Eleonora Tinto

Diversity of cultured heterotrophic bacteria from polar sea waters
and sea ice ecosystems: A unique source for blue biotechnology

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Eleonora Tinto

Diversity of cultured heterotrophic bacteria from polar sea waters and sea ice ecosystems: A unique source for blue biotechnology

MSc in Marine Biology

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To Whom It May Concern:

As supervisor of the Master's thesis of student ELEONORA TINTO, I must inform you that the project was carried out in a positive manner, with the student showing the necessary commitment to achieve the objectives of her work. She performed excellent work and part of her master's will derive in a future publication. Thus, from observing the work and analyzing her Master's thesis, entitled "Diversity of heterotrophic bacteria in polar sea water and sea ice ecosystems: A unique source for blue biotechnology', my judgment is favorable to its presentation.

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PARECER

Como coorientadora de Tese de Mestrado da aluna ELEONORA TINTO cumpre-me informar que o projecto decorreu duma forma positiva, tendo a aluna demonstrado o empenho necessário à obtenção dos objectivos do trabalho que realizou.

Assim, do acompanhamento que fiz do trabalho e a análise do respectiva Tese de Mestrado, intitulada "Diversity of cultured heterotrophic bacteria from polar sea waters and sea ice ecosystems: A unique source for blue biotechnology", é favorável o meu parecer quanto à sua entrega.

Faro, 26 de setembro de 2023

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Statement of authorship

Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Abstract

The discovery of the CRISPR-Cas system, a prokaryotic defense mechanism, has revolutionized genomics and biotechnology. This system uses Cas nucleases to precisely cut DNA sequences by relying on a protospacer adjacent motif (PAM). Cas9, especially SpCas9 from *Streptococcus pyogenes*, has been widely studied for genome engineering due to its well-understood biochemistry and short PAM sequence. However, its limitations in the recognition of PAM sequences have led researchers to look for natural orthologs of Cas9. This study focuses on the remote areas of the polar regions, where extreme conditions result in unique environments. During three expeditions - PI-ICE (2019) and ANTOM-2 (2022) in Antarctica, and QikIce in the Arctic (2023) – seawater, sea ice and brine samples were collected. Subsequent cultivation showed differences in bacterial growth and taxa enrichment between seawater and sea ice heterotrophic bacteria. Furthermore, the taxonomic analysis of 199 colonies revealed similarities and variations in their biodiversity across habitats, depths and regions. In particular, in ANTOM-2, seawater samples from different depths showed a prevalence of Gammaproteobacteria in deeper layers, while Alphaproteobacteria dominated the photic zones. Dominant genera included *Sulfitobacter*, *Pseudoalteromonas* and *Shewanella*. Five out of seven deep-sea genera were found in photic zones, including *Bacillus*, *Pseudoalteromonas*, *Pseudomonas*, *Psychroserpens* and *Winogradskyella*. In contrast, sea ice isolates from PI-ICE showed an absence of Alphaproteobacteria, with Flavobacteriia and Gammaproteobacteria dominating. Notable genera were *Flavobacterium*, *Psychrobacter* and *Chryseobacterium*. During the QikIce expedition, Bacilli and Flavobacteriia were predominant, while three genera differed from those found in Antarctica: *Planomicrobium*, *Maribacter* and *Octadecabacter*. In the final phase of the study, the design of two primer pairs led to the amplification of potential Cas9 sequences from three *Flavobacterium* isolates, opening up new avenues for future efforts. This research highlights the importance of exploring extreme environments, such as polar regions, for biotechnological discoveries and expanding the CRISPR-Cas toolbox.

Keywords: Bacterial diversity, Polar regions, Photic layers, Deep layers, Sea ice, Cas9

Resumo

A descoberta do sistema CRISPR-Cas, um mecanismo de defesa para procariotas, revolucionou rapidamente o mundo da biologia, biotecnologia e biomedicina. Essa tecnologia particular de edição genómica utiliza a capacidade de uma família de proteínas, chamadas nucleases Cas, para cortar o genoma em sequências de nucleotídeos específicas. Esta clivagem é possível somente quando uma sequência de reconhecimento PAM (Motivo Adjacente ao Protospacer) está presente antes do local de edição, garantindo, também, que o DNA autêntico não seja danificado.

Em particular, a Cas9 foi estudada para engenharia genómica muito mais do que qualquer outra proteína pertencente à família Cas, especialmente a SpCas9, descoberta em *Streptococcus pyogenes*. Isso deve-se à sua bioquímica bem conhecida e à presença de uma breve sequência PAM. Apesar de anos de estudo e otimização, a Cas9 apresenta limitações relacionadas ao reconhecimento das sequências PAM, exigindo uma seleção cuidadosa do local, para evitar ligações e clivagens indesejadas.

Para superar estas limitações, os investigadores estão a explorar ativamente novos ortólogos de Cas9 que ocorrem naturalmente. Esta investigação está em constante crescimento, em paralelo com a exploração da biodiversidade marinha. Este estudo faz parte desta investigação, com foco num ambiente extremo e único: as regiões polares.

Durante este projeto, bactérias foram cultivadas a partir de um total de 33 amostras coletadas em três diferentes expedições polares. A primeira expedição, chamada PI-ICE, ocorreu na Antártida de 4 de fevereiro a 11 de março de 2019. Foram consideradas duas amostras de gelo marinho, coletadas perto à entrada do Mar de Weddell, através do Estreito de Bransfield.

A segunda expedição, conhecida como ANTOM-2, teve lugar de 27 de janeiro a 5 de fevereiro de 2022. Durante esta expedição, foram recolhidas amostras de água do mar a diferentes profundidades, em cinco estações ao longo da costa ocidental da Península Antárctica. No total, foram analisadas 25 amostras, incluindo 12 amostras de estratos fóticos, 10 de estratos mesopelágicos e 3 de estratos batipelágicos.

A terceira e mais recente expedição, denominada QikIce, teve lugar no Ártico de 22 a 25 de abril de 2023. Em particular, foram recolhidas seis amostras de diferentes fontes: uma do mar, três de secções de gelo marinho e duas de salmoura. Estas amostras foram recolhidas a sudoeste de Qikiqtarjuaq, também conhecida como Ilha de Broughton.

Durante o nosso estudo, observámos diferenças significativas na capacidade de cultivo entre as amostras de água do mar recolhidas durante a expedição ANTOM-2 e as amostras de gelo marinho recolhidas durante a expedição PI-ICE. Utilizámos meios de cultura padrão para o cultivo. As bactérias das amostras de água do mar foram cultivadas em meios ricos em nutrientes constituídos por caldo marinho e ágar, enquanto as das amostras de gelo marinho foram cultivadas num meio pobre em nutrientes chamado R2A.

Em particular, as amostras de água do mar apresentaram um crescimento limitado das colónias, enquanto que as amostras de gelo marinho apresentaram um crescimento abundante. Além disso, as bactérias recolhidas na água do mar de diferentes profundidades apresentaram variações significativas. Em geral, o número de unidades formadoras de colónias (CFU/ml) tendeu a diminuir à medida que se passava das zonas fóticas para as zonas mais profundas.

Foi selecionado um total de 199 colónias das três expedições, 119 da expedição ANTOM-2, 64 da expedição PI-ICE e 16 da expedição QuikIce. Posteriormente, as células foram lisadas e as sequências do gene 16S rRNA foram amplificadas, utilizando os iniciadores específicos para bactérias 358F e 907R. Após verificação da qualidade dos produtos de PCR por eletroforese em gel, procedeu-se à sequenciação. Uma vez obtidas as sequências purificadas das estirpes isoladas, a taxonomia de cada uma foi determinada por comparação com as sequências bacterianas na base de dados SILVA rRNA.

