

Microbial tracers involved in Antarctica cloud condensation and ice nuclei

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Dissertation carried out as part of the Master in Biodiversity, Genetics and Evolution
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Dedicated to my

mother Nataša,

grandmother Jelena,

and grandfather Milan,

For their immeasurable support,

guidance and love.

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Resumo

A Antártida é o continente terrestre mais frio, seco e isolado. No entanto, as alterações climáticas estão a aumentar significativamente a temperatura e a precipitação em extensas zonas do continente, particularmente, na Península Antártida. O derretimento da superfície e a precipitação na Antártida são bastante influenciados pelas alterações extremas do clima, incluindo os rios atmosféricos (RAs) que transportam o calor e a humidade das latitudes médias em direção aos polos. Os RAs também podem facilitar o transporte de aerossóis que têm funções importantes na condensação das nuvens e em processos de nucleação de gelo. Os bioaerossóis têm um papel importante na formação das nuvens da Antártida, no entanto, é preciso desenvolver-se mais investigações para perceber a sua contribuição no processo atmosférico. Este trabalho, tem como objetivo, providenciar um estudo compreensivo das composições microbiológicas da precipitação em forma de chuva ou de neve, assim como da precipitação após atingir a superfície, como seja a neve dos glaciares. Com o uso de métodos de cultivo, quarenta e oito estirpes bacterianas foram identificadas através da sequenciação do gene 16S rRNA. Em cada tipo de amostra recuperou-se uma diversidade de estirpes bacterianas distintas, através do uso de métodos dependentes de cultivo. Quatro das estirpes isoladas foram identificadas como potenciais novas espécies, ressaltando a importância destes ambientes extremos para o avanço do conhecimento sobre a biodiversidade de bioaerossóis em amostras de precipitação da Antártida. Adicionalmente, estirpes bacterianas conhecidas pela sua atividade de nucleação de gelo foram identificadas nas amostras estudadas. Dadas as limitações dos métodos de cultura na captura da biodiversidade real, a otimização de métodos independentes da cultura e a extração de eADN de amostras da precipitação, também foram realizadas. Esta investigação fornece conhecimento acerca da diversidade dos microrganismos cultiváveis nas amostras analisadas, sugerindo o seu potencial papel na formação de nuvens e precipitação. Outros estudos são no entanto necessários para compreender o potencial papel destes microrganismos nas alterações climáticas na Antártica.

Palavras-chave: Antártida, Península Antártica, Rios atmosféricos, bioaerossóis, microbiana, nucleação de gelo, condensação de nuvens, biodiversidade cultivável, precipitação, mudanças climáticas

Abstract

Antarctica is the Earth's coldest, driest and most isolated continent. However, climate change is leading to a significant increase in temperature and precipitation over large parts of the continent, particularly over the Antarctic Peninsula (AP). Surface melt and anomalous precipitation over Antarctica are strongly controlled by extreme weather events, including atmospheric rivers (ARs) carrying heat and moisture from midlatitudes polewards. ARs can also facilitate transport of aerosols playing a role in cloud condensation and ice nucleation processes. Bioaerosols play an important role in Antarctic cloud formation, yet further research is necessary to understand their contribution to atmospheric processes. Therefore, this work aims to provide a comprehensive study of the microbiological composition of precipitation - rainfall, snowfall, and surface snow precipitation - with glacier snow collected for comparison. Using culturable methodologies, forty-eight bacterial strains were identified by 16S rRNA gene sequencing. Each sample type had a distinct culturable biodiversity, with notable differences between rainfall and snowfall. Four strains identified as potentially new species were found in the samples, highlighting the potential of extreme environments to foster unique biodiversity. Additionally, bacterial strains known for their ice nucleating activity (INA) were present in the samples. Given the limitations of culturing methods in capturing true biodiversity, optimization of culture-independent methodologies and eDNA extraction from precipitation samples were also conducted. This research provides insights into the culturable diversity of microorganisms in the analysed samples, suggesting their potential roles in cloud condensation and precipitation formation. Additional studies in the field are needed to grasp the potential role of these microorganisms in Antarctic climate change.

Keywords: Antarctica, Antarctic Peninsula, Atmospheric rivers, bioaerosols, microbial, ice nucleation, cloud condensation, culturable biodiversity, precipitation, climate change

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List of Abbreviations

AAR	Aerosol Atmospheric River
AP	Antarctic Peninsula
AR	Atmospheric River
C	Celsius
CCN	Cloud Condensation Nuclei
CIIMAR	Centro Interdisciplinar de Investigação Marinha e Ambiental
cm dNTP	Centimetre
eDNA	Deoxynucleotide Triphosphates
FCUP	Environmental Deoxyribonucleic Acid
G	Faculty of Sciences of the University of Porto
GS	Gamma-distributed
GRS	Glacier Snow
HKY	Grisp Research Solutions
HQ	Hasegawa-Kishino-Yano
I	High Quality
ID	Invariable Sites
INA	Identification
INP	Ice Nucleating Activity
I3S	Ice Nucleating Particles
KGI	Research and Innovation in Health
MA	King George Island
ML ml	Marine Agar
NC	Maximum Likelihood
NCBI	Millilitre
NCP	Negative Control
ng PCA	National Centre for Biotechnology Information
PCR	No-Cleaning Protocol
PI	Nanogram
R2A	Plate Count Agar
RP	Polymerase Chain Reaction
SP	Principal Investigator
SS	Reasoner's Agar
TN	Rain Precipitation
UV WGS	Snow Precipitation
rRNA	Surface Snow
µL	Tamura-Nei
µM	Ultraviolet
UV	Whole Genome Sequencing
WGS	Ribosomal Ribonucleic Acid
rRNA	Microlitre
µl	Micrometre
µM	

1. Introduction

1.1. Geoclimatic characteristics of Antarctica

Antarctica, covering an area of 14.2 million square kilometres in the Southern Hemisphere, is Earth's most isolated continent, distinguished by its extreme environmental conditions (De Blij, 1978). It is the coldest and driest region on the planet, where rapidly evolving environmental processes make it a critical focus of contemporary climate change research (Convey, 2006).

Almost entirely situated south of the Antarctic Circle (66º 33' 39" S latitude), Antarctica experiences extreme variations in light due to Earth's 23.5-degree axial tilt. During the austral summer, the South Pole receives 24 hours of continuous daylight, while the austral winter is marked by perpetual darkness. This unique pattern of solar insolation significantly influences the region's environmental characteristics.

Despite receiving high levels of solar radiation during the polar summer, Antarctica's high albedo—the reflectivity of ice and snow—causes most of the sunlight to be reflected into the atmosphere, reinforcing its position as the coldest place on Earth. The lowest temperature ever recorded was −89.2°C, at Vostok Station, on July 21, 1983 (Turner et al., 2009a). The extreme cold also contributes to Antarctica's arid conditions. As a result, Antarctica is classified as a polar desert, receiving only minimal annual precipitation.

The topographical relief of the continent is marked by extensive ice formations, including its massive ice sheets and floating ice shelves, which extend into the surrounding ocean. These ice shelves serve as seaward extensions of the continental ice mass, while areas of fast ice remain anchored to the coast or ice shelves, unmoved by winds or currents (Turner et al., 2009b). The continent is surrounded by the Southern Ocean, which plays a crucial role in its climatic and environmental conditions (Meredith & Brandon, 2016). Throughout the austral winter, the Antarctic sea ice expands drastically, covering the area of17.6 million km² in the winter of 2024; this was however the second lowest value measured in 46 years of satellite observations (NSIDC, 2024).

Antarctica has the highest average elevation of any continent, with a mean elevation of 2,200 meters. East Antarctica is dominated by the high, domed Polar Plateau and is a largely land-based ice sheet, while West Antarctica is defined by a faulted, rugged terrain that is largely marine-based. The Transantarctic Mountains form the boundary between these two regions (Siple, 1956; Turner et al., 2009b) (Figure 1).

West Antarctica extends toward the Antarctic Peninsula (AP), the most prominent extension of mainland Antarctica into mid-latitudes. The Peninsula reaches toward South America, separated from Tierra del Fuego by roughly 1,000 kilometres across the Drake Passage. The Bransfield Strait lies between the AP and the South Shetland Islands, an
Antarctic archipelago with a total area of 3,687 km². The largest in the South Shetland Archipelago, the King George Island (KGI), hosts numerous research stations conducting interdisciplinary research, with nine of these stations being staffed yearround (Simões et al., 2004).

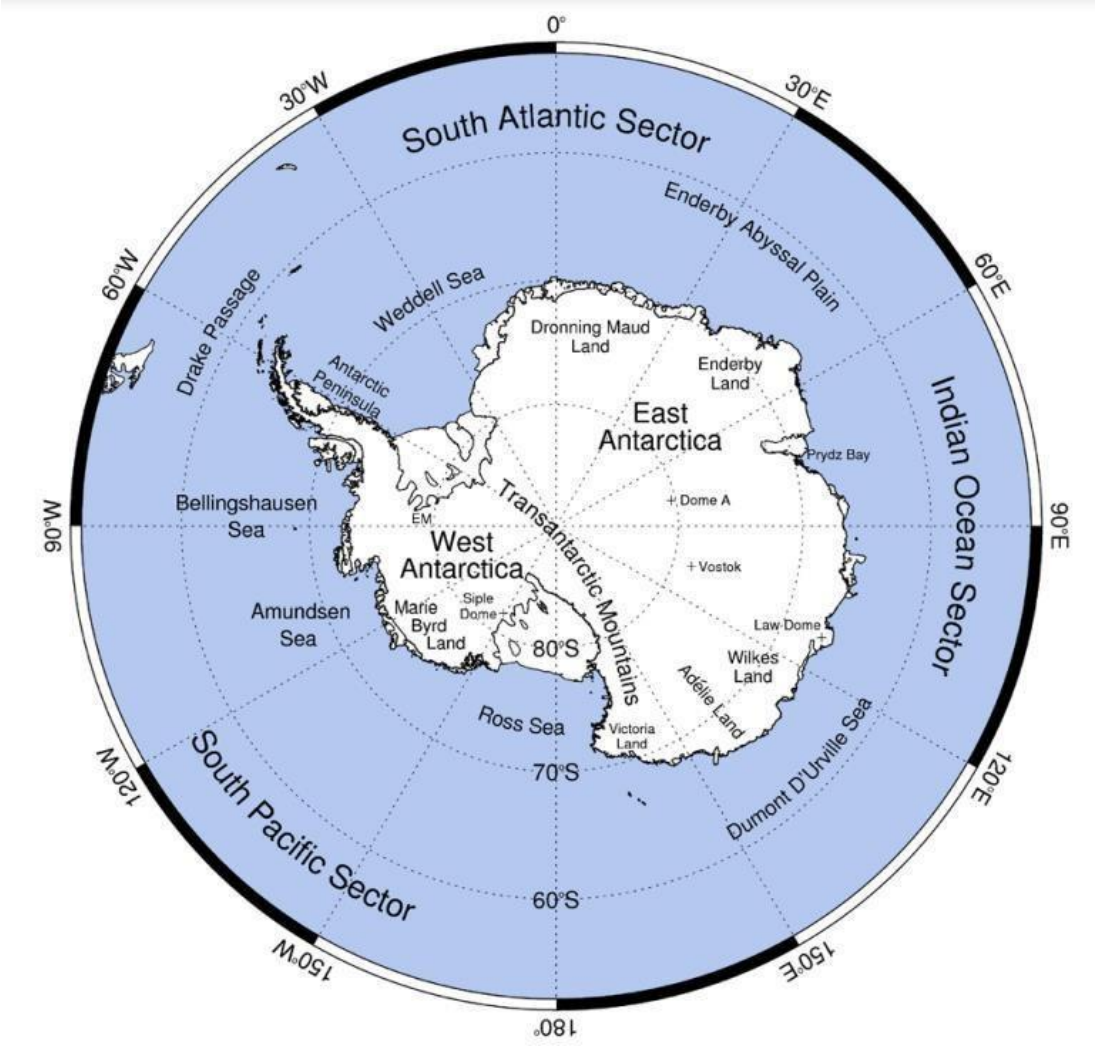


Figure 1 - A map of Antarctica constructed by Turner et al., 2009b. The map represents the authors' selected topographic features and locations. The continent is surrounded by the Southern Ocean, directly connected with the Pacific, Atlantic and Indian Ocean section

1. 2. Climate change over Antarctica

The onset of continental ice formation in Antarctica dates back approximately 36 million years (Zachos et al., 1992). A further cooling around 14 million years ago led to the development of a thicker and more stable ice sheet, that is thought to have persisted through Earth's subsequent temperature fluctuations (Flower & Kennett, 1994). Since then, several climate epochs have left marks on the ice core, allowing us to trace changes in Antarctic climate over millions of years. Mercer (1968) found that the West Antarctic Ice sheet melted away during a period where the sea temperatures were 6-7°C degrees higher than today, suggesting future temperature rises could have impacts on Antarctic ice stability.

Modern studies focus primarily on understanding changes observed during the 20th and 21st centuries, in the context of anthropogenic climate change. The past 50 years, which have been monitored through continuous instrumental records, are of particular interest (Mayewski et al., 2009).

Studies reveal varying climate trends across the continent. Cooling has been observed in East Antarctica since the 1970s (Chapman & Walsh, 2007). On the other hand, the Antarctic Peninsula has experienced one of the most pronounced warming trends globally over the past 50 years (Vaughan et al., 2003). Vernadsky Station (AP) saw a 2.8°C increase in annual mean surface air temperature from 1951 to 2000. The late 1970s saw additional warming linked to stratospheric ozone loss. Observed warming since the 1950s and cooling since the late 1990s are consistent with natural decadal scale climate variability according to Turner et al. (2016). Still, they note that under medium emission scenarios, climate models project that anthropogenic warming in the AP will match or exceed the late 20th-century warming by the end of this century. This is further supported by Bracegirdle et al. (2008) who presented a new assessment of Antarctic climate change throughout the 21st century. They project surface warming averaged across the continent to be 0.34°C per decade, with a standard deviation of 0.10°C.

Climate is projected to change in the next hundreds of years, yet climate changes happening at present can be observed in extreme warm events. February 2020 was marked by record-high temperatures of 18.3 °C, measured at Esperanza Station, Antarctic Peninsula. These temperature extremes were part of a heatwave that swept across Antarctica between February 6th and 11th and were associated with climate change (González-Herrero et al., 2022). Extreme weather events in recent years have surpassed previous benchmarks, and it is expected weather extremes will increase both on a global, and local Antarctic scale (Fischer et al., 2021; Siegert et al., 2023).

More climate parameters are also being affected by climate change. Precipitation over Antarctica is projected to change by the end of the current century, although the extent of change remains uncertain (Lynch et al., 2006). According to Bracegirdle et al. (2008), precipitation increases will be greatest along the coast of Antarctica, while the most significant increases relative to the current climate—up to 25%—are observed in the interior region of East Antarctica (Figure 2). While most of the precipitation over the Antarctic continent falls as snowfall, rain events have been frequently reported in the north-western part of the AP (Vignon et al., 2021). In the Arctic, Bintanja et al. (2018) project a shift from snowfall-dominated to rainfall-dominated precipitation by the end of the 21st century. If Antarctica follows the patterns of changing precipitation, it could result in increased surface melting, alterations in surface mass balance, and significant impacts on the entire ecosystem and its biodiversity (Nicolas et al., 2017; Ropert-Coudert et al., 2015). However, the future of precipitation change in Antarctica remains uncertain.

