (Figure 1 and Table 2).

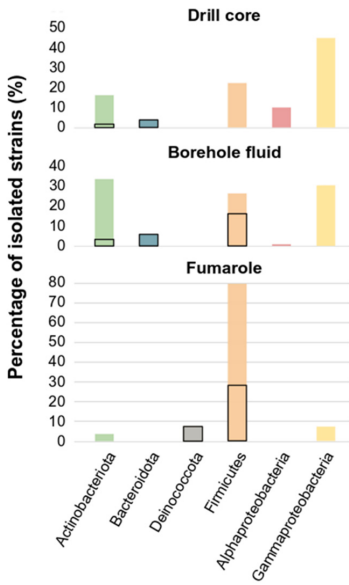

Figure 1. Taxonomical distribution at the phylum level (classes for Proteobacteria) of the bacterial isolates from Surtsey island by sampling sites. The percentages represent the relative cultivable bacterial abundance. Thermophilic strains (≤60 °C) are framed in bold.

Although many enrichment cultures showed growth in anaerobic conditions, subsequent growth was unsuccessful and most of the isolates were aerobic and heterotrophic bacteria. Thermophilic strains represented almost 30% of the strain collection, with 58 strains isolated at temperatures above 60 °C.

The isolates were identified by comparative analysis of their partial 16S rRNA gene sequences against the NCBI reference sequences (RefSeq) database. They were assigned to different families of five bacterial phyla (Figure 1), representing 42 different genera (Tables 2 and S6). No Archaea isolates were obtained in pure culture under the culture conditions tested. The isolates were assigned to Actinobacteriota (43 strains), Bacteroidota (8 strains), Alphaproteobacteria (6 strains), Gammaproteobacteria (56 strains), Deinococcota (4 strains), and Firmicutes (78 strains) (Table 2). Firmicutes were mostly isolated from fumarole samples (80%), Gammaproteobacteria were mostly isolated from the drill core samples (46%), and Actinobacteriota were mostly isolated from borehole fluid (35%) (Figure 1). Most of the thermophilic strains belonged to the phylum Firmicutes,

while eight strains of thermophilic Bacteroidetes and four strains of thermophilic Actinobacteria were isolated. In addition, four strains were isolated at 80 °C, and all belonged to the phylum Deinococcota. Only strains that belong to Rhodobacteraceae, Enterobacter, Micrococcus, and Microbacterium were isolated on the medium SO, while one strain of Sphingomonadaceae was isolated on the medium YPS. Enrichment cultures targeting iron, sulfur, sulfate reducer microorganisms, and methanogens showed growth, but only *Cryobacterium*, *Frigoribacterium*, *Leifsonia*, *Acinetobacter*, *Micrococcus*, and species that belong to the Enterobacterales were isolated on the media M and I and the rest of the enrichments could not be maintained by the dilution-to-extinction technique to isolate strains.

3.2. Phylogeny of the Isolates and Habitat of the Closest Relatives

The 16S rRNA genes of the isolates were partially sequenced. After trimming the end of the sequences to increase quality, the sizes of the sequence were mostly between 400 and 600 bp and sequence quality along all sequences was above 90%. Few sequences were smaller than 400 bp and thus were unambiguously assigned at the family level. To overcome this, taxonomic assignment of partial 16S rRNA gene sequences obtained from the 55 representative sequences of the isolates was performed using both the BLASTn on NCBI using the RefSeq database and SILVA Incremental Aligner (SINA 1. 2. 11) tool with Silva database version 138.1 [43] (Figure 2 and Figure S1, and Table S6).

Few isolated strains obtained in this study potentially represent novel species, according to the percentage value used as a boundary for species delineation of 98.65% 16S rRNA sequence similarity [53–55]. Twelve thermophilic strains belonging to the Actinobacteriota and the Bacteroidota showed sequence similarity below 98% and were related to *Rhodothermus marinus* (NR_074728) (95 to 96.41% sequence similarity) and *Rubrobacter xylanophilus* (NR_074552) (96.75–97.57%). The closest neighbor sequences corresponded to uncultured *Rhodothermus* species detected in seawater (EU249949) and *Rubrobacter xylanophilus* DSM 9941 isolated from hot spring water samples (CP000386) (Figure 2). The novel strains of *Rhodothermus* were characterized, and the name *Rhodothermus bifroesti* was attributed to the new species [56]. In addition, other isolated strains potentially represent novel species. Their sequences fell close to 98% of sequence similarity and were related to the genera *Planococcus* (97.80%), *Halomonas* (97.87–98.18%), *Microbacterium* (98.11–98.97%), and *Polaromonas* (98.01%) (Figure 3, Table S6). The closest neighbor sequences were detected in permafrost soil (JQ684228), seawater and deep-sea sediment (KP975360, AY582931), soil or continental Antarctic lake (CBVQ010000169, FR691402), and glacial sediment (JF719329) (Figure 2).

Other thermophilic strains were isolated in this study and showed sequence similarity percentages higher than 98%, with the closest related sequences usually detected from thermal environments. They belonged to the genera *Thermus* within the phylum Deinococcota and to *Geobacillus*, *Ureibacillus*, *Brevibacillus*, *Caldalkalibacillus*, and *Planifilum* within the Firmicutes. The only strains isolated at 80 °C were closely related with *Thermus thermophilus* (NR_113293.1), a hyperthermophilic bacterium that was originally isolated in saline hot spring [57]. The closest neighbor sequence was detected in a hydrothermal vent (AE017221) (Figure 2). Three strains showed 99.62% sequence similarity with *Geobacillus subterraneus* (NR_025109.1, NR_132400.1), a hydrocarbon-oxidizing thermophilic bacterium isolated from a petroleum reservoir located at 1200–2730 m b.s. [58]. Other strains isolated in this study were identified as *Geobacillus* sp. and fell into the *Geobacillus* thermoleovorans group. They were closely related to *Geobacillus thermoparaffinivorans* (KP218042), previously isolated from a crude oil deep reservoir [59]. Seven non-thermophilic isolated strains identified as *Paeniglutamicibacter* sp. were also closely related to sequences previously detected in oil-brine from an oilfield reservoir (NR_026237.1). Four strains were closely related to *Ureibacillus thermosphaericus* isolated from the air (NR_119203.1, X90640), while six strains were identified as *Brevibacillus* spp. and showed a high sequence similarity percentage with species previously isolated from hot spring (NR_117986.1) or hot water (ATNE01000001). One isolated strain was closely related at 99.93% sequence similarity to *Planifilum yunna-*

nense (NR_043563.1), a thermophilic thermoactinomycete that has been isolated from hot spring [60]. In addition, six other thermophilic strains isolated from borehole fluids showed 99.45% sequence similarity with *Caldalkalibacillus uzonensis* (NR_043653.1; DQ221694), an alkali-thermophilic bacterium isolated from hot spring [61].

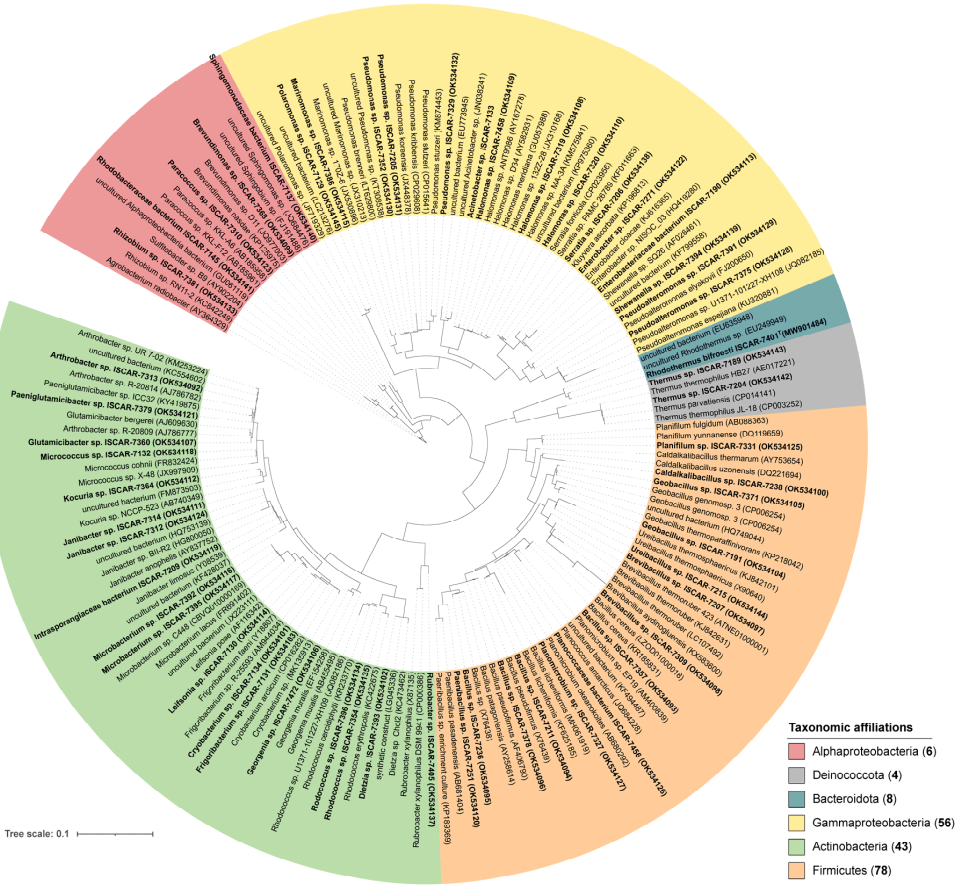

Figure 2. Maximum likelihood phylogeny of the partial 16S rRNA gene sequences placing the cultured bacteria from the subsurface of Surtsey island. The final alignment contained 158 sequences, 55 from this study (in bold), and was generated using the SILVA SINA alignment tool and the SILVA reference alignment. The tree was constructed using RAxML under the GTR GAMMA model of evolution.

Non-thermophilic strains isolated in this study were also closely related to alkaliphilic bacteria. Their sequences were closely related to *Dietzia natronolimnaea* (NR_116683.1), which has been isolated from soda lake [62], and to *Bacillus pseudofirmus* (X76439, AF406790, NR_026139.1) and *Bacillus patagoniensis* (AY258614, X76438, NR_025741.1), which were both recently reclassified to the genus *Alkalihalobacillus* [63].

Twenty-six isolates were closely related to species previously reported in marine habitats, including deep-sea sediments. These comprised *Planomicrobium* (NR_113593.1, AB680292), *Paracoccus* (NR_113921.1, AB185961), *Pseudoalteromonas* (NR_044837, FJ200650), *Sulfitobacter* (NR_043547.1, AY902204), *Marinomonas* (NR_116234.1, JX530896, JX310213), *Shewanella* (NR_040951.1, KF799558), and *Halomonas* (NR_116997.1, NR_027185.1, KP975360, AY582931) (Figure 2).

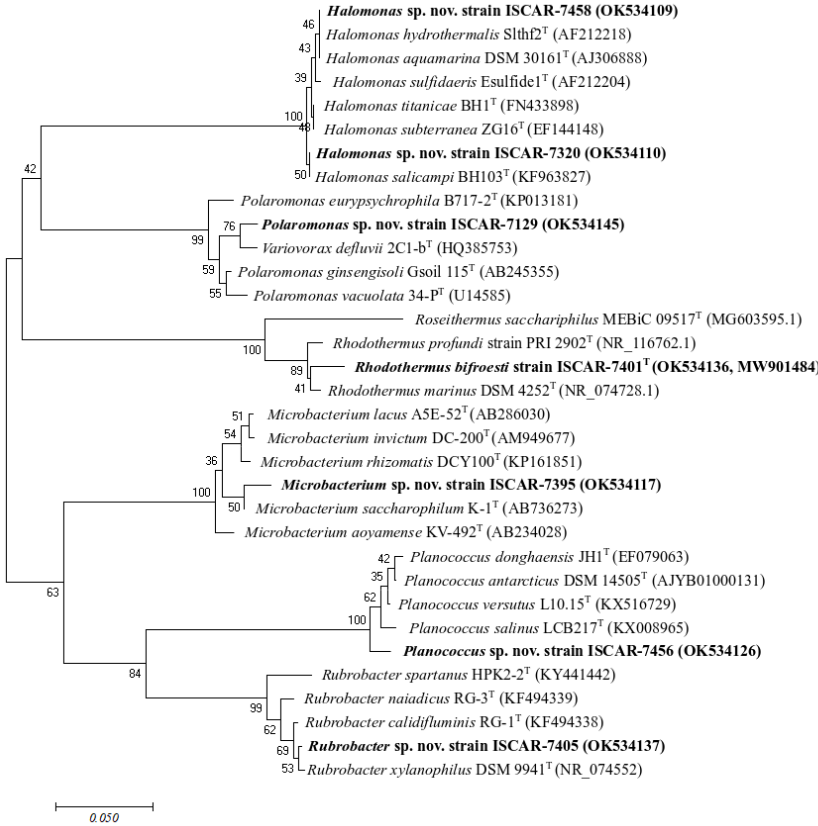

Figure 3. Phylogenetic tree based on partial 16S rRNA gene sequences showing the relationship between the novel species isolated in this study and closest cultured type strains. GenBank accession numbers are given in parentheses. The tree is based on the clustal_w and the neighbour-joining method with 1000 bootstraps using a total of 224 positions in the final dataset. Bar, 0.05 represented the nucleotide substitution per position.

Few isolated strains identified in this study belonging to the Intrasporangiaceae family were closely related to *Janibacter limnosus* (NR_026362.1). Although this species has been previously reported as endemic from deep-sea sediments along with *Rhodococcus*, *Arthrobacter*, *Kocuria*, and *Dietzia* [64], the closest neighbor sequences were detected in a wastewater treatment plant (Y08539) and metazoan gut's (HQ753139, AY837752). While some of the isolated strains were closely related to sequences that could be potential contaminants (e.g., *Paenibacillus pasadenensis* (AB681404), *Rhodococcus* from group 2 (KC422675), *Glutamicibacter bergerei* (AJ609630), and *Micrococcus cohnii* (FR832424, NR_117194.1)), other strains isolated in this study were closely related to sequences that were previously detected in the environment. This involved the permafrost (e.g., *Rhodococcus cercidiphylli* (KR233770), and *Cryobacterium arcticum* (CP016282, MK135913) and soil (e.g., uncultured *Arthrobacter* (KM253224, KC554602, NR_041546.1), uncultivated *Kocuria* (FM873503), *Leifsonia poae* (AF116342), *Bacillus licheniformis* (MH061919), *Frigoribacterium faeni* (Y18807), *Rhizobium* sp. RN11-2 (KC842249), and *Brevundimonas* sp. D11 (JQ977003)).

Overall, our results showed that many of the isolated species were closely related to sequences previously reported in marine (e.g., seawater, sediment, and deep-sea hydrothermal vent), alkaline (e.g., submarine alkaline or terrestrial hot springs, soda lake, and salty soil), and subsurface environments (e.g., petroleum reservoirs), while others showed

percentages of 16S rRNA gene sequence similarity below 98%, suggesting that they might represent novel species.

3.3. Comparison of the Bacterial Diversity Observed by Amplicon Sequencing from Environmental Samples and Cultured Diversity

The culture-dependent (isolation) and -independent (metabarcoding) approaches showed different taxonomic distributions at the phylum level (Figure 4a,b). As expected, the number of phyla obtained by the culture-dependent approach was much lower than observed using the molecular method. Of the 49 phyla detected in the 16S rRNA gene amplicon datasets, only 5 were recovered in the culture collection (Figure 4a,b). However, those five phyla represented more than 80% of the ASVs obtained by amplicon sequencing (Figure 4a).

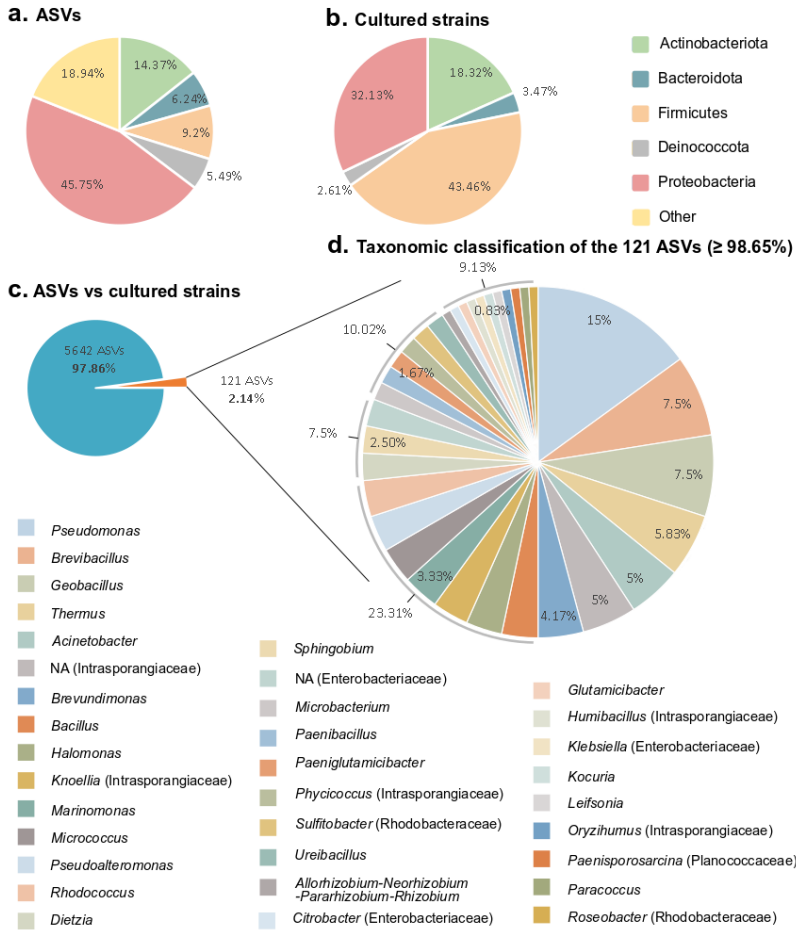

Figure 4. For each phylum, number of ASVs obtained by high throughput sequencing (**a**) and cultured strains (**b**). (**c**) The fraction of the in situ diversity represented by cultured strains, and taxonomic classification (**d**). An ASV was considered to be represented by the cultured strains if the ASV had equal or higher than 98.65% sequence similarity with the 16S rRNA gene sequence of cultured strains. The taxonomic classification of the ASVs represented by the cultured strains at the genus level. Family given in parenthesis for Enterobacteriaceae, Intrasporangiaceae, Planococcaceae and Rhodobacteraceae. NA. Not assigned genera.

To determine if the cultured strains were detected in the subsurface of Surtsey island, we used a culture-independent method (Figure S2). The partial 16S rRNA gene sequences of the isolates were aligned against Amplicon Sequence Variants (ASVs) from the 16S rRNA gene amplicon datasets (accession number ERP126178), which were obtained from the same samples as for cultivation [37] (Figure S2). Only the ASVs matching at percentages higher than 98.65% sequence similarity with sequences from the cultured strains were kept for investigation (Figure 4d and Table 3; bold values). This threshold was selected for species delimitation, assuming that the same species was detected using culture-dependent and -independent methods at an equal or higher percentage. Among a total of 5642 unique ASVs from the amplicon datasets, only 121 ASVs (≥98.65% sequence similarity) were represented by the culturable strains, which correspond to 2.14% of the in situ diversity (Figure 4c). This fraction belongs to 34 different genera, such as *Pseudomonas*, *Brevibacillus*, *Geobacillus*, *Thermus*, *Acinetobacter*, a non-assigned genus from the Intrasporangiaceae family, and *Brevundimonas*, accounting for half of the 121 ASVs (Figure 4c,d). This suggests that some of the species isolated in this study were also detected in the subsurface of Surtsey island using high-throughput sequencing.

Table 3. Heatmap comparing partial 16S rRNA gene sequences from the isolated strains to ASVs from 16S rRNA amplicon gene sequencing datasets from [37] (EMBL-EBI, accession number PRJEB42339). F, Fumaroles; BF, Borehole fluids; DC, Drill cores; Control, extraction blanks. Values in bold correspond to sequence similarity percentages above 98.65%, potentially indicating that the same isolated species has been detected in the subsurface using culture-independent method.

Phylogenetic Class	Genus	F	BF	DC	Control	Closest Sequence Similarity Percentage (Megablast)	Isolated From
Actinobacteriota	*Arthrobacter*	0	1	0	0	97.794	BF and F
	Dietzia	0	2	2	0	**99.265–100**	DC
	Georgenia	0	0	0	0		DC
	Glutamicibacter	0	0	0	1	**100**	BF
	Intrasporangiaceae	3	7	6	1	**100**	BF
	Kocuria	0	1	1	0	**99.259**	BF
	Leifsonia	0	2	2	0	**98.684**	BF
	Microbacterium lacus	0	0	2	1	**99.029**	DC
	Micrococcus	1	2	4	2	**99.457–100**	BF and DC
	Paeniglutamicibacter	1	1	2	0	**100**	BF
	Rhodococcus group 1	1	1	2	1	**100**	BF
	Rhodococcus group 2	1	2	2	0	**100**	BF
	Rubrobacter	2	0	1	0	93.605	BF and DC
Bacteroidota	*Rhodothermus*	0	2	0	0	95.588	BF and DC
Deinococcota	*Thermus*	0	4	5	1	**98.693–100**	F
Firmicutes	*Bacillus (para)licheniformis*	0	1	3	0	**99.495–100**	BF and F
	Bacillus cereus	2	2	2	1	**100**	BF, F and DC
	Bacillus group 1	0	0	1	0	97.024	BF and DC
	Bacillus group 2	0	0	0	0	/	BF
	Brevibacillus	0	1	8	0	**99.052–100**	BF and F
	Caldalkalibacillus	0	2	0	1	98.529	BF
	Geobacillus	0	2	2	0	**99.074–99.537**	BF
	Geobacillus thermoleovorans group	0	2	5	0	**99.487–100**	F
	Paenibacillus	0	0	2	0	**100**	DC
	Planifilum	0	0	0	0	/	BF

Table 3. *Cont.*

Phylogenetic Class	Genus	F	BF	DC	Control	Closest Sequence Similarity Percentage (Megablast)	Isolated From
	Planococcaceae	0	2	2	0	97.674	BF
	Planomicrobium	0	1	0	0	**98.897**	BF
	Ureibacillus	0	0	2	0	**100**	F and BF
Alphaproteobacteria	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	0	1	1	0	**99.034**	DC
	Brevundimonas	1	1	5	1	**99.457–100**	BF
	Paracoccus	0	0	1	0	**100**	DC
	Rhodobacteraceae	0	0	3	0	**100**	DC
	Sphingobium	2	1	1	0	**99.425–100**	DC
Gammaproteobacteria	*Acinetobacter*	4	4	5	2	**99.254–100**	BF
	Enterobacteriaceae	2	3	4	2	**99.533–100**	F, BF and DC
	Halomonas	0	4	0	0	**99.265–100**	BF
	Marinomonas	0	1	3	0	**99.052–100**	BF
	Polaromonas	0	2	3	1	98.276	BF
	Pseudoalteromonas	2	4	4	1	**100**	BF
	Pseudomonas group 1	2	7	3	0	**99.306–100**	DC
	Pseudomonas group 2	0	2	2	2	**98.529–98.897**	F
	Pseudomonas group 3	3	4	7	0	**98.907–100**	BF
	Serratia	0	0	0	1	97.619	F
	Shewanella	0	0	1	0	96.691	DC

Table 3 indicates in which sample type the 121 ASVs were detected, in addition to the samples origin of the isolates. The sample types correspond to the 16S rRNA amplicon gene sequencing of fumarole, borehole fluid, and drill core samples (Table 3, F, BF, and DC) as well as the sequencing of blank as extraction controls (Table 3, Control). For example, ASVs detected in the borehole fluid and drill core samples possibly represent the same species as the *Dietzia* sp. isolated in this study from a drill core sample (Table 3). Likewise, other cultured species were detected in the samples using a culture-independent approach. These species include *Kocuria*, *Leifsonia*, *Paeniglutamicibacter*, and *Rhodococcus* from group 2; *Bacillus* (*para*)*licheniformis*, *Brevibacillus*, *Geobacillus*, and *Geobacillus* from the thermoleovorans group; and *Paenibacillus*, *Ureibacillus*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Paracoccus*, *Sphingobium*, *Halomonas*, *Marinomonas*, and *Pseudomonas* from groups 1 and 3; and the cultured strains that belong to the family Rhodobacteraceae (Table 3). The latter species were not detected in the blank extraction controls (Table 3; Control), indicating that they might be endemic to the subsurface of the island.

Surprisingly, we cultivated genera that were not detected using metabarcoding (≤98.65%). They belong to *Arthrobacter*, *Georgenia*, *Rubrobacter*, *Rhodothermus*, *Caldalkalibacillus*, *Planifilum*, *Planomicrobium*, *Polaromonas*, *Serratia*, and *Shewanella*. Indeed, if no ASVs matched with a cultured strain sequence above 98.65%, ASVs with the highest score were kept in Table 3 as an indication of the closest taxa (Table 3, light values). Two species isolated in this study, *Georgenia* and *Planifilum*, did not match any detected ASVs, while other isolated species aligned with ASVs at low sequence similarity. These include *Arthrobacter* (97.78%), *Rubrobacter* (93.6%), *Rhodothermus* (95.58%), *Bacillus* from group 1 (97%), *Caldalkalibacillus* (98.53%), *Polaromonas* (98.27%), *Shewanella* (96.7%), and the cultured strain from the Planococcaceae family (97.67%).

Many of the species isolated in this study matched at high sequence similarity percentages with ASVs detected in the extraction blanks. These include *Microbacterium lacus*, *Micrococcus*, and *Rhodococcus* from group 1; *Thermus*, *Caldalkalibacillus*, *Brevundimonas*, *Acine-*

tobacter, *Polaromonas*, *Pseudoalteromonas*, and *Pseudomonas* from group 2; and the cultured strains belonging to the families Intrasporangiaceae and Enterobacteriaceae. While those taxa were detected in the extraction blanks, they were frequently detected at a much higher abundance in the samples. In contrast, the strain identified as *Glutamicibacter* sp. was only detected in the extraction blanks at 100% sequence similarity, suggesting that it is a potential contaminant.

4. Discussion

Most of the isolates cultured in this study were highly related to aerobic bacteria, and only a few taxa were isolated under anaerobic conditions. Among the 270 enrichment conditions tested, two nutrient-rich media, 166 and MB, were the most successful in the isolation of strains (Table 2). No archaea were isolated, and all of the bacterial isolates were heterotrophic. Our results suggest that the media and conditions tested were ineffective for isolating archaea and chemolithoautotrophs, although those taxa were detected in the subsurface of the Surtsey volcano [37]. The vast majority of the bacterial diversity in our samples seems to be uncultivable with our cultivation techniques, as it has been previously reported [17,19,20,65]. Archaeal cultivation is even more challenging [66]. Our results are comparable with other findings and could be explained by insufficient care during sampling, transportation, incorrect storage of the samples used as inoculum, or inadequate cultivation conditions and isolation methods [67]. In this study, many isolates could not be maintained or subsequently cultivated, especially colonies obtained in anaerobic conditions. Colonies obtained using the Hungate roll-tube technique [68,69] that could not grow after being transferred on solid or liquid media represent a good example. This could be due to a lack of information on the habitat's physicochemical parameters, which are necessary for establishing cultivation settings that mimic in situ environmental conditions. For example, trace micronutrient substances or growth factors originally present in the samples could be essential for growth. The utilization of 0.22 µm filtered borehole fluids for media preparation may have thus facilitated the second transfer of colonies and aided in the maintenance of the subcultures. However, the volume of sterilized fluids was insufficient to serve as cultivation medium basis. In addition, some microorganisms can only be cultivated in the presence of other cells. Syntrophic relationships, competition, or inhibition between microorganisms can also exclude the cultivation and isolation of specific taxa. Lowering the nutrient content of the rich media used for enrichment could have improved the diversity of culturable microorganisms (e.g., [70]). In this study, fast-growing bacteria may have been favored due to the nutrient-rich substrate and short incubation time. The multiplication of experiments, methods (e.g., acclimation step, use of antibiotics, innovative cultivation techniques for co-cultivation, simulation of the natural environment, or single-cell isolation), media (wide range of carbon and energy sources), and culture conditions (e.g., temperature and pressure) may overcome these challenges.