Os resultados taxonómicos revelaram que, na expedição ANTOM-2, a classe Gammaproteobacteria dominou nas camadas profundas, enquanto a classe Alphaproteobacteria foi predominante nas zonas fóticas. Os três géneros mais comuns entre os isolados desta expedição foram *Sulfitobacter*, *Pseudoalteromonas* e *Shewanella*. Além disso, cinco dos sete géneros identificados nas águas profundas também estavam presentes nas zonas fóticas, incluindo *Bacillus*, *Pseudoalteromonas*, *Pseudomonas*, *Psychroserpens* e *Winogradskyella*.

Em comparação com os resultados obtidos a partir dos isolados de gelo marinho recolhidos na expedição PI-ICE, a classe Alphaproteobacteria esteve ausente, enquanto as classes predominantes foram Flavobacteria e Gammaproteobacteria. Em termos de géneros, os mais abundantes foram *Flavobacterium* e *Psychrobacter*, seguidos de *Chryseobacterium*.

Na expedição QikIce, embora o número de colónias isoladas fosse limitado, foram identificadas diferenças taxonómicas em comparação com as expedições antárcticas. As classes mais abundantes foram Bacilli e Flavobacteriia. Em termos de géneros, foram encontrados três géneros que não foram observados nas outras duas expedições. Por ordem de abundância, estes foram *Planomicrobium*, *Maribacter* e *Octadecabacter*.

Posteriormente, para determinar a possível relação filogenética entre os nossos isolados, as sequências foram alinhadas utilizando o programa bioinformático MEGA11 (Molecular Evolution Genetics). Os dados obtidos foram utilizados para construir a árvore filogenética utilizando o método Neighbour-Joining (NJ).

Em particular, foram identificadas algumas ligações entre isolados de diferentes habitats e regiões polares. No que diz respeito aos isolados da Antárctida, alguns dos géneros *Flavobacterium* e *Pseudomonas* da água do mar apresentaram agrupamentos independentes com isolados dos mesmos géneros do gelo marinho. Foi também encontrada uma semelhança entre os isolados do género *Planomicrobium* da água e do gelo marinho do Ártico. Além disso, foi encontrada uma ligação entre os isolados de água do mar do Antártico dos géneros *Psychroserpens* e *Bacillus* e os seus homólogos encontrados no gelo marinho do Ártico.

Por fim, foram concebidos primers específicos para amplificar potenciais genes Cas9 presentes no ADN extraído de certos isolados, pertencentes ao género *Flavobacterium* de amostras de gelo e de água do mar da Antárctida. O primeiro par de primers foi concebido utilizando como modelo as sequências Cas9 de *F. frigidarium* e *F. psychrophilum*, identificadas com o programa CRISPR-CasFinder, com o objetivo de amplificar uma sequência de aproximadamente 1600 pb. O segundo par de primers foi concebido com base na sequência Cas9 de *F. pectinovorum*, com o objetivo de amplificar uma sequência de aproximadamente 1100 pb.

Os dados da amplificação indicaram a possível presença dos genes Cas9 em dois isolados de gelo marinho. Em particular, os produtos de PCR analisados em géis mostraram a presença de duas bandas individuais ténues de cerca de 1000 e 1500 pb. Além disso, outro isolado apresentou uma única banda mais espessa e claramente visível de cerca de 500 pb, que poderia representar um fragmento mais curto.

Os resultados preliminares deste estudo revelaram semelhanças e variações na biodiversidade de bactérias heterotróficas em diferentes ambientes, profundidades e regiões polares. Em particular, foram identificadas bactérias que são atualmente de grande interesse para a investigação em biotecnologia azul.

Além disso, a descoberta da presença de sequências que contêm o gene Cas9 constitui um ponto de partida para futuras investigações. A integração de dados metagenómicos poderia enriquecer

ainda mais esta informação, permitindo uma maior precisão e eficiência no estudo da presença e função dos genes Cas9 em ambientes polares.

Este estudo sublinha a importância crucial da exploração de ambientes extremos, como as regiões polares, para conduzir descobertas no campo da biotecnologia e expandir o conjunto de instrumentos relacionados com o CRISPR-Cas.

Palavras-chave: Diversidade bacteriana, Regiões polares, Estratos fóticos, Estratos profundos, Gelo marinho, Cas9.

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Contents

ABSTRACT I

RESUMO II

ACKNOWLEDGEMENT OF THE THESIS VI

CONTENTS VII

LIST OF FIGURES IX

LIST OF TABLES X

LIST OF ABBREVIATIONS X

CHAPTER 1 - GENERAL INTRODUCTION 1

- 1.1. RELEVANCE OF POLAR ECOSYSTEMS IN THE CONTEXT OF CLIMATE CHANGE 1
  - 1.1.1. Overview of Polar Regions 1
  - 1.1.2. The effects of global change on the Polar regions 2
- 1.2. CHARACTERIZE AND ADAPTATION OF PSYCHROPHILIC MICROORGANISMS 3
  - 1.2.1. Molecular adaptation 3
    - *1.2.1.1. Genome structure 3*
    - *1.2.1.2. Proteins and enzymes 4*
  - 1.2.2. Physiological adaptations 4
    - *1.2.2.1. Membrane function 4*
    - *1.2.2.2. Cryoprotectants and antifreeze proteins 5*
- 1.3. THE POLAR OCEAN MICROBIOME 6
  - 1.3.1. Seasonal Diversity of Microorganisms in Polar Seawater and Sea Ice 6
    - *1.3.1.1. Microbial Communities of the Seawater 6*
    - *1.3.1.2. Microbial Communities of the Sea Ice 7*
  - 1.3.2. Culturing and Molecular Approaches to Assess Bacteria Taxonomy 8
- 1.4. POLAR MICROORGANISMS AS A UNIQUE SOURCE FOR BLUE BIOTECHNOLOGY 10
  - 1.4.1. CRISPR/Cas technology 10
    - *1.4.1.1. Bacterial Applications 12*
    - *1.4.1.2. Limitations 13*
- 1.5. PROJECT AND OBJECTIVES 14

REFERENCES 16

CHAPTER 2 - MANUSCRIPT 28

- ABSTRACT 29
- 2.1. INTRODUCTION 30
- 2.2. METHODS 32

2.2.1. Study Area and Sampling 32

2.2.2. Culturing and isolation of bacteria 33

2.2.2.1. Conventional Culturing Strategy in Medium 33

2.2.2.2. Isolation of bacteria 34

2.2.3. Bacterial cell lysis and 16S rRNA PCR amplification from colony cultures 35

2.2.4. Sequencing of the 16S rRNA gene and bioinformatics analysis 36

2.2.5. Taxonomy and phylogenetic analyses of isolated bacteria 36

2.2.6. Primer design for the detection of Cas9 in the cultured bacteria 37

2.2.6.1. Analysis of Cas9 Genes in Public Uncultured Metagenomic Assembled Genomes (MAGs) 37

2.2.6.2. Primer Design for PCR 38

2.2.7. DNA Extraction and Cas9 Quantification 38

2.2.8 PCR amplification using the Primers designed 40

2.2.8.1 Primers Rehydration 40

2.2.8.2. PCR amplification for the detection of Cas9 40

2.3. RESULTS 42

2.3.1. Divergent cultured bacteria abundance and colony forming units (CFUs) in seawater and sea ice samples from the Antarctic Peninsula 42