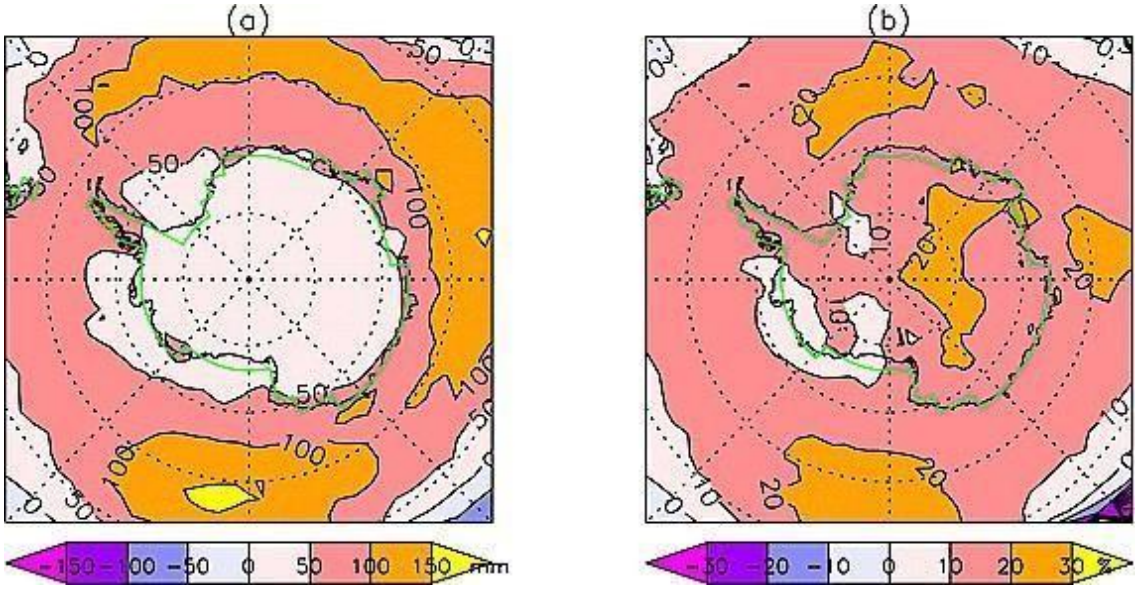


Figure 2 - Weighted ensemble mean projections for changes in the annual mean precipitation rate during the 21st century (Bracegirdle et al., 2009). Difference between the mean precipitation rates for 2080–2099 and 2004–2023, as (a) absolute change in millimetres per year and (b) percentage change

1. 3. Atmospheric Rivers in Polar regions

Atmospheric rivers (ARs) are relatively infrequent but play a crucial role in transporting water vapour across midlatitudes, accounting for over 90% of the poleward water vapour transport (Nash et al., 2018). Their influence extends to the Antarctic continent, where extreme precipitation events contribute significantly to annual precipitation variability. In Antarctica, these extreme events explain 70% of the precipitation variance, rising to over 90% in certain regions (Turner et al., 2019).

The concept of atmospheric rivers was first introduced by Zhu and Newell in 1996, who developed an algorithm to study water vapour fluxes in the troposphere. Since then, research interest in ARs has increased, particularly regarding their impacts on polar systems (Ralph et al., 2017). ARs are known to carry heat, contributing to record-high temperatures in the Antarctic Peninsula, which exacerbates surface ice melt and threatens ice-shelf stability (Gorodetskaya et al., 2023) (Figure 3).

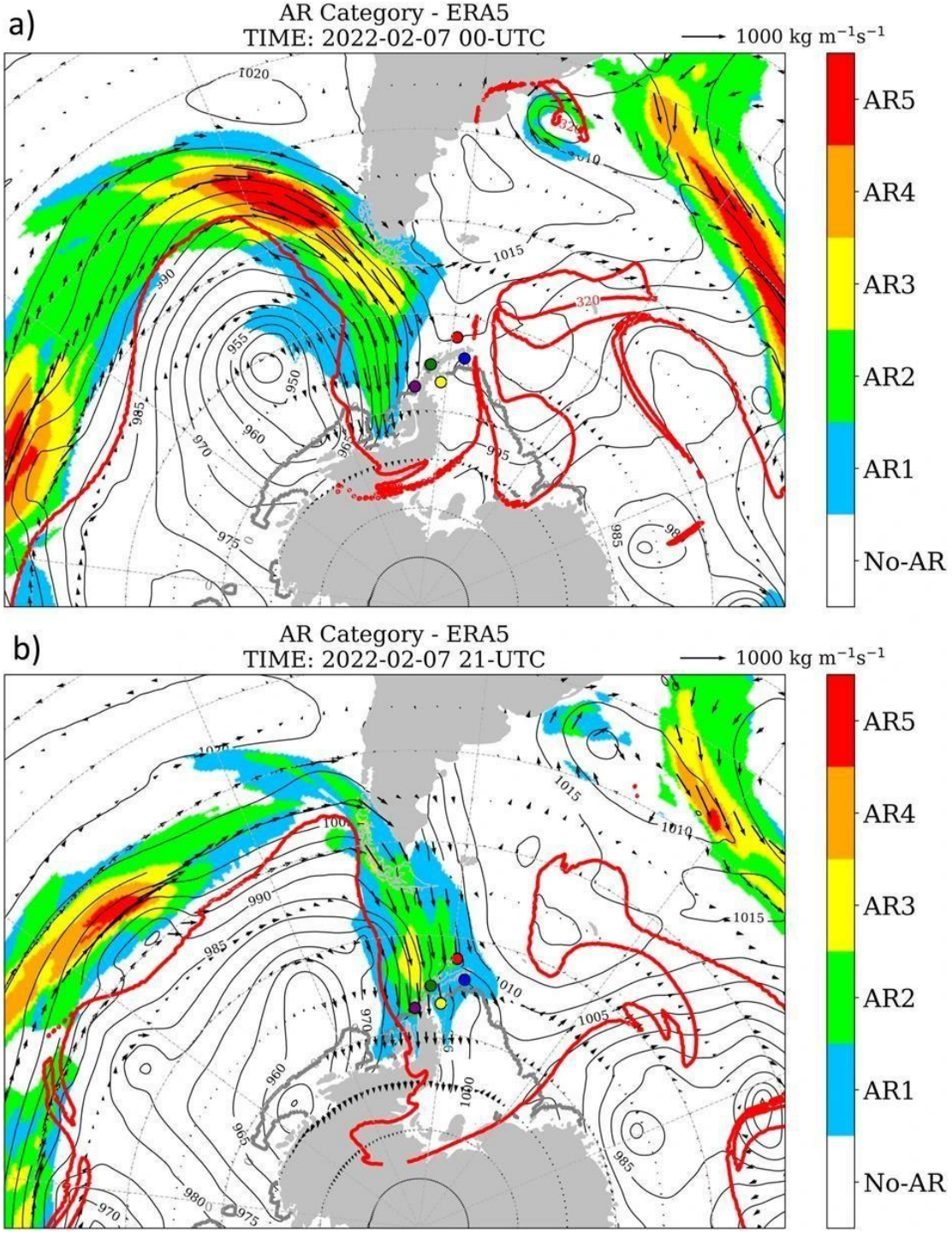


Figure 3 - An example of an atmospheric river, an extreme weather event linked to elevated surface temperatures and melt in the AP during February 2022 (Gorodetskaya et al., 2023).

AR behaviour and characteristics in both the present and future carry a lot of uncertainty. O'Brien et al. (2022) projected an increase in atmospheric river frequency, size, and number under strong radiative forcing, emphasising the need to address uncertainty in AR research for future climate studies.

In addition to transporting heat and moisture, ARs also facilitate the long-range transport of aerosols. This led Chakraborty et al. (2021) to expand the "atmospheric river" concept by developing a global algorithm for detecting aerosol atmospheric rivers (AARs).

Recently, Lapere et al. (2024) identified the presence of polar aerosol atmospheric rivers, highlighting their role in the polar aerosol budget.

1.4. Aerosols and Their Impacts

Aerosols are small liquid, solid or mixed particles suspended in the atmosphere, which interact with clouds and radiation, and can significantly affect warming in polar regions (Schmale et al., 2021). Their interactions introduce considerable uncertainty in global climate models, particularly for the Arctic and Antarctic (Szopa et al., 2021). Understanding the aerosol budget in polar regions is crucial, especially given the rapid transport of aerosols from mid-latitudes to the poles (Lapere et al., 2024)

While the sources of aerosols have changed in comparison with industrial and preindustrial times, the Southern Hemisphere has been identified as an area where pristine aerosols, those unaffected by human activity, are most likely to be localized (Hamilton et al., 2014). Aerosols of natural origin are primarily generated through complex emission processes by biological organisms, influenced by biogeochemical cycles, as well as through the production of sea spray (Mallet et al., 2023; Figure 4).

Additionally, long-range transport via ARs and air mass intrusions contribute to aerosol levels (Jumelet et al., 2020). Aerosols, including bioaerosols, have an important role in climate modulation, mainly as particles involved in precipitation processes and the formation of clouds (Kumari & Yadav, 2024).

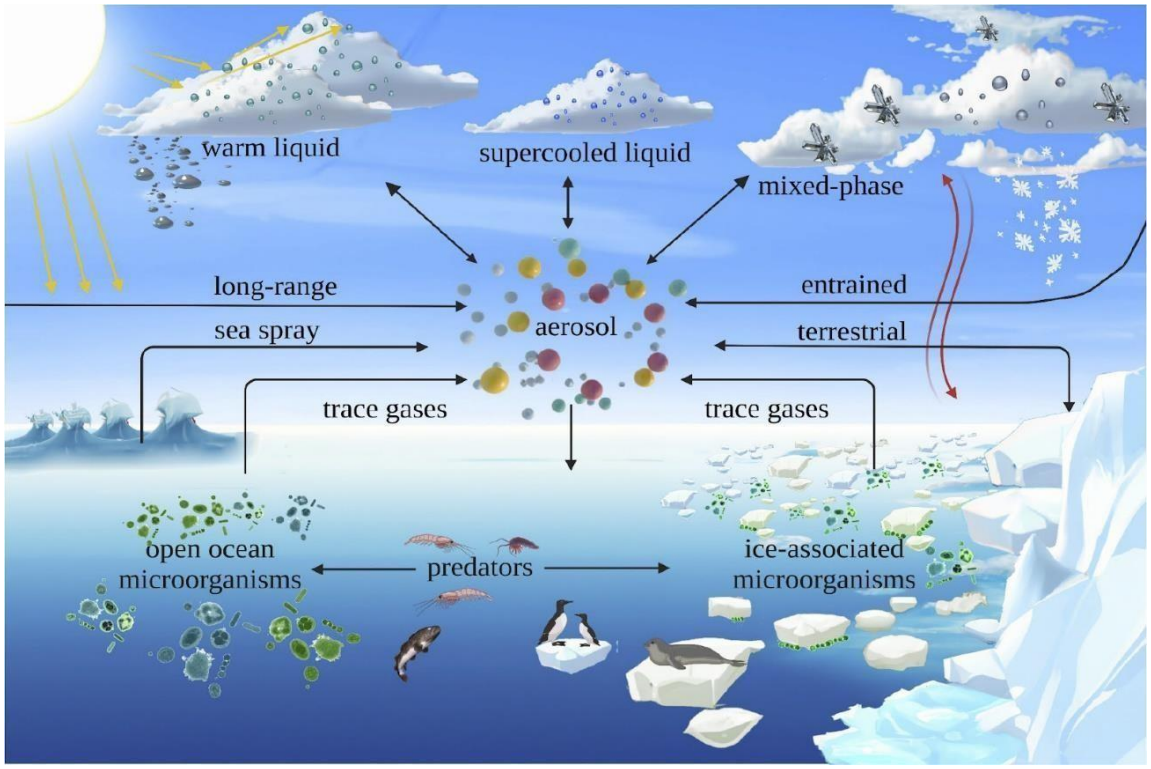


Figure 4 - Sources of biological aerosols in the Southern Ocean and Antarctica, and their interactions with cloud formation and atmospheric processes (Mallet et al., 2023).

1. 5. Clouds, INPs and CCNs

Clouds play a critical role in the global climate by regulating the temperature and moisture structure of the atmosphere, impacting the energy balance of the surface Earth

system (Quante, 2004). Wang (2010) defines the clouds in the atmosphere as “condensed forms of water (water droplets and ice particles) suspended in air”. Ice crystals in clouds play a role in cloud microphysical properties and are particularly important in precipitation formation, thereby affecting the global hydrological cycle and contributing to climate change.

The main classifications of clouds are based on their altitude level, physical forms and phase of water in the clouds, with the latter being most relevant to this topic. Clouds can be all liquid, all ice-phase, or mixed-phase clouds, constituted of both liquid and ice particles (Curry et al., 1996).

The initiation of ice formation in clouds, ice nucleation, occurs through two mechanisms: homogeneous and heterogeneous ice nucleation. Homogeneous ice nucleation happens in the absence of freezing catalysts, with water remaining supercooled at temperatures well below 0°C, freezing only at temperatures below −39°C. Heterogeneous ice nucleation is facilitated by freezing catalysts such as aerosol particles with ice-nucleating activity (INA), which are crucial for natural freezing processes (Morris et al., 2004). These particles with INA are capable of lowering the energy barrier of the phase transition and are called ice nucleating particles (INPs) (Kanji et al., 2017; Knopf et al., 2018). Moreover, cloud condensation nuclei (CCN) are needed to facilitate vapor condensation (gas to liquid phase transformation), and form clouds.

INPs originate from various sources, including desert dust, marine aerosols, industrial emissions, and biological aerosols (bioaerosols). Because bioaerosols can promote ice crystal formation in clouds at relatively warms temperatures, they represent a unique class of INPs (Huang et al., 2021). Notable bioaerosols include bacteria, pollen, fungal spores, algae, lichens, archaea, viruses, and plant or animal fragments or biological molecules—such as proteins, lipids and polysaccharides (Després et al., 2012; Szyrmer, & Zawadzki, 1997).

Bacteria have a high ice-nucleating potential, making them of special interest as INPs in the atmosphere. In addition to their role as INPs, bacteria, along with other compounds like organic acids, sugars, and polysaccharides, can serve as CCN, providing surfaces for water vapour to condense and thus facilitating cloud formation (Delort et al., 2010).

1.6. Ice nucleation ability of Bacteria

Bacterial ice nucleation was first discovered in cultures of *Pseudomonas syringae* Van Hall 1904, isolated from decaying leaves that could freeze at relatively high temperatures, ranging from -1.8°C to -3.8°C (Maki et al., 1974). Subsequent research identified additional species with high INA, such as *Erwinia herbicola* Löhnis 1911 and *P. fluorescens* Migula 1895.

Ice-nucleating bacteria are characterised by the presence of a single INA gene, which appears to confer ice nucleation activity (the Ina+ phenotype). The protein product of this gene was first identified in *P. syringae* (Figure 5), which has the *inaZ* gene. *P. fluorescens* possesses the *InaW* gene. Despite substantial differences between these genes, they share a common origin and exhibit a notable similarity: both have a central region with 120 contiguous repeats of an eight-codon motif (Warren, 1987). Fragments of *E. herbicola* cells have also identified to be capable of catalysing cell-free ice nucleation (Phelps et al., 1986; Deininger et al., 1988). A further study identified additional strains and genera with the ability to catalyse ice formation, such as *Stenotrophomonas* Palleroni and Bradbury 1993, yet the molecular base of the process is still not known (Failor et al., 2017).