Closest relatives to our isolated strains were previously detected in soil, seawater, and geothermal active sites near the surface (e.g., hot springs and hydrothermal vents) (Figure 2). This observation supports the hypothesis based on 16S rRNA gene amplicon sequencing results that microbial cells from the surrounding environment are transported into the subsurface of Surtsey island by fluid inflow (meteoric water and seawater inflow) and that the colonization of young basalt has been driven by the ability of some microorganisms to adapt to a new environment and to disperse across ecosystems [37]. Our culture-based approach brings insight into the question of whether the cells die, survive, or adapt to the extreme environmental conditions that exist within the volcano since seawater bacteria were alive and cultured from borehole fluid or drill core samples (e.g., *Pseudoalteromonas*, *Halomonas*, and *Marinomonas*, Table 3). In addition, alkaliphilic bacteria (e.g., *Thioalkalimicrobium*) were previously detected in the subaerial deposits of the Surtsey volcano [37]. The isolation of alkaliphilic bacteria (e.g., *Caldalkalibacillus*) from the subsurface of Surtsey confirmed that the environmental conditions occurring at some depths are highly saline and alkaline. This could be the result of the biotic or abiotic dissolution of

some minerals or basaltic glass at low fluid–rock ratios [33]. It suggests that these groups of adapted microorganisms are present and active within the high-temperature geothermal system of Surtsey (range of 50–150 °C) and could have significant impacts on subsurface geochemistry [71].

As expected from other environmental microbiome research, a disparity was observed between the microbial communities identified in the samples using cultivation-independent methods in comparison with the cultured strains [72]. Among the total ASVs detected using a culture-independent approach (metabarcoding), only 2.14% were recovered in the cultured collection (Figure 4). The threshold of 98.65% sequence similarity was selected for species delimitation. Because some species share a high level of 16S rRNA gene sequence similarity (>99%), it is challenging to differentiate two species using 16S rRNA gene sequences alone. However, many comparative studies investigated large datasets to determine an optimal 16S rRNA gene sequence similarity threshold for microbial species demarcation, considering DNA–DNA hybridization (DDH) and average nucleotide identity (ANI) values, and taking into account the effect of the taxonomic group [55]. These studies suggested that 98.65% of 16S rRNA gene sequence similarity can be used as an adequate threshold for differentiating two species. The high proportion of ASVs detected in subsurface environment samples that remained uncultured could be explained by several reasons. The DNA detected in the samples might come from dead cells, prohibiting their detection using culture-dependent methods [67]. Additionally, as previously mentioned, the cultivation methods applied may be unsuitable for the cultivation of specific microbial taxa, and the application of unappropriated elements such as the composition of the medium, temperature, and oxidative stress can proscribe their growth [73]. Therefore, culturing efforts to characterize microbial communities should include a variety of methodologies and media. Still far too few microorganisms were isolated from extreme environments, especially autotrophic anaerobic microorganisms discovered using molecular techniques. The number of previously uncultured taxa may be increased with the application of innovative culturing methods, the addition of growth factors in the media, and the use of in situ cultivation methods in their original habitats [74–76]. At the same time, some of the cultivated genera were not detected with a culture-independent approach and could represent members of the rare biosphere. For example, *Planifilum*, which belongs to the Thermoactinomycetaceae family, was isolated from borehole fluid but was not detected using metabarcoding (Table 3). This observation has been made in a variety of environments [77,78] and suggests that cultivation not only provides a small percentage of what was detected in the sequencing data but also could increase the total diversity present in a sample.

When investigating low biomass samples from subsurface environments using a molecular approach, the incorporation of contaminants is very high during sample collection and processing [79,80]. Based on the datasets obtained through the activity of the Census of Deep Life (CoDL) and the Deep Carbon Observatory on low biomass subsurface environments, a list of genera was published and identified as "typical" potential contaminants that were usually associated with molecular reagents or potential human contamination [80]. Although the approach to removing all of the listed genera from a dataset has been proven effective [81–83], it can still result in the removal of "species" that may be indigenous and ecologically important. In this study, we isolated species that belong to genera identified as contaminants in previous studies [80,84]. These comprise *Brevibacillus*, *Dietzia*, *Kocuria*, *Paenibacillus*, *Paracoccus*, *Pseudomonas*, *Rhodococcus*, and *Sphingobium*. However, a distinction between species should be made to differentiate the false from the true contaminants. Nonetheless, comparing ASVs and 16S rRNA gene sequences from cultured strains can bring evidence. For example, sequences from the strains of *Bacillus cereus* isolated in this study were blasted at 100% sequence similarity with ASVs that were detected in almost all the samples, including the blank extraction controls (Table 3), and were closely related with sequences previously detected in human (LZOD01000018). In this case, we assume that this taxon is likely a contamination. Likewise, the isolated *Serratia* is most likely a contaminant,

as it was only detected in the blank extraction controls (Table 3) and was closely related with sequences detected in human (Figure S1). While culture-independent methods may be affected by possible inherent contamination problems of subsurface samples, the question of whether a microorganism is truly indigenous may be answered by a comparison with a culture-dependent approach. Cultured strains may have the advantage of representing microorganisms that are adapted to subsurface conditions.

5. Conclusions

To conclude, our study is the first to report the culturable microbial diversity from the basaltic subsurface of Surtsey island. In total, we tested 270 enrichments using different temperatures and media. We isolated 195 strains, representing 42 different genera. Most of the isolates were aerobic and heterotrophic bacteria, and few novel species were isolated. In this perspective, a bacterium isolated in this study, *Rhodothermus bifroesti*, has been characterized [56]. The comparison of our cultured strains and ASVs from 16S rRNA amplicon gene datasets obtained from the same samples [37] revealed that isolates were detected using both culture-dependent (isolation) and -independent methods (metabarcoding). This study provided a valuable supplementary description of the microbial diversity in the subsurface of Surtsey. Both culture-independent and dependent methods are essential to improving our insight into the microbial communities inhabiting such an extreme and complex volcanic environment.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microorganisms10061177/s1, Figure S1: Maximum likelihood 16S rRNA gene sequence phylogenetic tree of the cultured strains using ARB; Figure S2: Sampling and workflow of experiment. Table S1: Medium 166 modified from [39] (without proline). Grunnur base from medium 162 from [85]. Table S2: Medium YPS. Table S3: Medium sulfate reducer (SO). Table S4: Medium iron reducer (I). Table S5: Medium methanogen (M). Table S6: Culture collection represented by 55 selected strains.

Author Contributions: Conceptualization, P.B., P.V. and V.Þ.M.; methodology, P.B., P.V. and V.Þ.M.; formal analysis, P.B.; investigation, P.B., J.F. and A.M.; writing—original draft preparation, P.B.; writing—review and editing, P.B., P.V. and V.Þ.M.; visualization, P.B.; supervision, V.Þ.M.; project administration, V.Þ.M.; funding acquisition, V.Þ.M., P.V. and P.B. All authors have read and agreed to the published version of the manuscript.

Funding: The drilling operation was funded by International Continental Scientific Drilling Program (ICDP) through the Surtsey Underwater volcanic System for Thermophiles, Alteration processes and INnovative Concretes (SUSTAIN) project (led by M.D. Jackson). This research was funded by the Icelandic Research Fund (IRF, RANNÍS) through the grant of excellence IceSUSTAIN, number 163083-051 (led by M.T. Gudmundsson and V.Þ. Marteinsson), and the doctoral student grant ELiBSS, number 206582-051 (led by P. Bergsten).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Partial 16S rRNA gene sequences obtained in this study have been deposited in the GenBank databases under accession numbers OK534092 to OK534145.

Acknowledgments: The authors thank all members of the SUSTAIN onsite, science teams, and the Surtsey Research Society for their contributions to the drilling project and sampling, especially M.T. Gudmundsson, M.D. Jackson, A.M. Klonowski, B. Kleine, T. Weisenberger, S. Prause, and C.F. Gorny.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Magnabosco, C.; Lin, L.H.; Dong, H.; Bomberg, M.; Ghiorse, W.; Stan-Lotter, H.; Pedersen, K.; Kieft, T.L.; van Heerden, E.; Onstott, T.C. The Biomass and Biodiversity of the Continental Subsurface. *Nat. Geosci.* **2018**, *1*, 707–717. [CrossRef]
2. Huber, J.A.; Johnson, H.P.; Butterfield, D.A.; Baross, J.A. Microbial Life in Ridge Flank Crustal Fluids. *Environ. Microbiol.* **2006**, *8*, 88–99. [CrossRef] [PubMed]

3. Nakagawa, S.; Inagaki, F.; Suzuki, Y.; Steinsbu, B.O.; Lever, M.A.; Takai, K.; Engelen, B.; Sako, Y.; Wheat, C.G.; Horikoshi, K. Microbial Community in Black Rust Exposed to Hot Ridge Flank Crustal Fluids. *Appl. Environ. Microbiol.* **2006**, *72*, 10. [CrossRef] [PubMed]
4. Lever, M.A.; Rouxel, O.; Alt, J.C.; Shimizu, N.; Ono, S.; Coggon, R.M.; Shanks, W.C.; Lapham, L.; Elvert, M.; Prieto-Mollar, X.; et al. Evidence for Microbial Carbon and Sulfur Cycling in Deeply Buried Ridge Flank Basalt. *Science* **2013**, *339*, 6125. [CrossRef]
5. Jungbluth, S.P.; Lin, H.T.; Cowen, J.P.; Glazer, B.T.; Rappé, M.S. Phylogenetic Diversity of Microorganisms in Subseafloor Crustal Fluids from Holes 1025C and 1026B along the Juan de Fuca Ridge Flank. *Front. Microbiol.* **2014**, *5*, 119. [CrossRef]
6. Robador, A.; Jungbluth, S.P.; LaRowe, D.E.; Bowers, R.M.; Rappé, M.S.; Amend, J.P.; Cowen, J.P. Activity and Phylogenetic Diversity of Sulfate-Reducing Microorganisms in Low-Temperature Subsurface Fluids within the Upper Oceanic Crust. *Front. Microbiol.* **2015**, *5*, 748. [CrossRef]
7. Baquiran, J.P.M.; Ramírez, G.A.; Haddad, A.G.; Toner, B.M.; Hulme, S.; Wheat, C.G.; Edwards, K.J.; Orcutt, B.N. Temperature and Redox Effect on Mineral Colonization in Juan de Fuca Ridge Flank Subsurface Crustal Fluids. *Front. Microbiol.* **2016**, *7*, 396. [CrossRef]
8. Cowen, J.P.; Giovannoni, S.J.; Kenig, F.; Johnson, H.P.; Butterfield, D.; Rappé, M.S.; Hutnak, M.; Lam, P. Fluids from Aging Ocean Crust That Support Microbial Life. *Science* **2003**, *299*, 120–123. [CrossRef]
9. Orcutt, B.N.; Bach, W.; Becker, K.; Fisher, A.T.; Hentscher, M.; Toner, B.M.; Wheat, C.G.; Edwards, K.J. Colonization of Subsurface Microbial Observatories Deployed in Young Ocean Crust. *ISME J.* **2011**, *5*, 692–703. [CrossRef]
10. Jungbluth, S.P.; Grote, J.; Lin, H.T.; Cowen, J.P.; Rappé, M.S. Microbial Diversity within Basement Fluids of the Sediment-Buried Juan de Fuca Ridge Flank. *ISME J.* **2013**, *7*, 161–172. [CrossRef]
11. Jungbluth, S.P.; Bowers, R.M.; Lin, H.T.; Cowen, J.P.; Rappé, M.S. Novel Microbial Assemblages Inhabiting Crustal Fluids within Mid-Ocean Ridge Flank Subsurface Basalt. *ISME J.* **2016**, *10*, 2033–2047. [CrossRef] [PubMed]
12. Parks, D.H.; Rinke, C.; Chuvochina, M.; Chaumeil, P.-A.; Woodcroft, B.J.; Evans, P.N.; Hugenholtz, P.; Tyson, G.W. Recovery of Nearly 8,000 Metagenome-Assembled Genomes Substantially Expands the Tree of Life. *Nat. Microbiol.* **2017**, *2*, 1533–1542. [CrossRef] [PubMed]
13. Lynch, M.D.J.; Neufeld, J.D. Ecology and Exploration of the Rare Biosphere. *Nat. Rev. Microbiol.* **2015**, *13*, 217–229. [CrossRef] [PubMed]
14. Hug, L.A.; Baker, B.J.; Anantharaman, K.; Brown, C.T.; Probst, A.J.; Castelle, C.J.; Butterfield, C.N.; Hernsdorf, A.W.; Amano, Y.; Ise, K.; et al. A New View of the Tree of Life. *Nat. Microbiol.* **2016**, *1*, 16048. [CrossRef]
15. Thompson, L.R.; Sanders, J.G.; McDonald, D.; Amir, A.; Ladau, J.; Locey, K.J.; Prill, R.J.; Tripathi, A.; Gibbons, S.M.; Ackermann, G.; et al. A Communal Catalogue Reveals Earth's Multiscale Microbial Diversity. *Nature* **2017**, *551*, 457–463. [CrossRef]
16. Nunoura, T.; Chikaraishi, Y.; Izaki, R.; Suwa, T.; Sato, T.; Harada, T.; Mori, K.; Kato, Y.; Miyazaki, M.; Shimamura, S.; et al. A Primordial and Reversible TCA Cycle in a Facultatively Chemolithoautotrophic Thermophile. *Science* **2018**, *359*, 559–563. [CrossRef]
17. Amann, R.I.; Ludwig, W.; Schleifer, K.H. Phylogenetic Identification and in Situ Detection of Individual Microbial Cells without Cultivation. *Microbiol. Rev.* **1995**, *59*, 143–169. [CrossRef]
18. Hugenholtz, P.; Goebel, B.M.; Pace, N.R. Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity. *J. Bacteriol.* **1998**, *180*, 4765–4774. [CrossRef]
19. Rappé, M.S.; Giovannoni, S.J. The Uncultured Microbial Majority. *Annu. Rev. Microbiol.* **2003**, *57*, 369–394. [CrossRef]
20. Steen, A.D.; Crits-Christoph, A.; Carini, P.; DeAngelis, K.M.; Fierer, N.; Lloyd, K.G.; Cameron Thrash, J. High Proportions of Bacteria and Archaea across Most Biomes Remain Uncultured. *ISME J.* **2019**, *13*, 3126–3130. [CrossRef]
21. Thorarinsson, S.; Þórarinsson, S. The Surtsey Eruption: Course of Events and the Development of the New Island. *Surtsey Res. Prog. Rep.* **1965**, *1*, 51–55.
22. Jakobsson, S.P.; Moore, J.G. The Surtsey Research Drilling Project of 1979. *Surtsey Res. Prog. Rep.* **1982**, *9*, 76–93.
23. Jakobsson, S.P.; Thors, K.; Vésteinsson, Á.T.; Ásbjörnsdóttir, L. Some Aspects of the Seafloor Morphology at Surtsey Volcano: The New Multibeam Bathymetric Survey of 2007, Surtsey Research. *Surtsey Res. Prog. Rep.* **2009**, *12*, 9–20.
24. Jakobsson, S.P.; Moore, J.G. Hydrothermal Minerals and Alteration Rates at Surtsey Volcano, Iceland. *Geol. Soc. Am. Bull.* **1986**, *97*, 648–659. [CrossRef]
25. Takai, K.; Nakamura, K.; Toki, T.; Tsunogai, U.; Miyazaki, M.; Miyazaki, J.; Hirayama, H.; Nakagawa, S.; Nunoura, T.; Horikoshi, K. Cell Proliferation at 122 °C and Isotopically Heavy CH4 Production by a Hyperthermophilic Methanogen under High-Pressure Cultivation. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 10949–10954. [CrossRef] [PubMed]
26. Ólafsson, M.; Jakobsson, S.P. Chemical Composition of Hydrothermal Water and Water-Rock Interactions on Surtsey Volcanic Island: A Preliminary Report. *Surtsey Res.* **2009**, *12*, 29–38.
27. Marteinsson, V.; Klonowski, A.; Reynisson, E.; Vannier, P.; Sigurdsson, B.D.; Ólafsson, M. Microbial Colonization in Diverse Surface Soil Types in Surtsey and Diversity Analysis of Its Subsurface Microbiota. *Biogeosciences* **2015**, *12*, 1191–1203. [CrossRef]
28. Jackson, M.D.; Couper, S.; Stan, C.V.; Ivarsson, M.; Czabaj, M.W.; Tamura, N.; Parkinson, D.; Miyagi, L.M.; Moore, J.G. Authigenic Mineral Texture in Submarine 1979 Basalt Drill Core, Surtsey Volcano, Iceland. *Geochem. Geophys. Geosyst.* **2019**, *20*, 3751–3773. [CrossRef]

29. Jackson, M.D.; Gudmundsson, M.T.; Bach, W.; Cappelletti, P.; Coleman, N.J.; Ivarsson, M.; Jónasson, K.; Jørgensen, S.L.; Marteinsson, V.; McPhie, J.; et al. Time-Lapse Characterization of Hydrothermal Seawater and Microbial Interactions with Basaltic Tephra at Surtsey Volcano. *Sci. Drill.* **2015**, *20*, 51–58. [CrossRef]
30. Jackson, M.D.; Gudmundsson, M.T.; Weisenberger, T.B.; Rhodes, J.M.; Stefánsson, A.; Kleine, B.I.; Lippert, P.C.; Marquardt, J.M.; Reynolds, H.I.; Kück, J.; et al. SUSTAIN Drilling at Surtsey Volcano, Iceland, Tracks Hydrothermal and Microbiological Interactions in Basalt 50 Years after Eruption. *Sci. Drill.* **2019**, *25*, 35–46. [CrossRef]
31. Weisenberger, T.B.; Gudmundsson, M.T.; Jackson, M.D.; Gorny, C.F.; Türke, A.; Kleine, B.I.; Marshall, B.; Jørgensen, S.L.; Marteinsson, V.T.; Stefánsson, A.; et al. *Operational Report for the 2017 Surtsey Underwater Volcanic System for Thermophiles, Alteration Processes and INnovative Concretes (SUSTAIN) Drilling Project at Surtsey Volcano, Iceland*; GFZ German Research Centre for Geosciences: Potsdam, Germany, 2019; 240p. [CrossRef]
32. Schipper, C.I.; Le Voyer, M.; Moussallam, Y.; White, J.D.L.; Thordarson, T.; Kimura, J.I.; Chang, Q. Degassing and Magma Mixing during the Eruption of Surtsey Volcano (Iceland, 1963–1967): The Signatures of a Dynamic and Discrete Rift Propagation Event. *Bull. Volcanol.* **2016**, *78*, 33. [CrossRef]
33. Kleine, B.I.; Stefánsson, A.; Kjartansdóttir, R.; Prause, S.; Weisenberger, T.B.; Reynolds, H.I.; Sveinbjörnsdóttir, Á.E.; Jackson, M.D.; Gudmundsson, M.T. The Surtsey Volcano Geothermal System: An Analogue for Seawater-Oceanic Crust Interaction with Implications for the Elemental Budget of the Oceanic Crust. *Chem. Geol.* **2020**, *550*, 119702. [CrossRef]
34. McPhie, J.; White, J.D.L.; Gorny, C.; Jackson, M.D.; Gudmundsson, M.T.; Couper, S. Lithofacies from the 1963-1967 Surtsey Eruption in SUSTAIN Drill Cores SE-2a, SE-2b and SE-03. *Surtsey Res.* **2020**, *14*, 85–90. [CrossRef]
35. Moore, J.C.; Jackson, M.D. Observations on the Structure of Surtsey. *Surtsey Res.* **2020**, *14*, 33–45. [CrossRef]
36. Prause, S.; Weisenberger, T.B.; Cappelletti, P.; Grimaldi, C.; Rispoli, C.; Jónasson, K.; Jackson, M.D.; Gudmundsson, M.T. Alteration Progress within the Surtsey Hydrothermal System, SW Iceland—A Time-Lapse Petrographic Study of Cores Drilled in 1979 and 2017. *J. Volcanol. Geotherm. Res.* **2020**, *392*, 106754. [CrossRef]
37. Bergsten, P.; Vannier, P.; Klonowski, A.M.; Knobloch, S.; Gudmundsson, M.T.; Jackson, M.D.; Marteinsson, V.T. Basalt-Hosted Microbial Communities in the Subsurface of the Young Volcanic Island of Surtsey, Iceland. *Front. Microbiol.* **2021**, *12*, 2789. [CrossRef]
38. Wolin, E.A.; Wolin, M.J.; Wolfe, R.S. Formation of methane by bacterial extracts. *J. Biol. Chem.* **1963**, *238*, 2882–2886. [CrossRef]
39. Hjorleifsdottir, S.; Skirnisdottir, S.; Hreggvidsson, G.O.; Holst, O.; Kristjansson, J.K. Species Composition of Cultivated and Noncultivated Bacteria from Short Filaments in an Icelandic Hot Spring at 88 °C. *Microb. Ecol.* **2001**, *42*, 117–125. [CrossRef]
40. Button, D.K.; Schut, F.; Quang, P.; Martin, R.; Robertson, B.R. Viability and Isolation of Marine Bacteria by Dilution Culture: Theory, Procedures, and Initial Results. *Appl. Environ. Microbiol.* **1993**, *559*, 881–891. [CrossRef]
41. Walsh, P.S.; Metzger, D.A.; Higuchi, R. Chelex® 100 as a Medium for Simple Extraction of DNA for PCR-Based Typing from Forensic Material. *BioTechniques* **1991**, *10*, 506–513. [CrossRef]
42. Skirnisdottir, S.; Hreggvidsson, G.O.; Hjörleifsdottir, S.; Marteinsson, V.T.; Petursdottir, S.K.; Holst, O.; Kristjansson, J.K. Influence of Sulfide and Temperature on Species Composition and Community Structure of Hot Spring Microbial Mats. *Appl. Environ. Microbiol.* **2000**, *66*, 2835–2841. [CrossRef] [PubMed]
43. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. *Nucleic Acids Res.* **2013**, *41*, D590–D596. [CrossRef] [PubMed]
44. Kozlov, A.M.; Darriba, D.; Flouri, T.; Morel, B.; Stamatakis, A. RAxML-NG: A Fast, Scalable and User-Friendly Tool for Maximum Likelihood Phylogenetic Inference. *Bioinformatics* **2019**, *35*, 4453–4455. [CrossRef]
45. Miller, M.A.; Pfeiffer, W.; Schwartz, T. Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees. In Proceedings of the 2010 Gateway Computing Environments Workshop (GCE 2010), New Orleans, LA, USA, 14 November 2010.
46. Letunic, I.; Bork, P. Interactive Tree of Life (ITOL) v5: An Online Tool for Phylogenetic Tree Display and Annotation. *Nucleic Acids Res.* **2021**, *49*, W293–W296. [CrossRef] [PubMed]
47. Thompson, J.D.; Higgins, D.G.; Gibson, T.J. CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. *Nucleic Acids Res.* **1994**, *22*, 4673–4680. [CrossRef]
48. Saitou, N.; Nei, M. The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425. [CrossRef] [PubMed]
49. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef]
50. Caporaso, J.G.; Lauber, C.L.; Walters, W.A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S.M.; Betley, J.; Fraser, L.; Bauer, M.; et al. Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms. *ISME J.* **2012**, *6*, 1621–1624. [CrossRef]
51. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-Resolution Sample Inference from Illumina Amplicon Data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef]
52. McMurdie, P.J.; Holmes, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef]
53. Goris, J.; Konstantinidis, K.T.; Klappenbach, J.A.; Coenye, T.; Vandamme, P.; Tiedje, J.M. DNA-DNA Hybridization Values and Their Relationship to Whole-Genome Sequence Similarities. *Int. J. Syst. Evol. Microbiol.* **2007**, *57*, 81–91. [CrossRef] [PubMed]