2.3.2. Diversity of cultured bacteria from seawater and sea ice from polar regions 44

2.3.2.1. Taxonomic identification by 16S rRNA gene analyses 44

2.3.2.2. NCBI BLAST Analysis: Closest matches for sequences belonging to the most abundant genera 54

2.3.2.3. Association of morphological characteristics with some genera of interest 56

2.3.3. Phylogenetic analyses of the 199 isolates 57

2.3.4. Design and testing of primers for Cas9 gene detection in polar bacterial taxa 61

2.4. DISCUSSION 65

2.4.1. Higher cultivability in Antarctic sea ice than in seawater and in photic layers 65

2.4.2. Diversity of cultured bacteria from seawater and sea ice from polar regions 66

2.4.2.1 Differences between bacteria across ocean layers in seawater (ANTOM-2 expedition) 66

2.4.2.2. Differences between bacteria in different Antarctic polar habitats (seawater and sea ice) 69

2.4.2.3. Comparative analyses of Antarctic vs Arctic bacterial isolates 71

2.4.3. Phylogenetic analyses revealed potential different species and ecotypes 73

2.4.4. Detection of the Cas9 gene in polar bacterial isolates 74

2.5. CONCLUSIONS 75

2.6. ACKNOWLEDGEMENTS OF THE PAPER 76

REFERENCES 77

ANNEXES 86

Appendix I – Tables CFU/ml from ANTOM-2 and PI-ICE and Number of colonies from QikIce 86

Appendix II – Tables Taxonomy by 16S rRNA gene analyses (SILVA) 88

Appendix III – Tables Closest matches for sequences belonging to the most abundant genera (NCBI) 92

List of figures

FIGURE 1.1. THE ARCTIC (LEFT) AND ANTARCTIC (RIGHT) POLAR REGIONS. 1
FIGURE 1.2. COMMON PHYSIOLOGICAL ADAPTATIONS IN A PSYCHROPHILIC PROKARYOTE 6
FIGURE 1.3. SCHEMATIC OVERVIEW OF THE ENVIRONMENTAL FACTORS INFLUENCING SEASONAL SUCCESSION OF MARINE BACTERIOPLANKTON IN POLAR REGIONS 7
FIGURE 1.4. BACTERIAL COMMUNITY STRUCTURE OF SEA ICE BASED ON 16S RRNA GENE SURVEYS. 8
FIGURE 1.5. A) CRISPR LOCUS OVERVIEW. B) ELEMENTS OF THE CRISPR/CAS9 SYSTEM. 11
FIGURE 1.6. EXPLOITATION OF ENDOGENOUS AND ENGINEERED CRISPR-CAS SYSTEMS IN BACTERIA 12
FIGURE 1.7. GENERA FROM WHICH 79 CAS9 PROTEINS HAVE BEEN DERIVED 14
FIGURE 2.1. (A) MAP OF THE PI-ICE EXPEDITION. (B) MAP OF SELECTED STATIONS OF THE ANTOM-2 EXPEDITION AND THE FIVE VERTICAL PROFILES (C) LOCATION OF STATION QI0200 NEAR THE ISLAND OF QIKIQTARJUAQ. (D) SEA ICE CORE, BRINE AND SEAWATER FROM WHICH SAMPLES WERE COLLECTED DURING THE QIKICE EXPEDITION 33
FIGURE 2.2. GRAPHICAL OVERVIEW OF DNA EXTRACTION PROCESS WITH POWERSOILPRO KIT 40
FIGURE 2.3. CFU/ML VARIATION AT DIFFERENT DEPTHS FROM ANTOM-2 PLATES 43
FIGURE 2.4. CFU/ML VARIATION AT DIFFERENT SITES FROM PI-ICE PLATES. 44
FIGURE 2.5. DISTRIBUTION OF BACTERIAL ISOLATES FROM ANTOM-2 EXPEDITION BY CLASS AND GENUS BASED ON DEPTH 46
FIGURE 2.6. COMPARISON OF GENUS ABUNDANCE AND OCCURRENCE ACROSS ANTARCTIC SAMPLING STATIONS 48
FIGURE 2.7. PERCENTAGE IDENTITY RANGE WITHIN GENERA OF INTEREST FROM ANTOM-2 49
FIGURE 2.8. DISTRIBUTION OF ISOLATES FROM PI-ICE EXPEDITION BY CLASS AND GENUS BASED ON THE STATION 50
FIGURE 2.9. PERCENTAGE IDENTITY RANGE WITHIN GENERA OF INTEREST FROM PI-ICE 51
FIGURE 2.10. DISTRIBUTION OF ISOLATES FROM THE QIKICE EXPEDITION BY CLASS AND GENUS BASED ON LOCATION 53
FIGURE 2.11. MORPHOLOGY OF COLONIES FROM PI-ICE AND QIKICE EXPEDITIONS. 57
FIGURE 2.12. CIRCULAR NEIGHBOUR-JOINING PHYLOGENETIC TREE 58
FIGURE 2.13. RECTANGULAR NEIGHBOUR-JOINING PHYLOGENETIC TREE. 60
FIGURE 2.14. ELECTROPHORETIC GEL SHOWING PCR PRODUCTS AMPLIFIED WITH 16S RRNA PRIMERS (POSITIVE CONTROL) AND THE PRIMERS DESIGNED TO AMPLIFY CAS9 AT AN ANNEALING TEMPERATURE OF 55 °C 63
FIGURE 2.15. ELECTROPHORETIC GEL SHOWING THE PCR PRODUCTS AMPLIFIED WITH DESIGNED PRIMERS AT AN ANNEALING TEMPERATURE OF 50 °C 64

List of tables

TABLE 2.1. RELATIVE ABUNDANCE OF CLASSES AND GENERA AT DIFFERENT DEPTHS DURING ANTOM-2 47
TABLE 2.2. RELATIVE ABUNDANCE OF CLASSES AND GENERA AT TWO STATIONS DURING PI-ICE 51
TABLE 2.3. RELATIVE ABUNDANCE OF CLASSES AND GENERA AT DIFFERENT LOCATIONS DURING QIKICE 53
TABLE 2.4. PERCENTAGE IDENTITY OF EACH GENUS FROM QIKICE 54
TABLE 2.5. PRIMERS DESIGNED FOR CAS9 DETECTION 62

List of Abbreviations

ABI	Application Binary Interface
ACT	Alignment, Classification and Tree Service
BLAST	Basic Local Alignment Search Tool
BR	Brine
DCM	Deep Chlorophyll Maximum
DE3	Depth 3
DE4	Depth 4
DE5	Depth 5
IMG	Integrated Microbial Genome system
MEGA11	Molecular Evolutionary Genetics Analysis Version 11
MQ	Milli-Q Water
NA	Not Available
NCBI	National Center for Biotechnology Information
NJ	Neighbour-Joining method
QIK	QikIce expedition
SI	Sea Ice
SRF	Surface
ST or St	Station
STD	Standard Deviation
SW	Sea Water

Chapter 1 - General Introduction

1.1. Relevance of Polar Ecosystems in the Context of Climate Change

The polar regions, comprising the Arctic and Antarctic, are extreme environments characterized by low temperatures, low availability of carbon sources, and extreme seasonality in light conditions (Hodson et al., 2008; Verde et al., 2016). Situated at the Earth's highest latitudes, these regions play a crucial role as regulators of global climate. One of their significant impacts lies in their influence on the generation and maintenance of marine currents that distribute cold waters across different latitudes. Thus, the presence of sea ice contributes significantly to climate regulation through its reflective properties and thermal insulation effects (Meredith et al., 2019).

1.1.1. Overview of Polar Regions

The Arctic and Antarctic regions (Fig. 1.1) have a large geographical coverage, encompassing 20% of the global ocean surface and 69% of the world's glacier area. Moreover, they include land areas with the most persistent winter snow cover (Meredith et al., 2019).