Moreover, some *Pseudomonas* Migula 1894 strains can produce biosurfactants, found to be very important in lowering atmospheric critical supersaturations required for the activation of aerosols into CCNs, influencing cloud droplet size and increasing cloud lifetime and albedo (Ahern et al., 2007).

Interestingly, *P. syringae* was found to be an epiphyte commonly living on plant leaves. Lindow (1983) associated it with frost injury in numerous plant species, which could be a strategy by the plant pathogen to access nutrients within plants (Morris et al., 2004).

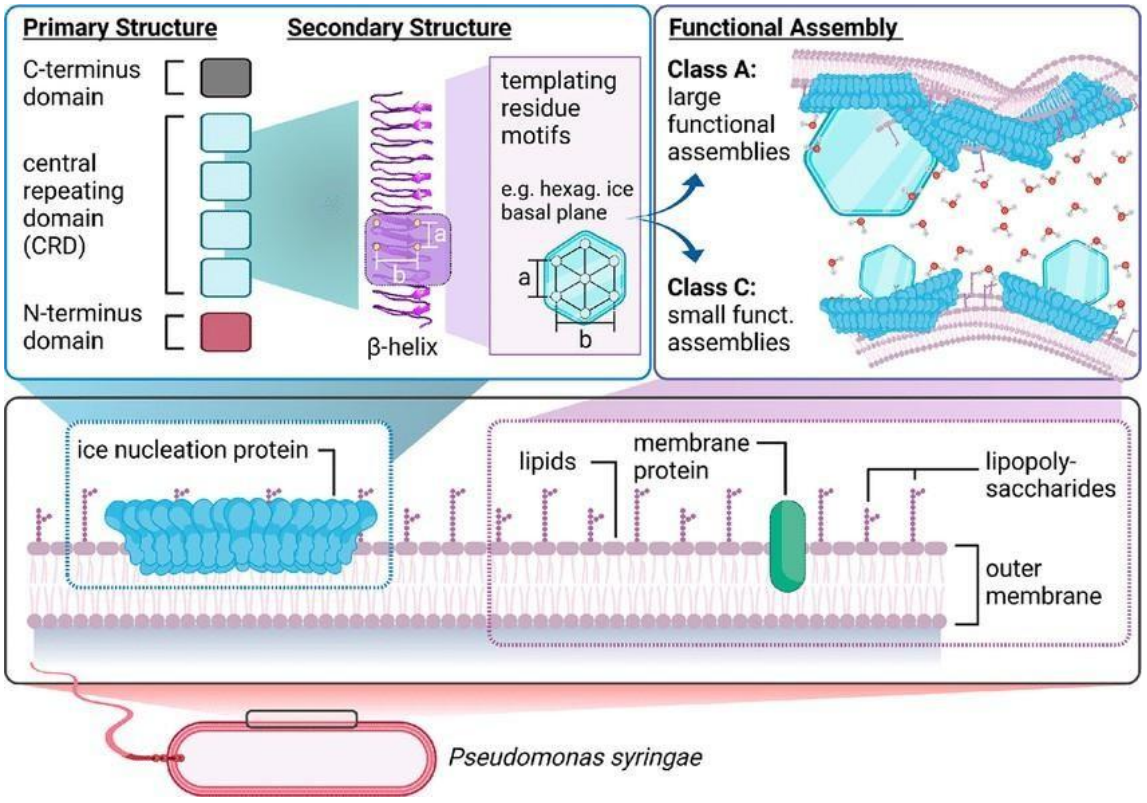


Figure 5 - Proposed structure and functional mechanism of ice-nucleation protein of *Pseudomonas syringae* (Lukas et al., 2022).

1.7. Long-distance transport and the Aeromicrobiome

The role of precipitation in the long-distance dissemination of plant pathogens and INA particles remains inadequately understood. Monteil et al. (2014) demonstrated that the presence of *Pseudomonas syringae* in precipitation is statistically associated with biological, chemical, and climatic parameters of both the precipitation water and the air mass carrying it, noting that *P. syringae* is favoured by warmer conditions. Additionally, they found that snowfall and rainfall differ in their ability to deposit *P. syringae*. Failor et al. (2017) observed that precipitation with higher concentrations of total INPs active at −8°C was linked to precipitation originating from air masses that travelled longer distances. Failor et al. (2017) hypothesize that the correlation between INP concentration and the distance travelled by air masses is due to the increased uptake of INP from Earth's surfaces through upward air fluxes.

Biological material is ubiquitous in the atmosphere and plays a significant role in influencing cloud properties. Antarctica is the most isolated continent on the planet, making it an ideal location for research on the airborne transport and survival of microorganisms (Pearce et al.,

2009). Moreover, studies suggest that microorganisms are present and metabolically active in clouds. However, organisms surviving in these conditions must possess extreme characteristics to endure such environments.

The existing literature on biological INPs and CCNs shows the necessity of interdisciplinary approaches, combining atmospheric and biological knowledge, in order to understand the transport and roles of bioaerosols in Antarctica in the light of climate and biodiversity interactions.

1.8. The Methodological approach and Aim of study

We employed microbial culturable methodologies on precipitation (rainfall and snowfall) surface snow after precipitation events, and glacier snow in order to isolate microbial strains, identify them and study their phylogeny. Subsequently, we explored the potential role of the identified microorganisms in ice nucleation and cloud condensation processes by reviewing existing literature on the topic.

In addition, the optimization of culture-independent methodologies for extracting environmental DNA (eDNA) from precipitation samples was performed for future microbial community analyses. Combining these approaches allows us to utilize the advantages of each method and is a common approach in studies of microbial diversity (Pontes et al., 2007).

This study aims to enhance our understanding of microbial and aerosol contributions to Antarctic climate dynamics, highlighting the interplay between biological and atmospheric processes. Such kind of studies are necessary to understand the complex interactions between the Earth's system and its biodiversity in the rapidly changing global climate.

2. Methodology

2.4. Site description

This study focuses on King George Island (KGI) (Figure 6), the largest of the South Shetland Islands located 120 km northwest of the AP. KGI covers an area of 1250 km2, having 90 per cent of the surface permanently glaciated (Simoes et al., 1999).

Some of the most significant fluctuations of climatic conditions in the southern hemisphere are affecting KGI (Kejna et al., 2013). The island's position in the Southern Ocean, at the northern entrance to Antarctica, makes it a remarkable locality for inspecting the land-ocean-atmospheric interactions and climatic changes of the region.

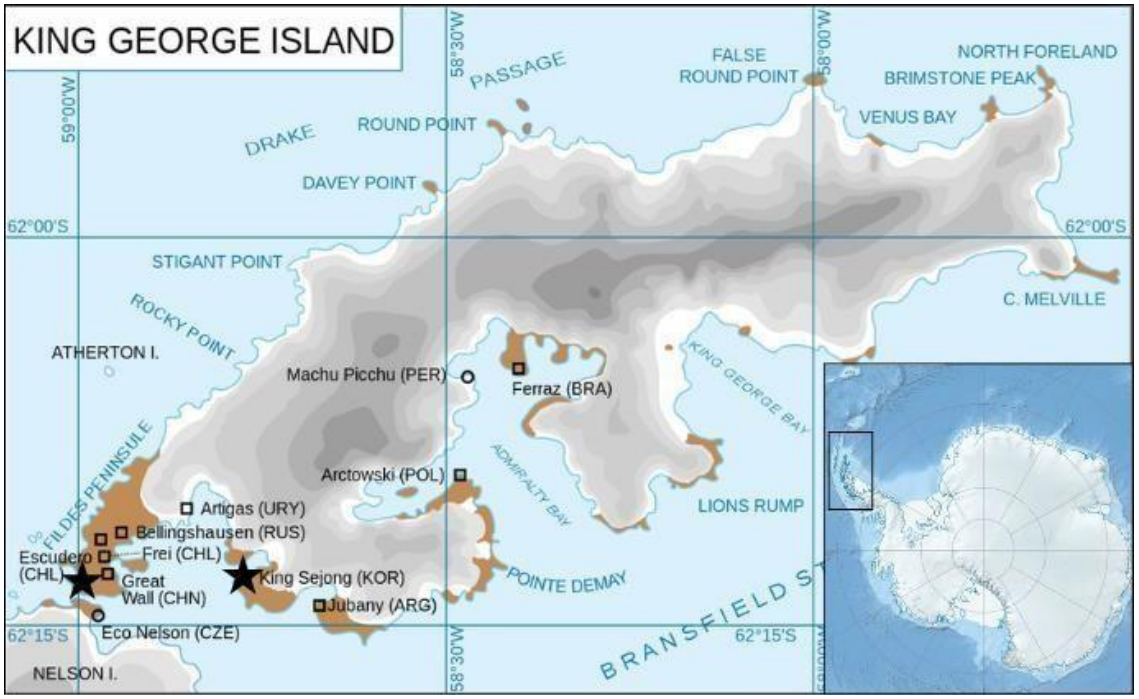


Figure 6 - King George Island, located at the northern tip of the Antarctic peninsula. The research stations where the sampling was performed are marked with a star symbol. Adapted from Giovanni Fattori, CC BYSA 3.0 http://creativecommons.org/licenses/by-sa/3.0/ , via Wikimedia Commons.

Two sampling campaigns in KGI were performed in the austral summer period, in February 2022 and February 2023 as part of the Portuguese Polar Program projects APMAR and APMAR2 (PI Irina Gorodetskaya, http://atlace.web.ua.pt/apmar_tulip/). The first sampling campaign in 2022 was performed in the region of Professor Julio Escudero Base (Figure 6), a Chilean Antarctic research station. The collection site in the 2023 sampling was at the Korean Antarctic research station King Sejong and its proximity. In both campaigns, the sampling was also performed on Bellingshausen Dome / Collins Glacier, which is located on the western side of KGI, near the Fildes Peninsula.

2.5. Sampling

2.5.1. Pre-sampling Sterilisation and Sample Handling

Prior to the onset of sample collection, measures of sterilisation were conducted to minimise contamination via the sampling material and cross-contamination between samples. Sterile gloves were used while handling the sampling equipment. The sampling tubes were washed using bleach or 90% alcohol. After the sampling, each sample was labelled with identification codes, including sampling locality, sampling start and end time, sample types and volumes. Field-trip log sheets were filled with sampling information after each collection.

2.5.2. Sampling for Culturable Methodologies

Precipitation samples were collected using plastic funnels attached to 15 ml and 50 ml vials (Figure 7). Rainfall samples were obtained during light precipitation events, with two 15

ml vials collected specifically for culturable methodologies. Additionally, three samples of snowfall were gathered during snowfall events. These samples were combined to reach a total volume of 50 ml, also for use in culturable methodologies. Each sample was collected in duplicate.

Surface snow samples were collected from Collins Glacier, these samples were collected by scooping the upper 2 cm of snow after a mixture of rainfall and snowfall in the previous days.



Figure 7 – Sampling setup. On the left: a setup used for direct collection of rainfall and snowfall; and on the right: collection of surface snow after precipitation events, ©Irina Gorodetskaya

The collection site in the 2023 campaign was the proximity of King Sejong station. Two surface snow samples were collected in the south of the station, directly following snowfall. These samples were collected by scooping the upper 2 cm of snow. In addition, glacier snow samples from Collins Glacier were collected from the surface and at several depths of a snow pit, reaching 80 cm depth. Four glacier snow samples were collected into 50 ml sterile vials.

2.5.3. Sampling for eDNA Optimization and Analysis

The samples of surface snow just after precipitation events for eDNA analysis were collected using 1-litre plastic bottles, maximising the sample volume. The sample collection was done from the top snow layer using sterilised equipment and gloves in clean zones, away from the station and animal activity to minimise contamination from local microbial communities. Collected thawed snow was filtered through 0.22 µm pore size filters using Sterivex cartridges. After filtration, the samples were preserved at -80°C. During transport to Portugal, the samples were kept at approximately +4°C.

eDNA rain samples were also collected at CIIMAR in December of 2023 in order to optimize eDNA isolation. During a strong precipitation event, rainfall was collected using 1-litre sterile plastic bottles. The collected precipitation was filtered through 0.22 µm pore size filters using Sterivex cartridges for eDNA extraction. After filtration, the samples were preserved at -80°C, for further eDNA extraction.

2.5.4. Post-sampling Handling and Transportation

After sampling, the samples were transported from Antarctica to Punta Arenas, Chile, as personal luggage in a frigobox. Upon arrival, they were stored in a refrigerator at +4ºC for

three days. The samples were then transported from Punta Arenas to Porto, Portugal, as checked luggage in a frigobox, with the journey lasting approximately 20 hours. Following their arrival to Porto, the samples were stored at +4ºC in a home refrigerator from March 4th until June 9th, a period of three months. Subsequently, the samples were transported to CIIMAR's microbiological laboratory.

2.6. Culture-dependent Analysis

Samples collected during precipitation events in February of 2022 were analysed in the microbiological laboratory, following a schema illustrated in Figure 8.