54. Richter, M.; Rosselló-Móra, R. Shifting the Genomic Gold Standard for the Prokaryotic Species Definition. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 19126–19131. [CrossRef] [PubMed]
55. Kim, M.; Oh, H.S.; Park, S.C.; Chun, J. Towards a Taxonomic Coherence between Average Nucleotide Identity and 16S RRNA Gene Sequence Similarity for Species Demarcation of Prokaryotes. *Int. J. Syst. Evol. Microbiol.* **2014**, *64*, 346–351. [CrossRef] [PubMed]
56. Bergsten, P.; Vannier, P.; Mougeolle, A.; Rigaud, L.; Marteinsson, V.T. Rhodothermus Bifroesti Sp. Nov., a Thermophilic Bacterium Isolated from the Basaltic Subsurface of the Volcanic Island Surtsey. *Int. J. Syst. Evol. Microbiol.* **2022**, *72*, 005214. [CrossRef] [PubMed]
57. Oshima, T.; Imahori, K. Description of Thermus Thermophilus (Yoshida and Oshima) Comb. Nov., a Nonsporulating Thermophilic Bacterium from a Japanese Thermal Spa. *Int. J. Syst. Bacteriol.* **1974**, *24*, 102. [CrossRef]
58. Nazina, T.N.; Tourova, T.P.; Poltaraus, A.B.; Novikova, E.V.; Grigoryan, A.A.; Ivanova, A.E.; Lysenko, A.M.; Petrunyaka, V.V.; Osipov, G.A.; Belyaev, S.S.; et al. Taxonomic Study of Aerobic Thermophilic Bacilli. *Int. J. Syst. Evol. Microbiol.* **2001**, *51*, 433–446. [CrossRef]
59. Elumalai, P.; Parthipan, P.; Narenkumar, J.; Anandakumar, B.; Madhavan, J.; Oh, B.T.; Rajasekar, A. Role of Thermophilic Bacteria (Bacillus and Geobacillus) on Crude Oil Degradation and Biocorrosion in Oil Reservoir Environment. *3 Biotech* **2019**, *9*, 79. [CrossRef]
60. Zhang, Y.X.; Dong, C.; Biao, S. Planifilum Yunnanense Sp. Nov., a Thermophilic Thermoactinomycete Isolated from a Hot Spring. *Int. J. Syst. Evol. Microbiol.* **2007**, *57*, 1851–1854. [CrossRef]
61. Zhao, W.; Zhang, C.L.; Romanek, C.S.; Wiegel, J. Description of Caldalkalibacillus Uzonensis Sp. Nov. and Emended Description of the Genus Caldalkalibacillus. *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 1106–1108. [CrossRef]
62. Duckworth, A.W.; Grant, S.; Grant, W.D.; Jones, B.E.; Meijer, D. Dietzia Natronolimnaios Sp. Nov., a New Member of the Genus Dietzia Isolated from an East African Soda Lake. *Extremophiles* **1998**, *2*, 359–366. [CrossRef]
63. Patel, S.; Gupta, R.S. A Phylogenomic and Comparative Genomic Framework for Resolving the Polyphyly of the Genus Bacillus: Proposal for Six New Genera of Bacillus Species, Peribacillus Gen. Nov., Cytobacillus Gen. Nov., Mesobacillus Gen. Nov., Neobacillus Gen. Nov., Metabacillu. *Int. J. Syst. Evol. Microbiol.* **2020**, *70*, 406–438. [CrossRef] [PubMed]
64. Ettoumi, B.; Chouchane, H.; Guesmi, A.; Mahjoubi, M.; Brusetti, L.; Neifar, M.; Borin, S.; Daffonchio, D.; Cherif, A. Diversity, Ecological Distribution and Biotechnological Potential of Actinobacteria Inhabiting Seamounts and Non-Seamounts in the Tyrrhenian Sea. *Microbiol. Res.* **2016**, *180*, 366–376. [CrossRef] [PubMed]
65. Hugenholtz, P.; Pitulle, C.; Hershberger, K.L.; Pace, N.R. Novel Division Level Bacterial Diversity in a Yellowstone Hot Spring. *J. Bacteriol.* **1998**, *186–187*, 71–80. [CrossRef]
66. Sun, Y.; Liu, Y.; Pan, J.; Wang, F.; Li, M. Perspectives on Cultivation Strategies of Archaea. *Microb. Ecol.* **2020**, *73*, 770–784. [CrossRef]
67. Hahn, M.W.; Koll, U.; Schmidt, J. Isolation and Cultivation of Bacteria. In *The Structure and Function of Aquatic Microbial Communities*; Springer: Cham, Switzerland, 2019.
68. Hungate, R.E. The Anaerobic Mesophilic Cellulolytic Bacteria. *Bacteriol. Rev.* **1950**, *14*, 1–49. [CrossRef] [PubMed]
69. Clark, H. Culturing Anaerobes. *Nat. Res.* **2019**, *2019*.
70. Cho, J.C.; Giovannoni, S.J. Cultivation and Growth Characteristics of a Diverse Group of Oligotrophic Marine Gammaproteobacteria. *Appl. Environ. Microbiol.* **2004**, *70*, 432–440. [CrossRef]
71. D'Hondt, S.; Pockalny, R.; Fulfer, V.M.; Spivack, A.J. Subseafloor Life and Its Biogeochemical Impacts. *Nat. Commun.* **2019**, *10*, 1–13. [CrossRef]
72. Demko, A.M.; Patin, N.V.; Jensen, P.R. Microbial Diversity in Tropical Marine Sediments Assessed Using Culture-Dependent and Culture-Independent Techniques. *Environ. Microbiol.* **2021**, *23*, 6859–6875. [CrossRef]
73. Tang, L. Culturing Uncultivated Bacteria. *Nat. Methods* **2019**, *16*, 1078. [CrossRef]
74. Jung, D.; Liu, L.; He, S. Application of in Situ Cultivation in Marine Microbial Resource Mining. *Mar. Life Sci. Technol.* **2021**, *3*, 148–161. [CrossRef]
75. Jung, D.; Machida, K.; Nakao, Y.; Kindaichi, T.; Ohashi, A.; Aoi, Y. Triggering Growth via Growth Initiation Factors in Nature: A Putative Mechanism for in Situ Cultivation of Previously Uncultivated Microorganisms. *Front. Microbiol.* **2021**, *12*, 1107. [CrossRef] [PubMed]
76. Berdy, B.; Spoering, A.L.; Ling, L.L.; Epstein, S.S. In Situ Cultivation of Previously Uncultivable Microorganisms Using the Ichip. *Nat. Protoc.* **2017**, *12*, 2232–2242. [CrossRef] [PubMed]
77. Crespo, B.G.; Wallhead, P.J.; Logares, R.; Pedrós-Alió, C. Probing the Rare Biosphere of the North-West Mediterranean Sea: An Experiment with High Sequencing Effort. *PLoS ONE* **2016**, *11*, e0159195. [CrossRef] [PubMed]
78. Pédron, J.; Guyon, L.; Lecomte, A.; Blottière, L.; Chandeysson, C.; Rochelle-Newall, E.; Raynaud, X.; Berge, O.; Barny, M.A. Comparison of Environmental and Culture-Derived Bacterial Communities through 16S Metabarcoding: A Powerful Tool to Assess Media Selectivity and Detect Rare Taxa. *Microorganisms* **2020**, *8*, 1129. [CrossRef]
79. Kallmeyer, J. Contamination Control for Scientific Drilling Operations. *Adv. Appl. Microbiol.* **2017**, *98*, 61–91. [CrossRef]
80. Sheik, C.S.; Reese, B.K.; Twing, K.I.; Sylvan, J.B.; Grim, S.L.; Schrenk, M.O.; Sogin, M.L.; Colwell, F.S. Identification and Removal of Contaminant Sequences from Ribosomal Gene Databases: Lessons from the Census of Deep Life. *Front. Microbiol.* **2018**, *9*, 840. [CrossRef]

81. Orsi, W.D.; Edgcomb, V.P.; Christman, G.D.; Biddle, J.F. Gene Expression in the Deep Biosphere. *Nature* **2013**, *499*, 205–208. [CrossRef]
82. Inagaki, F.; Hinrichs, K.U.; Kubo, Y.; Bowles, M.W.; Heuer, V.B.; Hong, W.L.; Hoshino, T.; Ijiri, A.; Imachi, H.; Ito, M.; et al. Exploring Deep Microbial Life in Coal-Bearing Sediment down to ~2.5 Km below the Ocean Floor. *Science* **2015**, *349*, 420–424. [CrossRef]
83. Reese, B.K.; Zinke, L.A.; Sobol, M.S.; LaRowe, D.E.; Orcutt, B.N.; Zhang, X.; Jaekel, U.; Wang, F.; Dittmar, T.; Defforey, D.; et al. Nitrogen Cycling of Active Bacteria within Oligotrophic Sediment of the Mid-Atlantic Ridge Flank. *Geomicrobiol. J.* **2018**, *35*, 468–483. [CrossRef]
84. Salter, S.J.; Cox, M.J.; Turek, E.M.; Calus, S.T.; Cookson, W.O.; Moffatt, M.F.; Turner, P.; Parkhill, J.; Loman, N.J.; Walker, A.W. Reagent and Laboratory Contamination Can Critically Impact Sequence-Based Microbiome Analyses. *BMC Biol.* **2014**, *12*, 87. [CrossRef] [PubMed]
85. Degryse, E.; Glansdorff, N.; Piérard, A. A Comparative Analysis of Extreme Thermophilic Bacteria Belonging to the Genus Thermus. *Arch. Microbiol.* **1978**, *117*, 189–196. [CrossRef] [PubMed]

Supplementary

Culturable Bacterial Diversity from the Basaltic Subsurface of the Young Volcanic Island of Surtsey, Iceland

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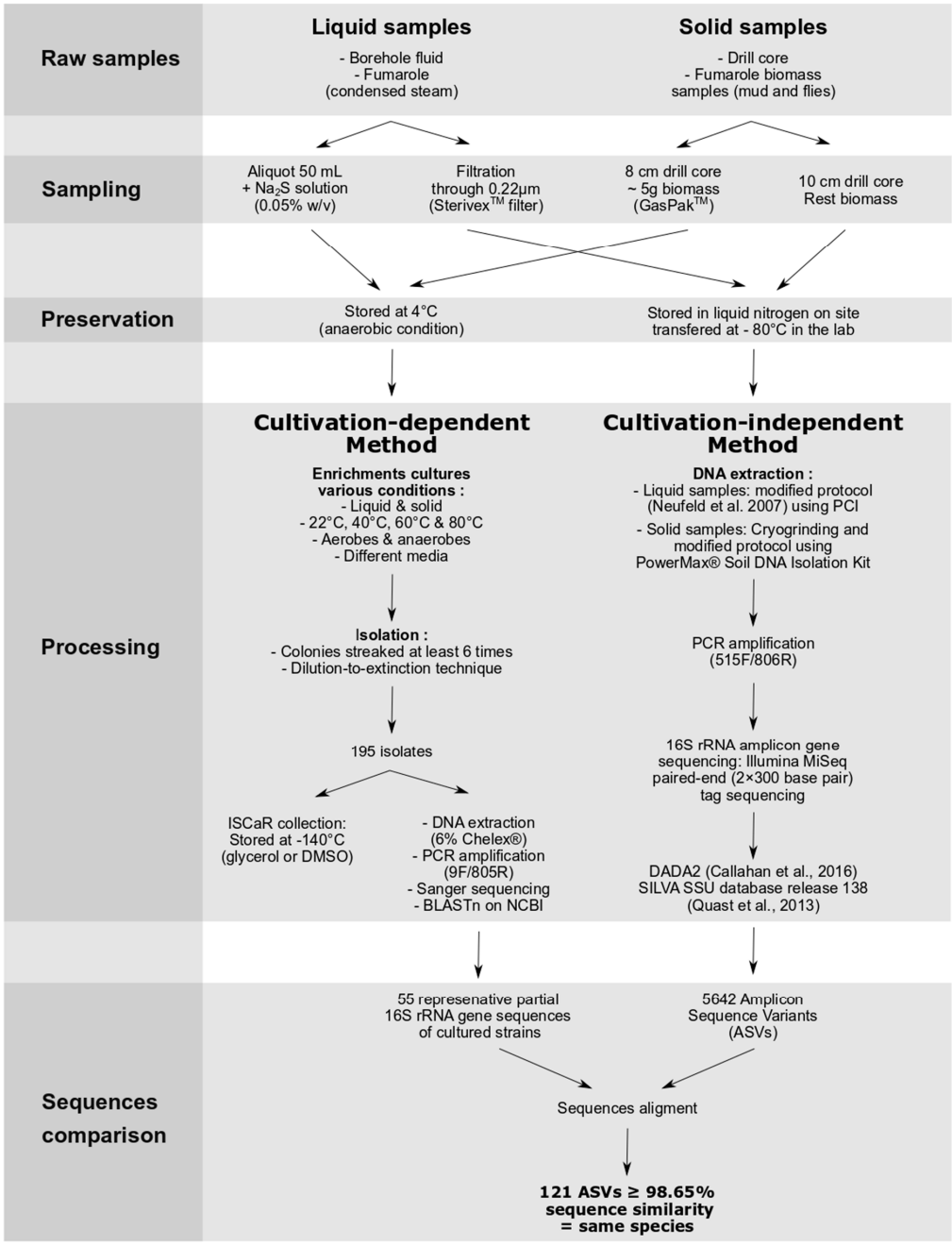
Supplementary text:

Using another method, a supplementary phylogenetic tree was constructed for comparison. The ARB software package (http://www.arb-home.de) (Ludwig et al., 2004) was used with the SILVA SSU Release 138.1 database (Pruesse et al., 2012; Quast et al., 2013) (Fig. S1). Already aligned sequences using SINA were imported in ARB (Fasta_wgap.ift). The sequence alignments were not manually refined. The 151 sequences were added to the SILVA database tree using "ARB parsimony (quick add marked) and the ecoli filter. The closest neighbors were selected (442 sequences) and the tree was built using the sequence data using the maximum likelihood (PhyML-20130708 DNA algorithm) with default parameters. No outgroup was selected.

Supplementary figures:

Figure S1. Maximum likelihood 16S rRNA gene sequence phylogenetic tree of the cultured strains.

Figure S2. Sampling and workflow of experiment.



Supplementary tables:

Table S1. Medium 166 modified from (Hjorleifsdottir et al., 2001) (without proline). Grunnur base from medium 162 from (Degryse et al., 1978).

For 1L of liquid media:	
NaCl	20 g
K_2HPO_4	0.3 g
Yeast extract	1 g
Peptone	1 g
Tryptone	1 g
Glucose	0.5 g
Amidon (starch)	0.5 g
Na-pyruvate	0.6 g
Na_2CO_3	0.18 g
Base "Grunnur"	100 mL
Hot tap water	900 mL
pH adjusted to 7-7.5	
Grunnur (for 1L):	
Titriplex I (nitrilotriacetic acid)	1.32 g
$CaSO_4 \times 2H_2O$	0.4 g
$MgCl_2 \times 6H_2O$	2.0 g
Trace elements (Wolfe´s mineral solution)	5 ml
Ironcitrate solution	5 ml
pH adjusted to 7,2	
Ironcitrate (for 1L):	
Na_3citrate $\times 2H_2O$	2.94 g
$FeCl_3 \times 6H_2O$	2.7 g

Table S2. Medium YPS.

• **Solution A (autoclave)**	For 970 mL
NaCl	10 to 30 g
NH_4Cl	0.5 g
$MgSO_4$ x 7 H_2O	3.4 g
$MgCl_2$ x 6 H_2O	4.18 g
KCl	0.33 g
$FeSO_4$ x 7 H_2O	0.01 g
Na_2SeO_3 x 5 H_2O	1 mg
PIPES	3 g
resazurin (0.1% w/v)	few drops
ASW supplemented by:	
Yeast extract	0.5 g
Peptone	0.5 g
pH at 7	
• **Solution B (autoclave)**	5 mL
K_2HPO_4	0.14 g
• **Solution C (autoclave)**	5 mL
$CaCl_2$ x 2 H_2O	0.50 g
• **Trace element solution (medium 141) (filtration)**	10 mL
• **Extra solutions (filtration)**	
Vitamin solution (see medium 141)	10 mL
elemental sulfur	10 g

After sterilization by autoclave or filtration (0.22 μm) and cooling, the solutions were pooled.

Table S3. Medium Sulfate reducer (SO).

• **Solution A (autoclave)**	For 960 mL
NaCl	10 to 30 g
NH_4Cl	0.5 g
$MgSO_4$ x 7 H_2O	3.4 g
$MgCl_2$ x 6 H_2O	4.18 g
KCl	0.33 g
$FeSO_4$ x 7 H_2O	0.01 g
Na_2SeO_3 x 5 H_2O	1 mg
PIPES	3 g
resazurin (0.1% w/v)	few drops
ASW supplemented by:	
Na_2SO_4	2 g
Yeast extract	0.2 g
L-lactate	0.5 g
Na-pyruvate	0.5 g
L-ascorbate	0.5 g
pH at 7.5	
• **Solution B (autoclave)**	5 mL
K_2HPO_4	0.14 g
• **Solution C (autoclave)**	5 mL
$CaCl_2$ x 2 H_2O	0.50 g
• **Trace element solution (medium 141) (filtration)**	10 mL
• **Extra solutions (filtration)**	
$NaHCO_3$ (0.1% w/v)	20 mL

After sterilization by autoclave or filtration (0.22 μm) and cooling, the solutions were pooled.

Table S4. Medium Iron reducer (I).

• **Solution A (autoclave)**	For 950 mL
NaCl	10 to 30 g
NH_4Cl	0.5 g
$MgSO_4$ x 7 H_2O	3.4 g
$MgCl_2$ x 6 H_2O	4.18 g
KCl	0.33 g
$FeSO_4$ x 7 H_2O	0.01 g
Na_2SeO_3 x 5 H_2O	1 mg
PIPES	3 g
resazurin (0.1% w/v)	few drops
ASW supplemented by:	
Fe(III) citrate	10 g*
Na-acetate	2.5 g
pH at 8	
• **Solution B (autoclave)**	5 mL
K_2HPO_4	0.14 g
• **Solution C (autoclave)**	5 mL
$CaCl_2$ x 2 H_2O	0.50 g
• **Trace element solution (medium 141) (filtration)**	10 mL
• **Extra solutions (filtration)**	
$NaHCO_3$ (0.2% w/v)	20 mL
Vitamin solution (see medium 141)	10 mL
Na_2WO_4 x 2 H_2O (0.1% w/v) (stored under N_2)	0.25 mL

* First dissolve ferric citrate by heating the water under continuous stirring. After cooling to room temperature adjust the pH to 6.0, then add and dissolve the remaining ingredients to prepare solution A. After sterilization by autoclave or filtration (0.22 μm) and cooling, the solutions were pooled.

Table S5. Medium Methanogen (M).

• **Solution A (autoclave)**	For 945 mL
NaCl	10 to 30 g
NH_4Cl	0.5 g
$MgSO_4$ x 7 H_2O	3.4 g
$MgCl_2$ x 6 H_2O	4.18 g
KCl	0.33 g
$FeSO_4$ x 7 H_2O	0.01 g
Na_2SeO_3 x 5 H_2O	1 mg
PIPES	3 g
resazurin (0.1% w/v)	few drops
ASW supplemented by:	
Yeast extract	0.2 g
pH at 7.5	
• **Solution B (autoclave)**	5 mL
K_2HPO_4	0.14 g
• **Solution C (autoclave)**	5 mL
$CaCl_2$ x 2 H_2O	0.50 g
• **Trace element solution (medium 141) (filtration)**	10 mL
• **Extra solutions (filtration)**	
$NaHCO_3$ (0.2% w/v)	20 mL
Vitamin solution (see medium 141)	10 mL
Methanol	5 mL
Coenzyme M (stored under N_2)	0.5 g/L

After sterilization by autoclave or filtration (0.22 μm) and cooling, the solutions were pooled.

Table S6. 16S rRNA gene sequences from the 55 selected cultured strains representing the culture collection.

Seq Num	ISCAR num	Accession number (Genbank)	Table 2 groups (SILVA)	bps	Blast seq	Description NCBI	Query cover	Per. Identity (BLASTn)	lca_tax_slv
1	7133	did not passed quality	Acinetobacter	147	>NR_113346.1	Acinetobacter lwoffii strain JCM 6840	0,99	1	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter;
4	7313	OK534092	Arthrobacter	810	>NR_041546.1	Arthrobacter humicola strain KV-653	1	0,9975	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Micrococcaceae;Arthrobacter;
7	7357	OK534093	Bacillus cereus	534	>NR_121761.1	Bacillus toyonensis strain BCT-7112	1	1	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
13	7211	OK534094	Bacillus (para)licheniformis	453	>NR_118996.1	Bacillus licheniformis strain DSM 13	1	1	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
17	7236	OK534095	Bacillus group 2	446	>NR_025741.1	Bacillus patagoniensis strain PAT 05	1	1	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
22	7378	OK534096	Bacillus group 1	448	>NR_026139.1	Bacillus pseudofirmus strain DSM 8715	1	0,9912	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
27	7207	OK534097	Brevibacillus thermoruber	444	>NR_112213.1	Brevibacillus thermoruber strain DSM 7064	1	0,9977	Bacteria;Firmicutes;Bacilli;Brevibacillales;Brevibacillaceae;Brevibacillus;
28	7309	OK534098	Brevibacillus	552	>NR_112213.1	Brevibacillus thermoruber strain DSM 7064	0,99	0,9982	Bacteria;Firmicutes;Bacilli;Brevibacillales;Brevibacillaceae;Brevibacillus;
29	7368	OK534099	Brevundimonas	536	>NR_113586.1	Brevundimonas vesicularis strain NBRC 12165	1	0,9981	Bacteria;Proteobacteria;Alphaproteobacteria;Caulobacterales;Caulobacteraceae;Brevundimonas;

35	7238	OK534100	Caldalkalibacillus	543	>NR_043653.1	Caldalkalibacillus uzonensis strain JW/WZ-YB58	1	0,9945	Bacteria;Firmicutes;Bacilli;Caldalkalibacillales;Caldalkalibacillaceae;Caldalkalibacillus;
36	7134	OK534101	Cryobacterium	528	>NR_170455.1	Cryobacterium soli strain GCJ02	1	0,9924	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Microbacteriaceae;Cryobacterium;
37	7393	OK534102	Dietzia	1160	>NR_117963.1	Dietzia cercidiphylli strain X0053	1	1	Bacteria;Actinobacteriota;Actinobacteria;Corynebacteriales;Dietziaceae;Dietzia;
38	7131	OK534103	Frigoribacterium	473	>NR_115033.1	Frigoribacterium faeni strain DSM 10309	1	0,9979	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Microbacteriaceae;Frigoribacterium;
39	7191	OK534104	Geobacillus thermoleovorans group	353	>NR_114089.1	Geobacillus kaustophilus NBRC 102445	1	1	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Geobacillus;
41	7371	OK534105	Geobacillus	533	>NR_132400.1	Geobacillus subterraneus subsp. aromaticivorans strain Ge1	0,99	0,9962	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Geobacillus;
51	7472	OK534106	Georgenia	484	>NR_112820.1	Georgenia muralis strain NBRC 103560	1	0,9877	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Bogoriellaceae;Georgenia;
55	7360	OK534107	Glutamicibacter	553	>NR_025612.1	Glutamicibacter bergerei strain Ca106	1	1	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Micrococcaceae;Glutamicibacter;
56	7319	OK534108	Halomonas	1231	>NR_114866.1	Halomonas glaciei strain DD 39	1	0,9903	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;Halomonas;
61	7458	OK534109	Halomonas	544	>NR_027185.1	Halomonas sulfidaeris Esulfide1	1	0,9818	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;Halomonas;
62	7320	OK534110	Halomonas	513	>NR_027185.1	Halomonas sulfidaeris Esulfide1	1	0,9787	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;Halomonas;

66	7314	OK534111	Janibacter	522	>NR_026362.1	Janibacter limosus strain DSM 11140	1	0,9981	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Intrasporangiaceae;Janibacter;
68	7364	OK534112	Kocuria	566	>NR_026451.1	Kocuria palustris strain TAGA27	1	0,9929	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Micrococcaceae;Kocuria;
69	7190	OK534113	Enterobacteriaceae	230	>NR_118121.1	Pantoea rwandensis strain LMG 26275	1	1	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;
70	7130	OK534114	Leifsonia	341	>NR_115031.1	Leifsonia poae strain DSM 15202	1	1	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Microbacteriaceae;Leifsonia;
71	7386	OK534115	Marinomonas	526	>NR_116234.1	Marinomonas foliarum strain IVIA-Po-155	1	0,9924	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Marinomonadaceae;Marinomonas;
73	7392	OK534116	Microbacterium lacus	531	>NR_041516.1	Microbacterium ginsengisoli strain Gsoil 259	0,99	0,9811	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Microbacteriaceae;Microbacterium;
76	7395	OK534117	Microbacterium lacus	1356	>NR_041563.1	Microbacterium lacus strain A5E-52	1	0,9897	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Microbacteriaceae;Microbacterium;
78	7132	OK534118	Micrococcus	342	>NR_117194.1	Micrococcus cohnii strain WS4601	1	0,9942	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Micrococcaceae;Micrococcus;
80	7209	OK534119	Intrasporangiaceae	196	>NR_164959.1	Janibacter massiliensis strain Marseille-P4121	1	1	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Intrasporangiaceae;
82	7251	OK534120	Paenibacillus	534	>NR_113987.1	Paenibacillus pasadenensis strain NBRC 101214	1	0,9981	Bacteria;Firmicutes;Bacilli;Paenibacillales;Paenibacillaceae;Paenibacillus;

90	7379	OK534121	Paeniglutamicibacter	559	>NR_026237.1	Paeniglutamicibacter sulfureus strain DSM 20167	1	0,9946	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Micrococcaceae;Paeniglutamicibacter;
91	7271	OK534122	Enterobacter	598	>NR_111998.1	Pantoea agglomerans strain JCM1236	1	1	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;Enterobacter;
92	7310	OK534123	Paracoccus	560	>NR_113921.1	Paracoccus marinus strain NBRC 100637	1	0,9964	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Paracoccus;
94	7312	OK534124	Janibacter	471	>NR_108472.1	Phycicoccus badiiscoriae strain Sco-B23	1	1	Bacteria;Actinobacteriota;Actinobacteria;Micrococcales;Intrasporangiaceae;Janibacter;
95	7331	OK534125	Planifilum	1375	>NR_043563.1	Planifilum yunnanense strain LA5	1	0,9993	Bacteria;Firmicutes;Bacilli;Thermoactinomycetales;Thermoactinomycetaceae;Planifilum;
96	7456	OK534126	Planococcaceae	407	>NR_156838.1	Planococcus versutus strain L10.15	1	0,978	Bacteria;Firmicutes;Bacilli;Bacillales;Planococcaceae;
97	7327	OK534127	Planomicrobium	1189	>NR_113593.1	Planomicrobium okeanokoites strain NBRC 12536	1	0,9975	Bacteria;Firmicutes;Bacilli;Bacillales;Planococcaceae;Planomicrobium;
103	7375	OK534128	Pseudoaltero monas	592	>NR_114191.1	Pseudoaltero monas undina strain NBRC 103039	1	1	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Pseudoalteromonadaceae;Pseudoalteromonas;
104	7391	OK534129	Pseudoaltero monas	549	>NR_114191.2	Pseudoaltero monas undina strain NBRC 103040	1	1	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Pseudoalteromonadaceae;Pseudoalteromonas;

110	7352	OK534130	Pseudomonas group 1	603	>NR_025103.1	Pseudomonas brenneri strain CFML 97-391	1	1	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;
114	7205	OK534131	Pseudomonas group 3	365	>NR_152710.1	Pseudomonas turukhanskensis strain IB1.1	1	0,9945	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;
116	7329	OK534132	Pseudomonas group 2	1218	>NR_134795.1	Pseudomonas zhaodongensis strain NEAU-ST5-21	1	0,9984	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;
119	7381	OK534133	Allorhizobium - Neorhizobium - Pararhizobium -Rhizobium	557	>NR_116445.1	Rhizobium rosettiformans W3	1	0,9911	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium;
120	7396	OK534134	Rhodococcus group 1	512	>NR_116275.1	Rhodococcus cercidiphylli strain YIM 65003	1	1	Bacteria;Actinobacteriota;Actinobacteria;Corynebacteriales;Nocardiaceae;Rhodococcus;
124	7354	OK534135	Rhodococcus group 2	494	>NR_145886.1	Rhodococcus degradans strain CCM 4446	1	1	Bacteria;Actinobacteriota;Actinobacteria;Corynebacteriales;Nocardiaceae;Rhodococcus;
129	7401	OK534136	Rhodothermus	1255	>NR_074728.1	Rhodothermus marinus DSM 4252	1	0,9536	Bacteria;Bacteroidota;Rhodothermia;Rhodothermales;Rhodothermaceae;Rhodothermus;
134	7405	OK534137	Rubrobacter	578	>NR_074552.1	Rubrobacter xylanophilus strain DSM 9941	1	0,9692	Bacteria;Actinobacteriota;Rubrobacteria;Rubrobacterales;Rubrobacteriaceae;Rubrobacter;
138	7206	OK534138	Serratia	420	>NR_025339.1	Serratia fonticola strain DSM 4576	1	0,9976	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Yersiniaceae;Serratia;

140	7394	OK534139	Shewanella	1247	>NR_040951.1	Shewanella kaireitica strain c931	1	0,9912	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Shewanellaceae;Shewanella;
141	7137	OK534140	Sphingomonadaceae	294	>NR_026304.1	Sphingobium xenophagum strain BN6	1	1	Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;
142	7145	OK534141	Rhodobacteraceae	404	>NR_043547.1	Sulfitobacter litoralis strain Iso 3	1	1	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;
143	7204	OK534142	Thermus	278	>NR_037066.1	Thermus thermophilus HB8	1	1	Bacteria;Deinococcota;Deinococci;Thermales;Thermaceae;Thermus;
144	7189	OK534143	Thermus	317	>NR_037066.1	Thermus thermophilus HB8	1	1	Bacteria;Deinococcota;Deinococci;Thermales;Thermaceae;Thermus;
150	7215	OK534144	Ureibacillus	480	>NR_119203.1	Ureibacillus thermosphaericus strain P-11	1	1	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Ureibacillus;
151	7129	OK534145	Polaromonas	251	>NR_109102.1	Variovorax defluvii strain 2C1-b	1	0,9801	Bacteria;Proteobacteria;Gammaproteobacteria;Burkholderiales;Comamonadaceae;Polaromonas;

Paper III

Rhodothermus bifroesti sp. nov., a thermophilic bacterium isolated from the basaltic subsurface of the volcanic island Surtsey.