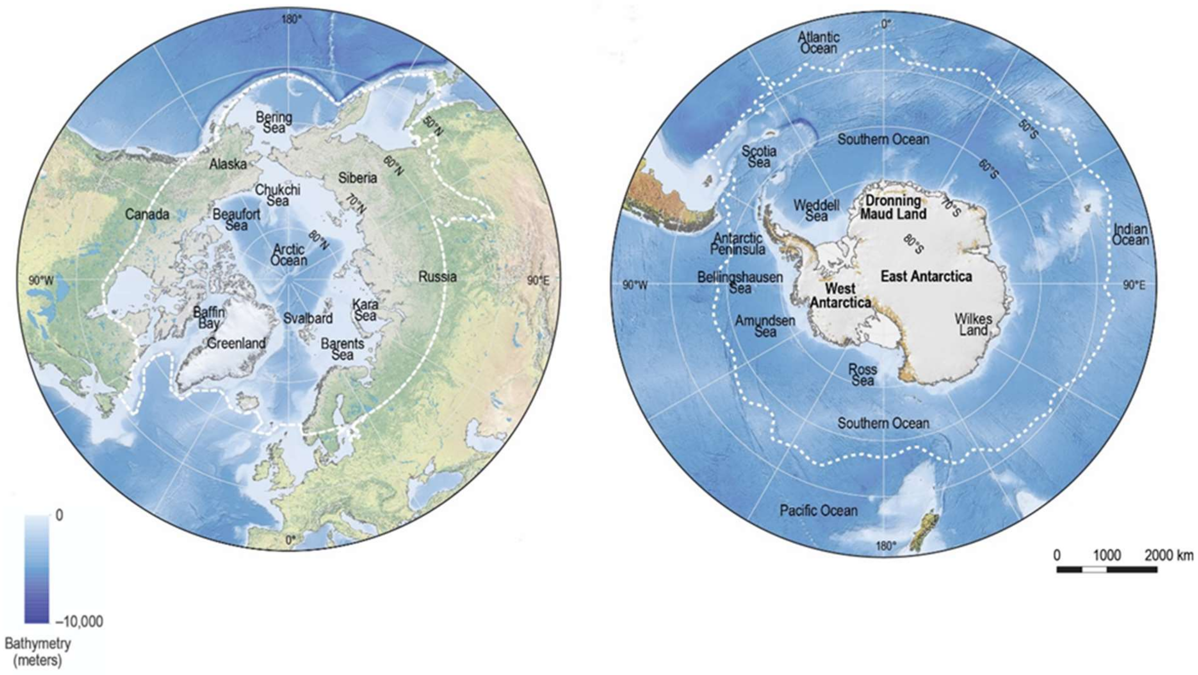

Figure 1.1. The Arctic (left) and Antarctic (right) polar regions *(Adapted from* Meredith et al., 2019 using Biorender*).*

1.1.2. The effects of global change on the Polar regions

Currently, polar regions serve as indicators of global climate change due to their sensitivity to atmospheric and oceanic conditions. Surface water temperatures have been steadily increasing over much of the Arctic, while significant increases in Antarctic temperatures have been concentrated in the Antarctic Peninsula region (Turner et al., 2007; Walsh, 2009; Maksym, 2019). This region has experienced some of the most pronounced increases in near-surface air temperature globally over the past 50 years (Turner et al., 2005), despite a temporary pause in this upward trend over the past two decades.

Satellite observations since 1979 have recorded a steady decline in the extent of sea ice, which represents the total area of the Arctic with at least 15% sea ice concentration, in all months of the year (Barber et al., 2017; Comiso et al., 2017; Stroeve & Notz, 2018). Meanwhile, permafrost thawing is releasing significant amounts of greenhouse gases into the atmosphere, including methane and other short-lived hydrocarbons with devastating effects on atmospheric concentrations (Schuur et al., 2015). Climate model projections suggest that the Arctic Ocean could experience summer without sea ice by the second half of this century (Notz & Community, 2020). This dramatic change is expected to have significant consequences for the dynamics of phytoplankton blooms and the cycling of carbon in surface waters.

In addition, polar regions, especially the Arctic, are highly vulnerable to ocean acidification caused by anthropogenic CO_2 emissions. This vulnerability is aggravated by the increased solubility of CO_2 in cold waters, leading to a decrease in pH (acidification) and carbonate ion (CO_3^{2-}) concentration (Shadwick et al., 2013). As revealed by recent studies, the occurrence of aragonite undersaturation in approximately 20% of surface waters in the Canada and Makarov Basins, accompanied by significant sea ice melt, highlights the ecological concerns for organisms that rely on calcium carbonate ($CaCO_3$) for the formation of their shells and skeletal structures (Robbins et al., 2013).

Finally, anthropogenic pollutant inputs have significantly impacted the Arctic and Antarctic, transforming them into pollutant sinks (Xie et al., 2022). These regions exhibit a wide range of pollutants, including persistent organic pollutants (POPs), which are known for their persistence, high toxicity, potential for bioaccumulation, and tendency for long-range environmental transport (Arnot et al., 2011; S. M. Bengtson Nash et al., 2017). In addition, emerging organic contaminants (EOCs) are widespread in both Arctic and Antarctic environments and biota (S. Bengtson Nash, 2011; Wania, 2007; Wania & Dugani, 2003).

1.2. Characterize and adaptation of psychrophilic microorganisms

Psychrophiles are microorganisms that thrive in cold environments and exhibit optimal growth at temperatures approximately below 15°C (Morita, 1975). To survive and grow in these extreme conditions, they have developed adaptive strategies to maintain essential cellular functions at low temperatures (Tehei & Zaccai, 2005). These mechanisms enable them to withstand stressors such as desiccation, radiation, excessive UV exposure, pH fluctuations, osmotic pressure changes, and limited nutrient availability (Morgan-Kiss et al., 2006; Tehei & Zaccai, 2005).

The review paper *'Some like it cold: understanding the survival strategies of Psychrophiles'* (De Maayer et al., 2014) effectively summarizes psychrophilic microorganisms' molecular and physiological adaptations. The essential points of this adaptation are presented below.

1.2.1. Molecular adaptation

1.2.1.1. Genome structure

The study of the genome structure of psychrophilic microbes reveals interesting features related to cold adaptation. Some psychrophiles exhibit genomic regions with high G+C content, particularly those associated with informative proteins such as transfer RNA (tRNA), elongation factors and RNA polymerase (Ayala-Del-Río et al., 2010; Rabus et al., 2004). Furthermore, genomic redundancy, characterized by multiple copies of tRNA species and increased diversity of chaperones (Math et al., 2012), suggests that high translational and post-translational processing capacity may be critical for adaptation and survival in cold environments (De Maayer et al., 2014).

The prevalence of features that contribute to genome plasticity, such as plasmids and mobile genetic elements, has also been noted. Many of these elements and the genes they carry are directly linked to cold adaptation traits, including unsaturated fatty acid biosynthesis (Allen et al., 2009; Math et al., 2012). Finally, horizontal gene transfer (HGT) plays a role in the acquisition of cold survival traits and explains the similarities and differences observed between organisms in low-temperature environments (Zhao et al., 2010).

1.2.1.2. Proteins and enzymes

Psychrophilic enzymes have high structural flexibility, which can be global or restricted to specific regions, and lower thermostability, so they can function at low temperatures (D'Amico et al., 2006; Paredes et al., 2011). Several mechanisms contribute to the achievement of these properties, although their applicability varies between psychrophilic proteins (Chattopadhyay, 2006).