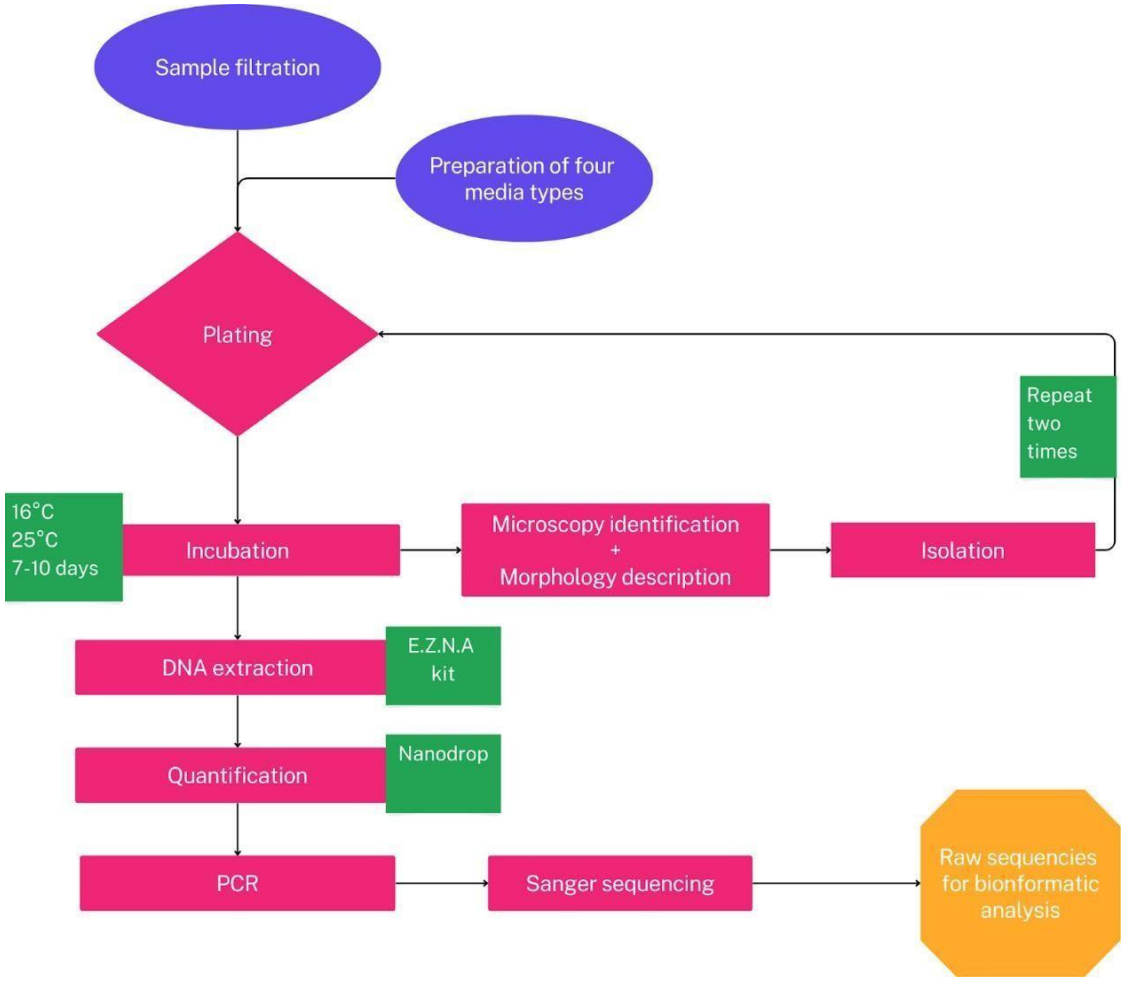


Figure 8 - A schematic illustration of culture-based methodology workflow

Four types of media were used: Plate Count Agar (PCA), PCA diluted 10 times, Reasoner's 2A agar (R2A) and Marine Agar diluted 10 times (MA10). PCA is a nonselective medium, adequate for the growth of the majority of bacteria. Agar is a gelatinous substance derived from seaweed that is used as a solidifying agent. To promote bacterial growth, this media also contains tryptone as a source of amino acids, vitamin factors from yeast extract, and glucose used as an energy source. PCA10 is composed of the same ingredients as Plate Count Agar, diluted 10x. MA10 is a medium containing all the nutrients necessary to cultivate the majority of marine bacteria, this medium was diluted 10x. It differs from PCA by using agarose as a solidifying agent, and by having additional salts and nutrients especially

designed for the growth of marine bacteria. Reasoner's 2A agar (R2A) is used to grow bacteria from potable water and environmental samples. It typically contains protease peptone, casamino acids, yeast extract, dextrose, soluble starch, dipotassium phosphate, magnesium sulphate, sodium pyruvate and agar. Using a variety of growth media ensures capturing the highest possible diversity strains using culturing methods.

For half of the Petri dishes, 10 ml of the sample was filtered using 0.22 µm filters and a peristaltic pump, concentrating the present bacteria. The filters were then transferred to the media plates. For the other half of Petri dishes, 100 µl of the sample was spread on the surface of the plates. For each sample, these steps were repeated on the four growth media types. The plates were incubated at 16°C and 25°C for approximately 7-10 days, or until maximum growth was observed. The species that did not exhibit growth at 25°C were transferred to 16°C. The temperatures were chosen as belonging to the optimal temperature range for the growth of obligative and facultative psychrophilic bacteria (Harder & Veldkamp, 1971). The grown cultures were transferred to +4°C for preservation.

The morphologies of the colonies were identified and described using a microscope, and each isolate was photographed (Figure 9). Obtaining information about the morphological characteristics is essential to eliminate potential contamination and to select the distinct strains for the next isolation and sequencing steps.

Distinct isolates were transferred to new plates containing the same media as initially used and incubated at the same temperatures for maximum growth. The process was repeated once again, to obtain a substantial biomass and ensure the pureness of colonies.

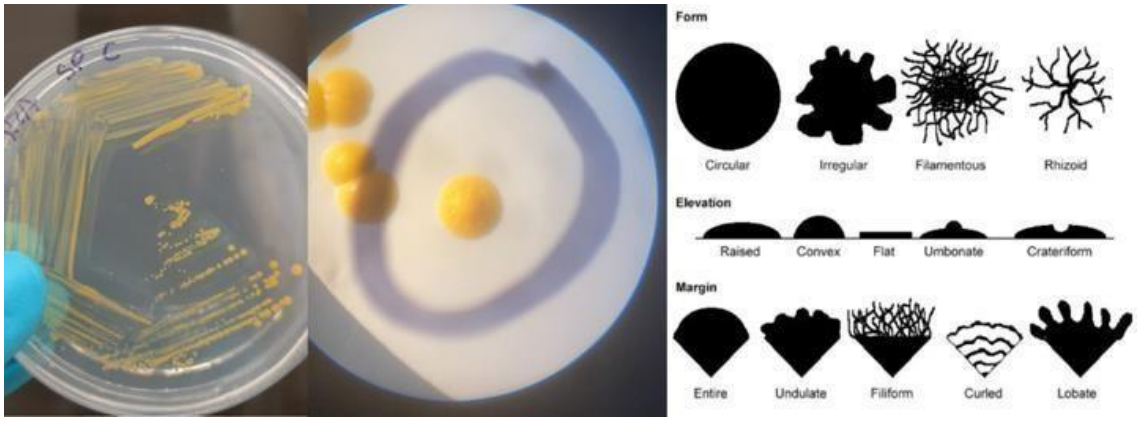


Figure 9 - Two bacterial colonies that were recovered from snowfall and glacier snow samples (Photo: K. Vučković) and described using a scheme of bacterial colony morphology

The total DNA of the bacterial isolates was extracted using the E.Z.N.A Bacterial DNA kit, which allows for the isolation of high-quality DNA from various gram-positive and gram-negative prokaryotes. It processes up to 1 x 10^9 cells per column using optimised lysis conditions. The cell wall is broken down by lysozyme and bead beating, followed by protease digestion. The sample is then applied to a HiBind DNA spin-column, where two quick wash steps remove contaminants. DNA was eluted in an Elution Buffer, ready for direct use in downstream applications.

DNA concentration was quantified using Nanodrop, with 2 µL of sample volume.

PCR amplification was performed with the use of “27F - AGA GTT TGA TCA TGG CTC A” forward and “1492R - TAC GGT TAC CTT GTT ACG ACT T” reverse primers targeting the 16S rRNA gene. Studying bacterial phylogeny and taxonomy has long been marked by the use of the 16S rRNA due to its presence in all bacteria with a highly conserved sequence

(Janda et al., 2007). For the PCR reaction mixture, we used one µl of DNA per isolate, five µl of DreamTaq polymerase, one µl of each primer, and two µl of DNAfree water.

To visualise the quality of the amplifications we utilised gel electrophoresis and BioRad imaging system with ImageLab software (Figure 10). For several samples, we observed streaks located in an unexpected position (most visible in M215 and M218 in the upper image in Figure 10). In situations where there is an abundance of template DNA in the PCR reaction, non-amplified high-molecular-weight DNA could cause unusual streaking. Therefore, the original samples were diluted 5 or 10 times, and the PCR was performed again.

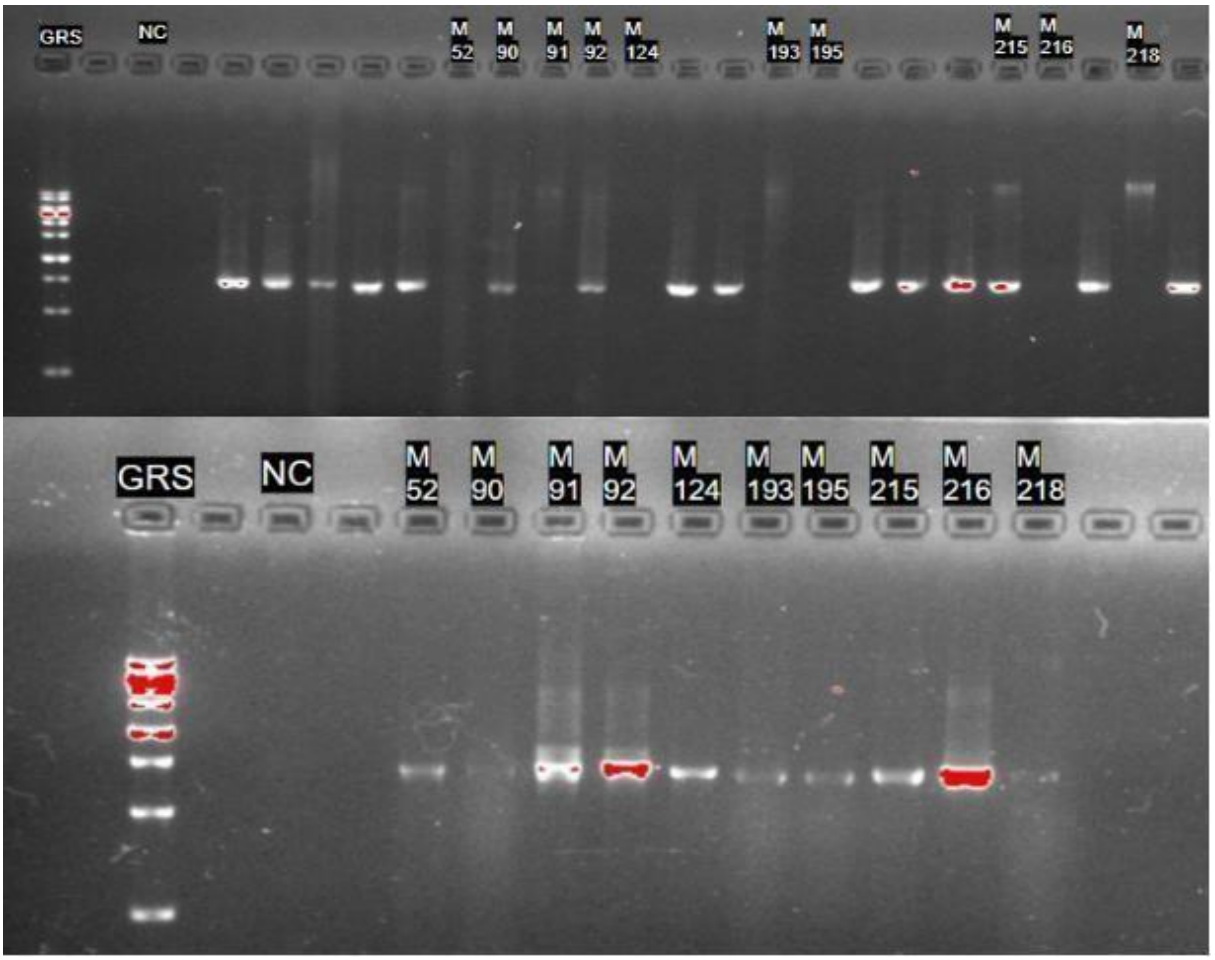


Figure 10 - Visualisation of the PCR products using gel electrophoresis. Upper image - First PCR reaction. Lower image - Repeated PCR reaction using 1:5 dilutions. GRS - Ladder. NC - negative control

Sanger sequencing of PCR products was done at the Institute for Research and Innovation in Health (I3S). The products were purified using Exo-Sap-IT. The ExoSAPIT Express reagent, which includes modified Exonuclease I and Shrimp Alkaline Phosphatase in a special buffer, efficiently removes excess primers and dNTPs from PCR mixtures. It is a quicker and more precise alternative to traditional methods like spin columns or beads and does not interfere with downstream applications (thermofisher.com/exosapit).

The sequencing reaction was prepared using The BigDye™ Terminator v3.1 Cycle Sequencing Kit. Purification sequence reaction using Sephadex G50-fine. Capillary electrophoresis was used for reading the sequences, later analysed using Sequencing Analysis software version 7 (Applied Biosystems).

2.7. Culture-independent Analysis

2.7.1. Optimization of eDNA Extraction

To maximise the extraction of eDNA from precipitation samples, several extraction kits and different DNA concentrations were tested on rainfall samples collected at CIIMAR in December of 2023. The eDNA was extracted using DNeasy PowerWater Kit, DNeasy PowerSoil Pro Kit and DNeasy PowerSoil Pro Kit protocol with no cleaning steps. The concentration was established using Qubit™ dsDNA HS Assay Kit, with sample volumes of 10µl and 15µl.

PCR amplification was performed with the use of primers Parada et al. (2016) utilized for targeting 16rDNA genes of marine microbe communities. For the PCR reaction working solution, we used 2 µl of DNA per isolate, five µl of Bioline polymerase, 0.5 µl of each primer, and two µl of DNA-free water. The main advantage of Bioline polymerase is increased affinity for DNA, thereby improving amplification in cases with limited amounts of template DNA.

To visualise the quality of the amplifications, the PCR products were run in gel electrophoresis and visualized the results in the BioRad Imaging System, with ImageLab software version 4.0.

Secondly, the optimally performing kits were employed for DNA extraction of precipitation samples collected in King George Island in 2022 and 2024. The chosen protocol was optimised by reducing the elution buffer volume and repeating the elution step to concentrate the sample. The concentration was again established using Qubit™ dsDNA HS Assay Kit, this time with sample volumes of 20µl.

2.8. Bioinformatic Analysis

After obtaining the raw sequences starting from the forward and reverse primer, they were analysed using Geneious Prime software, version 2024.0.4. The first step is to check the quality index of the sequences; only sequences with above 50% high quality (HQ) index were analysed. The reverse primer sequence was transferred to the same orientation as the forward primer sequence using the *Reverse Complement* tool. Forward and reverse sequences of each isolate were used to form consensus sequences, which were then trimmed (Align/Assemble -> De Novo Assemble ->Trim before Assembly -> Trim sequences -> Options: ✓ Annotate new trimmed regions).

After forming a consensus sequence, the sequences were scanned for mismatches between the forward and reverse reads. In places where we observed gaps, mismatches or unidentified nucleotide bases, we altered the bases by comparing them with the consensus sequence of higher quality. Finally, the processed consensus sequences were extracted to a separate folder, ready for further analysis.

The consensus sequences were compared using NCBI's BLAST - The Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This program determines the statistical

significance of matches between nucleotide or protein sequences and sequence databases. The sequences were compared to a specifically curated Bacteria and Archaea: 16S ribosomal RNA database, which is a part of the NCBI RefSeq Targeted Loci Project (McVeigh, 2024). This dataset contains curated 16S ribosomal RNA gene sequences that correspond to bacteria and archaea-type sequences.

For more precise identification, the sequences were also compared to EzBioCloud databases (https://www.ezbiocloud.net/identify). While BLASTN gives identity value which is match/total length, EzBioCloud gives similarity value which is match / (match + mismatch). By comparing the results from these databases, the isolates were identified to the genus or species level. A threshold of 98.65 % 16S rRNA gene sequence similarity was used as the threshold for differentiating two species (Kim et al., 2014). Strains that showed less than the threshold of pairwise similarity were considered as possible new species.

Visualisation of the percentage of representation of each bacterial phyla and genera across all samples was done using R packages (R Core Team, 2024). The chosen chart for representation was a pie chart with an excluded centre, known as a “doughnut pie chart”. The library used was “ggplot2” and “dplyr”.

Moreover, the genus diversity in distinct sample types was represented with a stacked column chart. In addition to the “ggplot2” library (Wickham, 2016), the “tidyr” (Wickham et al., 2024) and “cowplot” (Wilke, 2024) packages were employed (see Appendix).