Pauline Bergsten, Pauline Vannier, Alan Mougeolle, Louise Rigaud, and Viggó Þór Marteinsson

Published in *International Journal of Systematic and Evolutionary Microbiology* (2022*) Volume 72*.

Candidate contribution:
Conceived and designed the study, performed fieldwork, performed laboratory work, analyzed data, wrote the manuscript, reviewed and edited the manuscript and corresponded during the publication process.

INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Bergsten *et al.*, *Int. J. Syst. Evol. Microbiol.*
DOI 10.1099/ijsem.0.005214

MICROBIOLOGY SOCIETY

Rhodothermus bifroesti sp. nov., a thermophilic bacterium isolated from the basaltic subsurface of the volcanic island Surtsey

Pauline Bergsten[1,2], Pauline Vannier[1], Alan Mougeolle[1], Louise Rigaud[1] and Viggó Thór Marteinsson[1,3,*]

Abstract

Novel thermophilic heterotrophic bacteria were isolated from the subsurface of the volcanic island Surtsey off the south coast of Iceland. The strains were isolated from tephra core and borehole fluid samples collected below 70 m depth. The Gram-negative bacteria were rod-shaped (0.3–0.4 µm wide, 1.5–7 µm long), aerobic, non-sporulating and non-motile. Optimal growth was observed at 70 °C, at pH 7–7.5 and with 1% NaCl. Phylogenetic analysis identified the strains as members of the genus *Rhodothermus*. The type strain, ISCAR-7401^T, was genetically distinct from its closest relatives *Rhodothermus marinus* DSM 4252^T and *Rhodothermus profundi* PRI 2902^T based on 16S rRNA gene sequence similarity (95.81 and 96.01%, respectively), genomic average nucleotide identity (73.73 and 72.61%, respectively) and digital DNA–DNA hybridization (17.6 and 16.9%, respectively). The major fatty acids of ISCAR-7401^T were iso-$C_{17:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{15:0}$ (>10 %). The major isoprenoid quinone was MK-7 while phosphatidylethanolamine, diphosphatidylglycerol, an unidentified aminophospholipid and a phospholipid were the predominant polar lipid components. Based on comparative chemotaxonomic, genomic and phylogenetic analyses, we propose that the isolated strain represents a novel species of the genus *Rhodothermus* with the name *Rhodothermus bifroesti* sp. nov. The type strain is ISCAR-7401^T (=DSM 112103^T=CIP 111906^T).

INTRODUCTION

The order *Rhodothermales*, together with the *Balneolales*, form the superclass *Rhodothermae*, which represents a deep lineage within *Bacteroidetes*/*Chlorobi* group [1–3]. It currently contains four families that exhibit different characteristics: *Salinibacteraceae* (extremely halophilic), *Salisaetaceae* (halophilic; can be extremely alkalophilic), *Rubricoccaceae* (mesophilic) and *Rhodothermaceae* (thermophilic) [3–6]. To date, the members of the order *Rhodothermales* are described as chemoheterotrophic aerobes, requiring NaCl for growth (0.5 to>15%). They were isolated from salty environments and often form red-, orange- to pink-pigmented colonies. The cells are Gram-stain-negative, non-spore-forming, rod-shaped and either non-motile or mobile with flagella.

The genus *Rhodothermus*, a member of the family *Rhodothermaceae*, was first described in 1988, with *Rhodothermus marinus* as the type species, originating from submarine alkaline hot springs in Iceland [7]. This thermophilic and moderately halophilic organism grows at a temperature range of about 54–77 °C (optimum at 65 °C) and a salinity range of 0.5–6.0% NaCl [7]. Many different *Rhodothermus* strains have been isolated in similar geothermal habitats worldwide [8–10]. In 1996, Sako and collaborators suggested the addition of *Rhodothermus obamensis* [11], a thermophilic bacterium isolated from a shallow marine hydrothermal vent in Japan. However, Silva *et al.* demonstrated that the two species were synonymous, based on 16S rRNA gene sequencing comparison,

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Keywords: *Rhodothermus*; *Rhodothermaceae*; subsurface; volcanic island Surtsey; thermophile; genome; Iceland.
Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA—DNA hybridization.
Accession number for the genome: JAGKTL000000000. The unassembled sequencing data (accession SRR14119291), assembled genome (accession JAGKTL000000000) and the full 16S rRNA gene (accession MW901484) were assigned to the NCBI BioProject: PRJNA718815 and BioSample: SAMN18570965.
Three supplementary tables and 11 supplementary figures are available with the online version of this article. The annotated genes from the genome of strain ISCAR-7401T in this study can be found on Figshare at https://figshare.com/s/748a2928b2e6066776e8https://figshare.com/s/748a2928b2e6066776e8 and https://figshare.com/s/12b6ef2b844efcb227c9.

DNA–DNA hybridization values and fatty acid profiles [9, 12]. At the time of writing, the genus *Rhodothermus* comprises only one additional validly named species, *Rhodothermus profundi*. This organism was isolated from a deep-sea hydrothermal vent in the Pacific Ocean [13]. *R. profundi* strains share characteristics with *R. marinus* in being aerobic, thermophilic and neutrophilic heterotrophs. Cells are rod-shaped, Gram-stain-negative and are non-spore-forming, non-motile, oxidase-negative and catalase-positive. However, *R. profundi* does not exhibit red pigments and grows at a higher range of temperatures (55–80 °C, optimum at 70–75 °C) than *R. marinus*.

Here, we describe strains isolated from the subsurface of the young island Surtsey, located on the volcanic rift zone south of Iceland. The strains are related to the genus *Rhodothermus* while differing from *R. marinus* and *R. profundi* in genetic and phenotypic features. We propose to position the strains within a novel species with the name *Rhodothermus bifroesti* sp. nov. The type strain is ISCAR-7401^T.

ISOLATION AND ECOLOGY

The novel strains were isolated from the subsurface of Surtsey island, located in the Vestmannaeyjar archipelago on the southern offshore extension of the Icelandic volcanic rift zone (63° 18′ 8.878″ N; 20° 35′ 54.784″ W). The island was formed between 1963 and 1967 by underwater basaltic eruptions from the seafloor [14–16]. Cultivation and strain isolation was part of an effort supported by the International Continental Scientific Drilling Program (ICDP) among other project partners, to characterize the subsurface edifice of the island in terms of volcanology, mineralogy, hydrology, chemistry and microbiology [17–19]. Of many bacteria isolated while studying the cultivable part of the subsurface prokaryotic diversity, the strains described here were selected because of their low 16S rRNA gene sequence similarity (~96 %) to other species. Their closest relatives were bacterial species belonging to the genus *Rhodothermus* [7, 10, 13, 20, 21]. Eight strains had similar phenotypic characteristics. Two of them were isolated from a tephra drill core collected at 70 m depth in the volcano (*in situ* temperature: ~100 °C; strains ISCAR-7403 and 7404). The others were isolated from borehole fluid samples collected at 75 (ISCAR-7397 and 7398), 80 (ISCAR-7399 and 7400) and 160 m depth (ISCAR-7401^T and 7402). Enrichment cultures for each sample (rock pieces and subsurface fluids) were set up in liquid medium 166 [20] supplemented with 2 % NaCl (Table S1, available in the online version of this article). After 3 days of incubation at 60 °C, about 0.1 ml of the enrichment cultures were spread on the same medium containing agar (14 g l^{-1}). Colonies appeared after 48 h of incubation and strains were purified by streaking at least six times. Four of the purified strains, ISCAR-7401^T, 7403, 7399, and 7397 were selected for further studies and strain ISCAR-7401^T for full characterization. *R. marinus* DSM 4252^T and *R. profundi* PRI 2902^T were used as reference strains during this study.

GENOME FEATURES AND 16S RRNA PHYLOGENY

Genomic DNA from strains ISCAR-7401^T, 7403, 7399 and 7397 was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre). The 16S rRNA gene was amplified using OneTaq Hot Start DNA Polymerase according to manufacturer's instructions (New England BioLabs) using multiple primers: 27F (5′-AGAGTTTGATC-CTGGCTCAG-3′) and 805R (5'- GACTACCAGGGTATCT AATCC-3′), 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 1046R (5′-CGACAGCCATGCANCACCT-3′) and 784F (5'- AGGATTAGATACCCTGGTA-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The amplified 16S rRNA gene fragments were purified by using Exonuclease I and Shrimp Alkaline Phosphatase (New England BioLabs) and sequenced with an ABI 377 DNA sequencer by using BigDye Terminator Cycle Sequencing Ready Reaction kit according to the manufacturer (PE Applied Biosystems). Sequencher 5.2.4 software (Gene Codes Corporation) was used for sequence quality check and assembly. Nearly complete 16S rRNA gene sequences were obtained (1265–1267 bp).

The genome of strain ISCAR-7401^T was sequenced by using Illumina MiSeq (2×200 bp) and produced 1981862 raw reads. The genome assembly is available from the NCBI BioProject PRJNA718815. Raw sequences were trimmed with Trimmomatic (version 0.38) [22] and FastQC was used to confirm the quality of the trimming. SPAdes (version 3.12.0) [23] was used for the assembly. QUAST 5.0.2 (http://quast.sourceforge.net/quast.html) and CheckM (version 1.0.18) were used to assess the quality of the assembly and the genome, respectively. The average nucleotide identity (ANI) values between pairs of genomes and genome G+C contents were obtained by using the ANI Calculator from EzBioCloud [24] and the digital DNA–DNA hybridization (dDDH) values were obtained by using the Genome-to-Genome Distance Calculator 2.1 online tool from the DSMZ (https://ggdc.dsmz.de/) [25]. The results of formula 2 were adopted.

The draft assembly contained eight contigs of 2884752 bases in total (N50:738146). This was considerably smaller than the genomes of *R. marinus* DSM 4252^T (3.26 Mb) and *R. profundi* PRI 2902^T (3.14 Mb) (Table 1). The G+C content was 56.19 mol%. Genome completeness was at 99.44 % and no contamination was detected. The genome sequence of strain ISCAR-7401^T has been deposited at DDBJ/ENA/GenBank under the accession number JAGKTL000000000. The version described in this paper is JAGKTL010000000.

Genome annotation was performed using RASTtk version 1.073 [26] and Kbase (annotate genome/assembly) [27]. The 2720 predicted open reading frames included 2511 coding sequences (Table S2). The genes had 1701 distinct functions, including 1374 genes with an SEED annotation ontology.

ANI values of 95–96% (dDDH value of 70% or 98.65% 16S rRNA sequence similarity) have been described as a boundary for species delineation [28–30]. The ANI values

Table 1. Phylogenetic and genomic characteristics that differentiate strain ISCAR-7401^T from its three most closely related type strains

Strains: 1, ISCAR-7401^T; 2, *Rhodothermus marinus* DSM 4252^T (NR_029282.2); 3, *Rhodothermus profundi* strain PRI 2902^T (NR_116762); 4, *Roseithermus sacchariphilus* MEBiC 09517 ^{T} (MG603595.1). Columns are ordered by decreasing values of average nucleotide identity.

Characteristic	1	2	3	4
Genome size (Mb)	2.88	3.26	3.14	4.81
Number of contigs (≥1000 bp)	8	1	18	1
G+C content (mol%)	56.19	64.3	59.11	68.65
Average nucleotide identity to strain 1 (%)	100	73.73	72.61	67.95
digital DNA–DNA hybridization (Generalized linear model-based*) to strain 1 (%)	100	17.6 [15.5–19.9]	16.9 [14.8–19.2]	19.2 [17.1–21.6]
16S rRNA gene similarity (%)	100	95.81	96.16	89.73

*Model-based confidence intervals are specified in square brackets.

for ISCAR-7401^T with *R. marinus* DSM 4252^T, *R. profundi* PRI 2902^T and *Roseithermus sacchariphilus* MEBiC 09517^T were 73.73, 72.61 and 67.95%, respectively (Table 1). The dDDH values for ISCAR-7401^T with the same strains were 17.6, 16.9 and 19.2%, respectively (Table 1). These values and the difference in genome sizes supported a genus status of strain ISCAR-7401^T within the genus *Rhodothermus*.

Partial 16S rRNA gene sequences of about 1265 bp from the seven other strains showed more than 99% sequence similarity among them and with ISCAR-7401^T (Table S3). A complete 16S rRNA gene sequence of 1523 bp was extracted from the genome assembly of strain ISCAR-7401^T. Phylogenetic analysis was performed using the complete 16S rRNA gene sequence of strain ISCAR-7401^T and revealed 96.16% similarity to *R. profundi* PRI 2902^T (NR_116762), 95.81% to *R. marinus* DSM 4252^T (NR_029282.2) and 89.73% to *Roseithermus sacchariphilus* MEBiC 09517^T (MG603595). Sequences were aligned using the ClustalW program [31]. Phylogenetic trees were reconstructed using the neighbour-joining [32] (Fig. 1), maximum-likelihood and maximum-parsimony (Figs S1 and S2) algorithms in MEGA7 [33], based on 1000 bootstrap replications and a total of 1354 positions

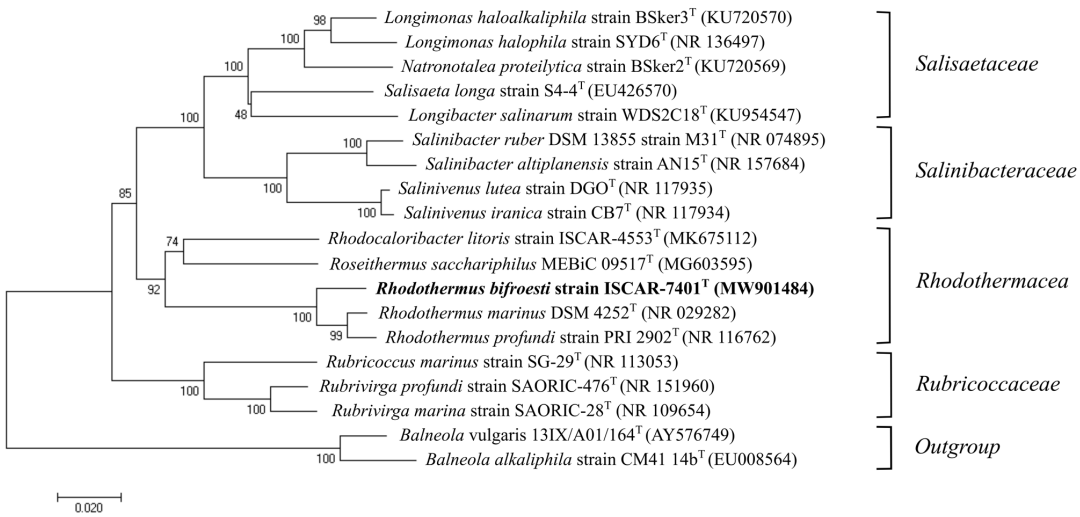

Fig. 1. Phylogenetic tree based on the complete 16S rRNA gene sequences (1523 bp) showing the relationship between strain ISCAR-7401^T and members of the order *Rhodothermales*. GenBank accession numbers are given in parentheses. The tree is based on the ClustalW and the neighbour-joining method with 1000 bootstraps using a total of 1354 positions in the final dataset. The sequences of *Balneola* species were used as an outgroup. Bar, 0.02 represented the nucleotide substitution per position.

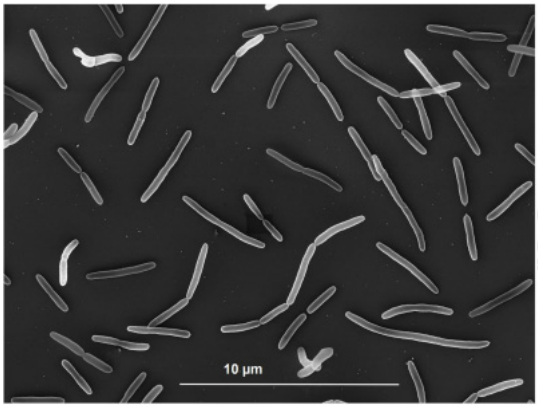

Fig. 2. SEM image of strain ISCAR-7401^T grown for 48 h at 70 °C on solid medium 166 with 1 % NaCl, pH 7.5. Bar, 10 µm; EHT, 20 kV.

in the final dataset. *Balneola* species, from the family *Crenotrichaceae*, served as the outgroup. The trees showed the same topology, where strain ISCAR-7401^T was positioned next to *R. marinus* and *R. profundi*, suggesting that it was a member of the family *Rhodothermaceae* while more distant from the related genera of *Roseithermus* and *Rhodocaloribacter* (Figs 1, S1 and S2).

PHYSIOLOGY AND CHEMOTAXONOMY

The novel strains were routinely cultivated in medium 166 containing 1% NaCl at 70 °C, with or without agar [34] (Table S1). *R. marinus* DSM 4252^T was cultivated under the same conditions while *R. profundi* PRI 2902^T did not grow in medium 166 and so was grown in half strength marine broth [35]. The temperature, NaCl concentration and pH ranges for growth were tested in liquid medium 166 with 1% NaCl for 2–7 days. The temperatures tested were at 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C (Fig. S5). The range of NaCl concentrations was tested in medium 166 containing 0–7% (at intervals of 1%), as well as at pH 5.0, 5.5, 6.0, 7.0, 7.5, 8.0, 9.0, 9.5 and 10.0 (Figs S6 and S7). Negative controls were used for all tested culture conditions. For detailed growth assessment, samples were acquired at 1 or 2 h intervals for 30 h and optical density was measured at 580 nm using a Novaspec III Spectrophotometer (Biochrom). Growth curves were used to calculate the growth rates of ISCAR-7401^T at each condition.

Gram-staining was performed on fresh cells using the BBL Gram Stain Kit (BD) following the manufacturer's instructions. Catalase and oxidase activities were evaluated in triplicate by the addition of 3% (v/v) H_2O_2 solution to the cells and an oxidase reagent kit (Difco BBL), respectively. Single-carbon source assimilation was tested on minimal medium as described in Alfredsson *et al.* [7]. The minimal medium consisted of modified agar medium 166 at pH 7.5 and 1% NaCl, without peptone, tryptone, glucose, starch or Na-pyruvate and with yeast extract reduced to 0.01% (w/v). In addition, the medium contained 4 mM NH_4Cl, 10 mM phosphate buffer (KH_2PO_4, Na_2HPO_4) at pH 7.2, 4 ml of vitamin solution [36] and a final concentration of 0.4% (w/v) for sugars and 0.2% (w/v) of other carbon sources. Growth was tested in duplicate for 1 week at 70 °C on the following carbon sources: D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, raffinose, D-rhamnose, sucrose, acetate, citrate, L-malic acid, pyruvate, succinate, L-aspartate, L-glutamate, L-alanine, L-arginine, L-asparagine, L-glutamine, L-proline, L-serine, L-threonine, L-valine and glycerol. For *R. marinus* DSM 4252^T and the novel strains, susceptibility to antibiotics was tested in triplicate on plates with medium 166 at pH 7.5 containing 1 % NaCl at the optimum growth conditions using antibiotic discs (Oxoid; 30 µg per disc): rifampicin, gentamicin and kanamycin. Susceptibility to streptomycin was tested at the same conditions while in liquid medium with the addition of the antibiotic at 100 µg ml^{-1}, 30 µg ml^{-1} and 10 µg ml^{-1}. For *R. profundi* PRI 2902^T, susceptibility to antibiotics was tested using the same procedure in half strength marine broth, with or without agar. Anaerobic growth was tested on agar plate medium 166, pH 7.5 and 1% NaCl at 70 °C in a BD GasPak for 1 week.

A polarized light microscope Olympus BX51 (×100, 1.30 oil, Ph3) and a scanning electron microscope (SEM; Supra25 Gemini FE-SEM, Zeiss) were used to observe the cell size and morphology of strain ISCAR-7401^T (Figs 2 and S4). Cells were grown at 70 °C in liquid medium 166, pH 7.5 and 1% NaCl. For SEM, exponentially grown cells were fixed in phosphate buffer (25 mM, pH 7) with 2% glutaraldehyde for 15 min at room temperature and at 4 °C overnight. Samples were washed twice in 1 ml of the same buffer. After careful resuspension, samples were placed on poly-L-lysine coated coverslips for 1 h. They were subsequently dehydrated in increasing concentrations of ethanol (10, 30, 50, 70, 90 and 100%) for 15 min each, and critical point dried using a Leica EM CPD300. They were gold-palladium-coated with argon gas using an Edwards Sputter Coater S150B and visualized with FE-SEM at an electron high tension (EHT) voltage of 20 kV.

The colonies of strains ISCAR-7401^T, 7403, 7399 and 7397 appeared to be circular with entire margins, 2.5–3.5 µm in diameter, flat, translucent and pinkish-pigmented after 3 days of incubation at 70 °C on agar plate medium 166, pH 7.5 and 1% NaCl (Table S1). In addition, the colonies displayed an iridescent characteristic exhibiting all spectral colours ranging from red to blue only under the condition of oblique illumination (light coming from a certain angle) (Fig. S3). To the best of our knowledge, this feature has never been reported among thermophilic bacteria. The novel strains were Gram-stain-negative and their cells were rod-shaped, measuring approximately 0.3–0.4 µm wide and 1.5–7 µm long (Fig. 2). They were strictly aerobic, non-motile, non-sporulating, oxidase-negative and catalase-negative (Table S3). Ranges of growth of strains ISCAR-7401^T, 7403, 7399, and 7397 were estimated after 2–7 days for all strains at 44–77

Table 2. Phenotypic characteristics that distinguish strain ISCAR-7401^T from the type strains of the genus *Rhodothermus*

Strains: 1, ISCAR-7401^T; 2, *Rhodothermus marinus* DSM 4252^T; 3, *Rhodothermus profundi* strain PRI 2902^T. Data were obtained in the present study unless indicated otherwise. –, Negative; +, positive; /, no growth was obtained on minimal medium.

Characteristic	1	2	3
Pigmentation	Pinkish	Orange-red	Colourless
Appearance	Translucent	Opaque	Opaque
Rod size (width×length; μm)	0.3–0.4×1.5–7.0	0.5×2.0–2.5	0.5×1.5–3.5†
Optimum growth temperature (°C)	70	67	70†
Growth at/with:			
50 °C	+	+	–
80 °C	–	–	+
0 % NaCl (w/v)	+	–	–
6 % NaCl (w/v)	–	+	–
Utilization of:			
D-Fructose, acetate, L-alanine	–	+	/
Maltose, L-glutamate	+	+	/
L-Asparagine, L-glutamine	+	–	/
Resistance to:			
Rifampicin	+	–	–
Gentamicin	–	+	+
Catalase reaction	–	+	+
Polar lipids*	PE, DPG, APL, AL, PLs, GLs	PE, DPG, PG, PL, GL‡	DPG, PE, PG, PLs, GLs†
Fatty acids (>10 %)	iso-$C_{17:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$	iso-$C_{17:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$, anteiso-$C_{15:0}$	iso-$C_{17:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$, anteiso-$C_{15:0}$
Menaquinones	MK-7 (98.8%), MK-8 (1.2%)	MK-7 (96.7%), MK-6 (2.7%)§	MK-7 (100%)†
Isolation source	Hot basaltic subsurface	Costal submarine hot spring¶	Deep-sea hydrothermal vent†

*DPG, diphosphatidylglycerol; GL, unidentified glycolipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unidentified phospholipid; AL, unidentified aminolipid; APL, unidentified aminophospholipid; L, unidentified lipid.
†Data from [13].
‡Data from [39].
§Data from [37].
¶Data from [7].

°C (optimum, 70 °C), at pH 5.5–9.5 (optimum, pH 7.0–7.7) and with NaCl 0–5.5% (w/v; optimum, 1%). Weak growth was observed at NaCl 6% for strain ISCAR-7403 (Table S3). All the novel strains grew using D-galactose, glucose, lactose, maltose, D-mannose, raffinose, D-rhamnose, sucrose, pyruvate, L-asparagine, L-aspartate, L-glutamine and L-glutamate as sole sources of carbon for energy and growth, except for strain ISCAR-7403 that did not grow on maltose and L-glutamate. L-Asparagine and L-glutamine supported weak growth for strains ISCAR-7399 and 7403. D-Fructose, acetate, citrate, L-malic acid, L-alanine, L-arginine, L-proline, L-serine, L-threonine and L-valine were not utilized by any of the novel strains. All the strains were resistant to aminoglycoside antibiotics (rifampicin, kanamycin, streptomycin) except for gentamicin (Table 2).

The analyses of cellular fatty acids (MIDI System and GC-MS), quinones (HPLC and ultra-high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry) and polar lipids (two-dimensional silica gel thin-layer chromatography) composition were carried out on freeze-dried cells from actively growing liquid cultures (medium 166, 1% NaCl at 70 °C) by the Identification Service of Leibniz-Institute DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (details in supplementary information) [33–39]. The reference strains,

Table 3. Cellular fatty acid compositions of strain ISCAR-7401^T and closely related species from the genus *Rhodothermus*

Strains: 1, ISCAR-7401^T; 2, *Rhodothermus marinus* DSM 4252^T; 3, *Rhodothermus profundi* strain PRI 2902^T. Data were obtained in the present study. Values are percentages of total fatty acids.

Fatty acid	1	2	3
Saturated:			
$C_{16:0}$	4.44	3.06	2.34
$C_{17:0}$	0.58	0.41	–
$C_{18:0}$	0.82	0.70	0.50
Saturated branched:			
iso-$C_{14:0}$	0.55	1.70	1.07
iso-$C_{15:0}$	11.20	6.68	7.46
anteiso-$C_{15:0}$	16.31	13.48	10.28
iso-$C_{16:0}$	7.68	15.00	19.72
iso-$C_{17:0}$	40.07	32.78	32.66
anteiso-$C_{17:0}$	16.13	18.79	15.98
iso-$C_{18:0}$	2.23	6.61	9.17
iso-$C_{19:0}$	–	0.24	0.41
anteiso-$C_{19:0}$	–	0.55	0.41

R. marinus DSM 4252^T and *R. profundi* PRI 2902^T, were also sent for parallel analysis of fatty acids. For comparison, all the strains were grown in half strength marine broth at 70 °C. The fatty acids of strain ISCAR-7401^T mainly consisted of carbons with 15 or 17 chain lengths such as iso-$C_{17:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ (>10%). Chains containing 16 carbons were also abundant, including iso-$C_{16:0}$ (7.68%) and $C_{16:0}$ (4.44%) (Table 3, Figs S9–S11). The respiratory quinones detected in strain ISCAR-7401^T were MK-7 (98.8%) and MK-8 (1.2%). The polar lipid profile of strain ISCAR-7401^T consisted of diphosphatidylglycerol, phosphatidylethanolamine, one unidentified aminophospholipid, one unidentified aminolipid, three unidentified glycolipids and two unidentified phospholipids (Table 2, Fig. S8). The whole-cell sugar analysis revealed that ribose, xylose and glucose were the main components, while minor amounts of arabinose and galactose were also detected.

Colonies of the novel strains differed from those of the reference strains. Both *R. marinus* DSM 4252^T and *R. profundi* PRI 2902^T produced opaque colonies that were red-orange and colourless, respectively, while the novel strains produced pinkish and translucent colonies (Table 2). The novel strains were catalase-negative in contrast with both reference strains. The optimum growth temperature of strains ISCAR-7401^T, 7403, 7399 and 7397 was 70 °C, which was similar to *R. profundi* PRI 2902^T as previously reported [13], while higher than that of *R. marinus* DSM 4252^T, which grew best at 67 °C. Under the same laboratory conditions, *R. marinus* DSM 4252^T grew at the same temperature and pH ranges as strains ISCAR-7401^T, 7403, 7399 and 7397 but at a higher NaCl concentration range of 1–6%. *R. profundi* PRI 2902^T showed a higher temperature range than the others strain, which could not grow at 50 °C but grew at 80 °C. The proposed species is the only described *Rhodothermus* species that can grow without NaCl. The novel strains were resistant to rifampicin and sensitive to gentamicin, which differentiate them from *R. marinus* DSM 4252^T and *R. profundi* PRI 2902^T, which were sensitive to rifampicin and resistant to gentamicin (Table 2).