Several psychrophilic enzymes have been found to contain lower levels of arginine and proline. These amino acids form rigid structures through the formation of numerous hydrogen bonds and salt bridges, and their loss in proteins favours conformational flexibility (Huston et al., 2008; Michaux et al., 2008). Psychrophilic proteins also exhibit compositional changes, such as increased levels of asparagine, methionine and glycine. Glycine clustering near the catalytic site enhances local mobility, while a higher lysine-to-arginine ratio reduces hydrogen bonding and salt bridge formation (Mavromatis et al., 2002; Michaux et al., 2008; Siddiqui et al., 2006). Comparative analyses reveal variations in the three-dimensional structures of psychrophilic proteins. These proteins have extensive outer loops with lower proline content, leading to less compact and more flexible structures. The catalytic site and surrounding molecular structures exhibit increased flexibility and mobility, potentially reducing catalytic energy costs (Bauvois et al., 2008; Sonan et al., 2007). Furthermore, high-resolution models have shown that psychrophilic proteins have larger cavities with a higher number of hydrophilic groups. These cavities bind more water molecules, thereby reducing the energy required and lowering the ideal temperature for enzyme activity (Jung et al., 2008; Paredes et al., 2011).

1.2.2. Physiological adaptations

1.2.2.1. Membrane function

The membrane of psychrophilic microorganisms shows specific adaptations, revealed by the upregulation of genes involved in membrane biogenesis, fatty acid and lipopolysaccharide biosynthesis, peptidoglycan biosynthesis, glycosyltransferases and outer membrane proteins (Durack et al., 2013; Frank et al., 2011; Gao et al., 2006). General membrane transport proteins are regulated to compensate for the reduced diffusion rate under cold conditions, while peptide transporters contribute to nutrient uptake and peptidoglycan biosynthesis (Bakermans et al., 2007; Cacace et al., 2010; Durack et al., 2013). In contrast, genes encoding proteins and outer membrane structures are generally repressed in the cold (Fig. 1.2) (Durack et al., 2013; Piette et al., 2011). Carotenoid pigments are another type of modulator of membrane fluidity. They have been found in Antarctic bacteria and are thought to buffer membrane fluidity and maintain homeoviscosity during temperature changes (Fig. 1.2) (Chattopadhyay, 2006; Rodrigues & Tiedje, 2008). Wax esters also contribute to cold-regulated membrane fluidity (Ayala-Del-Río et al., 2010).

1.2.2.2. Cryoprotectants and antifreeze proteins

Several cryoprotective mechanisms are used to protect cells from freezing and osmotic imbalance (Klähn & Hagemann, 2011). In particular, the accumulation of compatible solutes such as glycine, betaine, sucrose and mannitol lowers the cytoplasmic freezing point, protecting against freezing, desiccation and hyperosmolarity (Fig. 1.2) (Casanueva et al., 2010; Cowan, 2009). In addition, trehalose, a disaccharide, acts as a cryoprotectant by preventing protein denaturation, scavenging free radicals and stabilizing cell membranes in cold conditions (Kandror et al., 2002).

Some psychrophiles can also produce antifreeze proteins (AFP), which control ice crystal growth by lowering the freezing point of the aqueous based cytoplasm (thermal hysteresis), and ice nucleation proteins (INs), which facilitate ice crystal formation at temperatures near the melting point. These AFP and IN proteins play a critical role in preventing supercooling of water and controlling ice crystal formation, respectively (Celik et al., 2013; Kawahara, 2002).

Another cryoprotective mechanism is the production of exopolysaccharides (EPS), which lower the freezing point, trap water and nutrients, facilitate biofilm formation and protect extracellular enzymes (De Los Ríos et al., 2004; Mancuso Nichols et al., 2005; Qin et al., 2007). EPS can alter the microstructure of ice, reducing its permeability and increasing salt retention. This significantly affects organisms' ability to colonize and thrive in cold environments, giving them a distinct advantage in icy habitats (Ewert & Deming, 2011; Krembs et al., 2011).

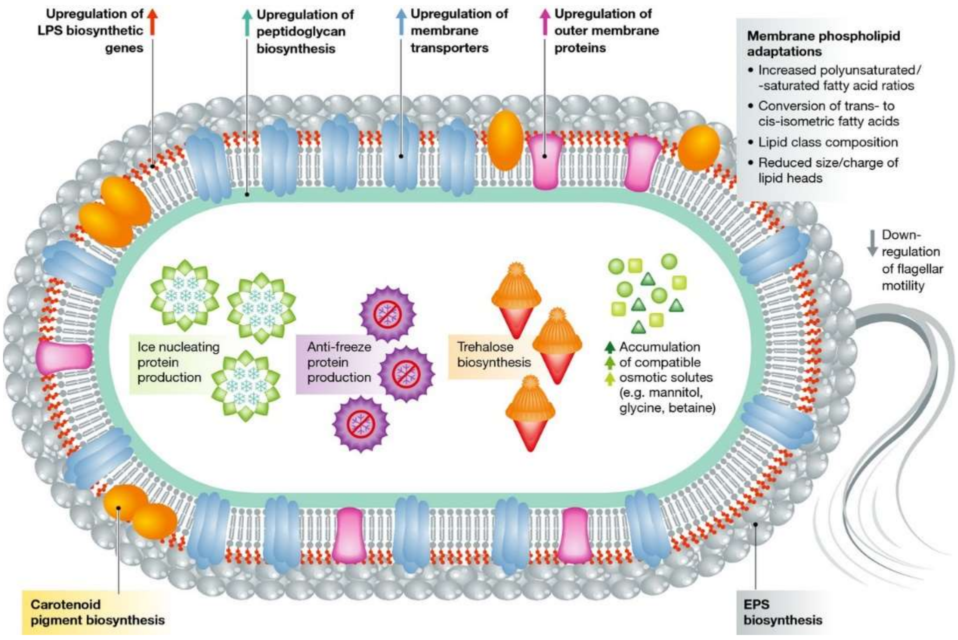

Figure 1.2. Common physiological adaptations in a psychrophilic prokaryote *(Source:* De Maayer et al., 2014*)*.

1.3. The polar ocean microbiome

1.3.1. Seasonal Diversity of Microorganisms in Polar Seawater and Sea Ice

Life in polar regions faces unique challenges due to the extremes of prolonged winters characterized by ice cover, darkness, low water temperatures, and contrasting summer conditions with extended sunlight exposure. These environmental factors result in significant seasonal variations in microbial properties and the composition of bacterial communities in polar regions (Alonso-Sáez et al., 2008; Ghiglione et al., 2012).

1.3.1.1. Microbial Communities of the Seawater

Microbes in the polar oceans, particularly those inhabiting the upper water column, are subject to a constantly changing environment (John Wiley & Sons, 2016). During winter, the limited availability of light limits primary productivity, resulting in minimal carbon export from the surface layers. This condition favours the growth of heterotrophic bacteria and archaea, such as mixotrophs and chemolithotrophs (Fig. 1.3) (Boetius et al., 2015; Riedel et al., 2007). In particular, the ammonia-oxidising MGI Crenarchaeota can comprise up to 16% of bacterioplankton cells in polar surface waters in winter (Alonso-Sáez et al., 2008). Winter bacterial blooms have also been observed, with the *Janthinobacterium* (Betaproteobacteria) population accounting for 20% of bacterial cells in February, although they are barely observed before or after this period (Alonso-Sáez et al., 2014).

In spring and summer, the greater availability of light and higher temperatures stimulates phytoplankton growth (Fig. 1.3). These blooms thrive under the ice cover and in the vicinity of the ice melt, and once the ice has melted, they extend into the open sea, causing significant changes in bacterioplankton communities. Several populations of the *Roseobacter* clade, Alteromonadales and Bacteroidetes show pronounced seasonal variation in polar regions (Alonso-Sáez et al., 2008; Ghiglione et al., 2012).