2.9. Phylogenetic Analysis

In order to gain a deeper insight into the phylogenetic relationship of the identified microbial strains, the consensus sequences were compared to the NCBI databases once again. For each of the strains, three of the sequences from the database that showed the highest nucleotide similarity to the consensus sequences were downloaded in .fasta format, and imported into Geneious Prime 2024.0.4. (https://www.geneious.com/). The sequences from the strains and their according sequences from the NCBI database were aligned using MUSCLE alignment. The alignment was exported in Mega format and imported into MEGA software, version 11.0.13, for phylogenetic analysis (Tamura et al., 2007).

Using the *Find Best DNA model* tool and employing the default settings, we tested the optimal statistical method for our dataset. Using the *Construct/Test Maximum-Likelihood tree* tool, we set the model to the ones obtained in the previous step, keeping in mind the rates among sites and numbers of discrete gamma categories. The phylogeny test was done using the Bootstrap method with 1000 or 2000 bootstraps.

In order to visualise the produced trees in MEGA 11.0.13, we employed different software. IToL (https://itol.embl.de/) was used for a clearer representation of the identified genera, using various colours for marking the branches.

A phylogenetic tree (Newick) viewer (http://etetoolkit.org/treeview/) was employed to explore the different branch lengths. The result was a total of three trees.

The bioinformatic and phylogenetic analysis workflow is presented in Figure 11.



Figure 11 - A schematic illustration of bioinformatic and phylogenetic analysis workflow

3. Results

3.4. Taxonomy and Biodiversity

A total of 28 bacterial isolates were identified from precipitation and surface snow samples in the campaign in 2022 (M22_X sample IDs in Table 1). Additionally, 20 bacterial isolates were found in surface snow and glacier samples collected in 2023 (M23_X sample IDs in Table 1). Additional information about sample ID can be found in Table 5 (see Appendix).

Growth was obtained for both temperatures tested, and all four media tested presented microbial growth. While 15 strains were identified at the species level, the other 33 were identified until the genus level.

Table 1 - Identification of bacterial strains using 16s rRNA sequence databases. RP - rain precipitation, SP - snow precipitation, SS - surface snow. GS - glacier snow. Temperatures and growth media for each strain are shown in the columns on the right.

Sample ID	Identification	Sample type/ID	T(°C)	Media
M22_01	*Stenotrophomonas rhizophila* Wolf et al. 2002	RP/1	16	PCA
M22_02	*Stenotrophomonas rhizophila Wolf et al. 2002*	RP/1	16	PCA10
M22_03	*Stenotrophomonas rhizophila Wolf et al. 2002*	RP/1	25	PCA10
M22_04	*Pseudomonas sp. Migula 1894*	RP/1	16	PCA
M22_05	*Stenotrophomonas sp. Palleroni and Bradbury 1993*	RP/1	25	MA10
M22_07	*Paenibacillus sp. Ash et al. 1994*	RP/2	16	R2A
M22_09	*Spirosoma sp. Migula 1894*	RP/2	16	PCA10
M22_11	*Spirosoma sp. Migula 1894*	RP/2	16	MA10
M22_12	*Stenotrophomonas rhizophila* Wolf et al. 2002	RP/2	25	R2A
M22_14	*Paenibacillus sp. Ash et al. 1994*	SP/1	16	PCA10
M22_16	*Mucilaginibacter glaciei Yang et al. 2021*	SP/1	16	R2A
M22_17	*Sphingomonas glacialis Zhang et al. 2011*	SP/1	16	R2A
M22_19	*Mucilaginibacter sp. Pankratov et al. 2007*	SP/1	16	R2A
M22_20	*Sphingomonas glacialis Zhang et al. 2011*	SP/1	16	MA10
M22_25	*Paenibacillus sp. Ash et al. 1994*	SS/1.1	25	PCA10

M22_27	*Sphingomonas sp. Yabuuchi et al. 1990*	SS/1.1	16	R2A
M22_30	*Paenibacillus sp. Ash et al. 1994*	SS/1.1	25	R2A
M22_31	*Mucilaginibacter glaciei Yang et al. 2021*	SS/1.1	25	R2A
M22_34	*Sphingomonas faeni Busse et al. 2003*	SS/1.1	25	PCA10
M22_42	*Sphingomonas faeni Busse et al. 2003*	SS/1.1	25	R2A
M22_43	*Sphingomonas sp.* Yabuuchi et al. 1990	SS/1.1	16	PCA10
M22_44	*Sphingomonas sp.* Yabuuchi et al. 1990	SS/1.2	25	R2A
M22_47	*Sphingomonas sp.* Yabuuchi et al. 1990	SS/1.2	16	PCA
M22_49	*Sphingomonas sp.* Yabuuchi et al. 1990	SS/1.2	25	R2A
M22_53	*Sphingomonas sp.* Yabuuchi et al. 1990	SS/1.2	25	R2A
M22_55	*Sphingomonas sp.* Yabuuchi et al. 1990	SS/1.2	25	PCA10
M22_56	*Sphingomonas sp.* Yabuuchi et al. 1990.	SS/1.2	16	MA10
M22_57	*Sphingomonas sp.* Yabuuchi et al. 1990	SS/1.2	25	PCA10
M23_08	*Acinetobacter sp. Brisou and Prévot 1954*	SS/1.1	25	R2A
M23_15	*Acinetobacter sp. Brisou and Prévot 1954*	GS/1	25	R2A
M23_34	*Mesorhizobium sp. Jarvis et al. 1997*	GS/1	25	R2A
M23_35	*Pseudomonas yamanorum Arnau et al. 2015*	GS/1	25	R2A
M23_52	*Pseudomonas yamanorum Arnau et al. 2015*	GS/3	25	MA10
M23_90	*Pseudomonas sp. Migula 1894*	GS/4	25	MA10
M23_92	*Stenotrophomonas sp. Palleroni and Bradbury 1993*	SS/2.1	25	R2A
M23_124	*Kocuria sp. Stackebrandt et al. 1995*	SS/2.1	25	MA10
M23_190	*Acinetobacter johnsonii Bouvet and Grimont 1986*	SS/2.2	25	MA10

M23_192	*Pseudomonas sp. Ash et al. 1994*	SS/2.2	25	MA10
M23_193	*Sphingomonas sp. Yabuuchi et al. 1990*	SS/2.2	25	R2A
M23_195	*Sphingomonas sp. Yabuuchi et al. 1990*	SS/2.2	25	R2A
M23_203	*Acinetobacter johnsonii Bouvet and Grimont 1986*	SS/2.3	25	MA10
M23_205	*Bacillus sp. Cohn 1872*	SS/2.3	25	MA10
M23_206	*Paenisporosarcina sp. Krishnamurthi et al. 2009*	SS/2.3	25	R2A
M23_215	*Kocuria sp. . Stackebrandt et al. 1995*	SS/2.3	25	MA10
M23_216	*Acinetobacter sp. Brisou and Prévot 1954*	SS/2.3	25	R2A
M23_217	*Kocuria sp. . Stackebrandt et al. 1995*	SS/2.3	25	R2A
M23_218	*Kocuria sp. Stackebrandt et al. 1995*	SS/2.3	25	R2A
M23_219	*Acinetobacter johnsonii Bouvet and Grimont 1986*	SS/2.3	25	R2A

The identified bacteria strains were associated with 11 different genera and 11 different families, indicating a high level of cultured biodiversity in the samples.

Pseudomonas, a bacterial genus well known for its ice nucleation activity, was identified in rain precipitation samples, surface snow, and glacier snow samples (Table 1).

The strains which were identified to genus level showed high levels of percentage identity (above 99%) with two or more organisms compared in the database, therefore the species of the strain could not be determined. In other cases, the strains showed less than 98.65% similarity - the threshold for differentiating two species by 16S rRNA gene sequences. Four of the strains fall into this category and are indicated in Table 2. They were identified as potentially new species belonging to *Spirosoma* and *Paenibacillus* genera. The strains were grown at 16°C, which is considered the optimal temperature for psychrotolerant bacteria (Table 1).

Strain	ID NCBI	Query Cover	Similarity	EzBioCloud	Query Cover	Similarity
MAC 22_07	*Paenibacillus paridis /endophyticus*	97/ 100	99,06/98,30	*Paenibacillus paridis/ sinopodophylli*	99,5/100	98,58/97,81
MAC 22_09	*Spirosoma spitsbergense/ luteum*	93/98	97,06/96,59	*Spirosoma spitsbergense*	100	97,38
MAC 22_11	*Spirosoma spitsbergense /luteum*	94/98	97,06/96,60	*Spirosoma spitsbergense*	100	97,31
MA 22_14	*Paenibacillus paridis/endophyticus*	97/ 100	99,06/98,30	*Paenibacillus paridis*	99,5	98,58

Table 2 - Description of the analysed strains with the 16S rDNA sequence similarity below 98.65%

Table 3 indicates a taxonomic lineage obtained with the NCBI Taxonomy Database, containing a curated classification and nomenclature for all of the organisms in the public sequence databases. The data was used for inspecting the biodiversity of samples in class and phylum level.

Table 3 - Lineage of the strains identified in samples collected in 2022 and 2023, according to the Taxonomy Browser of NCBI

Genus	Family	Order	Class	Phylum
Pseudomonas	Pseudomonadaceae	Pseudomonadales	Gammaproteobacteria	Pseudomonadota
Paenibacillus	Paenibacillaceae	Bacillales	Bacilli	Bacillota
Sphingomonas	Sphingomonadaceae	Sphingomonadales	Alphaproteobacteria	Pseudomonadota
Mucilaginibacter	Sphingobacteriaceae	Sphingobacteriales	Sphingobacteriia	Bacteroidota
Spirosoma	Cytophagaceae	Cytophagales	Cytophagia	Bacteroidota
Stenotrophomonas	Lysobacteraceae	Lysobacterales	Gammaproteobacteria	*Pseudomonadota*
Mesorhizobium	*Phyllobacteriaceae*	*Hyphomicrobiales*	*Alphaproteobacteria*	*Pseudomonadota*
Kocuria	*Micrococcaceae*	*Micrococcales*	*Actinomycetes*	*Actinomycetota*
Acinetobacter	*Moraxellaceae*	*Moraxellales*	*Gammaproteobacteria*	*Pseudomonadota*
Paenisporosarcina	*Planococcaceae*	*Bacillales*	*Bacilli*	*Bacillota*
Bacillus	*Bacillaceae*	*Bacillales*	*Bacilli*	*Bacillota*

The phylum diversity is illustrated through a comparative analysis of two pie charts generated using R Studio (Figure 12), titled "Phylum Diversity 2022" and "Phylum Diversity 2023." These charts depict the phylum diversity across all sample types for the respective years. The most prominent observation is the dominance of *Pseudomonas* (previously classified as Proteobacteria). Additionally, Bacillota were identified in both years, with a slightly higher percentage observed in 2022. Bacteroidota (formerly Bacteroidetes) were detected in the 2022 samples but were absent in 2023. Actinomycetota, not present in 2022, emerged in the 2023 samples. This phylum is related to strains identified as *Kocuria* sp. (Table 1).

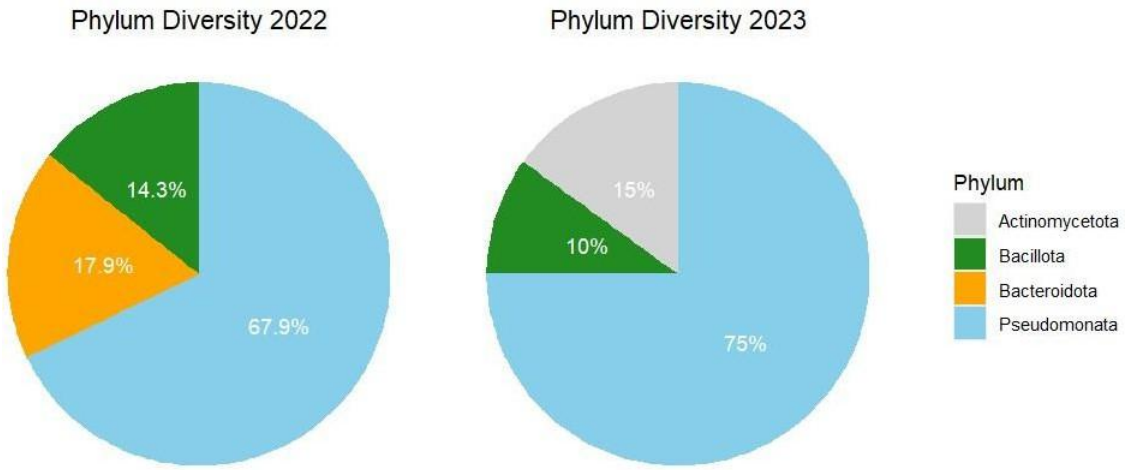


Figure 12 – The percentage of representation of each bacterial phyla across all samples in 2022 and 2023

In Figure 13, two pie charts are showcasing the class diversity among the samples in the two years respectively.

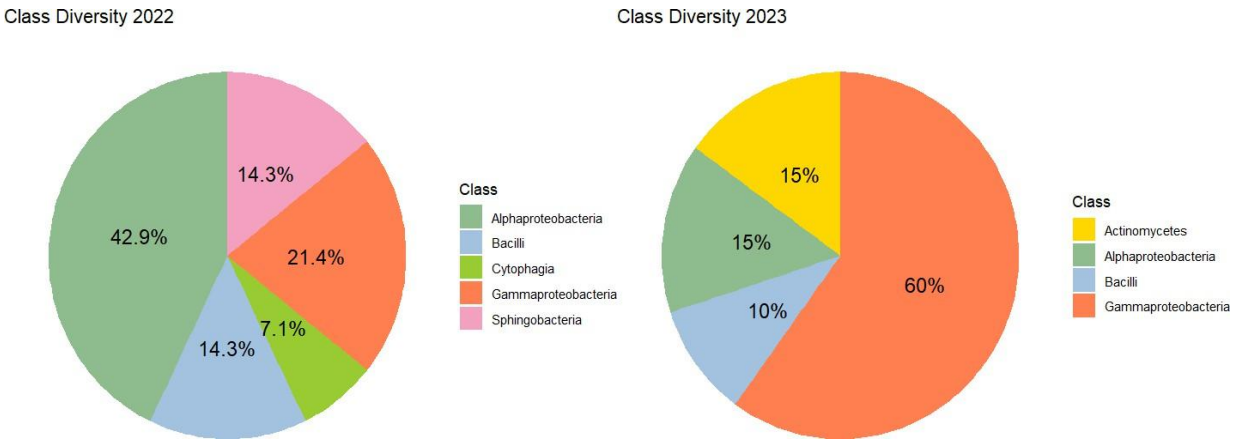


Figure 13 - The percentage of representation of each bacterial class across all samples in 2022 and 2023.

The class-level taxonomic diversity in the 2022 samples is predominantly represented by Alphaproteobacteria, constituting the majority of the microbial isolates. Gammaproteobacteria account for approximately one-fifth of this diversity. Other notable classes include Bacilli, Sphingobacteriia, and Cytophagia, contributing to the culturable microbial composition recovered in less significant proportions (Figure 13).

While inspecting the second chart, a clear dominance of Gammaproteobacteria is observed, followed by Alphaproteobacteria and Actinomycetes. Therefore, the two main classes in the two years are reversed in their relative abundance, even keeping in mind that the same media were used (Figure 3). However, the sample types analysed in the two years are different, hence observing differences was expected. Bacilli are also present in 2023, yet with a smaller representation compared to 2022.