The most abundant type of fatty acid was also iso-$C_{17:0}$ in *R. marinus* DSM 4252^T (32.78%) and *R. profundi* PRI 2902^T (32.66%). However, this type of fatty acid was detected in strain ISCAR-7401^T at a higher percentage (40.07%), as well as for the types anteiso-$C_{15:0}$ and iso-$C_{15:0}$. In addition, strain ISCAR-7401^T showed lower abundance of iso-$C_{16:0}$ and iso-$C_{18:0}$ and no chains containing 19 carbons were detected (Table 3). The respiratory quinones detected in strain ISCAR-7401^T (MK-7 at 98.8% and MK-8 at 1.2%) differed from *R. profundi* PRI 2902^T (MK-7 at 100%) and *R. marinus* DSM 4252^T (MK-7 at 96.7% and MK-6 at 2.7%), as previously reported [13, 37]. In addition, aminophospholipid and aminolipid were detected in strain ISCAR-7401^T in contrast to the other species of *Rhodothermus*.

To conclude, strain ISCAR-7401^T had obvious phenotypic characteristics that differentiated it from the other species of *Rhodothermus*, such as in pigmentation and translucid appearance of colonies (Fig. S3), the absence of catalase activity and the ability to grow without NaCl (Table 2). Considering the size of its genome, the dDDH and ANI values, the low 16S RNA gene sequence similarity to the closest relative species (Fig. 1, Table 1) and based on chemotaxonomic differences in the composition of polar lipids and cellular fatty acids, strain ISCAR-7401^T represents a novel species of the genus *Rhodothermus*, for which the name *Rhodothermus bifroesti* sp. nov. is proposed.

DESCRIPTION OF *RHODOTHERMUS BIFROESTI* SP. NOV.

Rhodothermus bifroesti (bi.froes'ti. N.L. gen. n. *bifroesti*, named after the burning rainbow bridge Bifröst in Norse mythology).

The species name refers to its thermophilic and iridescent characteristic on agar plate medium 166, as well as to its origin. The strain was isolated from the subsurface of Surtsey island, the 'Surtr's island' in Iceland, named after a giant fire from Norse mythology, which corresponds to 'the (volcanic) fire of the Underworld' [38].

Cells are Gram-stain-negative, strictly aerobic, non-motile, non-sporulating, rod-shaped (0.3–0.4 µm wide, 1.5–7 µm long), oxidase-negative and catalase-negative. Colonies are pinkish, irregular with a smooth edge, appearing translucent and measuring 2.5–3.4 mm in diameter after incubation at 70 °C for 2 days. On agar plate medium 166 containing 1% NaCl, they display an iridescent characteristic exhibiting all

spectral colours ranging from red to blue only under the condition of oblique illumination. Growth occurs at 44–77 °C (optimum, 70 °C), pH 5.5–9.5 (optimum, pH 7.0–7.5) and with 0–5.5% NaCl (w/v; optimum, 1%). D-Galactose, glucose, lactose, maltose, D-mannose, raffinose, D-rhamnose, sucrose, pyruvate, L-asparagine, L-aspartate, L-glutamine and L-glutamate are used as sole sources of carbon and energy for growth. Does not use D-fructose, acetate, citrate, L-malic acid, L-alanine, L-arginine, L-proline, L-serine, L-threonine or L-valine. Resistant to rifampicin, kanamycin, streptomycin and sensitive to gentamicin. The predominant polar lipids are phosphatidylethanolamine, diphosphatidylglycerol, unidentified aminophospholipid and unidentified phospholipids. The major respiratory quinone is MK-7. The major fatty acids are iso-$C_{17:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{15:0}$ (>10%).

The type strain, ISCAR-7401^T (=DSM 112103^T=CIP 111906^T), was isolated from borehole fluid collected at 160 m depth below the surface of Surtsey island, a young volcanic island located south of Iceland. The DNA G+C content of the type strain is 56.19 mol%.

Funding information
The sampling and samples processing was funded with a grant of excellence from the Icelandic Science fund, ICF-RANNÍS IceSUSTAIN (163083-051). The drilling operation was funded with International Continental Scientific Drilling Program (ICDP) through the SUSTAIN program and the IceSUSTAIN grant to M. D. J., M. T. G. and V. T. M. A doctoral student grant from RANNÍS (206582–051) was attributed to P. B.

Acknowledgements
The authors would like to thank all members of the SUSTAIN onsite, science teams and the Surtsey Research Society for their contributions to the drilling project and sampling, especially M. T. Gudmundsson, M. D. Jackson, A. M. Klonowski, B. Kleine, T. Weisenberger, S. Prause and C.F. Gorny. We are grateful to S. H. Björnsdóttir and J. Matthíasson for the contributions of sample preparation and SEM images. We also thank S. H. Björnsdóttir for valuable discussions.

Author contributions
Conceptualization: V.T.M., P.V. and P.B.; data curation: P.B.; funding acquisition: V.T.M., P.V. and P.B.; investigation: P.B., P.V., A.M. and L.R.; methodology: P.V. and P.B.; project administration: V.T.M., P.V. and P.B.; writing – original draft: P.B.; writing – review and editing: P.B., P.V. and V.T.M.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Cavalier-Smith T, Chao EEY. Multidomain ribosomal protein trees and the planctobacterial origin of neomura (eukaryotes, archaebacteria). *Protoplasma* 2020;257:621–753.
2. Munoz R, Rosselló-Móra R, Amann R. Revised phylogeny of Bacteroidetes and proposal of sixteen new taxa and two new combinations including *Rhodothermaeota* phyl. nov. *Syst Appl Microbiol* 2016;39:281–296.
3. Park MJ, Oh JH, Yang SH, Kwon KK. *Roseithermus sacchariphilus* gen. nov., sp. nov. and proposal of *Salisaetaceae* fam. nov., representing new family in the order Rhodothermales. *Int J Syst Evol Microbiol* 2019;69:1213–1219.
4. Ludwig W. Family I. *Rhodothermaceae* fam. nov. In: *Bergey's Manual of Systematics of Archaea and Bacteria*. Wiley, 2015.
5. Park S, Akira Y, Kogure K. The family *Rhodothermaceae*. In: *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*. Springer, 2014. pp. 849–856.
6. Björnsdóttir SH, Pétursdóttir SK, Gudmundsdóttir EE, Olgudóttir E, Stefansson SK, *et al*. *Rhodocaloribacter litoris* gen. nov., sp. nov., isolated from an intertidal hot spring. *Int J Syst Evol Microbiol* 2021;71.
7. Alfredsson GA, Kristjansson JK, Hjorleifsdottir S, Stetter KO. *Rhodothermus marinus*, gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarine hot springs in Iceland. *Microbiology* 1988;134:299–306.
8. Nunes OC, Donato MM, Da Costa MS. Isolation and characterization of *Rhodothermus* strains from S. Miguel, Azores. *Syst Appl Microbiol* 1992;15:92–97.
9. Moreira L, Fernanda Nobre M, Sa-Correia I, Da Costa MS. Genomic typing and fatty acid compositioTyping and Fatty Acid Composition of Rhodothermus marinus. *Syst Appl Microbiol* 1996;19:83–90.
10. Petursdottir SK, Hreggvidsson GO, Da Costa MS, Kristjansson JK. Genetic diversity analysis of *Rhodothermus* reflects geographical origin of the isolates. *Extremophiles* 2000;4:267–274.
11. Sako Y, Takai K, Ishida Y, Uchida A, Katayama Y. *Rhodothermus obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacteria. *Int J Syst Bacteriol* 1996;46:1099–1104.
12. Silva Z, Horta C, da Costa MS, Chung AP, Rainey FA. Polyphasic evidence for the reclassification of *Rhodothermus obamensis* Sako *et al.* 1996 as a member of the species *Rhodothermus marinus* Alfredsson *et al.* 1988. *Int J Syst Evol Microbiol* 2000;50 Pt 4:1457–1461.
13. Marteinsson VT, Bjornsdottir SH, Bienvenu N, Kristjansson JK, Birrien JL. *Rhodothermus profundi* sp. nov., a thermophilic bacterium isolated from a deep-sea hydrothermal vent in the Pacific Ocean. *Int J Syst Evol Microbiol* 2010;60:2729–2734.
14. Thorarinsson S, Þórarinsson S. The Surtsey eruption: course of events and the development of the new island. *Surtsey Research Progress Report* 1965;1:51–55.
15. Jakobsson SP, Moore JG. The Surtsey research drilling project of 1979. *Surtsey Research Progress Report* 1982;9:76–93.
16. Jakobsson SP, Thors K, Vésteinsson ÁT, Ásbjörnsdóttir L. Some aspects of the seafloor morphology at Surtsey volcano: the new multibeam bathymetric survey of 2007. *Surtsey Research Surtsey Research Progress Report* 2009;12:9–20.
17. Weisenberger TB, Gudmundsson MT, Jackson MDG, Türke A, Kleine BI, *et al*. *Operational Report for the 2017 Surtsey Underwater Volcanic System for Thermophiles, Alteration Processes and INnovative Concretes (SUSTAIN) Drilling Project at Surtsey Volcano Iceland*. Potsdam: GFZ German Research Centre for Geosciences; 2019. p. 240.
18. Jackson MD, Gudmundsson MT, Weisenberger TB, Rhodes JM, Stefánsson A, *et al*. SUSTAIN drilling at surtsey volcano, iceland, tracks hydrothermal and microbiological interactions in basalt 50 years after eruption. *Sci Dril* 2019;25:35–46.
19. Jackson MD, Gudmundsson MT, Bach W, Cappelletti P, Coleman NJ, *et al*. Time-lapse characterization of hydrothermal seawater and microbial interactions with basaltic tephra at surtsey volcano. *Sci Dril* 2015;20:51–58.
20. Bjornsdottir SH, Blondal T, Hreggvidsson GO, Eggertsson G, Petursdottir S, *et al*. *Rhodothermus marinus*: physiology and molecular biology. *Extremophiles* 2006;10:1–16.
21. Kristjansdottir T, Ron EYC, Molins-Delgado D, Fridjonsson OH, Turner C, *et al*. Engineering the carotenoid biosynthetic pathway in *Rhodothermus marinus* for lycopene production. *Metab Eng Commun* 2020;11:e00140.
22. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114–2120.
23. Nurk S, Bankevich A, Antipov D, Gurevich A, Korobeynikov A, *et al*. Assembling genomes and mini-metagenomes from highly chimeric reads. In: Deng M, Jiang R, Sun F and Zhang X (eds). *Research in Computational Molecular Biology Lecture Notes in Computer Science*. Berlin, Heidelberg: Springer; 2013. pp. 158–170.
24. Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y, *et al*. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene

sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017;67:1613–1617.

25. Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:60.
26. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, *et al*. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* 2008;9:75.
27. Arkin AP, Cottingham RW, Henry CS, Harris NL, Stevens RL, *et al*. KBase: The United States Department of Energy Systems Biology Knowledgebase. *Nat Biotechnol* 2018;36:566–569.
28. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, *et al*. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 2007;57:81–91.
29. Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci USA* 2009;106:19126–19131.
30. Kim M, Oh HS, Park SC, Chun J. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol* 2014;64:346–351.
31. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673–4680.
32. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–425.
33. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;33:1870–1874.
34. Hjorleifsdottir S, Skirnisdottir S, Hreggvidsson GO, Holst O, Kristjansson JK. Species composition of cultivated and noncultivated bacteria from short filaments in an Icelandic hot spring at 88 degrees C. *Microb Ecol* 2001;42:117–125.
35. ZoBell CE. Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *J Mar Res* 1941;4:41–75.
36. Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS. Methanogens: reevaluation of a unique biological group. *Microbiol Rev* 1979;43:260–296.
37. Tindall BJ. Lipid composition of *Rhodothermus marinus*. *FEMS Microbiol Lett* 1991;80:65–68.
38. Simek R. *Dictionary of Northern Mythology*. Cambridge: D.S. Brewer; 1993.
39. Nunes OC, Donato MM, Manaia CM, Da Costa MS. The polar lipid and fatty acid composition of *Rhodothermus* strains. *Systematic and Applied Microbiology* 1992;15:59–62.

Rhodothermus bifroesti sp. nov., a thermophilic bacterium isolated from the basaltic subsurface of the island Surtsey.

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Running title: *Rhodothermus bifroesti* sp. nov.

Subject category: New taxa-*Rhodothermaceae*

Supplementary Text.

Extended fatty acid analysis, quinones and polar lipids composition were carried out by the Identification service of Leibniz-institute DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Analysis of Cellular Fatty Acids by MIDI and GC-MS Cellular fatty acids

Cellular fatty acid were analyzed after conversion into fatty acid methyl esters (FAMEs) by saponification, methylation and extraction using minor modifications of the method of Miller (1982) and Kuykendall et al., (1988) (1,2). The fatty acid methyl esters mixtures are separated by gas chromatography and detected by a flame ionization detector using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.). Peaks are automatically integrated and fatty acid names and percentages calculated by the MIS Standard Software (Microbial ID).

For identity confirmation and to resolve summed features of the MIDI analysis, the analysis is supplemented by a GC-MS run on a Agilent GC-MS 7000D using an Agilent HP-5ms UI 30 m x 250 µm x 0,25 µm column with a helium flow of 1.2 ml with an injection of 1 µl with split ratio of 7.5:1. The oven program was as follows: initial temperature 170°C, ramp 3°C/min to 200°C, ramp 5°C/min to 270°C, ramp 120°C/min to 300°C and hold for 2 min. The inlet temperature was set to 170 °C and then linearly increased with 200 °C/min up to 350 °C and hold for 5min. The mass spectrometry parameters were set to aux temperature 230°C, source temperature 230°C, and electron impact ionization at 70 eV with mass range of m/z 40-600 or 40-800, respectively. Peaks were identified based on retention time and mass spectra. The position of single double bounds was confirmed by a derivatization to the corresponding dimethyl disulfide adduct (3). Branched-chain fatty acid positions, cyclopositions and multiple double bounds were determined by derivatization to their 3-pyridylcarbinol ("picolinyl") and/or 4,4-dimethyloxazoline (DMOX) derivatives (4–6).

Analysis of Respiratory Quinones

Respiratory quinones are extracted from freeze dried cell material using hexane and are further purified by a silica-based solid phase extraction. Purified samples are further analysed by HPLC

using a reverse phase column recording absorption spectra. 270 nm for ubiquinones and 326 nm for menaquinones are used for a relative quantification. For complex mixtures, samples are further analysed on an UHPLC-ESI-qTOF system.

Analysis of polar lipids

Polar lipids are extracted from freeze dried cell material using a choroform:methanol:0.3% aqueous NaCl mixture, polar lipids are recovered into the chloroform phase (modified after Bligh and Dyer, 1959 (7)).

Polar lipids are separated by two dimensional silica gel thin layer chromatography. The first direction is developed in chloroform:methanol:water, and the second in chloroform:methanol:acetic acid:water. Total lipid material is detected using molybdatophosphoric acid and specific functional groups detected using spray reagents specific for defined functional groups (8).

Supplementary Figures.

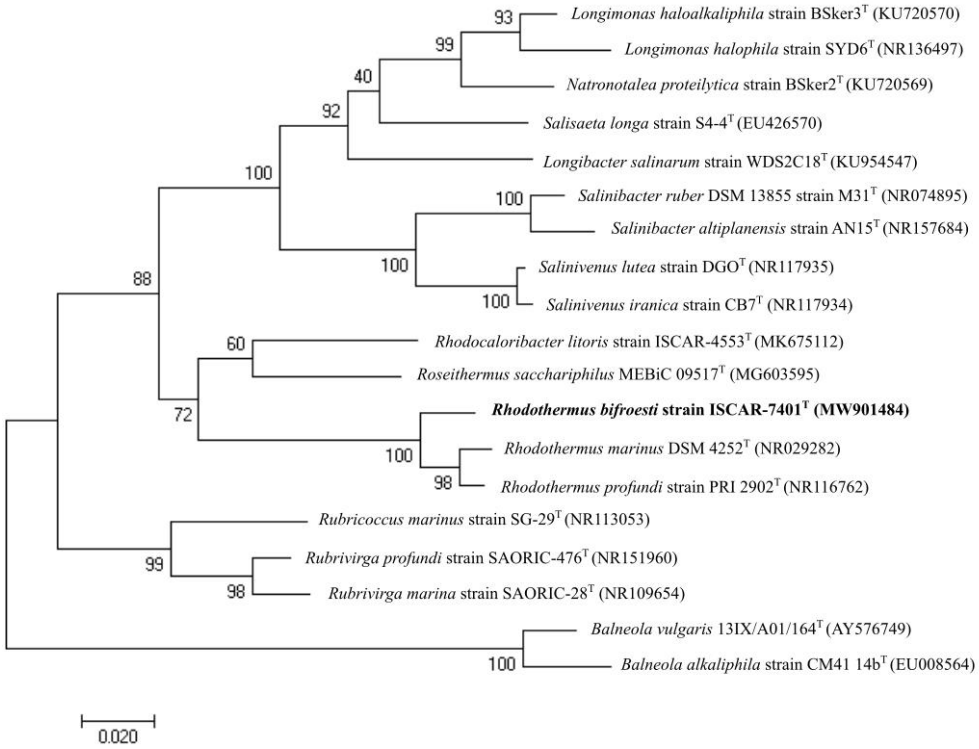

Fig. S1. Maximum Likelihood phylogenetic tree constructed based on the 16S rRNA gene sequences indicating the relationship of strain ISCAR-7401^T with the type strains within *Rhodothermaceae* and other species within the *Rhodothermales*. GenBank accession numbers are given in parentheses. Bootstrap was carried out 1000 replicates using a total of 1354 positions in the final dataset. The sequences of *Balneola* species were used as an outgroup. Bar, 0.02 represented the nucleotide substitution per position.

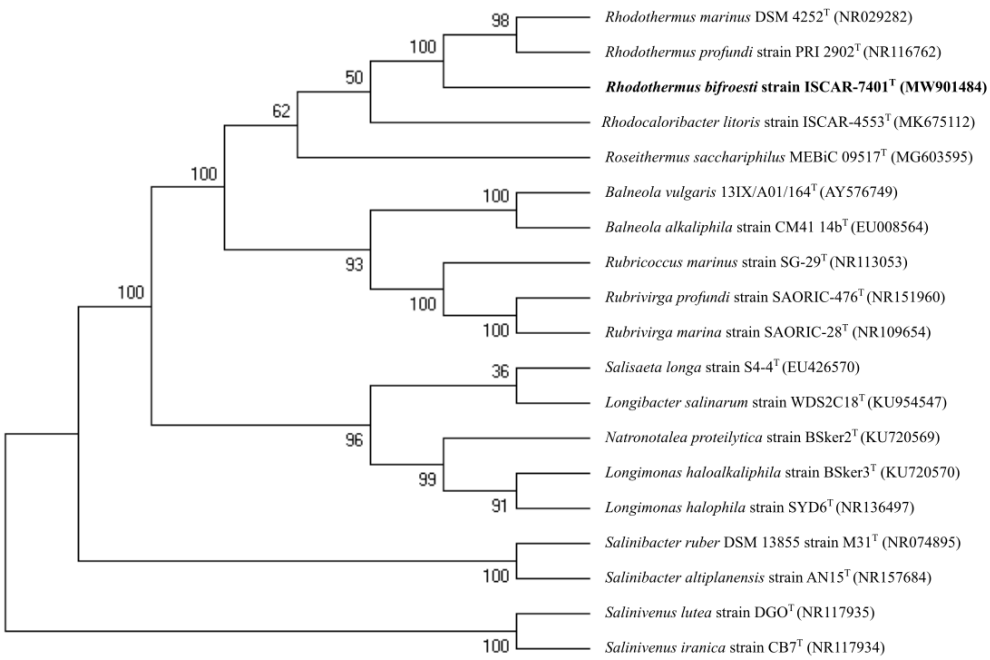

Fig. S2. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences of strain ISCAR-7401^T and closely related species. GenBank accession numbers are given in parentheses. Bootstrap was carried out 1000 replicates using a total of 1354 positions in the final dataset.

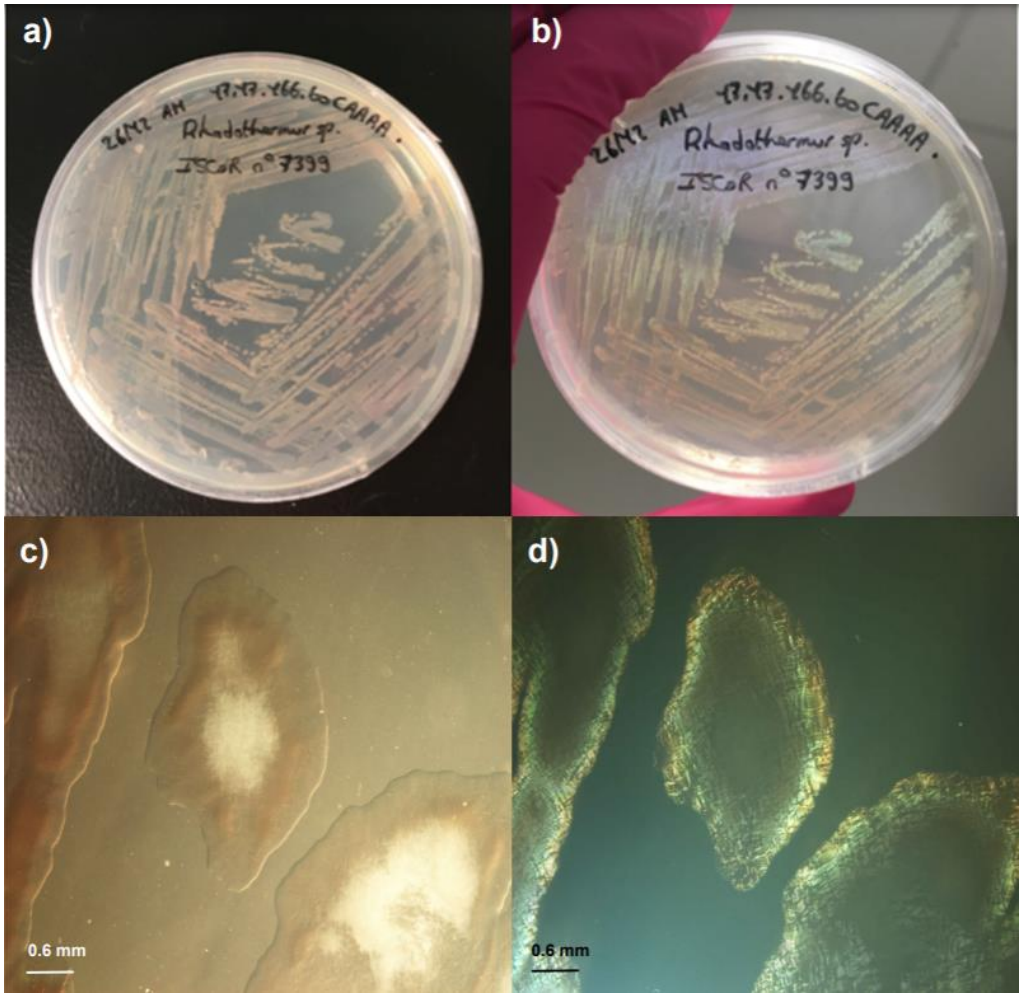

Fig. S3. Colonies of *Rhodothermus bifroesti* strain ISCAR-7399 at different scales and under different conditions of illumination. Colonies on a black background (a), under artificial light (b), observed with binocular microscope under condition of tran-illumination (c) and with a 60°angle-illumination (d), showing the iridescent characteristic of the species.

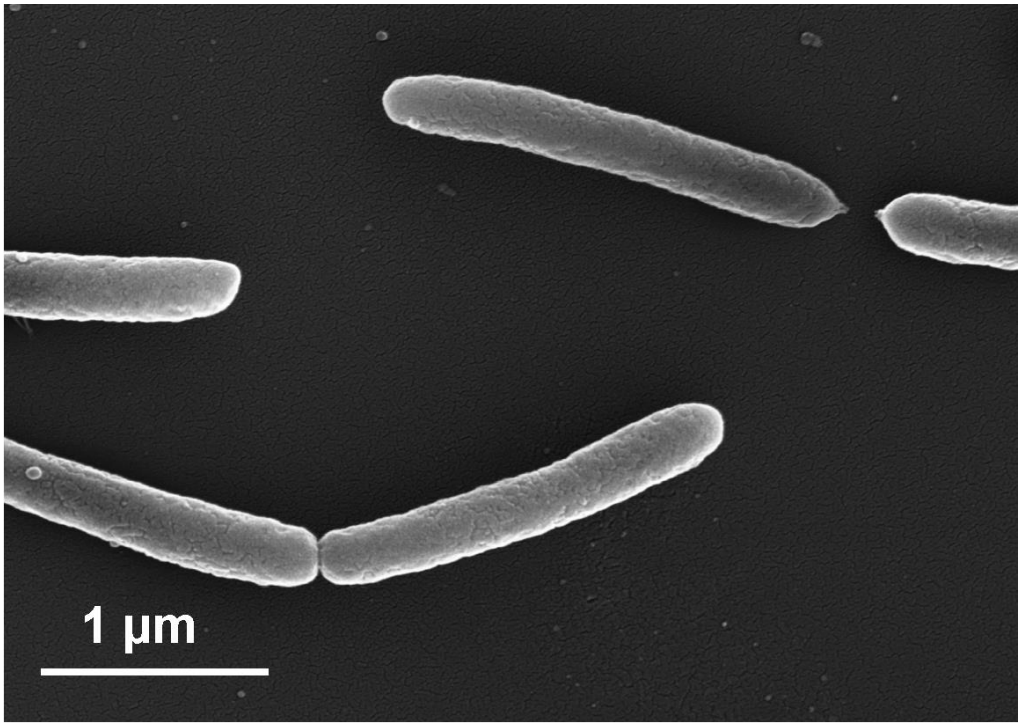

Fig. S4. Cell morphology of strain ISCAR-7401^{T} observed using scanning electron microscope Supra25 Gemini FE-SEM (ZEISS) at electron high tension voltage of 20 kV.

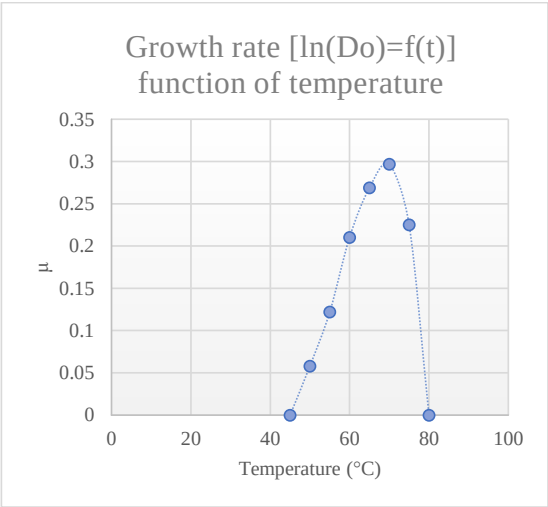

Fig. S5. Growth rates of the strain ISCAR-7401^{T} function of the temperature.