Indeed, Flavobacteriia and several Gammaproteobacteria showed a significant increase in the proportion of metaproteomes compared to winter (e.g. Alteromonadales increased from 1% to 13%) (Williams et al., 2012). Furthermore, the genus *Polaribacter* plays an important role as one of the dominant prokaryotic species in the Bacteroidetes community after phytoplankton blooms (Boetius et al., 2015; Bunse & Pinhassi, 2017). Limited information is available regarding the bacterioplankton community composition during autumn (Fig. 1.3) (Bunse & Pinhassi, 2017).

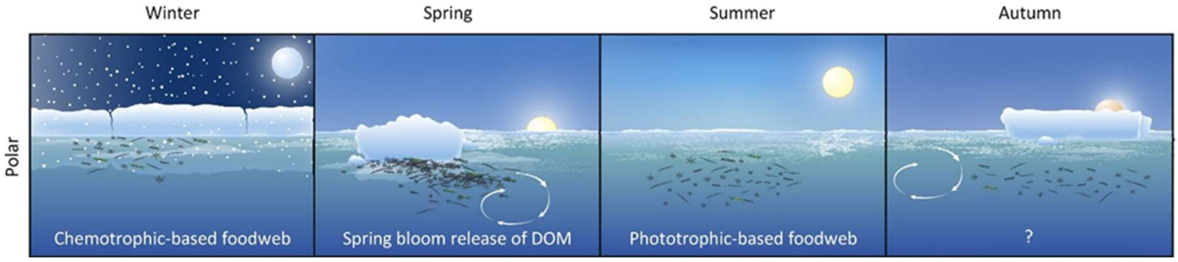

Figure 1.3. Schematic Overview of the Environmental Factors Influencing Seasonal Succession of Marine Bacterioplankton in Polar Regions. *Darkness and ice cover limit phytoplankton primary production in winter. In spring, phytoplankton and bacteria colonize the sea ice, seeding the water column when the ice cover breaks and giving rise to pronounced spring blooms (Source:* Bunse & Pinhassi, 2017*).*

1.3.1.2. Microbial Communities of the Sea Ice

The microbial communities within sea ice undergo dynamic fluctuations due to seasonal changes in temperature, from extreme cold in winter to above freezing during summer melt, and changes in the salinity of the brine trapped in the ice, from high salinity above saturation to freshwater levels in the meltwater (Collins et al., 2008). In winter, brine keeps water in a liquid state in the icy surface despite extremely low temperatures (Deming, 2007; Ewert & Deming, 2014; Junge et al., 2004). This phenomenon occurs because the underlying ocean gradually warms the lower ice layers, creating a favourable habitat. In spring and summer, phototrophs flourish within the brine pores and channels (Arrigo, 2014), establishing the essential base for a food web associated with the ice, which includes heterotrophic bacteria, protists, and small meiofauna (Gradinger, 2001). Bacterial communities in Arctic and Antarctic sea ice are dominated by the classes Flavobacteriia and Gammaproteobacteria, with smaller contributions from Alphaproteobacteria, Verrucomicrobia and Bacilli (Fig. 1.4). Within the Flavobacteriia class, the most abundant genera include *Polaribacter*, *Psychrobacter*, *Psychroflexus* and *Flavobacterium*. In the Gammaproteobacteria class, the dominant genera are *Glaciecola* and *Colwellia* (J. S. Bowman et al., 2011; Brinkmeyer et al., 2003; Cowie et al., 2014; Han et al., 2014).

Thus, although both poles share several bacterial genera, only *Polaribacter irgensii* (Eric Collins et al., 2010; Murray & Grzymski, 2007) and *Colwellia psychrerythraea* have been identified at the species level. Therefore, the extent of polar sea ice microbial diversification remains unknown (Staley & Gosink, 1999) .

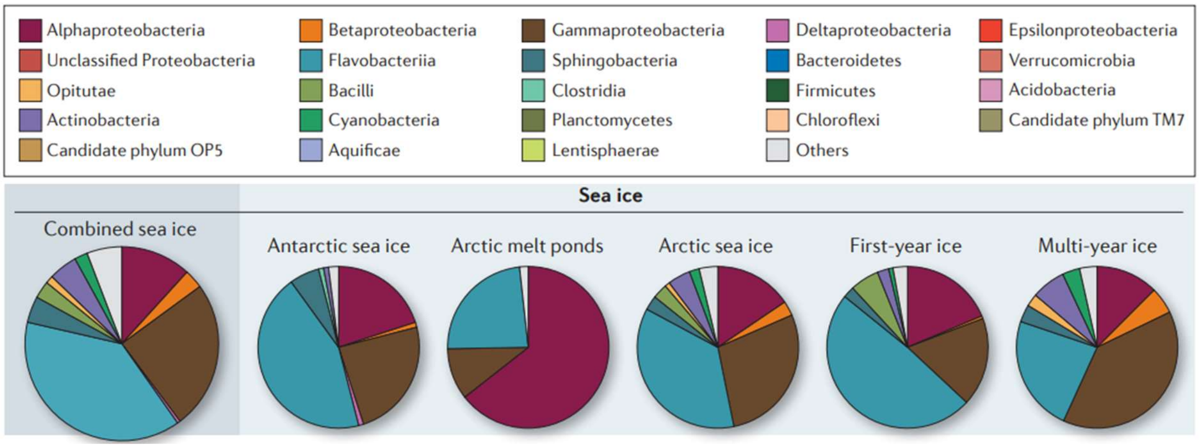

Figure 1.4. Bacterial community structure of sea ice based on 16S rRNA gene surveys. *Pie charts represent relative sequence abundances of different bacterial taxa (Source:* Boetius et al., 2015*).*

1.3.2. Culturing and Molecular Approaches to Assess Bacteria Taxonomy

Culturing and molecular approaches are two different methods used to assess bacterial diversity. Cultivation methods rely on inoculation and incubation of growth media with bacteria, and permits the isolation of individual organisms as well as their direct progeny (Salmonová & Bunešová, 2017). This approach has a number of limitations including the uniformity of bacterial distribution in samples, selectivity of culture media, and the time required to obtain results (Kirk et al., 2004; Mandal et al., 2011). In contrast, molecular techniques such as PCR amplification of the 16S ribosomal RNA gene sequence and metagenomics allow for direct analysis of all bacterial genetic material in a sample. These advanced approaches have revealed that a large number of bacterial phylogenetic groups are often underrepresented in approaches based on culturing (Zhang & Xu, 2008).

The 16S rRNA gene sequence is a culture-independent method, requiring only DNA from the bacteria under test (Sune et al., 2020). The technique has been widely used due to its universal application and the fact it captures most bacterial species, its functional stability, and the suitable size of product (1500 bp) for subsequent computational bioinformatic analysis (Janda & Abbott, 2007). Cumulative results from a limited number of studies have shown that this technique identifies genera in the majority of cases (>90%), while it is less reliable for the identification of species (65 to 83%), with 1-14% remaining unidentified (Drancourt et al., 2000; Mignard & Flandrois, 2006; Woo et al., 2003). On the other hand, metagenomics sequences DNA directly from environmental samples, facilitating the determination of microbial community composition at the species or strain level (Handelsman, 2004).

Recent studies have provided significant insights into polar microbial diversity using metagenomics. Notably, Royo et al. 2021 published the first metagenomics paper of polar microbial diversity reconstructing 530 bacterial and archaeal MAGs derived from 41 Arctic seawater samples, collected at various depths in different seasons during the Tara Oceans Polar Circle expedition. Together with the analyses of 33 metatranscriptomes, this study evaluated the ecology, metabolic potential and activity of resident bacteria and archaea in the Arctic polar regions and revealed 62 MAGs classified as key Arctic species, found only in the Arctic Ocean (Royo-Llonch et al., 2021). Subsequently the Ocean DNA MAG catalogue released a massive collection of 7,752 prokaryotic genomes derived from 129 metagenomes specifically obtained from polar regions (Nishimura & Yoshizawa, 2022).