By comparing sample types from 2022 on the genus level, a notable difference in biodiversity can be observed (Figure 14). The majority of strains identified in rain precipitation belong to the genus *Stenotrophomonas*, a group of Gammaproteobacteria previously recognized for their ice-nucleating potential. These bacteria were not found in snowfall or surface snow samples. Moreover, the only identified *Pseudomonas* strain was found in rain precipitation.

Snow precipitation differs from the other two samples of 2022 by the presence of *Mucilaginibacter* strains. Besides having characteristic species (Table 1), both rainfall and snowfall exhibited the presence of *Paenibacillus* (Figure 14).

On the other hand, surface snow of 2022 exhibits a largely reduced cultured biodiversity. Only one genera was identified, belonging to *Sphingomonas sp*. (Figure 14). Growth of *Sphingomonas sp*. was observed at 16 °C, on all three media apart from MA10 media.

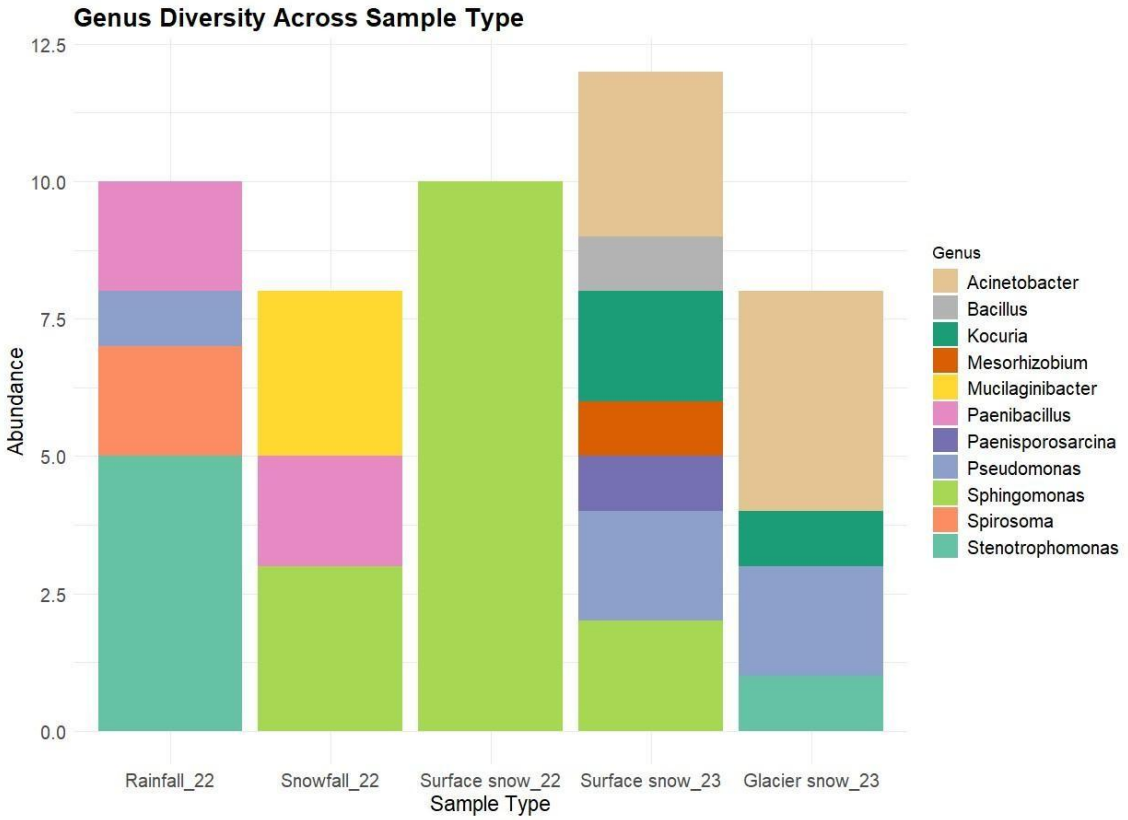


Figure 14 - Genus diversity identified in rainfall, snowfall, surface snow and glacier snow samples from 2022 and 2023

The genus level diversity of surface snow and glacier snow from the year 2023 is also illustrated in Figure 14. The surface snow sample exhibited a notable culturable biodiversity,

with seven recovered genera. Glacier snow samples show a reduced culturable biodiversity, having four genera identified. Three out of four genera identified in glacier snow were present in surface snow as well. The only genera present in glacier snow but not found in surface snowfall is *Stenotrophomonas*. *Sphingomonas* sp. Was identified in surface snow samples from both 2022 and 2023. Notably, *Pseudomonas* strains were identified in rainfall of 2022, and surface and glacier snow samples of 2023.

Although 16s rRNA databases are useful for the identifying stains by comparing them to the reference sequences, it does not allow for inferring information on the phylogenetic relationships between the cultured stains themselves. Therefore, the following chapter will focus on the phylogenetic relationships of the identified strains, as well as comparing them with closely related 16S rRNA sequences from the database.

3.5. Phylogenetic Analysis

Three phylogenetic trees were obtained for the different groups of samples analysed, using the Maximum-Likelihood statistical method in MEGA 11.0.13 (Figures 15, 16, and 17).

The first phylogenetic tree, constructed using the Maximum-Likelihood method, represents the bacteria identified in rainfall and snowfall precipitation samples collected in 2022 (Figure 15). The best-fitting model for this dataset was the Hasegawa-KishinoYano (HKY) model (Hasegawa et al., 1985) with gamma-distributed rate variation among sites (G) and a proportion of invariable sites (I), with the number of bootstrap iterations set to 1000. The tree includes rain and snow samples, with *Bremerella alba* chosen as outgroup due to their similar phylogenetic distance from all observed strains. *Bremerella alba* stands out as the most distant species compared to all strains.

Two major branches are observed in the tree, with the first branch consisting of strains belonging to the *Paenibacillus* genera. Notably, inside of this clade the *Paenibacillus* species form two distinct sub-branches according to the sample type. MAC22_30 and MAC 22_25, recovered from snowfall samples, are positioned next to *Paenibacillus tundrae,* whereas MAC22_14 and MAC22_07, isolated from rainfall, are positioned next to *Paenibacillus paridis.* This separation is possibly indicating the selectivity of bacterial species among the two different environments, discussed in further chapters.

The second clade consists of the remaining five genera identified. *Mucilianginibacter* and *Spirosoma* strains identified in our samples are more closely related, as opposed to *Spinhgomonas*, *Pseudomonas* and *Stenotrophomonas* forming a separate branch. MAC22_31 and MAC22_16, recovered from snowfall, are exhibiting a phylogenetic closeness to *Mucilaginibacter glacei.* Among the *Spinhgomonas* strains, MAC22_27 is positioned between *Sphingomonas faeni* and *Sphingomonas aurantiaca*, while MAC22_20 and MAC22_17 form a separate branch, most phylogenetically close to *Sphingomonas glacialis.* All three strains were recovered from snowfall samples.

The only *Pseudomonas* strain recovered in precipitation samples collected in 2022 was MAC22_04, exhibiting a close relation to *Pseudomonas versuta.*

Inside the *Stenotrophomonas* branch, we observe a notable differentiation between the identified strains. However, all the strains identified in this branch are the most closely related

to *Stenotrophomonas nematodicola,* contrasting with the results from the NCBI analysis (Table 1).

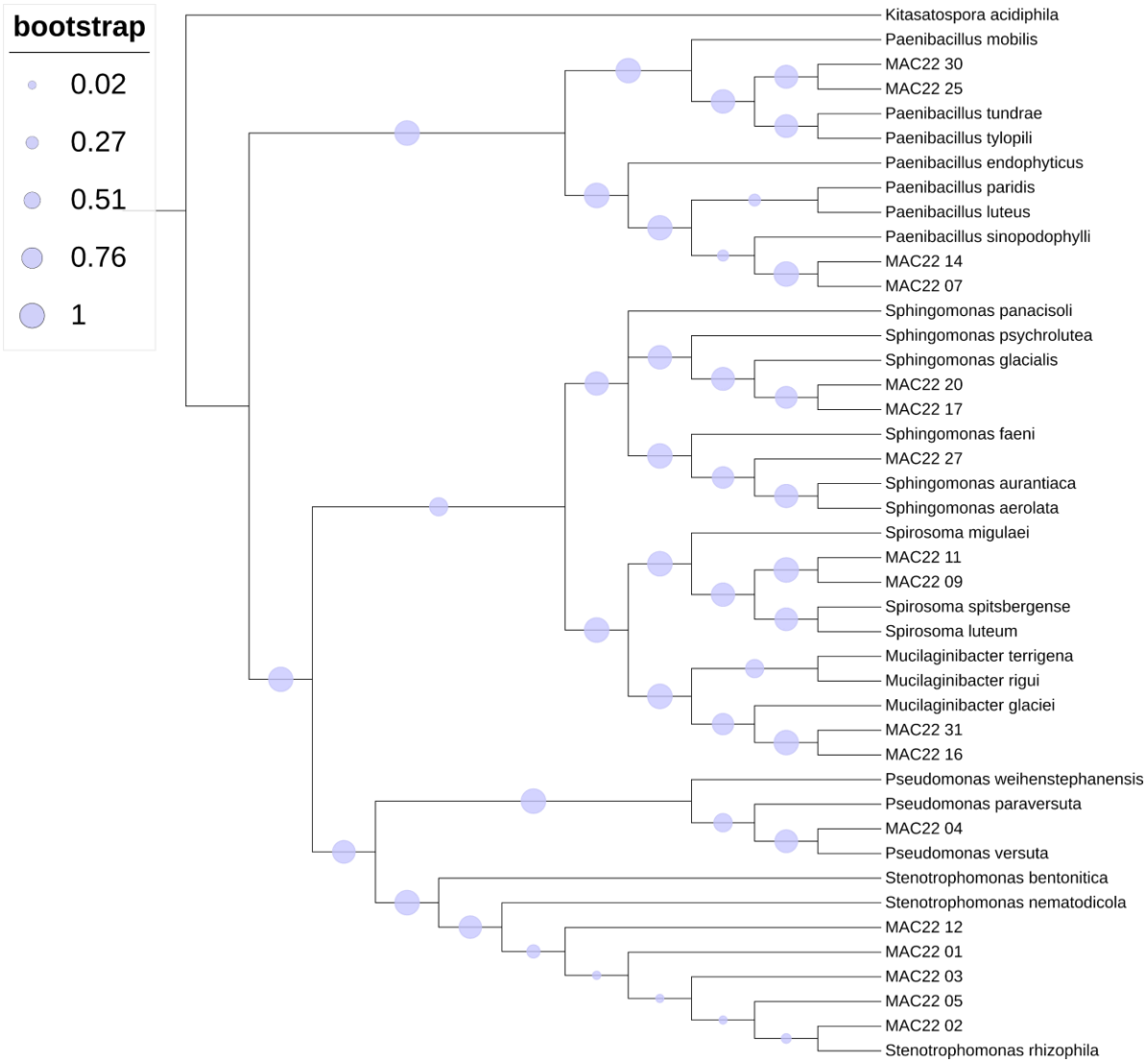


Figure 15 - Phylogenetic tree for 2022 rainfall and snowfall precipitation samples, using MaximumLikelihood methodology

An additional phylogenetic analysis was performed on four 2022 strains with less than 98.65% base pair similarity to known 16S rRNAgene sequences databases. These strains are indicated as possibly novel species (Table 2) originating from rainfall samples.

E. coli, a commonly used outgroup in microbiological analysis, was used in this phylogenetic tree (Figure 16). The Maximum-Likelihood statistical method that exhibited the best performance was the TN93 parameter substitution model (Tamura & Nei, 1993), and the phylogenetic analysis was performed with 1000 bootstrap iterations.

Two main branches were formed with the isolated strains 16S rRNA gene sequences. MAC22_09 and MAC22_11 are most closely related to *Spirosoma spitbergense* and *Spirosoma luteum*. *Spirosoma migulaei* exhibits a more distant phylogenetic relationship to the strains, most closely positioned next to the outgroup. MAC22_07 and MAC22_14 are seen in a second branch, exhibiting a close phylogenetic relation with *Paenibacillus paridis.*

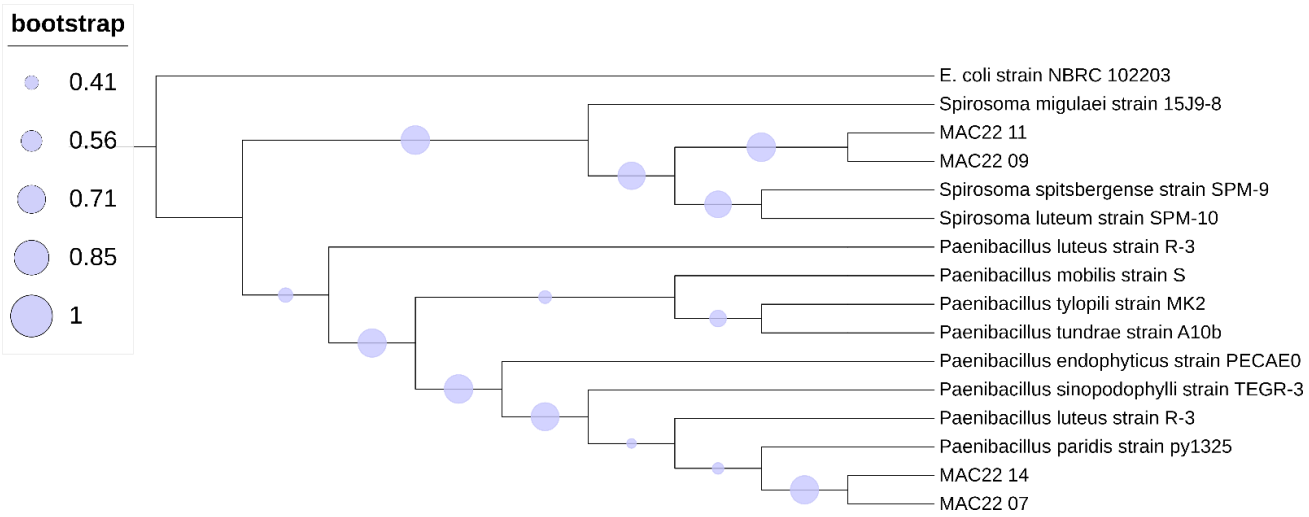


Figure 16 - Phylogenetic tree analysis of possibly new species recovered from 2022 samples, using Maximum-Likelihood methodology.

The phylogenetic relationships between microorganism groups identified in surface snow samples from 2022 and 2023 and glacier snow samples from 2023 were analysed collectively. This approach, considering the presence of some shared taxa across both sample types, provides more valuable insights into their phylogenetic relatedness.



Figure 17 - Phylogenetic tree analysis of surface and glacier snow strains recovered from surface snow samples from 2022 and 2023, and glacier snow samples from 2023, using Maximum-Likelihood methodology.

The Maximum-Likelihood statistical method that exhibited the best performance was the TN93 + G model (Figure 17). The bootstrap test utilised with 2000 iterations due to a larger dataset. *Bremerella alba* was designated as the outgroup.