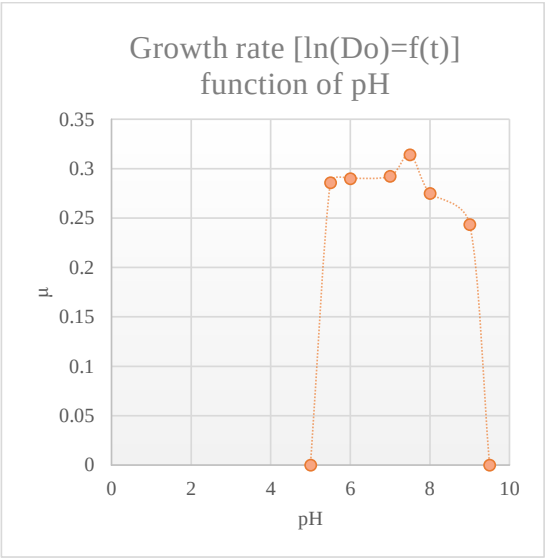

Fig. S6. Growth rates of strain ISCAR-7401^{T} function of pH.

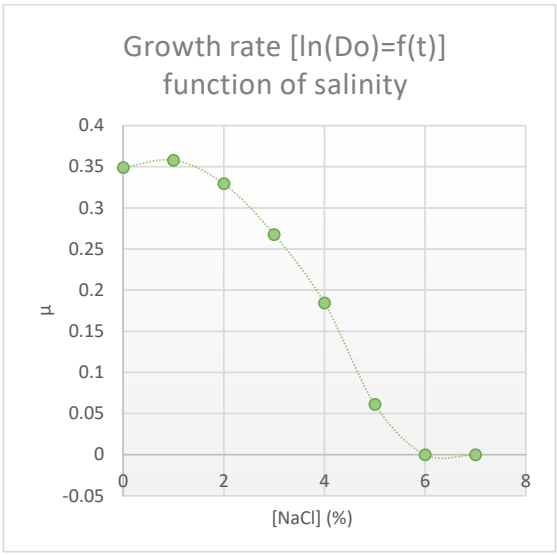

Fig. S7. Growth rates of strain ISCAR-7401^{T} function of salinity.

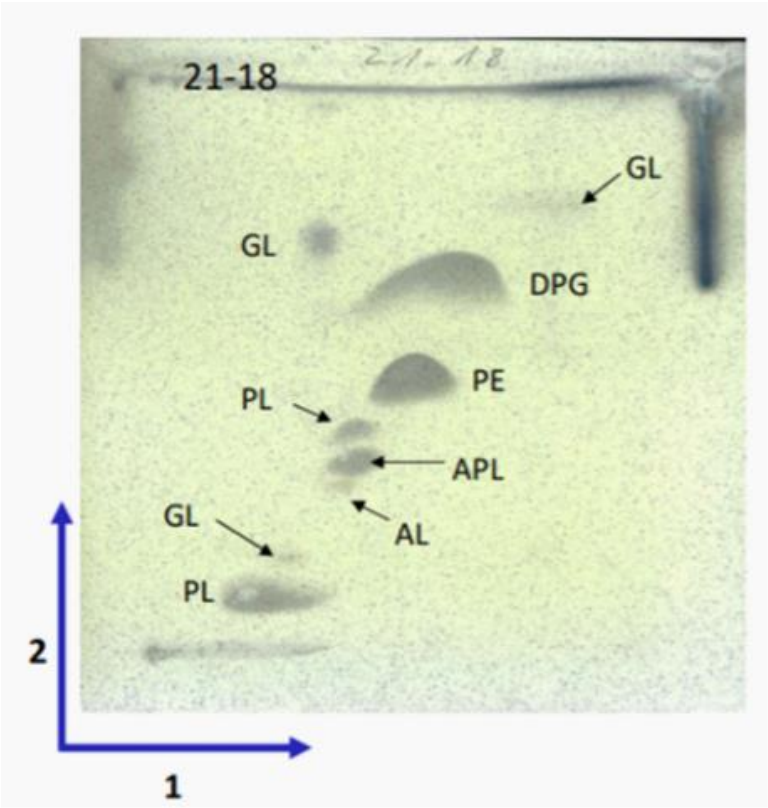

Fig. S8. Polar lipid profiles of strain ISCAR-7401^{T}. Lipid identification legends: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; AL, unidentified aminolipid; APL, unidentified aminophospholipid; GL, unidentified glycolipid; PL, unidentified phospholipid. 1, first dimension of TLC; 2, second dimension of TLC.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2	GC-MS
1.763	4.965E+8	0.027	----	7.007	SOLVENT PEAK	----	< min rt		
2.206	852	0.023	----	7.870		----	< min rt		
2.689	866	0.026	----	8.813		----	< min rt		
2.953	270	0.024	----	9.327		----			
3.210	297	0.026	----	9.826		----			
4.910	387	0.035	----	11.973		----			
6.953	636	0.031	0.982	13.619	14:0 iso	0.55	ECL deviates 0.000	Reference -0.005	OK
7.106	1062	0.036	----	13.730		----			
8.446	13211	0.040	0.964	14.623	15:0 iso	11.20	ECL deviates 0.000	Reference -0.004	OK
8.587	19256	0.040	0.963	14.713	15:0 anteiso	16.31	ECL deviates 0.000	Reference -0.004	OK
10.081	9166	0.043	0.952	15.627	16:0 iso	7.68	ECL deviates 0.000	Reference -0.004	OK
10.703	5317	0.045	0.949	15.999	16:0	4.44	ECL deviates -0.001	Reference -0.005	OK
11.800	48141	0.046	0.946	16.631	17:0 iso	40.07	ECL deviates 0.001	Reference -0.003	OK
11.961	19382	0.045	0.946	16.723	17:0 anteiso	16.13	ECL deviates 0.000	Reference -0.004	OK
12.444	692	0.044	0.945	17.001	17:0	0.58	ECL deviates 0.001	Reference -0.003	OK
13.557	2683	0.046	0.943	17.632	18:0 iso	2.23	ECL deviates 0.000	Reference -0.005	OK
14.204	987	0.041	0.943	17.999	18:0	0.82	ECL deviates -0.001	Reference -0.006	OK

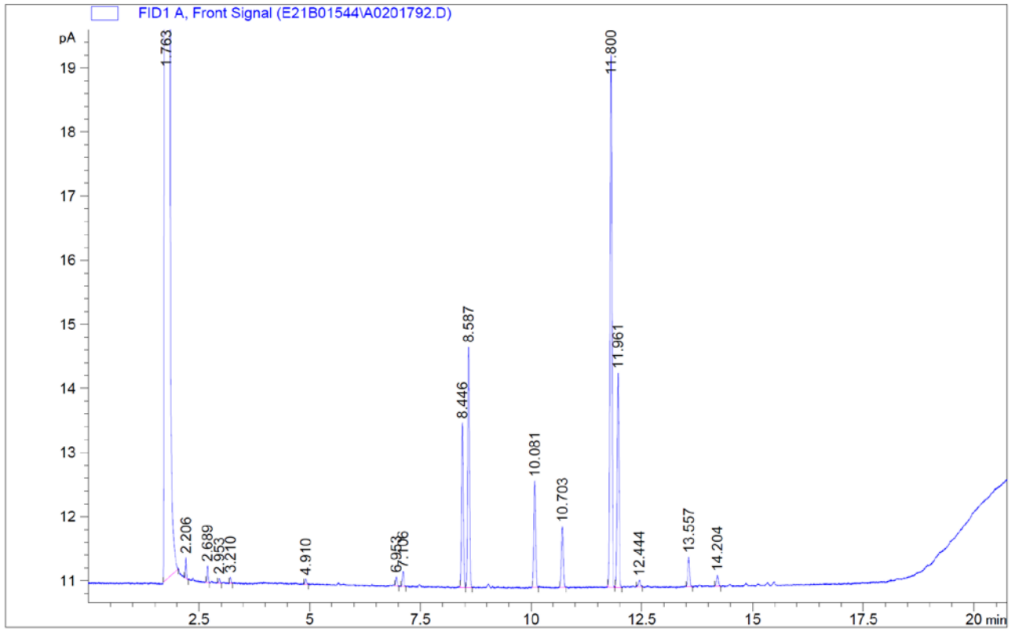

Fig. S9. Fatty acids analyses of *Rhodothermus bifroesti* strain ISCAR-7401^{T}.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2	GC-MS
1.763	4.924E+8	0.028	----	7.005	SOLVENT PEAK	----	< min rt		
2.205	952	0.022	----	7.868		----	< min rt		
2.690	2366	0.025	----	8.811		----	< min rt		
2.957	210	0.025	----	9.333		----			
3.215	336	0.029	----	9.834		----			
4.911	488	0.030	----	11.973		----			
6.954	3900	0.038	0.982	13.619	14:0 iso	1.70	ECL deviates 0.000	Reference -0.004	OK
7.107	1900	0.036	----	13.730		----			
8.447	15603	0.041	0.964	14.623	15:0 iso	6.68	ECL deviates 0.000	Reference -0.004	OK
8.587	31513	0.040	0.963	14.714	15:0 anteiso	13.48	ECL deviates 0.001	Reference -0.003	OK
10.081	35466	0.043	0.952	15.627	16:0 iso	15.00	ECL deviates 0.000	Reference -0.004	OK
10.702	7267	0.044	0.949	15.998	16:0	3.06	ECL deviates -0.002	Reference -0.006	OK
11.801	77994	0.046	0.946	16.631	17:0 iso	32.78	ECL deviates 0.001	Reference -0.003	OK
11.961	44723	0.045	0.946	16.723	17:0 anteiso	18.79	ECL deviates 0.000	Reference -0.004	OK
12.442	975	0.055	0.945	17.000	17:0	0.41	ECL deviates 0.000	Reference -0.004	OK
13.555	15777	0.047	0.943	17.631	18:0 iso	6.61	ECL deviates -0.001	Reference -0.006	OK
14.206	1668	0.049	0.943	18.000	18:0	0.70	ECL deviates 0.000	Reference -0.005	OK
15.321	565	0.041	0.942	18.636	19:0 iso	0.24	ECL deviates 0.002	Reference -0.004	OK
15.485	1325	0.043	0.942	18.729	19:0 anteiso	0.55	ECL deviates -0.002	Reference -0.007	OK

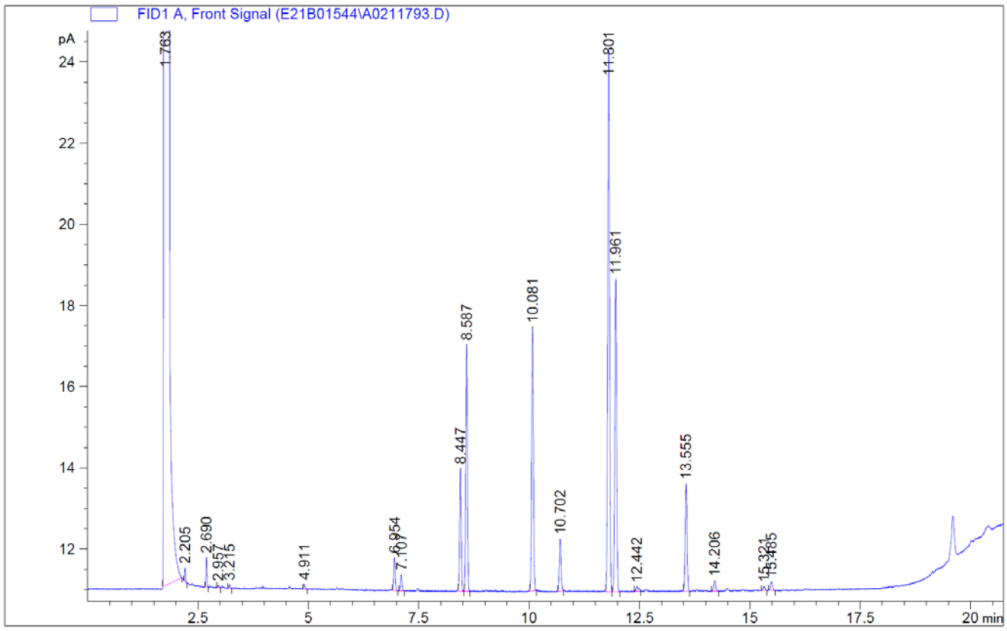

Fig. S10. Fatty acids analyses of *Rhodothermus marinus* DSM 4252^{T}.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2	GC-MS
1.763	4.938E+8	0.028	----	7.006	SOLVENT PEAK	----	< min rt		
2.206	583	0.023	----	7.869		----	< min rt		
2.690	1243	0.026	----	8.813		----	< min rt		
2.958	283	0.032	----	9.334		----			
3.214	320	0.026	----	9.834		----			
4.909	450	0.032	----	11.971		----			
6.954	2907	0.036	0.982	13.619	14:0 iso	1.07	ECL deviates 0.000	Reference -0.004	OK
8.447	20639	0.040	0.964	14.623	15:0 iso	7.46	ECL deviates 0.000	Reference -0.004	OK
8.588	28456	0.041	0.963	14.714	15:0 anteiso	10.28	ECL deviates 0.001	Reference -0.003	OK
10.081	55186	0.043	0.952	15.627	16:0 iso	19.72	ECL deviates 0.000	Reference -0.004	OK
10.702	6567	0.043	0.949	15.998	16:0	2.34	ECL deviates -0.002	Reference -0.006	OK
11.801	92022	0.046	0.946	16.631	17:0 iso	32.66	ECL deviates 0.001	Reference -0.003	OK
11.961	45055	0.045	0.946	16.723	17:0 anteiso	15.98	ECL deviates 0.000	Reference -0.004	OK
13.558	25916	0.047	0.943	17.632	18:0 iso	9.17	ECL deviates 0.000	Reference -0.004	OK
14.204	1402	0.045	0.943	17.998	18:0	0.50	ECL deviates -0.002	Reference -0.007	OK
14.850	607	0.037	----	18.367		----			
15.326	1155	0.047	0.942	18.638	19:0 iso	0.41	ECL deviates 0.004	Reference -0.001	OK
15.485	1170	0.043	0.942	18.729	19:0 anteiso	0.41	ECL deviates -0.002	Reference -0.007	OK

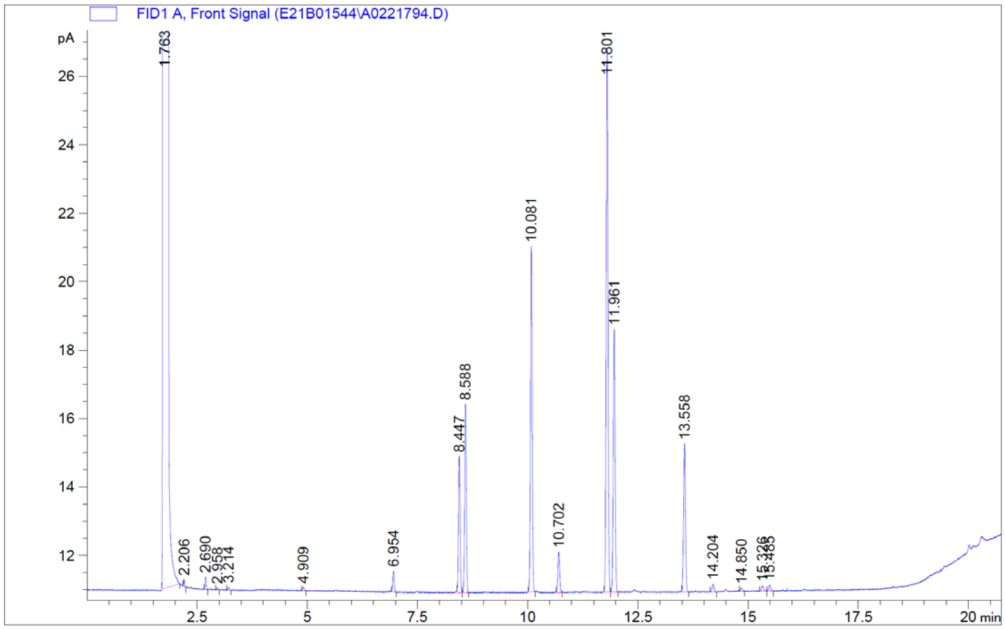

Fig. S11. Fatty acids analyses of *Rhodothermus profundi* strain PRI 2902^{T}.

Supplementary Tables.

Table S1. Media 166, modified from Hjorleifsdottir et al., 2001 (9) (without proline). Grunnur base from medium 162 from Degryse et al., 1978 (10).

For 1L of liquid media:	
NaCl	10 g
K_2HPO_4	0.3 g
Yeast extract	1 g
Peptone	1 g
Tryptone	1 g
Glucose	0.5 g
Amidon (starch)	0.5 g
Na-pyruvate	0.6 g
Na_2CO_3	0.18 g
Base "Grunnur"	100 mL
Hot tap water	900 mL
pH adjusted to 7-7.5	

Grunnur (for 1L):	
Titriplex I (nitrilotriacetic acid)	1.32 g
$CaSO_4$ x $2H_2O$	0.4 g
$MgCl_2$ x $6H_2O$	2.0 g
Trace elements (Wolfe´s mineral solution)	5 ml
Ironcitrate	5 ml
H_2O	1000 ml
pH adjusted to 7.2	

Ironcitrate (for 1L):	
Na_3citrate x $2H_2O$	2.94 g
$FeCl_3$ x $6H_2O$	2.7 g

Table S2. Annotated genes from genome of strain ISCAR-7401^T (see Excel file).

Table S3. Characteristics that differentiate the characterized strains of *Rhodothermus bifroesti*. 1, *Rhodothermus bifroesti* sp. nov. strain ISCAR-7401^T; 2, Strains ISCAR-7397; 3, Strains ISCAR-7399; 4, Strains ISCAR-7403. Data were obtained in the present study. All strains are aerobic, stain Gram-negative, produce pinkish, translucent colonies, grew optimally at 70°C, and are oxidase- and catalase-negative. All strains could grow without NaCl (w/v) and showed growth on D-galactose, glucose, D-lactose, D-mannose, D-raffinose, D-rhamnose, D-sucrose, pyruvate, L-asparagine, L-aspartate, and L-glutamine. None exhibited growth on D-fructose, acetate, citrate, L-malic acid, L-alanine, L-arginine, L-proline, L-serine, L-threonine, and L-valine. All the strains were resistant to rifampicin, kanamycin, and streptomycin and were sensitive to gentamicin. W, weak growth.

	1	**2**	**3**	**4**
Growth at 6% NaCl (w/v)	-	-	-	w
Growth at pH 9.5	w	+	+	+
Utilization of D-maltose, L-glutamate	+	+	+	-
Utilization of L-asparagine, L-glutamine	+	+	w	w
16S rRNA gene similarity (%) with strain ISCAR-7401^T	100	100	99.76	99.68

Supplementary references.

1. Miller LT. Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. Journal of Clinical Microbiology. 1982;16(3):584–6. doi: 10.1128/jcm.16.3.584-586.1982

2. Kuykendall LD, Roy MA, O'Neill JJ, Devine TE. Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. International Journal of Systematic Bacteriology. 1988;38(4):358–61. doi: 10.1099/00207713-38-4-358

3. Moss CW, Lambert-Fair MA. Location of double bonds in monounsaturated fatty acids of *Campylobacter* cryaerophila with dimethyl disulfide derivatives and combined gas chromatography-mass spectrometry. Journal of Clinical Microbiology. 1989;27(7):1467–70. doi: 10.1128/jcm.27.7.1467-1470.1989

4. Harvey DJ. Picolinyl esters as derivatives for the structural determination of long chain branched and unsaturated fatty acids. Biological Mass Spectrometry. 1982;9(1):33–8. doi: 10.1002/bms.1200090107

5. Spitzer V. Structure analysis of fatty acids by gas chromatography - Low resolution electron impact mass spectrometry of their 4,4-dimethyloxazoline derivatives - A review. Progress in Lipid Research. 1996;35(4):387–408. doi: 10.1016/S0163-7827(96)00011-2

6. Yu QT, Liu BN, Zhang JY, Huang ZH. Location of methyl branchings in fatty acids: Fatty acids in uropygial secretion of Shanghai Duck by GC-MS of 4,4-dimethyloxazoline derivatives. Lipids. 1988;23:804–810. doi: 10.1007/BF02536225

7. Bligh, E.G. and Dyer WJ. A rapıd method of total lıpıd extractıon and purıfıcatıon. Canadian Journal of Biochemistry and Physiology. 1959;37(8):911–7. doi: 10.1139/o59-099

8. Tindall BJ, Sikorski J, Smibert RA, Krieg NR. Phenotypic Characterization and the Principles of Comparative Systematics. In: Methods for General and Molecular Microbiology. 2007. doi: 10.1128/9781555817497.ch15

9. Hjorleifsdottir S, Skirnisdottir S, Hreggvidsson GO, Holst O, Kristjansson JK. Species composition of cultivated and noncultivated bacteria from short filaments in an icelandic hot spring at 88°C. Microbial Ecology. 2001;42(2):117–25. doi: 10.1007/s002480000110

10. Degryse E, Glansdorff N, Piérard A. A comparative analysis of extreme thermophilic bacteria belonging to the genus *Thermus*. Archives of Microbiology. 1978;117:189–196. doi: 10.1007/BF00402307

Paper IV

Microscopic investigations revealed putative biotic structures attached to basaltic drill cores collected from the subsurface of the Surtsey volcano.

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Manuscript

Candidate contribution:
Conceived and designed the study, performed fieldwork, performed laboratory work, analyzed data, wrote the manuscript, reviewed and edited the manuscript.

Microscopic investigations revealed putative biotic structures attached to basaltic drill cores collected from the subsurface of Surtsey volcano

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Keywords: Cell abundance, Scanning Electron Microscopy (SEM), Confocal laser scanning microscopy (CLSM), extreme environment, subsurface, oceanic basaltic crust, Iceland

Abstract

Surtsey, the youngest of the Vestmannaeyjar islands, is an oceanic volcano formed by explosive basaltic eruptions off Iceland's southern coast between 1963 and 1967. The International Continental Scientific Drilling Program (ICDP) SUSTAIN Expedition 5059 conducted three cored boreholes through the volcano's active hydrothermal-seawater system in the summer of 2017. Through previous molecular analyses and cultivation investigations, these cores revealed the first sight of the subsurface microbial diversity of the very young basaltic island associated with the newly formed oceanic crust. In this study, we evaluated the presence and distribution of cells as a function of the depth, the presence of putative microbial structures attached to the basaltic tuff and their organization at the micro-scale toward the substrate by using microscopic methods. This included epifluorescence, confocal laser scanning microscopy and scanning electron microscopy. We encountered many challenges due to the very low biomass present in the drill core samples and the presence of minerals. However, the results suggested that the number of cells was ranging from 5×10^4 and 1.36×10^6 cells per gram of rock, and the sample located at 65 m depth (a few meters below the coastal sea level) showed the highest number of cells. Furthermore, putative biotic structures attached to basaltic tuff were detected inside the numerous interconnected vesicles present in the basaltic glass. This suggests that early microbial colonization of the subaerial and submarine deposits already occurred barely 50 years after the eruptions terminated in a geothermal system with temperatures approaching the presumed thermal limit for functional life on Earth (~121°C).

Introduction

The subsurface represents the largest habitable region on Earth, possibly sheltering more than 70% of all prokaryotic cells (Edwards *et al.*, 2012a; Bar-On *et al.*, 2018; Magnabosco *et al.*, 2019). The limits of life in the subsurface mostly depend on the temperature, redox conditions and the availability of water (Baquiran *et al.*, 2016; Ramírez *et al.*, 2019). The rock porosity can also influence the distribution of microbial communities in such an environment, in terms of available space and permeability, which control the movement of cells and chemicals within the rock. For instance, sub-seafloor sediments are vertically stratified and therefore present very low porosity and permeability. On the contrary, basaltic

rocks that compose the oceanic crust are much more heterogeneous. Given its size and the difficulties to access it, the subsurface biosphere still remains largely unexplored (Edwards *et al.*, 2012b). Its characterization requires linking data from different fields of research such as geochemistry, mineralogy and microbiology. Different methods are used to study the subsurface biosphere including culture-depend and -independent methods and microscopic investigations.

Here, we take advantage of the drill core samples from 50 years old basalt that were collected in 2017 during the ICDP drilling operation on the Surtsey island to study microbe-basalt interactions through microscopic investigations (Jackson *et al.*, 2019b; Bergsten *et al.*, 2021). Surtsey is an underwater volcano that reached the surface and created an island in the south of Iceland after 3.5 years of basaltic eruptions that ended in 1979 (Jakobsson and Moore, 1982). Today, the still-active seawater-hydrothermal system reaches a temperature near 120°C at 100 m below surface (m b. s.), a maximal temperature very close to the known upper limit for functional microbial life (Takai *et al.*, 2008). The geological composition and volcanic structures of the island were studied over the years. After the drilling of SE-01 in 1979, the petrology of lithified tephra was described as mostly composed of tuff, and lava of alkali olivine basalt (Jakobsson and Moore, 1982, 1986). In 2019, the geological composition of the new drill cores, SE-02A, B and SE-03, revealed that since 1979 the alteration of glass, plagioclase, and olivine has produced additional smectite, clay minerals, principally nontronite, and authigenic zeolite and Al-tobermorite (Jackson *et al.*, 2019a; b; Weisenberger *et al.*, 2019; Prause *et al.*, 2020). Overall, the subsurface of Surtsey island can be geologically divided into three sections of lapilli tuff with different degrees of alteration: (i) the glassy lapilli tuff above the coastal sea level (~60 m measured depth), (ii) the more altered submarine lapilli tuff at the hydrothermal temperature maxima (65–138 m measured depth) and (iii) the less altered lapilli tuff at the base of the SE-02B borehole (148–180 m measured depth). Recent studies of the 1979 and 2017 drill core samples showed abundant microtubule structures in the basaltic glass that look like endolithic microborings (Jackson *et al.*, 2019a; Jackson, 2020). The observation of these structures in the drill core samples retrieved from the subsurface of Surtsey could indicate biotic alteration of the basalt (microbial microboring into the glass), as has previously been observed in other basalt samples (Fisk *et al.*, 2003; Staudigel *et al.*, 2008; Walton, 2008; Mcloughlin *et al.*, 2010).

The work focuses on the following questions: (i) can we estimate the cell abundance in the drill core samples as a function of the depth? (ii) can we detect cells and microbial structures such as biofilms and remnants of microbial activity? and (iii) if so, how organized are the cells toward the microstructures of the subtract (e.g. cavities, vesicles, tubules, cracks, etc.) and the minerals? To approach these questions, we used microscopic methods such as epifluorescence, Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM) on the drill core samples from the subsurface of the Surtsey volcano.

Material and Methods

Study site and samples collection

Located on the extension of the Icelandic rift zone, Surtsey island (63°18'10.8"N; 20°36'16.9"W) is a volcano that was formed after almost 4 years of underwater and basaltic eruptions from 1963 to 1969 (Thorarinsson and Þórarinsson, 1965; Jakobsson and Moore, 1982; Baldursson, S. & Ingadóttir, 2007; Jakobsson *et al.*, 2009). During the ICDP drilling operation on Surtsey island in 2017 (Jackson *et al.*, 2019b; Weisenberger *et al.*, 2019), drill

cores were collected and stored at 4°C under anaerobic conditions by applying GasPak™ (BD, USA) (Bergsten *et al.*, 2021). This study focuses on twenty-one drill core samples extracted at different depths from the subsurface of Surtsey island (Table 1). In the laboratory, the samples were broken in an anaerobic chamber (atmosphere: N2/CO2/H2: 80/10/10, Coy Laboratory Inc., USA) and only interior pieces of the drill cores were selected for microscopic investigations. Fragments with a diameter less than 5 cm were transferred to sterile 15 ml Falcon® tubes, sealed and put back in new GasPaks™. The subsamples were transported at 4°C to the institute of IPGP in Paris, where the analyses took place.