While Metagenomics offers valuable information, it also faces challenges such as the limited detection of low-abundance microorganisms, the requirement for advanced sequencing platforms, and the complexity of determining microbial abundance, influenced by various factors including environmental conditions (Zhang et al., 2021). Despite the significant advancements in molecular techniques, many environmental microorganisms remain uncultivable (Hug et al., 2016). Nonetheless, the study of cold-adapted microorganisms is of high interest due to their potential for biotechnological applications (Newman, 2016) and their ability to withstand environmental challenges such as radiation, desiccation, and extreme temperatures, which also impact global chemical cycles (Mykytczuk et al., 2013; Paulino-Lima et al., 2016; Pulschen et al., 2015; Rothschild & Mancinelli, 2001). Therefore, the cultivation of these microbes in a laboratory setting is crucial in order to gain insights into their growth preferences, metabolic activities, and physiological characteristics (Baker et al., 2015; Stewart, 2012; Vartoukian et al., 2010; Wade, 2002).

1.4. Polar microorganisms as a unique source for blue biotechnology

Blue biotechnology is concerned with exploring and harnessing the potential of marine organisms to develop new products of relevance for society. The discovery of organisms containing commercially interesting molecules and genes is increasing in parallel with the exploration of marine biodiversity. The global marine biotechnology market is already significant, spanning a broad range of commercial purposes including pharmaceutical, biofuel, and chemical industries (Hurst et al., 2016; Vierros et al., 2016).

As of 2017, a total of 12,998 genetic sequences from 862 marine species have been matched to patents with international protection filed under the Patent Cooperation Treaty. Microbial species represent 19% of all species listed in the World Register of Marine Species (WoRMS) but accounts for over 73% of all patented sequences in the databases (Blasiak et al., 2018). These data highlight the enormous potential of marine microbes for biological applications. A remarkable example of this potential lies in the discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) by Francisco Juan Martínez Mojica in the 1990s during his research on halophilic archaea in salt pans (Mojica et al., 1993). This observation paved the way for future CRISPR studies that led to today's gene-editing techniques.

1.4.1. CRISPR/Cas technology

CRISPR-Cas systems, including CRISPR-Cas9, function as an adaptive immune response system that protects prokaryotes from external agents and can be repurposed as a powerful genome-editing tool. The heart of this technology is the Cas nucleases, which are crucial for binding to and cutting DNA at specific sites in the genome. Exogenous DNA, entering a bacterium, is initially cut by the Cas nuclease, and inserted into the CRISPR DNA sequence, which contains repeated bacterial sequences interspersed with divergent spacer sequences from previously encountered phage regions (Fig. 1.5). The phage spacer and adjacent bacterial repeat sequences are transcribed into a crRNA (CRISPR RNA).

During a second infection, the crRNA recognizes the complementary sequence in the phage DNA. The bacterial portion of this hybrid RNA binds with a small RNA called tracrRNA (trans-activating crRNA) (Fig. 1.5). The tracrRNA recruits the Cas9 nuclease, which cuts the phage DNA at a region complementary to the crRNA, before the PAM (Protospacer-Adjacent Motif) recognition sequence (Fig. 1.5). This avoids inappropriate digestion of self-DNA (Maccarrone, 2019).

To enable gene editing applications, the two separate RNA components (crRNA and tracrRNA) can be combined to form a single guide RNA (sgRNA or gRNA). This determines the target genomic sequence based on complementarity with its spacer, while cleavage can only occur in the presence of PAM. Numerous type II CRISPR systems have been discovered in bacterial groups, but the SpCas9 system (derived from *Streptococcus pyogenes*) remains the most widely utilized. This is primarily due to its well-understood biochemistry and the presence of a short PAM sequence, 5′-NGG-3′, where 'N' represents any nucleotide base (Jinek et al., 2012).

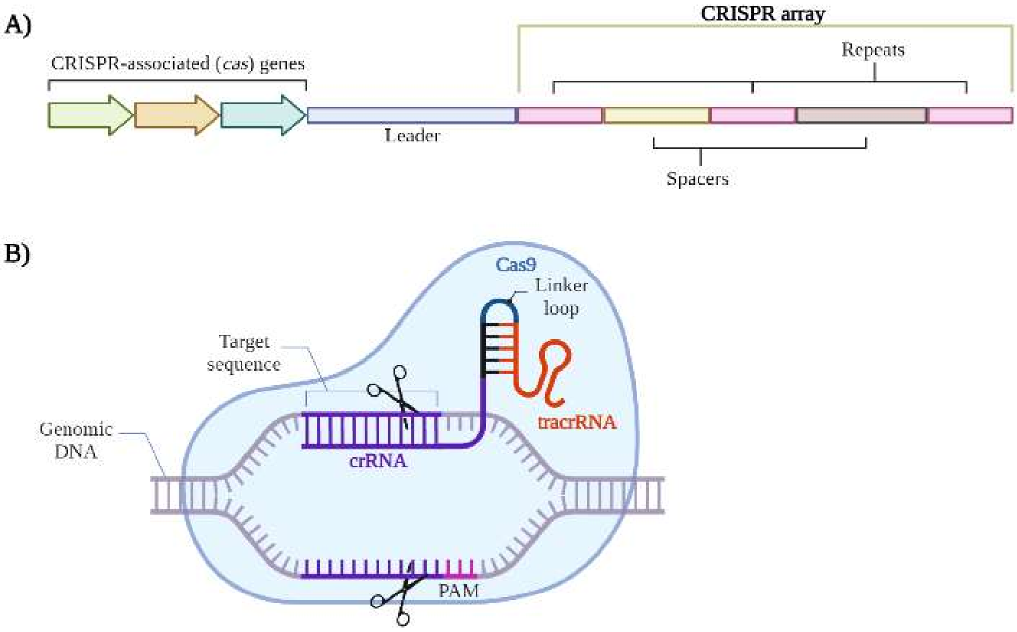

Figure 1.5. A) CRISPR locus overview. B) Elements of the CRISPR/Cas9 system *(Source: Prepared by the author using BioRender).*

1.4.1.1. Bacterial Applications

There is a broad range of applications using the CRISPR-Cas systems (see Figure 1.6 from the review *'Diversity of CRISPR-Cas immune systems and molecular machines'* (Barrangou, 2015). In the realm of food production, they can increase phage resistance in starter cultures, for example, by protecting yoghurt strains from bacteriophages. Additionally, they are useful in reducing the uptake and spread of unwanted plasmids containing antibiotic resistance genes. CRISPR-Cas systems also help maintain genomic integrity and stability by protecting cultures from mobile genetic elements such as transposons and prophages. In the industrial sector, CRISPR-Cas technologies are used for precise genome editing and the optimization of synthesis capacity and yield through the redirection of metabolic pathways. The approach enables efficient targeted gene modification to enhance desired traits.

The use of CRISPR activation (CRISPRa) and interference (CRISPRi) approaches provide precise control of gene expression, enabling the manipulation of gene activity. Another significant advantage of CRISPR-Cas systems is their self-targeting capability which facilitates the selective elimination of specific pathogens or contaminants.