The phylogenetic tree reveals two primary branches. The first branch holds *Paneisporacina*, *Kocuria*, and *Bacillus* species. Within this branch, strains MAC23_124 from glacier snow is positioned together with MAC23_218, from surface snow, whereas MAC23_215, isolated from surface snow, is located on a different branch. The closest related species to MAC23_215 is *Kocuria rhizophila*. MAC23_206 appears to be most closely related to *Paenisporacina antarctica*, contrasting with the database analysis, which identified *Paenisporacina indica* as the most similar species to MAC23_206. MAC23_205 is positioned on the same branch as *Bacillus safensis*.

The second major branch of the phylogenetic tree further subdivides into two smaller branches. The first sub-branch includes *Sphingomonas* and *Mesorhizobium* species. The total of strains from surface snow collected in 2022 correspond to *Sphingomonas* genus, indicating a significantly lower culturable biodiversity in comparison with all the other samples. Nonetheless, the sub-branch encompassing *Spinhomonas* species is divided into smaller minor sub-branches, revealing a genetic variability among the observed strains. MAC22_44 is positioned on a separate branch, most closely related to *Sphingomonas faeni,* commonly extracted from surface snow. In Table 1, it is noted that these strains were incubated at temperatures optimal for psychrotolerant species. MAC23_193 and MAC23_195, isolated from surface snow in 2023, are more closely related to *Spingomonas aurantiaca* and *Sphinogomnas ginsenosidivorax.*

The second sub-branch of the second major branch is further divided into three subbranches. The first sub-branch contains only *Stenotrophomonas*, with MAC23_92 positioned in the same branch as *Stenotrophomonas cyclobalanopsis*. The second subbranch consists of strains identified as *Pseudomonas* sp. MAC23_192, isolated from glacier snow, shows to be most closely related to *Pseudomonas cremicolorata,* while MAC23_90, also isolated from glacier snow, is most closely related to *Pseudomonas spelai.* MAC23_35 and MAC23_52, which were isolated from surface snow, form a separate branch, closely related to *Pseudomonas breneri.*

The final sub-branch contains *Acinetobacter* species. *Prolinoborus fasciculus*, a taxon with disputed species status, is also positioned within this branch. While Glaeser et al. (2020) suggest that the CIP 103579 strain (Figure 17) should be recognized as a member of *Acinetobacter lwoffii*, the phylogenetic analysis placed these two species on separate branches, with surface snow strain MAC23_217 positioned between them.

MAC23_190 and MAC23_203, recovered from glacier and surface snow samples respectively, are located on the same branch. *Acinetobacter oryzae* is identified as their closest related species. Meanwhile, MAC23_219, isolated from surface snow, is more closely related to *Acinetobacter johnsonii*.

MAC23_08 and MAC23_15 were excluded from the phylogenetic analysis due to having a HQ index lower than 50%.

3.1. Optimization of eDNA extraction Methodology

The culturable-based methodology can capture only a fraction of the microbial diversity present in the samples. Therefore, a full insight into the diversity and characteristics of bacteria in precipitation samples can be obtained by combining culture based and culture-independent methodologies.

To maximise the potential of eDNA extraction from low DNA concentration precipitation samples, several kits and different DNA concentrations were tested. The tests were performed on rainfall samples collected at CIIMAR, in December of 2023.

The DNeasy PowerWater Kit, with cleaning steps removed from the protocol, provided the best results. This protocol excluded steps designed to remove non-DNA particles, resulting in high contamination levels in the extracted DNA.

However, the complete PowerWater protocol yielded low results, with minor variability observed between using 10µg and 15µg of DNA and the Qubit dsDNA HS Assay Kit.

Table 4 - Values obtained in the DNA extraction optimization experiment. NCP - “No-cleaning” protocol.

Sample ID	Extraction Kit	Concentration(ng/µl)	Volume (µl)
MA23_1A	PowerWater	too low too low	10
			15
MA23_1B	PowerWater (NCP)	0.258	10
		0.273	15
MA23_2	PowerSoil	0.127	10
		0.133 too	15
MA22_1	PowerSoil	low	20
MA24_1A	PowerSoil (NCP)	0.150	20
MA24_1B	PowerSoil	0.336	20

The second-best performing protocol was PowerSoil, widely used for various environmental sample types. This protocol was utilized for DNA extraction from eDNA samples collected with Sterivex filters during the 2022 and 2024 campaigns.

Initial extractions from samples using the PowerSoil kit showed no detectable DNA , likely due to low sample volume filtrated (50 ml). The protocol was optimized by reducing the elution buffer volume and repeating the elution step to concentrate the sample. Post optimization, DNA quantification using Qubit recorded a concentration of 0.336ng/µl.

4. Discussion

This study aimed to characterize the culturable microbial community observed in two types of precipitation samples, rainfall and snowfall, as well as the in surface snow samples collected after precipitation events. Glacier snow samples collected from snow pits were used as a comparison with the precipitation samples. All samples were collected on King George Island in austral summer, February of 2022 and 2023.

Four different media types and two incubation temperatures were used in order to maximize the potential of culture-based methodology in capturing the culturable microbial diversity. This methodology proved to be effective in cultivating microorganisms in the samples studied, with all media and both temperatures exhibiting microbial growth. Forty-eight strains were identified, including genera well-known for ice nucleation activity (Table 1). Interestingly, key differences were noted between each sample type.

4.4. Microbial strains from Rainfall and Snowfall

In the two precipitation sample types collected in 2022 it was possible to culture different strains by using the same growth media. In the rainfall samples, the identified strains belonged to Pseudomonas, Bacteroidota and Bacillota phyla (Figure 12). The class diversity showed the presence of Gammaprotobacteria, Cytophagia and Bacilli (Figure 13). The strains were associated with four genera - *Paenibacillus, Pseudomonas, Spirosoma* and *Stenotrophomonas* (Table 1).

The strains identified in snowfall samples belong to the same phyla as observed in the rainfall samples. However, the class biodiversity shows a presence of Alphaproteobacteria, Bacilli and Sphingobacteriia (Figure 13). Genera observed in snowfall samples include *Muciliaginibacter, Paenibacillus,* and *Sphingomonas. Paenibacilus* was the only genus recorded in both types of precipitation samples (Table 1), suggesting that the type of precipitation has an impact on the type of bacteria strains present in the samples.

In fact, a previous study (Monteil et al., 2014) demonstrated that the presence of *Pseudomonas syringae* in precipitation is statistically associated with biological, chemical, and climatic parameters of precipitation water. These authors found that snowfall and rainfall differ in their ability to deposit *P. syringae*. Likewise, the samples analysed in this study suggest a different potential of precipitation in harbouring culturable microbial diversity, depending on the precipitation phase.

The meteorological factors of precipitation formation, such as temperature (Gregory et al., 1963) could potentially influence the presence of microorganisms. Moreover, as precipitation particles fall to the ground, they capture and remove atmospheric particles, including microorganisms, through a process known as precipitation scavenging (Loosmore & Cederwall, 2004). Raindrops and snowflakes can collect different types of microorganisms during this process (Bourcier et al., 2012; Péguilhan et al., 2021).

However, further studies are required to link the properties of precipitation with the presence of culturable microorganisms.

4.5. Microbial strains from Surface snow and Glacier snow

Comparing the diversity observed in precipitation samples (snowfall and rainfall samples) from 2022 with samples from surface snow following precipitation events from the same year (Figure 14), a lack of correspondence in the bacteria strains recovered was observed.

As discussed in the previous chapter, precipitation samples exhibit significant culturable biodiversity at the phylum, class, and genus levels. In contrast, in surface snow samples collected in 2022 only one genus was recovered - *Spingomonas* (Figure 14). The strains in surface snow samples show close phylogenetic relationships with *Spingomonas faeni*, *aerolata,* and *aurantiaca* (figure 17), previously been isolated from snow in Antarctica (Aislabie et al., 2000). The isolated strains in our study have been shown to grow at temperatures associated with psychrotolerant species (Table 1). *Spinhgomonas aerolata* has previously been isolated from 4200-year-old ice of Taylor Dome, Antarctica (Christner et al., 2000), showcasing its adaptation to the glacial environment conditions.

The lack of observed culturable biodiversity could signal surface snow, exposed to different temperature conditions, may not be as favourable for certain microorganisms. Freeze-thaw cycles, ultraviolet (UV) radiation, low temperatures, and exposure to oxygen create harsh conditions, leading to reduced biodiversity as only the most resilient microorganisms survive (Pearce, 2017). In addition, microorganisms in surface snow may quickly deplete available nutrients, limiting the growth of additional species. In contrast, freshly fallen precipitation may still contain a rich variety of nutrients (Verry et al., 1977) supporting a more diverse microbial community. However, it is also possible the bacteria in these samples were non-culturable for the media that was selected in this study.

On the other hand, the surface snow samples collected in 2023 recorded a high relative culturable biodiversity in comparison to the rest of the samples (Figure 14). There were three phyla present - Pseudomonadota, Bacillota and Actinomycetota. Furthermore, the classes identified in the surface snow samples were Gammaproteobacteria, Actinomycetes, Alphaproteobacteria and Bacilli. A total of seven genera were recovered, including *Acinetobacter, Bacillus, Kocuria, Mesorhizobium, Paenisporosarcina, Pseudomonas* and *Sphingomonas.*

Several factors may explain this difference in diversity. Firstly, the collection time after precipitation events varied between the two years. In the first year, surface snow was collected from Collins Glacier after mixed snowfall and rainfall events over several days. However, in the second year, surface snow was collected just after the precipitation events (see Appendix).

Analysis of glacier snow samples shows the presence of four genera - *Acinetobacter, Kocuria, Pseudomonas* and *Stenotrophomonas*. All of the genera were also identified in surface snow after precipitation events, suggesting a common origin or dispersal mechanism. Given that long-range transport is a source of aerosols in Antarctica (Lapere et al., 2024) it is possible

that the species identified in glacier snow were similarly introduced through the atmospheric pathways, while more information could be inferred using back-trajectory models (see Conclusions and Future research).

4.6. Potential INPs and CCNs

Aerosols are a major source of uncertainty in both global and polar climate systems. Therefore, accurately assessing the aerosol budget in polar regions, including the transport of aerosols from mid-latitudes to the poles, is crucial (Lapere et al., 2024; Schmale et al., 2021; Shaw, 1988). Aerosols contribute to cloud formation as cloud condensation nuclei and play a role in precipitation by acting as ice nuclei. Given the high ice-nucleation potential of aerosols of biological origin, this study aimed to identify potentially ice-nucleating bacteria in the analysed samples.

Failor et al. (2017) show that strains confirmed as Ice+ during standardised re-testing were almost exclusively Gammaproteobacteria.

On the class level, the bacteria isolated from samples analysed in this study shows that Gammaproteobacteria were the most abundant in surface snow and glacier snow in samples of 2023 and the second most abundant in precipitation and surface snow in samples of 2022. Alphaproteobacteria, most common among the precipitation samples, is typically identified in the atmosphere on the Antarctic coast (Cao et al., 2021; Els et al., 2019; Woo et al.,2013), implying possible roles in cloud formation.

Pseudomonos sp. were found in rain precipitation, surface snow samples, and glacier snow. The strains exhibited the highest phylogenetic similarity with *Pseudomonas versuta*, *cremicolorate*, *fulva*, *breneri*, *yamanorum*. Numerous studies highlight the INA potential of these species, *Pseudomonas syrynge* being the first identified INA bacteria (Maki et al., 1974).

Pseudomonas versuta was previously recovered from Antarctic soil (See-Too et al., 2017), as well as *Pseudomonas yamanorum* (Arnau et al., 2015).

Stenotrophomonas sp. was observed in the rain sample. Previous studies inspected the role of *Stenotrophomonas* in ice nucleation and detected at INA activity at temperatures of -8 ºC (Failor et al., 2017; Mortazavi et al., 2008).

Nejad et al. (2006) found that *Sphingomonas* spp. can be nucleators active between –4 and –6°C after growth at 15 to 25°C, and found that phosphate limitation had a strong negative effect on their ice nucleation ability.

Strains classified as *Bacillus* sp. strains were detected in surface snow samples collected in 2023. MAC23_205 has a notable phylogenetic similarity with *Bacillus safensis.* Some *Bacillus* species, with their ability to form spores and robust surface properties, can potentially serve as CCNs in the atmosphere (Delort et al., 2017; Du et al., 2023).

A study by Matulová et al. (2014) examined the ability of *Bacillus* sp. 3B6 to metabolise a variety of sugars, such as L-arabitol, D-fructose, sucrose, D-glucose, cellotetraose, cellulose, and starch, which could provide the energy necessary for its survival and metabolic activity in

warm cloud environments. These sugars are converted to exopolymeric substances (EPSs), which can help the bacterium withstand the challenging conditions found in clouds, including nutrient scarcity, low temperatures, freeze-thaw cycles, UV exposure, and changes in moisture levels due to evaporation and condensation. In this study, Malutova et al. conclude EPSs might also play a role in atmospheric processes by acting as secondary organic aerosols, potentially serving as effective cloud condensation nuclei.

Stephanie and Waturangi (2011) show that *Acinetobacter* species have been associated with a high potential for ice nucleation, exhibiting ice nucleation at −7.0 ºC. Moreover, *Acinetobacter haemolyticus*, also closely related to the samples, produces siderophores (Okujo et al., 1994).

This study shows interesting implications regarding INPs and CCNs in Antarctic samples. Further studies and improving study limits are necessary to understand the roles of these organisms in climate processes.

4.7. Stressful conditions Impacting the Aeromicrobiome

Precipitation samples can provide large insights into the cloud microbiome. Although the cloud environment allows for the presence of a high diversity of microorganisms, only certain organisms can thrive. The aerial environment imposes stress on the living organisms, requiring them to adapt to extreme conditions.

In the study of cloud water from Dôme Mountain, France, Amato et al. (2017) state that although there are prominent levels of diversity of microbial communities in clouds, only some microorganisms can maintain metabolic activity in clouds in spite stressful conditions. Many microorganisms identified in their study have close relationships with vegetation, epiphytes, parasites, and endosymbionts. Plant leaves, like clouds, are frequently exposed to shifts in temperature and humidity, as well as elevated levels of ultraviolet light. Therefore, Amato et al. hypothesised that airborne bacteria acquired physiological traits compatible with survival in clouds from the plant-associated lifestyle. *Pseudomonas,* the genus mostly identified with ice nucleation, for example, has been identified as a plant pathogen (Lindow, 1983), while the bacterial ice nucleation process has tight evolutionary ties with plants.

The rainfall, snowfall and surface snow samples show the presence of several genera identified with adaptations to an extreme environment. *Spirosoma* genus was found to have a high DNA damage resistance, such as the UV light caused damage (Lee et al., 2015). Interestingly, two of the potentially new species isolated from rainfall samples were identified as *Spirosoma* sp. and are most related to *Spirosoma spitsbergense* and *Spirosoma luteum* (Table 3), which were isolated from Arctic soil (Finster et al., 2009).

Sphingomonas, recovered from snowfall and surface snow samples, are psychrotolerant bacteria recovered from polar environments and air samples (Busse et al., 2023; Foght et al., 2004). They are know to have a resistance to numerous antibiotics which is one of the adaptations suitable for extreme conditions (Vaz-Moreira et al., 2011).