Table 1. Sample ID, borehole ID, core run number, depth, recovery of the full section of the core run and temperature.

Well	Core run no.	Depth (m)		Recovery (m)	Temperature (°C)	ICDP labelling system
		From	To			
SE-02-A	3	14.32	17.37	3.05	18.00	5059-1B-3Z-2,20-29
	6	23.47	26.52	3.05	22.00	5059-1B-6Z-3,21-30
	9	31.10	32.62	1.52	30.00	5059-1B-9Z-2,22-28
	12	38.72	41.77	3.05	40.00	5059-1B-12Z-2,25-30
	15	47.87	50.92	3.05	65.00	5059-1B-15Z-22-30
	18	57.02	60.07	3.05	85.00	5059-1B-18Z-3,24-30
	21	64.32	66.17	1.85	102.00	5059-1B-21Z-2,24-30
	24	69.22	71.62	2.40	110.00	5059-1B-24Z-3,22-30
	27	75.32	78.37	3.05	115.00	5059-1B-27Z-3,24-30
	30	84.47	87.52	3.05	120.00	5059-1B-30Z-3,24-30
	33	93.62	96.67	3.05	122.00	5059-1B-33Z-4,22-30
	36	102.77	105.82	3.05	122.00	5059-1B-36Z-3,24-30
	39	111.92	114.92	3.00	122.00	5059-1B-39Z-3,24-32
	42	121.07	124.12	3.05	111.00	5059-1B-42Z-3,22-30
	45	130.22	133.27	3.05	106.00	5059-1B-45Z-3,23-30
	49	139.37	142.32	2.95	85.50	5059-1B-49Z-4,22-30
SE-02-B	52	145.89	148.94	3.05	74.00	5059-1C-52Z-3, 0-9
	55	155.04	158.09	3.05	66.80	5059-1C-55Z-3, 22-30
	59	164.19	167.24	3.05	55.00	5059-1C-59Z-3, 15-22
	62	173.34	176.14	2.80	44.00	5059-1C-62Z-3, 11-19
	65	179.44	182.49	3.05	NA	5059-1C-65Z-2,0-9

Sample fixation

All material used during the experiments was cleaned using 70% ethanol (EtOH) and UV sterilized for 40 min. Using sterile tweezers, fragments of rock samples were transferred to 5 ml Eppendorf® tubes. Samples were fixed in 50% EtOH prepared with 1X Phosphate-Buffered Saline (PBS) (*v/v*) at -20°C for 24h.

Cell counting using epifluorescence microscopy

Small EtOH-fixed fragments were collected for each sampling depth from the drill core samples. The samples were crushed and stained with fluorescent dyes at a final concentration of 10 µM for 10 min. Both Syto™ 9 green fluorescent nucleic acid dye (SYTO9; Thermo Fisher Scientific, USA, # S34854) and 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, USA, # D1306) were tested. Citifluor was used as antifading (Citifluor Ltd., UK). Each slide was visualized under an epifluorescence microscope (Axiostar plus, Zeiss, Germany; magnification 1000× with 100× objective; λEx = 359 nm, λEm = 441 nm). Counting cells using epifluorescence microscopy was performed by selecting 15 random fields of view on a glass slide to calculate the arithmetic mean which was then used to estimate the total microbial abundance of a sample. However, too few cells were observed for the results to be meaningful.

Estimation of the number of cells based on DNA concentration

The number of cells was estimated based on the DNA concentration results from Bergsten *et al.*, 2021. In the last study, DNA was extracted from 15 g of rocks for each depth, resuspended in 50 µl of Tris buffer (10 mM, pH 8) and quantified using the Qubit fluorometer and high-sensitivity dsDNA reagents (Invitrogen™).

The concentration of DNA per gram of rock was estimated for each depth using the following equation:

$$\left[\frac{ng\ of\ DNA}{1\ gram\ of\ rocks}\right] = \frac{\text{DNA concentration } \left(\frac{\text{ng}}{\mu\text{l}}\right) \times \text{Volume of resuspension } (\mu\text{l})}{\text{Mass of rocks extracted (g)}}$$

Then, the cell number per gram of rock was estimated based on the DNA concentration. If it is considered that one prokaryotic cell contains 1 to 4 fg of DNA (Sessions and Hodgkin, 2013), then, it is possible to estimate the number of microbial cells potentially present in 1 gram of rock. To do so, the following equation was used:

$$\left[\frac{Cells}{1\ gram\ of\ rocks}\right] = \frac{\text{DNA concentration } \left(\frac{\text{ng}}{\text{g}}\right) \times 10 - 9}{\text{DNA quantity estimated in one cell (fg) } \times 10 - 15}$$

Confocal laser scanning microscopy (CLSM)

Several EtOH-fixed samples were selected for CLSM: (i) 5059-1B-9Z-2,22-28, labeled B9Z-Cu, which was extracted from SE-02A at 31,5 m depth corresponding to a temperature of 30°C, (ii) 5059-1B-49Z-4,22-30, labeled B49Z-Cu, which was extracted from SE-02A at 141 m depth where the temperature rises to 85°C and (iii) 5059-1C-65Z-2,0-9, labeled C65Z-Cu, extracted from SE-02B at 181 m depth where the temperature was around 37°C. The samples were selected to represent the three different sections of the drill core: the first sample corresponding to the glassy lapilli tuff section above the sea level, the second for the more altered submarine lapilli tuff section at the hydrothermal temperature maxima and the third representing the less altered lapilli tuff section at the base of the borehole.

Fragments of EtOH-fixed samples were transferred to a sterile mortar and crushed with a pestle to obtain a thin powder. The powder was transferred to a 1.5 ml Eppendorf® tube and

1 ml of a solution of fresh 50% EtOH was added. Samples were stained with 10 µM of SYTO9 for 10 min in ice and washed with 1 ml of PBS. DAPI was also tested. After removal of the supernatant, 100 µl of PBS were added, as well as a freshly prepared solution of 0.5% melted (37°C) agarose. After gentle homogenization, the solution was transferred to a glass slide with a well and put on ice for 5 min for agarose solidification. Antifading was added. Controls without staining were systematically performed for each sample. No fluorescence was observed in the SYTO9 fluorescence emission range for unstained samples. The aforementioned method was adjusted after several cell visualization attempts using CLSM. One strategy consisted of loading more powder material on the slide to concentrate the sample and the other used very small and friable fragments of drill core samples instead of powder. After staining, washing and addition of the agarose, the fragment was crushed in order to fit in the slide well.

CLSM images were obtained with excitation at 405 and 488 nm and fluorescence detection in the ranges of 425-475 and 500-530 nm using an Olympus FluoView FV1000 microscope (Tokyo, Japan) located at IPGP (Paris, France). The F10-ASW FluoView software (Olympus) was used to process the CLSM images.

Scanning Electron Microscopy (SEM)

Six drill core samples were selected for SEM, two samples for each of the three different sections of the drill core: 5059-1B-3Z-2, 20-29 and 5059-1B-9Z-2, 22-28 represent the glassy lapilli tuff section above the sea level, 5059-1B-21Z-2, 24-30 and 5059-1B-49Z-4, 22-30 for the more altered submarine lapilli tuff section at the hydrothermal temperature maxima, and 5059-1C-55Z-3, 22-30 and 5059-1C-65Z-2, 0-9, which correspond to the less altered lapilli tuff section at the base of the borehole.

Non-fixed samples stored at 4°C were delicately crushed in sterile mortar to obtain small grains of rocks (< 0.5 mm diameter) and dehydrated before SEM observations. To do so, four washes of 10 min in increasing concentrations of EtOH (30, 50, 80, and 100%) were performed. After air drying, samples were placed on carbon conductive tabs (PELCO Tabs™, 9mm) and were gold-coated. SEM was carried out using a Zeiss AURIGA 40 field-emission scanning electron microscope (Oberkochen, Germany), which is coupled with an Energy Dispersive X-ray analyzer (EDX) located at IPG. Images were acquired using secondary electron detectors (In-Lens for low current and SESI detector for high and low current) or with a backscattered electron detector detector (EsB) with accelerating voltage (EHT) ranging from 5 to 15 kV. Elemental analysis was carried out using an energy dispersive X-ray spectrometer Quantax 200 (125 eV resolution) from Bruker (Billerica, MA, USA).

Results and discussion

Estimation of the number of cells using microscopy

One of the main objectives of the study was to estimate the number of cells present in the drill core samples at each depth. However, the distinction between cells and minerals was difficult under the epifluorescence microscope and too few putative cells were detected in each sample for the cell estimation results to be meaningful. Indeed, counting cells from mineralized samples that host low biomass is challenging. The low cell abundance increases the probability that the cell count falls below the minimum detection limit (MDL), as they

must be statistically meaningful. If the cell count falls below the MDL, the data cannot be used for the global estimate, even though cells are present in the sample (Adhikari *et al.*, 2016). In addition, our method attempted to directly count cells from the rock powder. The separation of cells from the mineral matrix could have been helpful however for manual microscopic cell counting without interference with the minerals. The good recognition of cells using fluorescent dye surely depends on the absence of non-biological background signals and noise, which was present in the samples due to the unspecific binding of the dye to some minerals. Consequently, all these issues made the counting unreliable and it was not possible to estimate the number of cells present in the Surtsey drill core samples using this method. Some techniques were established such as crushing rocks into powder followed by gentle centrifugation (Lunau *et al.*, 2005), or cell extraction by layer density separation followed by filtration (Kallmeyer *et al.*, 2008). However, the cell separation could be incomplete, as cells can be strongly attached to the minerals (e.g. stuck between two layers of clay mineral), and this would greatly bias the results.

Cell numbers estimation based on DNA concentration

Another approach based on the DNA concentration results obtained for each sampling depth was applied to estimate the number of cells in the samples. The Surtsey subsurface drill core samples displayed recovery with low yields of quantifiable DNA in the range of 0.2–1.36 ng/g rocks (0.48 ng/g rocks on average), indicating extremely low standing biomass (Table 2) (Bergsten *et al.*, 2021). Based on these results, we estimated the number of cells per sample. All sampling depths showed an estimated cell number comprised of between 5×10^4 and 1.36×10^6 cells per gram of rock (Table 2). The sample 5059-1C-22Z-3, 0-10 showed a higher cell number estimated between 3.40×10^5 and 1.36×10^6. Overall, these results revealed that the Surtsey subsurface biosphere is an ecosystem that can be considered a low biomass habitat. Those results correlate with previous studies from analogous but far older basaltic environments, ranging from a few thousand years to 64 Ma (Fisk *et al.*, 2003; Mason *et al.*, 2010; Jungbluth *et al.*, 2013; Salas *et al.*, 2015; Jørgensen and Zhao, 2016; Meyer *et al.*, 2016; Zhang *et al.*, 2016).

Table 2. Estimation of the number of cells per gram of rock based on DNA yield.

ICDP labelling system	DNA concentration - Qubit (ds DNA HS) µg/ml	ng of DNA	Ng of DNAgram of rock	cells/gram if 4 fg of DNA in one cell	cells/gram if 1 fg of DNA in one cell
5059-1C-4Z-4, 0-10	0.172	8.6	0.573	1.43×10^5	5.73×10^5
5059-1C-9Z-3, 0-11	0.143	7.15	0.477	1.19×10^5	4.77×10^5
5059-1C-13Z-3, 0-9	0.16	8	0.533	1.33×10^5	5.33×10^5
5059-1C-17Z-3, 0-10	0.105	5.25	0.350	8.75×10^4	3.50×10^5
5059-1C-22Z-3, 0-10	0.408	20.4	1.360	3.40×10^5	1.36×10^6
5059-1C-27Z-3, 0-10	0.11	5.5	0.367	9.17×10^4	3.67×10^5
5059-1C-33Z-3, 0-13	0.089	4.45	0.297	7.42×10^4	2.97×10^5
5059-1C-36Z-3, 0-10	0.157	7.85	0.523	1.31×10^5	5.23×10^5
5059-1C-39Z-3, 10-20	0.149	7.45	0.497	1.24×10^5	4.97×10^5

5059-1C-42Z-3, 10-21	0.17	8.5	0.567	1.42×10^5	5.67×10^5
5059-1C-45Z-1, 81-91	0.15	7.5	0.500	1.25×10^5	5.00×10^5
5059-1C-49Z-4, 9-19	0.109	5.45	0.363	9.08×10^4	3.63×10^5
5059-1C-52Z-3, 10-20	0.104	5.2	0.347	8.67×10^4	3.47×10^5
5059-1C-55Z-3, 10-20	0.06	3	0.200	5.00×10^4	2.00×10^5
5059-1C-59Z-3, 22-33	0.095	4.75	0.317	7.92×10^4	3.17×10^5
5059-1C-62Z-3, 19-28	0.129	6.45	0.430	1.08×10^5	4.30×10^5
5059-1C-65Z-2,9-14	0.13	6.5	0.433	1.08×10^5	4.33×10^5
Control water	Too low	N.A.	N.A.	N.A.	N.A.
Control rock	Too low	N.A.	N.A.	N.A.	N.A.

N.A., Not Applicable.

Nonetheless, the DNA concentration obtained after extraction from 15 g of drill core does not represent the cell number of the environment. DNA from dead cells can be transported by fluid inflows (e.g., rain water, seawater, drilling fluid) within the subsurface, which could bias the estimation, as well as the uncertainty that all cells present in the samples were properly lysed during the extraction method (e.g. mineralized cells, cells difficult to access in the minerals, etc.). This method is used only to give an estimation of the cell number and compare it between the samples. Other methods such as qPCR could have provided a better estimation of the cell numbers.

Confocal laser scanning microscopy (CLSM)

In the study, we applied CLSM to investigate the cell distribution and the possible presence of biofilms in the drill core samples. Unfortunately, the presence of minerals complicates the use of fluorescence techniques. As with any other substrate, dyes can bind to minerals in non-specific ways. The binding to the inorganic surface results in false-positive signals, making the detection of the cells difficult. Moreover, the fluorescence of the minerals can also prevent the detection of true signals (Escudero *et al.*, 2018). Thus, the interpretation of the CLSM images should be carefully examined and evaluated.

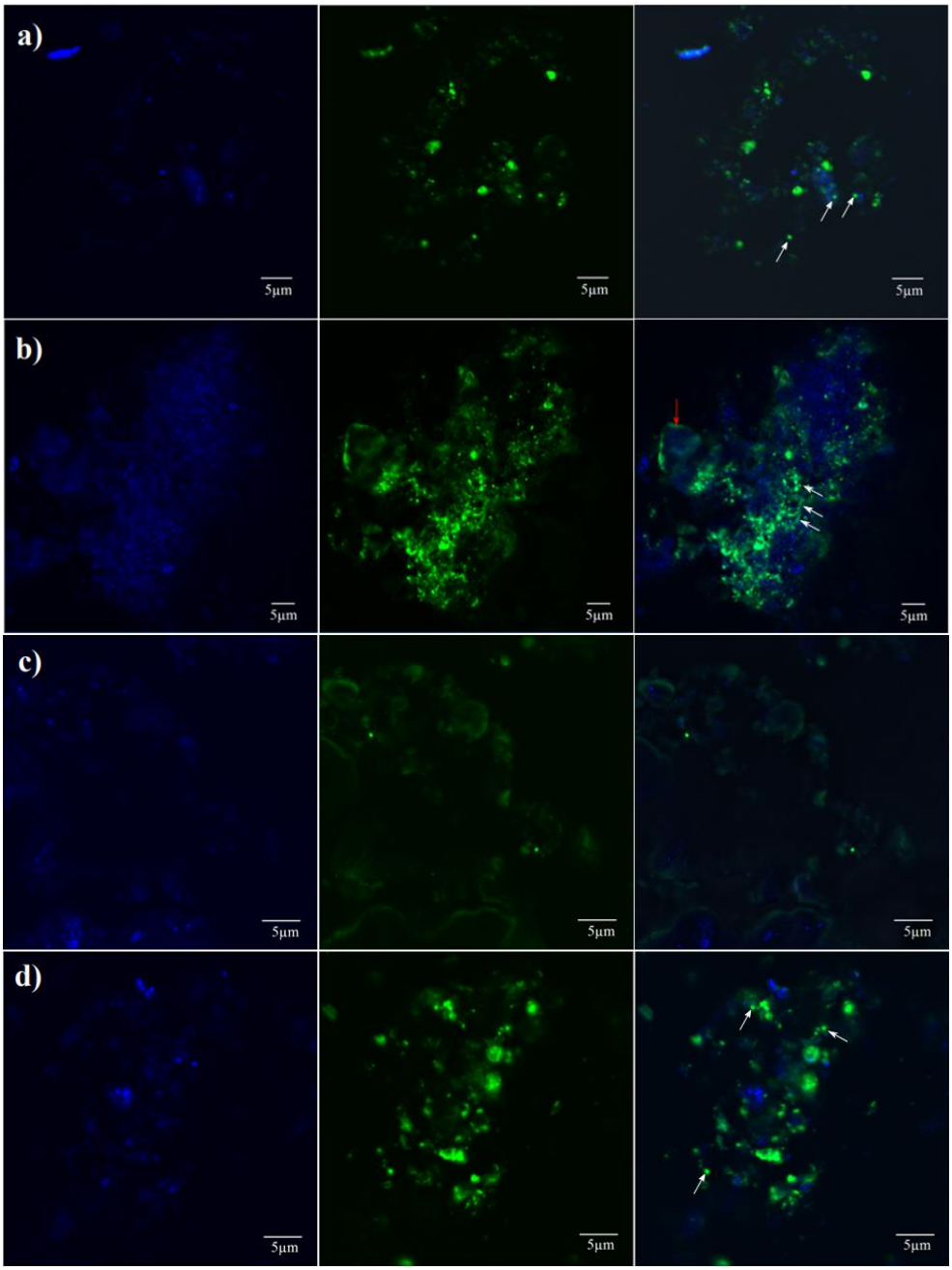

Figure 1. Confocal microscopy images from Surtsey drill cores. a) 5059-1B-9Z-2, 22-28, b) 5059-1B-21Z-2, 24-30, c) 5059-1C-55Z-3, 22-30 and d) 5059-1C-65Z-2,0-9. Blue: natural fluorescence of the minerals, green: SYTO9 staining. White arrows: possible cells. Red arrow: unspecific binding of the dye to the inorganic surface.

The first attempts to take CLSM images showed that the samples were not concentrated enough. Adjustments were performed to visualize signals and possible cells (Figure 1). Some minerals auto-fluoresce in blue. The SYTO9 signal, in some places, was visible on the edge of the minerals, indicating a false signal due to unspecific binding (Figure 1b, red arrows). Moreover, some forms of clay minerals, such as zeolite minerals, are known to bind with fluorescent dyes (Bonilla *et al.*, 2001). Furthermore, their shape and size can be confused with cells. The zeolite crystal has a spherical form and a size between 1 and 3 µm (Demirci *et al.*, 2014). However, the signals that most resembled microbial cells were around 1 µm in size and with a spherical shape (Figure 1, white arrows) and no biofilm was observed. The sample 5059-1B-21Z-2, 24-30 showed some fluorescent dots that appeared to be organized on the inside wall of the vesicles (Figure 1b, white arrows).

Confocal fluorescence microscopy techniques make it possible to study subsurface ecosystems at the micro-niche level, allowing the detection of microorganisms and extracellular polymeric substances (EPS), as well as the distribution of existing biofilms (Lecoeuvre *et al.*, 2021). Because of challenges such as unspecific binding and mineral fluorescence, it is essential to combine CLSM with other techniques such as SEM.

Scanning Electron Microscopy (SEM)

Fifty-five images of five drill core samples were taken using SEM. Unfortunately, no images were obtained from the sample 5059-1B-3Z-2,20-29. Only three images were obtained of the sample 5059-1B-49Z-4,22-30, which presented no signs of putative biotic structures. The following results are based on observations only and are not quantified by statistical methods. All lapilli tuff samples were porous with high water absorption (Jackson *et al.*, 2019b). All samples showed vesicles with spherical shapes, 10–80 µm in diameter, and platy clay mineral structures, with flower petal morphologies (Figure 2a). This indicates that the original volcanic glass has been altered (Jakobsson and Moore, 1986). The alteration of glass in clay minerals was previously observed in the drill cores (Weisenberger *et al.*, 2019). The distribution of microstructures varied with sampling depth. Micro-cracks were observed at all depths, which could increase pore connectivity by connecting isolated vesicles. This could greatly help fluid movement in the tuff deposit that is needed for the establishment and dissemination of microorganisms, also improving the transport of nutrients (Kieft *et al.*, 1998; Zhang *et al.*, 1998; Rebata-Landa and Santamarina, 2006).

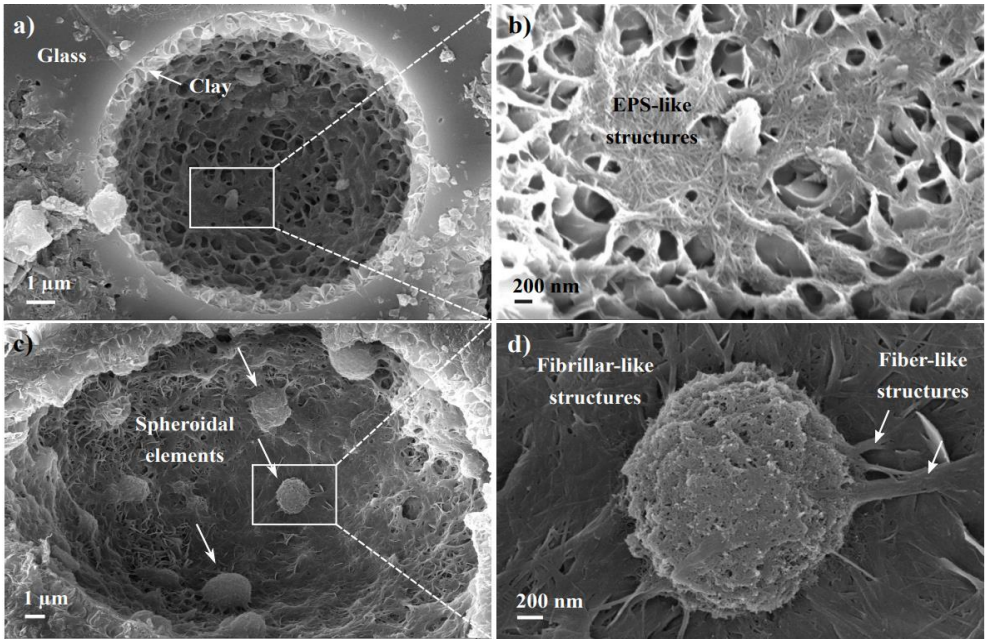

Figure 2. Scanning electron micrographs of the surface of small grains from the drill core sample 5059-1B-9Z-2, 22-28, corresponding to a depth of 32 meters in the subsurface of Surtsey volcano. a) and b) Vesicles in the volcanic glass are altered (presence of flaky clay minerals) and covered by a net of filaments, or EPS-like structures. c) and d) Fibrillar and fiber-like structures seem connected to mineralized spheroidal elements (EDX analyses: no carbon and no phosphate detected; data not show) (Bergsten *et al.*, 2021).

The drill core sample 5059-1B-9Z-2, 22-28 was collected from the borehole SE-02A at a depth of 32 meters, where the environmental temperature was around 30°C. The location of this sample was in the subaerial tuff deposit, above the coastal sea level. Some vesicles were covered on the inside by a net of filaments, or EPS-like structures, which were closely intertwined with each other (Figure 2b). Based on structural observation, these structures could be EPS representing a 'relic' of the past activity of a biofilm. Moreover, the morphology of the clay minerals inside and outside the vesicles covered by EPS-like structures seemed different, indicating differences in the altertation of the minerals. This could be the consequence of a past biofilm activity that would change the chemistry inside the vesicles, impacting the alteration of the minerals. However, no microbial cells were observed in the vesicles. Only small structures (around 300 nm in diameter) or debris were observed (figure 2b). In some other vesicles, spheroidal elements were detected, which could be putative microbial cells with a diameter of 2 µm (Figure 2c). Furthermore, these vesicles were covered by fibrillar-like structures (Figure 2d), which seem texturally different than the EPS-like structures observed in Figures 2a and b. These structures looked like a net of fibers with ramifications and specific directions. Indeed, some of the fiber-like structures seemed to be attached to the spheroidal structures. The cavity could be covered by a fibrillar-like extracellular matrix, which might facilitate the adhesion of microbial cells to the surface and lead to the formation of a biofilm. However, EDX analysis was realized on the spheroidal element and neither carbon nor phosphate was detected, but magnesium, aluminum and iron, suggesting inorganic matter. Yet, it has been shown that EPS could be the nucleation site for

carbonate precipitation (Han *et al.*, 2019), indicating that the spheroidal structures observed in Figure 2c, could represent mineralized microbial cells.

The drill core sample 5059-1B-21Z-2, 24-30 was collected from the borehole SE-02A at a depth of 65 meters. This sample was located just in the submarine tuff deposit below the sea level where the environmental temperature was around 100°C. Amorphous volcanic glass present in this sample seemed less organized than in the sample 5059-1B-9Z-2, 22-28, which could indicates that the cooling process was slower at this depth than at higher depth (Figure 3c). In some vesicles, the clay mineral layer covered the entire inside surface and in others, they were organized in patches (Figure 3a and b). This difference in the alteration degree of the clay minerals present in the vesicles could be explained by the different connections with the outside microenvironment. A vesicle isolated could grow clay minerals differently than a vesicle connected to the outside through cracks and fissures by fluid circulation. The differences of physico-chemical composition between vesicles could influence the microenvironments, the apparition of clay minerals and the establishment of microbial cells.

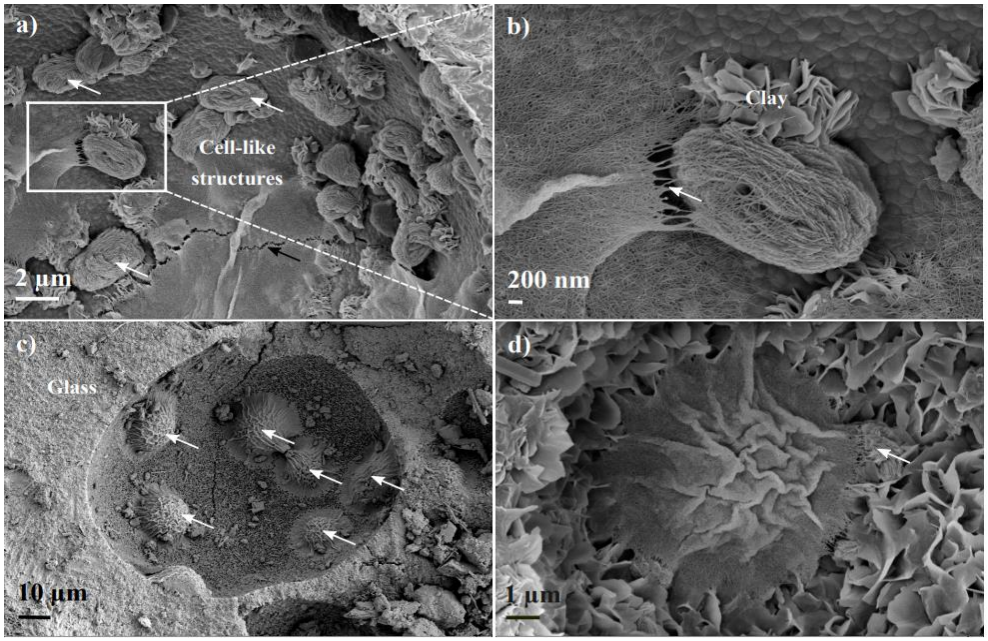

Figure 3. Scanning electron micrographs of the surface of small grains from the drill core sample 5059-1B-21Z-2, 24-30, corresponding to a depth of 65 meters in the subsurface of Surtsey volcano. a) and b) Patches of flaky clay minerals, mineralized ellipsoidal elements and soft net of filaments, or EPS-like structures can be observed inside of some vesicles presents in the volcanic glass. c) and d) Inside some other vesicles wrinkled dome structures can be observed, which seem to be made of a net of filaments (Bergsten *et al.*, 2021).