Paenibacillus strains identified in the snow precipitation samples in this study were closely related to *Paenibacillus tylopili* (Figure 15). This species produces spores, allowing for survival in extreme conditions (Kuisiene et al., 2008). However, the *Paenibacillus* species

found in rain precipitation were more closely related to *Paenibacillus paridis*, an endophyte associated with vegetation (Wang et al., 2020) supporting the hypothesis of Amato et al. (2017). Additionally, two of the potentially new species associated with *Paenibacillus* were identified in rainfall, highlighting the potential of precipitation ecosystem in harbouring undiscovered biodiversity,

(A microbial study by Mortazavi et al. (2014) conducted in the Arctic, found that *Paenibacillus* sp. is present in various snow types, including accumulated snow, windpack, blowing snow, snow hoar, fresh snow, and frost flowers.)

Two of the strains isolated from snowfall samples are most closely related to *Mucilaginibacter glacialis* (Figure 15), isolated from Tibetan snow (Yang et al., 2021). Vasconcelos et al. (2022) isolated *Mucilaginibacter* strains isolated from a mine dam with metal-contaminated soil. The species presented genes for resistance to metals (i.e., As, Zn, Co, Cd, and Mn) and cross-resistance to antibiotics, indicating their extremophilic adaptations. Mucilaginibacter was also previously isolated from precipitation samples (Lu et al., 2016; Woo et al., 2020).

Surface snowfall samples collected after precipitation in 2023 are also marked by *Kocuria* and *Mezorhizoium*, both genera connected to plants. A close phylogenetic relationship of MAC23_34 is seen with *Mesorhizobium australicum*, a Gramme-negative bacterium originally isolated from the root nodules of *Biserrula pelecinus* in Australia (Nandasena et al., 2009). *Kocuria rhizophila*, mostly identified in the samples, is an endophyte, but also as a fish pathogen affecting salmonids (Pękala et al., 2018).

The Acinetobacter sp. strains have the most relative abundance in surface snow and glacier snow samples. There are a few strains of Acinetobacter that show a close relation to the samples. For example, *Acinetobacter oryzae*, is a bacterium which has been isolated from a rice plant (Chaudhary et al., 2012).

It is evident that the strains found in the studied samples are associated with numerous extreme environments and have developed adaptations for the conditions of life in cloud ecosystems. Still, while precipitation samples can provide large insights into the cloud microbiome, they do not accurately represent it (Malard et al., 2022).

4.8. Study biases and Limitations

Global estimates of bacterial diversity range from 10^7 to 10^9 species (Curtis et al., 2002). However, culture-independent methods have revealed that certain bacterial groups and entire phyla have consistently evaded cultivation (Felske et al., 1999; Stewart, 2012). The “1% culturability paradigm” indicates 99% of environmental organisms are unculturable, yet Martiny (2019) calls for this concept to be revised. However, certain biases remain, as cultivation-based studies have shown a predominance of easily culturable phyla.

Culturable phylum diversity in the 2022 samples is consistent with findings from Malard et al. (2022), who collected air samples from 100 locations around the Southern Ocean and its islands, along with seven precipitation samples. The most abundant phyla identified were Pseudomonadota (formerly Proteobacteria), Bacillota (formerly Firmicutes), and Bacteroidota; yet bacteria in the Pseudomonadota phylum are easily culturable and might not reflect realistic diversity.

On the other hand, culture-independent methodologies are associated with another set of constraints. To fully understand the physiology, functionality, genomics, and potential biotechnological applications of microbes, effective cultivation and preservation techniques are crucial (Overmann et al., 2017; Prakash et al., 2021) in combination with culture-independent methodologies. Describing potentially new species also requires obtaining cultures and having a possibility for performing experiments with the recovered strains. Moreover, in this study, samples used for eDNA analysis required large sample volumes, difficult to collect during precipitation events in Antarctica. Challenges still exist in achieving adequate eDNA yields and filtering a sufficient amount of water volume for precise detection (Hunter et al., 2019). To overcome this limitation, this study used surface snow precipitation samples instead of relying only on direct precipitation collection, enlarging the sample volumes used to optimise the eDNA isolation protocol from precipitation samples.

In addition to laboratory practices, the sampling method could be advanced with an increased sample size and longer observational periods, providing insight into microbial seasonality. However, collecting data from Antarctica is highly demanding due to the remote location and extreme conditions of the working site. The transportation of samples from the sampling site to the laboratory imposes also limits on samples size. Moreover, as performing culture-based methodologies is lengthy not all samples from 2023 have been analysed, and the application of a less time-consuming methodology could facilitate the research. In future research, we will apply strategies for mitigating the imposed methodological limitations faced during this study.

4.9. Climate implications

Mitigating climate change impacts is one of the most significant tasks and challenges for humanity in the 21st century (Abbass et al., 2022). Climate change is already reshaping ecosystems globally, and the polar regions are particularly vulnerable to these transformations. As temperatures in the current century rise, Antarctica is predicted to experience shifts in precipitation patterns, with predictions of liquid precipitation increasing over almost the entire continent (Vignon et al., 2021). Such changes in the precipitation phase could impact local microbial communities, as rainfall and snowfall introduce distinct microbial populations. Moreover, rainfall precipitation was shown to carry possibly novel species (Table 2).

This study demonstrates how precipitation can serve as a vector for transporting diverse microbial species into Antarctic ecosystems. The findings suggest that microbial species delivered by rainfall may differ from those transported by snowfall, potentially altering the aeromicrobiome and the surface snow microbial communities. These microorganisms may not only colonise snow and ice but also influence critical atmospheric processes, such as cloud formation and ice nucleation, contributing to larger-scale climatic feedback loops. With a shift toward more frequent rainfall, as predicted, Antarctic ecosystems may face further disruptions. It is also critical to gain a better understanding of how precipitation-driven microbial transport may influence both atmospheric and terrestrial ecosystems in Antarctica, particularly under changing climate conditions.

5. Conclusions and Future Research

Antarctica and the Antarctic Peninsula remain vastly unexplored, particularly in terms of its unique biodiversity, and on the effects of climate change on these regions. Remote locations and extreme conditions hinder data collection, leading to study gaps and calling for interdisciplinary research efforts. Aerosols in the polar studies remain an uncertainty in global climate change models. This study combines biological and meteorological data and contributes to our understanding of how precipitation can serve as a vector for transporting diverse microbial species into Antarctic ecosystems. However, more research needs to be done by combining complementary culture and culture-independent molecular methodologies to understand the role these microorganisms might have in the aerosol budget and ice nucleation processes.

The collected and analysed samples show substantial culturable biodiversity, with similar identified strains with previous aeromicrobiome and polar microbiology studies. The observed differences in microbial diversity between rainfall and snowfall suggest that shifts in precipitation patterns, driven by climate change, may alter both the aeromicrobiome and surface snow microbial communities. This, in turn, could have implications for cloud formation and polar climate feedback mechanisms, making it crucial to further investigate clouds in the polar regions to mitigate climate change impacts.

While culturing methods have proven effective in harbouring polar and aerial microbial communities, utilising culture-independent methodologies is essential for obtaining complete insight into aerial bacterial diversity.

Advances in genome-sequencing technologies and metagenomics methods have drastically improved our knowledge in the field of microbial diversity (Hug et al., 2016). Sequencing environmental DNA (eDNA) will provide insights into the broader microbial community present in precipitation samples (Garlapati et al., 2019). Following the successful optimization of eDNA extraction, Illumina sequencing will be employed to analyse 16S rRNA and 18S rRNA metabarcoding data and infer the composition of microbial communities (Pascoal et al., 2022).

Using alternative sequencing approaches in future analysis, such as sequencing technology developed by Pacific Biosystems (PacBio), can expedite and enhance the analysis of culturable microbial communities (Kumar et al., 2022; Rhoads & Au, 2015).

Isolating and culturing species provides a greater understanding of their morphology, physiology and ecological roles (Keller & Zengler, 2004). Moreover, culture-based methodologies allow for obtaining whole genome sequencing (WGS) data from isolated bacteria, which can further be used for performing gene surveys across the genomes.

Future research will focus on expanding our understanding of ice nucleation through several advanced methodologies. Firstly, WGS of isolated microbial will be crucial for identifying genes involved in INA. We have conducted WGS on potentially new species such as MAC23_07, MAC23_09, MAC23_11, and MAC23_14, which are associated with *Paenibacillus* and *Spirosoma*. Further genome studies on these strains will be done to explore the molecular mechanisms underlying their potential roles in ice nucleation.

Employing biodiversity and phylogenetic analysis offers firsthand insights into the morphology, taxonomy and evolutionary relationships between microorganisms. However, several methods could be employed for directly assessing ice nucleating potential, including ice nucleating assays and the MicroPenguin technique (Kunert et al., 2018; Wieber et al., 2024).

Integrating meteorological data with biological data by computing back-trajectories representing air mass movements will be essential for tracing the sources of biological aerosols in Antarctica. Similar studies have been done by Grinn-Gofroń et al. (2016), tracking fungal spores in air force masses, or Smith et al. (2012) who looked at intercontinental air transport of microorganisms. Most specifically to the topic of this thesis, Monteil et al. (2014) mapped the occurrence of *P. syringae* in precipitation according to the origin of air masses. Most informative back-trajectory models which are typically used are the Lagrangian particle dispersion model FLEXPART or HYSPLIT (Gao et al., 2024; Makra et al., 2016). By linking meteorological conditions with biological findings, we aim to grasp a better understanding of the origins and distribution of polar aerosols.

Moreover, studies such as those by Malard et al. 2022 suggest that while the 'air-bridge' hypothesis may not fully explain microbial transport to Antarctica, but that each Antarctic Island has a distinctive role in these processes. These findings highlight the need for targeted conservation and biosecurity measures for each island's unique biodiversity.

Additional research campaigns are planned to enhance our knowledge of microbial communities in the Antarctic region. A cruise ship campaign in the Southern Ocean, scheduled for the austral spring, will add missing data on the seasonality and distribution of biological aerosols. This campaign will include stops between Southern Ocean islands and at Rothera Station in Antarctica. Furthermore, a sampling campaign on King Sejong Island is scheduled for January and February of 2025.

Interdisciplinary research and increased expeditions are necessary to explore and understand Antarctica's unique microbial biodiversity, which is critical for protecting it in the face of climate change and the global biodiversity crisis.

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Appendix

Sampling information:

Table 5 - Sampling information. RP - rainfall precipitation. SP - snowfall precipitation. SS - surface snow. GS - glacier snow

Sample name	Sample ID/year	Volume (ml)	Precipitation type
RP 10.4	RP1/2022	7	Fog/drizzle
RP 11.1	RP2/2022	15	Fog/drizzle
SP 13.2	SP1/2022	50	Snowfall
GS 12.1 + 12.2	SS1.1/2022	40	Surface snow on Collins glacier after a mix of snowfall and rainfall previous days
GS 13.3.1 + 13.3.2	SS1.2/2022	40	Surface snow on Collins glacier after a mix of snowfall and rainfall previous days
SSP 19-20	SS2.1/2023	40	Fresh surface snow, snowing before collection
SSP 27.2.4	SS2.2/2023	45	Fresh surface snow, snowing before collection
SSP 21.2	SS2.3/2023	50	Fresh surface snow, snowing before collection
GSD 6.5	GS1/2023	35	25-cm from bottom
GS 1.3	GS2/2023	50	Fourcade snow patch
GSD 6.6	GS3/2023	33	35-cm from bottom
GSS 6.2a	GS4/2023	40	Snow pit, combined

R-studio codes:

Pie chart:

```
install.packages("ggplot2")
install.packages("dplyr")

install.packages("cowplot")
library(ggplot2) library(dplyr)
library(cowplot)
```

```
data_2022 <- data.frame(
  Phylum = c("Pseudomonata", "Bacteroidota", "Bacillota"),  value =
c(19, 5, 4)
)

data_2023 <- data.frame(
  Phylum = c("Pseudomonata", "Actinomycetota", "Bacillota"),  value =
c(15, 3, 2)
)

combined_data <- rbind(data_2022, data_2023)

data_2022 <- data_2022 %>%  mutate(percentage =
value / sum(value) * 100)

data_2023 <- data_2023 %>%  mutate(percentage =
value / sum(value) * 100)

colors <- c(
  "Pseudomonata" = "#87CEEB",  # Sky Blue
  "Bacteroidota" = "#FFA500",  # Orange
  "Bacillota" = "#228B22",  # Forest Green
  "Actinomycetota" = "#D3D3D3"  # Light Gray
)

create_pie_chart <- function(data, title) {  ggplot(data,
aes(x = "", y = value, fill = Phylum)) +
geom_bar(width = 1, stat = "identity") +
coord_polar(theta = "y") +  theme_void() +
    geom_text(aes(label = paste0(round(percentage, 1), "%")),
position = position_stack(vjust = 0.5),
```

```
                color = "white") +         ggtitle(title)
+
      scale_fill_manual(values = colors) +         theme(plot.title
= element_text(hjust = 0.5),
            legend.position = "none")  # No legend on the pie charts
}

plot_2022 <- create_pie_chart(data_2022, "Phylum Diversity 2022") plot_2023 <-
create_pie_chart(data_2023, "Phylum Diversity 2023")

legend <- get_legend(
   ggplot(combined_data, aes(x = "", y = value, fill = Phylum)) +
geom_bar(stat = "identity") +         scale_fill_manual(values =
colors) +         theme(legend.position = "right")
)

combined_plot <- plot_grid(plot_2022, plot_2023, nrow = 1, align = 'hv') final_plot <-
plot_grid(combined_plot, legend, rel_widths = c(2, 0.5))

print(final_plot)
```

Stacked column:

```
# Load the required library library(ggplot2)

data <- data.frame(
  Sample = rep(c("Rainfall", "Snowfall", "Surface snow 2022", "Surface snow 2023",
"Glacier snow"),
               times = c(4, 3, 1, 7, 4)),
  Genus = c("Stenotrophomonas", "Spirosoma", "Pseudomonas", "Paenibacillus",
"Sphingomonas", "Paenibacillus", "Mucilaginibacter",
            "Sphingomonas",
```

```
        "Acinetobacter", "Bacillus", "Kocuria", "Mesorhizobium", "Paenisporosarcina",
"Pseudomonas", "Sphingomonas",
        "Stenotrophomonas", "Pseudomonas", "Kocuria", "Acinetobacter"),
  Abundance = c(5, 2, 1, 2,
                3, 2, 3,
                10,
                3, 1, 2, 1, 1, 2, 2,
                1, 2, 1, 4)
)

genus_colors <- c("Stenotrophomonas" = "#66c2a5",  # Soft green
                  "Spirosoma" = "#fc8d62",          # Coral
                  "Pseudomonas" = "#8da0cb",        # Soft blue
                  "Paenibacillus" = "#e78ac3",      # Light pink
                  "Sphingomonas" = "#a6d854",       # Light green
                  "Mucilaginibacter" = "#ffd92f",   # Bright yellow
                  "Acinetobacter" = "#e5c494",      # Soft brown
                  "Bacillus" = "#b3b3b3",           # Light grey
                  "Kocuria" = "#1b9e77",            # Teal green
                  "Mesorhizobium" = "#d95f02",      # Burnt orange
                  "Paenisporosarcina" = "#7570b3")  # Slate blue

ggplot(data, aes(x = Sample, y = Abundance, fill = Genus)) +
geom_bar(stat = "identity") +  scale_fill_manual(values =
genus_colors) +  theme_minimal() +
  ggtitle("Genus Diversity Across Sample Type") +  labs(x =
"Sample Type", y = "Abundance") +  theme(plot.title =
element_text(size = 16, face = "bold"),         axis.text =
element_text(size = 12),
```

```
      axis.title = element_text(size = 14),      legend.text =
element_text(size = 12))
```