Also, some vesicles that were "colonized by clay minerals" seemed to have lost their layer, making the inside nude. This might have occurred during the sample preparation. Ellipsoidal elements or cell-like structures were observed with a size of 2.5 to 3 µm long and 2 µm width (Figure 3a, white arrows). Since a few of these structures were present in the vesicle and showed the same size, it could represent microbial cells. However, the surface of the

structures seemed mineralized (Figure 3b). A net of filaments was also observed with a long tear or cut (Figure 3a, black arrow), indicating a soft structure. The presence of both putative cells and EPS-like structures could suggest the presence of a biotic activity. Moreover, wrinkled dome structures were observed in many vesicles at this depth (Figure 3c, white arrows) with a diameter size between 10 and 20 µm. A soft wrinkled structure seemed to cover a dome. This could be due to the sample preparation for SEM images, which included the graduate steps of dehydration. With closer inspection of the structure, a net of crisscrossed filaments was observed that appeared to cover the dome. These types of structures might be EPS forming the matrix of a biofilm and the dome would be a consortium of cells or very small colonies, which we called EPS-microcolony complexes.

An Energy Dispersive X-ray analysis was performed to confirm the biotic signature of the wrinkled domes (Figure 4). Energy Dispersive X-Ray Spectroscopy (EDS or EDX) analysis provided the elemental composition of the targeted structures, corresponding to the clay minerals and the wrinkled dome structure (Figure 4; blue and red areas). It revealed that the putative microcolonies were enriched in carbon, oxygen, sodium, phosphate and calcium compounds, which are cell-biomass-associated elements.

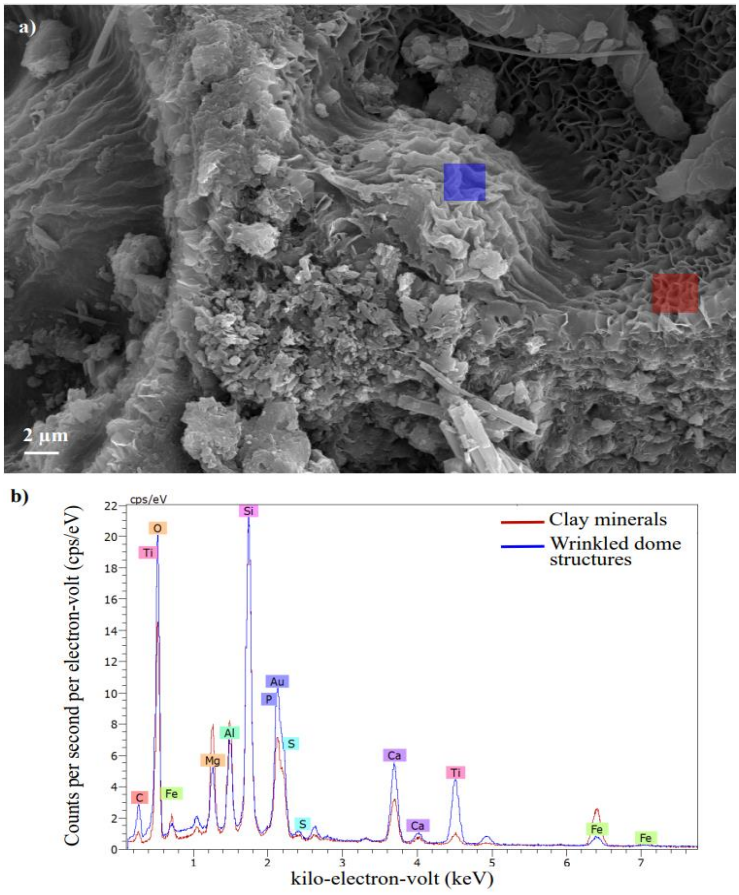

Figure 4. SEM image (a) showing regions of wrinkled dome structure (in blue) and clay minerals (in red) subject to EDX analysis (b). cps/Ev: counts per second per electron-volt,

keV: kilo-electron-volt. The EDS spectrum of the wrinkled dome shows enrichment of C, O, Na, P and Ca compounds.

The drill core sample 5059-1C-55Z-3, 22-30 was collected from the borehole SE-02B at a depth of 157 meters. This sample was located under the thermal barrier where the environmental temperature is around 64°C. In this sample, two types of filaments were observed: thin, flexible and tubular filaments (Figure 5a and b, white arrows) and thick, breakable and flat filaments (Figure 5c and d, white arrow). The flexible filaments seemed to be EPS unlike the breakable filament, which appeared similar to the structures identified as Al-tobermorite in the new drill core (Weisenberger *et al.*, 2019).

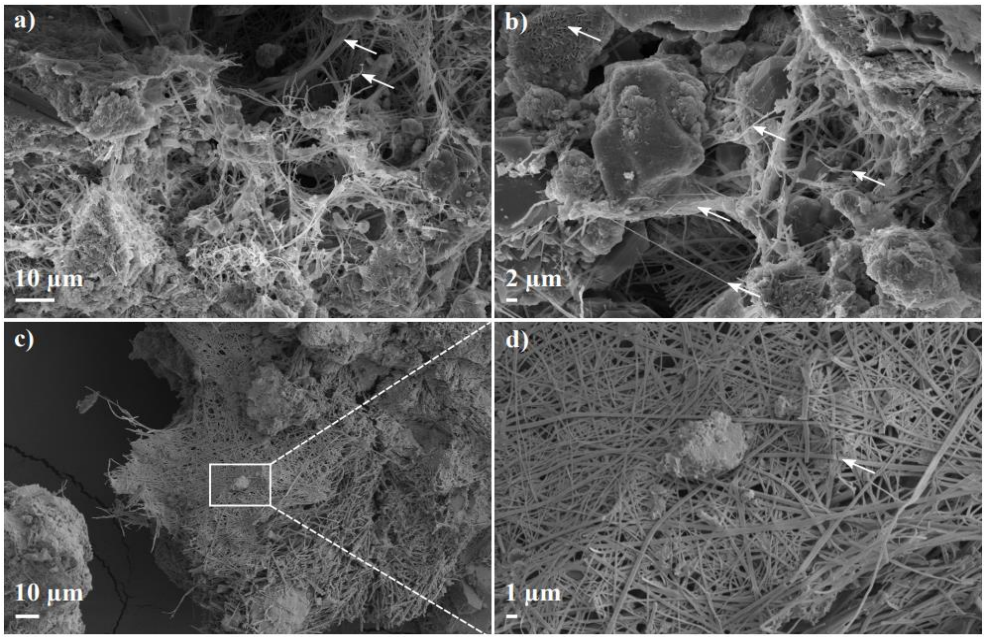

Figure 5. Scanning electron micrographs of the surface of small grains from the drill core sample 5059-1C-55Z-3, 22-30, corresponding to a depth of 157 meters in the subsurface of Surtsey volcano. a) and b) Thin, flexible and tubular filaments, or EPS-like structures. c) and d) Thick, breakable and flat filaments, which seems to be mineral structures.

The drill core sample 5059-1C-65Z-2,0-9 was collected from the borehole SE-02B at a depth of 181 meters. This sample was located at the bottom of the drill core, above the seafloor, where the environmental temperature was around 37°C. Vesicles in a spherical shape and differing in size (10 to 50 µm) were observed very close to each other. Spheroidal elements were observed inside some of them (Figure 6a, white arrows), which were consistent with bacterial form and size. A vesicle showed the presence of a biofilm-like structure where thin and flexible filaments forming a net were detected (Figure 6b, white arrows). These structures, which were likely EPS, seemed to be projected in three-dimension from central amass, where some cell-like structures and debris were observed (Figure 6b, pink arrows). Coccoidal shape elements with a size of 2 µm in diameter were observed. A soft texture net of filaments seemed to project from a central biofilm-like structure and covered the inside of the vesicle. Inside other vesicles, larger spheroidal elements were observed (2 to 5 µm in

diameter). These elements could be consistent with bacterial form (size and shape). As mentioned previously, the production of soluble EPS can initiate nucleation sites suitable for the growth of minerals. An article published in 2018, which studied the formation of some minerals mediated by a deep-sea bacterium (Li, Yao et al. 2019), showed a similar structure as the spheroidal elements present in Figure 6c. In the study, different bacterial components, such as native cells, soluble EPS, or small organic molecules, were used to influence the crystallization and growth of the minerals. They demonstrated through mineralization experiments that the bacteria did not only promote the mineralization but also influenced the shape. The spheroidal elements published in this study were very similar to the one observed in Figure 6c and were formed in the presence of components found in the supernatant of the bacterial culture. Thus, we suggest that the spheroidal structures observed in the vesicle in Figure 6c, could be abiotic but biologically mediated by small organic molecules.

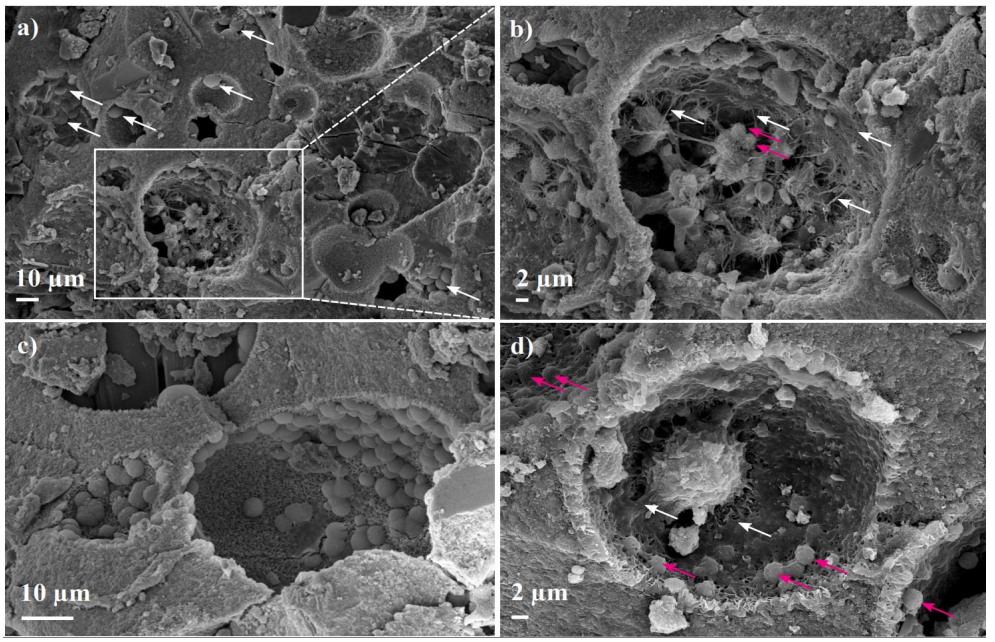

Figure 6. Scanning electron micrographs of the surface of small grains from the drill core sample 5059-1C-65Z-2,0-9, corresponding to a depth of 181 meters in the subsurface of the Surtsey volcano. a) Vesicles in the volcanic glass with inside spheroidal elements (white arrows). b) Magnification inside one of the vesicles where cell-like structures (pink arrows) and thin, flexible and tubular filaments, or EPS-like structures (white arrows), can be observed. c) Spheroidal elements inside vesicles and d) Magnification of another vesicle of the sample where a net of filaments (white arrows) covers the inside of the vesicle and spheroidal elements consistent with bacterial forms (pink arrows) can be observed.

Based on these observations, the structural strategy adopted by microorganisms inhabiting the subsurface rocks of Surtsey volcano appeared to be organized in biofilms with the production of EPS, or EPS-microcolony complexes, which appeared to be only located inside the numerous interconnected vesicles present in the volcanic glass.

Conclusion and Perspectives

In this study, we showed that the application of fluorescence techniques on the drill core samples from the subsurface of Surtsey volcano was challenging to (i) estimate the cell numbers, (ii) detect cells and microbial structures, and (iii) investigate their organization towards microstructures of the rocks and the minerals. The glassy basaltic tuff from the subsurface of Surtsey is porous and highly mineralized (Jackson *et al.*, 2019b; a; Prause *et al.*, 2020), and thus, the presence of minerals leads to some issues. First, minerals, such as zeolites crystals, were consistent with microorganism form and size, making their distinction difficult with actual cells. Secondly, some minerals auto-fluoresced, preventing the detection of true signals. And thirdly, dyes bonded in a non-specific way to these minerals, resulting in false-positive signals. Moreover, we worked with samples presenting very low biomass. Consequently, the detection of cells was a tedious task. Our estimation of the cell numbers based on counting using epifluorescence microscopy fell below the minimum detection limit. The cell numbers were thus estimated based on the DNA concentration and showed values ranging from 5×10^4 and 1.36×10^6 cells per gram of rock. A drill core sample located in the submarine tuff deposit (65 m depth, in situ temperature of 101.5°C) showed the highest number of estimated cells in the range of 3.40×10^5 to 1.36×10^6 cells per gram of rock. The CLSM images located at the same depth suggested that putative cells were situated inside vesicles in the basaltic glass. Furthermore, the analyses of the SEM images revealed putative biotic structures, such as microbial cells connected by nets of EPS, forming biofilm structures. Indeed, many vesicles at 65 m depth with a diameter size of 10–20 µm were found with wrinkled dome structures. Some evidence suggested that the observed structures were of biotic origin, including their appearance, size and the EDX analysis results. This cell cluster organization in EPS-microcolonies could be an adaptation strategy for microbial survival and protection from the extreme environmental conditions occurring in the subsurface of the Surtsey volcano. However, the interpretation is only based on observations and numerous questions still remain, such as: Do the inhabiting vesicles correspond to specialized micro-niches with different optimal conditions (e.g. aerobic versus anaerobic), different metabolisms and different microbial communities? Or are these niches interconnected to interchange metabolic products, thus generating a network of specialized metabolisms? Other methods should be tested to develop an effective protocol to separate cells from the mineral matrix before the cell count. In addition, performing qPCR on DNA extracted from the drill cores may give a better estimation of cell count. Moreover, different DNA dyes or fluorophores of higher sensitivity (e.g., SYBR-Green I) and with different wavelengths should be tested for better distinction between cell-derived fluorescence signals and nonspecific background fluorescence signals from minerals (Morono *et al.*, 2009). More efforts should be made to characterize the microbial communities attached to the submarine and subaerial basaltic tuff deposits of the Surtsey volcano using microscopic investigations, such as laser microdissection combined with whole-genome amplification (Gérard *et al.*, 2018). A subsurface observatory was deployed in September 2017 in the borehole SE-02B. It involved incubation chamber experiments that could lead to great discoveries such as the detection of biosignatures of microbe–mineral interactions on the glass and olivine samples retrieved from the observatory (Türke *et al.*, 2019).

Funding

The sampling and sample processing were funded with a grant of excellence from the Icelandic Science fund, ICF-RANNÍS IceSUSTAIN (163083-051). The drilling operation was funded with International Continental Scientific Drilling Program (ICDP) through the

SUSTAIN program and the IceSUSTAIN grant. A doctoral student grant from Jules Verne (ICE and Fire Interaction in Iceland) was attributed to P. Bergsten. “Icelandic subsurface microbial ecosystems and their implications for basalt alteration and associated biogeochemical cycles”.

Acknowledgments

The authors would like to thank all members of the SUSTAIN onsite, science teams and the Surtsey Research Society for their contributions to the drilling project and sampling. The Icelandic Science fund, ICF-RANNÍS Jules Verne fund (scientific and technical cooperation between Iceland and France) contributed to additional funds. The authors thank E. Gérard, A. Lecoeuvre, B. Ménez and S. Borensztajn for their contribution of sample preparation and SEM images. The authors also thank S. H. Björnsdóttir for reviewing the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

Adhikari, R.R., Glombitza, C., Nickel, J.C., Anderson, C.H., Dunlea, A.G., Spivack, A.J., et al. (2016) Hydrogen utilization potential in subsurface sediments. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2016.00008.

Baldursson, S. & Ingadóttir, Á. (2007) Nomination of Surtsey for the UNESCO World Heritage List. *Icelandic Institute of Natural History, Reykjavik*.

Baquiran, J.P.M., Ramírez, G.A., Haddad, A.G., Toner, B.M., Hulme, S., Wheat, C.G., et al. (2016) Temperature and redox effect on mineral colonization in Juan de Fuca Ridge Flank subsurface crustal fluids. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2016.00396.

Bar-On, Y.M., Phillips, R., and Milo, R. (2018) The biomass distribution on Earth. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1711842115.

Bergsten, P., Vannier, P., Klonowski, A.M., Knobloch, S., Gudmundsson, M.T., Jackson, M.D., and Marteinsson, V.T. (2021) Basalt-Hosted Microbial Communities in the Subsurface of the Young Volcanic Island of Surtsey, Iceland. *Frontiers in Microbiology* **12**:. doi: 10.3389/fmicb.2021.728977.

Bonilla, G., Tsapatsis, M., Vlachos, D.G., and Xomeritakis, G. (2001) Fluorescence confocal optical microscopy imaging of the grain boundary structure of zeolite MFI membranes made by secondary (seeded) growth. *Journal of Membrane Science*. doi: 10.1016/S0376-7388(00)00549-4.

Demirci, S., Ustaoğlu, Z., Yilmazer, G.A., Sahin, F., and Baç, N. (2014) Antimicrobial properties of zeolite-X and zeolite-A ion-exchanged with silver, copper, and zinc against a broad range of microorganisms. *Applied Biochemistry and Biotechnology*. doi: 10.1007/s12010-013-0647-7.

Edwards, K.J., Becker, K., and Colwell, F. (2012a) The deep, dark energy biosphere: Intraterrestrial life on earth. *Annual Review of Earth and Planetary Sciences*. doi: 10.1146/annurev-earth-042711-105500.

Edwards, K.J., Fisher, A.T., and Wheat, C.G. (2012b) The deep subsurface biosphere in igneous ocean crust: Frontier habitats for microbiological exploration. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2012.00008.

Escudero, C., Vera, M., Oggerin, M., and Amils, R. (2018) Active microbial biofilms in deep poor porous continental subsurface rocks. *Scientific Reports*. doi: 10.1038/s41598-018-19903-z.

Fisk, M.R., Storrie-Lombardi, M.C., Douglas, S., Popa, R., McDonald, G., and Di Meo-Savoie, C. (2003) Evidence of biological activity in Hawaiian subsurface basalts. *Geochemistry, Geophysics, Geosystems*. doi: 10.1029/2002GC000387.

Gérard, E., De Goeyse, S.D., Hugoni, M., Agogué, H., Richard, L., Milesi, V., et al. (2018) Key role of Alphaproteobacteria and Cyanobacteria in the formation of stromatolites of Lake Dziani Dzaha (Mayotte, Western Indian Ocean). *Frontiers in Microbiology*. doi: 10.3389/fmicb.2018.00796.

Han, Z., Wang, J., Zhao, H., Tucker, M.E., Zhao, Y., Wu, G., et al. (2019) Mechanism of biomineralization induced by bacillus subtilis J2 and characteristics of the biominerals. *Minerals*. doi: 10.3390/min9040218.

Jackson, M.D. (2020) Petrograpic and material observations of basaltic lapilli tuff, 1979 and 2017 Surtsey drill cores, Iceland. *Surtsey research*. doi: 10.33112/surtsey.14.4.

Jackson, M.D., Couper, S., Stan, C. V., Ivarsson, M., Czabaj, M.W., Tamura, N., et al. (2019a) Authigenic Mineral Texture in Submarine 1979 Basalt Drill Core, Surtsey Volcano, Iceland. *Geochemistry, Geophysics, Geosystems*. doi: 10.1029/2019GC008304.

Jackson, M.D., Gudmundsson, M.T., Weisenberger, T.B., Rhodes, J.M., Stefánsson, A., Kleine, B.I., et al. (2019b) SUSTAIN drilling at Surtsey volcano, Iceland, tracks hydrothermal and microbiological interactions in basalt 50 years after eruption. *Scientific Drilling* **25**: 35–46. doi: 10.5194/sd-25-35-2019.

Jakobsson, S.P. and Moore, J.G. (1986) Hydrothermal minerals and alteration rates at Surtsey volcano, Iceland. *Geological Society of America Bulletin* **97**: 648–659. doi: 10.1130/0016-7606(1986)97<648:HMAARA>2.0.CO;2.

Jakobsson, S.P. and Moore, J.G. (1982) The Surtsey Research Drilling Project of 1979. *Surtsey Research Progress Report* **9**: 76–93.

Jakobsson, S.P., Thors, K., Vésteinsson, Á.T., and Ásbjörnsdóttir, L. (2009) Some aspects of the seafloor morphology at Surtsey volcano: The new multibeam bathymetric survey of 2007, Surtsey Research. *Surtsey Research Progress Report* **12**: 9–20.

Jørgensen, S.L. and Zhao, R. (2016) Microbial inventory of deeply buried oceanic crust from a young ridge flank. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2016.00820.

Jungbluth, S.P., Grote, J., Lin, H.T., Cowen, J.P., and Rappé, M.S. (2013) Microbial diversity within basement fluids of the sediment-buried Juan de Fuca Ridge flank. *ISME Journal*. doi: 10.1038/ismej.2012.73.

Kallmeyer, J., Smith, D.C., Spivack, A.J., and D'Hondt, S. (2008) New cell extraction procedure applied to deep subsurface sediments. *Limnology and Oceanography: Methods*. doi: 10.4319/lom.2008.6.236.

Kieft, T.L., Murphy, E.M., Haldeman, D.L., Amy, P.S., Bjornstad, B.N., McDonald, E. V., et al. (1998) Microbial transport, survival, and succession in a sequence of buried sediments. *Microbial Ecology*. doi: 10.1007/s002489900120.

Lecoeuvre, A., Ménez, B., Cannat, M., Chavagnac, V., and Gérard, E. (2021) Microbial ecology of the newly discovered serpentinite-hosted Old City hydrothermal field (southwest Indian ridge). *ISME Journal*. doi: 10.1038/s41396-020-00816-7.

Lunau, M., Lemke, A., Walther, K., Martens-Habbena, W., and Simon, M. (2005) An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Environmental Microbiology*. doi: 10.1111/j.1462-2920.2005.00767.x.

Magnabosco, C., Biddle, J.F., Cockell, C.S., Jungbluth, S.P., and Twing, K.I. (2019) Biogeography, Ecology, and Evolution of Deep Life. In *Deep Carbon: Past to Present*. pp. 524–555. doi: 10.1017/9781108677950.017.

Mason, O.U., Nakagawa, T., Rosner, M., van Nostrand, J.D., Zhou, J., Maruyama, A., et al. (2010) First investigation of the microbiology of the deepest layer of ocean crust. *PLoS ONE*. doi: 10.1371/journal.pone.0015399.

Mcloughlin, N., Staudigel, H., Furnes, H., Eickmann, B., and Ivarsson, M. (2010) Mechanisms of microtunneling in rock substrates: Distinguishing endolithic biosignatures from abiotic microtunnels. *Geobiology*. doi: 10.1111/j.1472-4669.2010.00243.x.

Meyer, J.L., Jaekel, U., Tully, B.J., Glazer, B.T., Wheat, C.G., Lin, H.T., et al. (2016) A distinct and active bacterial community in cold oxygenated fluids circulating beneath the western flank of the Mid-Atlantic ridge. *Scientific Reports*. doi: 10.1038/srep22541.

Morono, Y., Terada, T., Masui, N., and Inagaki, F. (2009) Discriminative detection and enumeration of microbial life in marine subsurface sediments. *ISME Journal*. doi: 10.1038/ismej.2009.1.

Prause, S., Weisenberger, T.B., Cappelletti, P., Grimaldi, C., Rispoli, C., Jónasson, K., et al. (2020) Alteration progress within the Surtsey hydrothermal system, SW Iceland – A time-lapse petrographic study of cores drilled in 1979and 2017. *Journal of Volcanology and Geothermal Research*. doi: 10.1016/j.jvolgeores.2019.106754.

Ramírez, G.A., Garber, A.I., Lecoeuvre, A., D'angelo, T., Wheat, C.G., and Orcutt, B.N. (2019) Ecology of subseafloor crustal biofilms. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2019.01983.

Rebata-Landa, V. and Santamarina, J.C. (2006) Mechanical limits to microbial activity in deep sediments. *Geochemistry, Geophysics, Geosystems*. doi: 10.1029/2006GC001355.

Salas, E.C., Bhartia, R., Anderson, L., Hug, W.F., Reid, R.D., Iturrino, G., and Edwards, K.J. (2015) In situ detection of microbial life in the deep biosphere in Igneous ocean crust. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2015.01260.

Sessions, S.K. and Hodgkin, J. (2013) Genome Size. In *Encyclopedia of Genetics*. doi: 10.1016/B978-0-12-374984-0.00639-2.

Staudigel, H., Furnes, H., McLoughlin, N., Banerjee, N.R., Connell, L.B., and Templeton, A. (2008) 3.5 billion years of glass bioalteration: Volcanic rocks as a basis for microbial life? *Earth-Science Reviews*. doi: 10.1016/j.earscirev.2008.04.005.

Takai, K., Nakamura, K., Toki, T., Tsunogai, U., Miyazaki, M., Miyazaki, J., et al. (2008) Cell proliferation at 122°C and isotopically heavy CH4 production by a hyperthermophilic methanogen under high-pressure cultivation. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0712334105.

Thorarinsson, S. and Þórarinsson, S. (1965) The Surtsey eruption: Course of events and the development of the new island. *Surtsey Research Progress Report* **1**: 51–55.

Türke, A., Jackson, M.D., Bach, W., Kahl, W.A., Grzybowski, B., Marshall, B., et al. (2019) Design of the subsurface observatory at Surtsey volcano, Iceland. *Scientific Drilling*. doi: 10.5194/sd-25-57-2019.

Walton, A.W. (2008) Microtubules in basalt glass from Hawaii Scientific Driling Project #2 phase 1 core and Hilina slope, Hawaii: Evidence of the occurrence and behavior of endolithic microorganisms. *Geobiology*. doi: 10.1111/j.1472-4669.2008.00149.x.

Weisenberger, T.B., Gudmundsson, M.T., Jackson, M.D., Gorny, C.F., Türke, A., Kleine, B.I., et al. (2019) Operational Report for the 2017 Surtsey Underwater volcanic System for Thermophiles, Alteration processes and INnovative Concretes (SUSTAIN) drilling project at Surtsey Volcano, Iceland. *Potsdam: GFZ German Research Centre for Geosciences* 240. doi: 10.2312/ICDP.5059.001.

Zhang, C., Palumbo, A. V., Phelps, T.J., Beauchamp, J.J., Brockman, F.J., Murray, C.J., et al. (1998) Grain size and depth constraints on microbial variability in coastal plain subsurface sediments. *Geomicrobiology Journal*. doi: 10.1080/01490459809378074.

Zhang, X., Feng, X., and Wang, F. (2016) Diversity and metabolic potentials of subsurface crustal microorganisms from the western flank of the mid-atlantic ridge. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2016.00363.