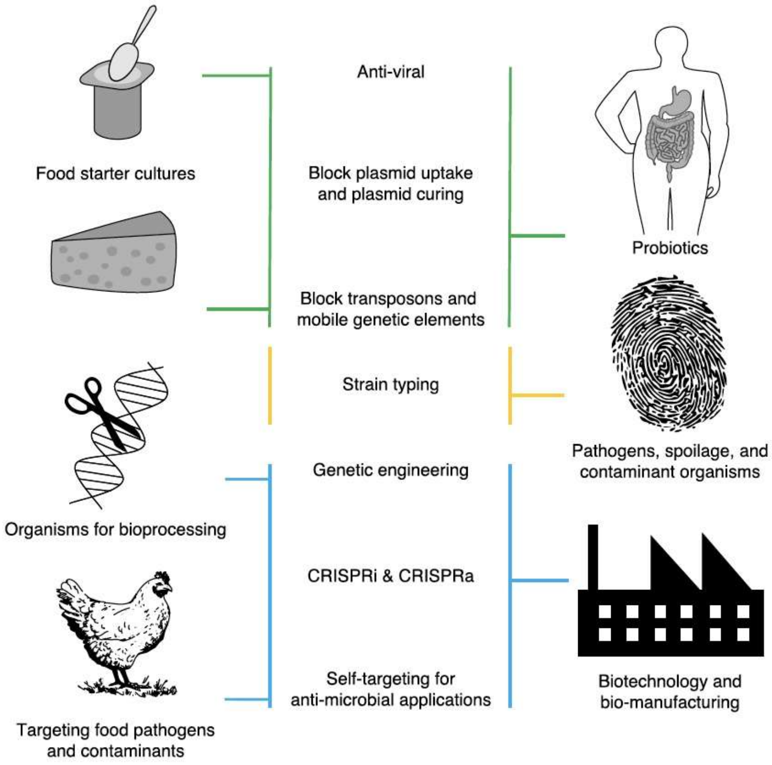

Figure 1.6. Exploitation of endogenous and engineered CRISPR-Cas systems in bacteria *(Source:* Barrangou, 2015*).*

1.4.1.2. Limitations

The range of sequences that Cas9 can address is limited by the recognition of PAM (protospacer adjacent motif) sequences. Furthermore, careful site selection is required to reduce off-target binding and cleavage, considering the level of tolerance for mismatches within the gRNA-PAM-target complex (Anzalone et al., 2020; Kim et al., 2019). This limitation is especially evident in therapeutic applications, where even infrequent genomic changes caused by off-targeting are undesirable (Kumar et al., 2019). In addition, precise site selection becomes critical in homology-directed repair (HDR) applications, where the proximity of the Cas nuclease cut site to the desired editing site greatly influences the outcome (Gasiunas et al., 2020). Although efforts are being made to overcome the limitations of SpCas9 targeting, the diversity offered by naturally occurring orthologues may improve the versatility of CRISPR-Cas systems by extending the range of editing options (Gasiunas et al., 2020).

The diversity of the genera from which 79 Cas9 proteins were identified in the article *'A catalogue of biochemically diverse CRISPR-Cas9 orthologs'* (Gasiunas et al., 2020) is shown in the table below (Fig. 1.7). To determine their protospacer adjacent motif (PAM) and guide RNA (gRNA) requirements, a cell-free biochemical screen was performed, revealing the existence of at least 7 different gRNA classes and 50 different PAM sequence requirements. Despite the large number of species considered in Gasiunas et al., 2020, no polar bacteria are represented in the study. Therefore, exploring this uncharted area has the potential to provide unprecedented insights into the field of CRISPR, offering the potential to discover unique CRISPR-Cas9 orthologs, expanding the range of its applications and advancing the understanding of this powerful genetic tool.

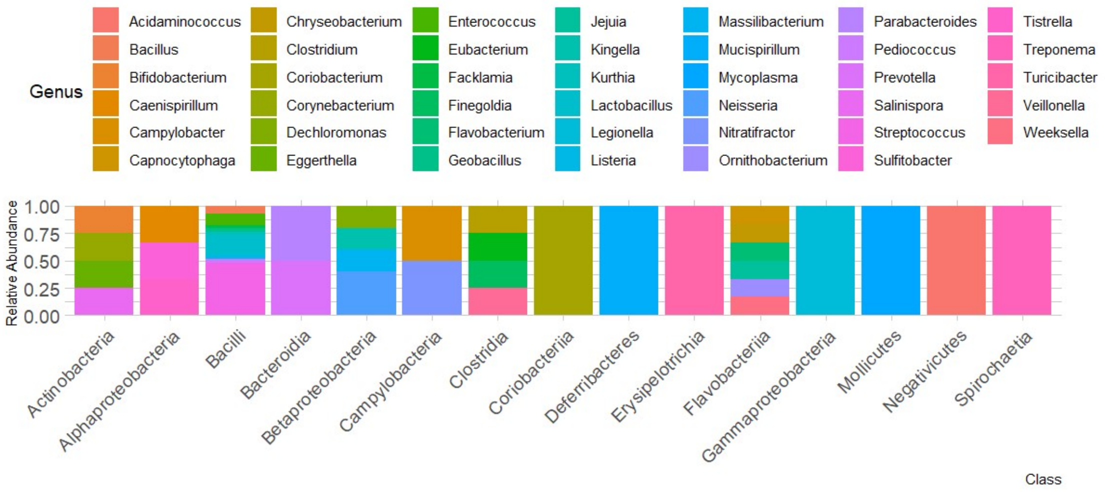

***Figure 1.7. Genera from which 79 Cas9 proteins have been derived** (Source:* Gasiunas et al., 2020*). Graph prepared by the author using RStudio.*

1.5. Project and Objectives

This research is part of the "Polar EcoGen" project, which stands for "Ecogenomics and Evolution of Key Polar Uncultured Prokaryotes". The project is funded by the Spanish National Plan I+D+I 2020 under the grant PID2020-116489RB-100, which covers the period from 2021 to 2024. The Principal Investigator leading this project is Dr Silvia G. Acinas from Institute of Marine Science (ICM)-CSIC in Barcelona, Spain.

The present research includes samples collected during three separate expeditions between 2019 and 2023: the Antarctic cruises (PI-ICE and ANTOM-2) and the Arctic cruise (QikIce).
The PI-ICE cruise was led by Dr Manuel Dall'Osto, in collaboration with Dr Dolors Vaqué, and Dr Elisa Berdalet all of them researches from the ICM-CSIC. This expedition took place along the Antarctic Peninsula from February to March 2019.
The ANTOM project, led by Dr Jordi Dachs and Dr Maria Vila, consisted of two cruises. ANTOM-1 covered the North and South Atlantic from December 2020 to January 2021, while ANTOM-2 focused on the Antarctic Ocean from January to February 2022.
The QikIce mission, carried out near Qikiqtarjuaq, Nunavut, from April to May 2023, was led by Dr Marcel Babin in collaboration with Dr. Silvia Acinas team with the participation of Dr. Pablo Sanchez from the ICM-CSIC.

The primary aim of this study is to improve our knowledge of the heterotrophic bacterial taxa inhabiting polar regions and to investigate the presence of Cas9 orthologs specifically adapted to this extraordinary environment. This will be achieved by pursuing the following objectives:

1. Investigate differences in the cultivability of bacteria collected from different depths, habitats and in different media.

2. Use a combination of culture-dependent and culture-independent techniques to assess microbial diversity in polar regions.

3. Measure the degree of sequence similarity between isolated strains.

4. Design primers to amplify Cas9 genes within the genomes of cultured bacteria.

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Diversity of cultured heterotrophic bacteria from polar sea waters and sea ice ecosystems: A unique source for blue biotechnology

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Abstract

The discovery of the CRISPR-Cas system, a prokaryotic defense mechanism, has revolutionized genomics and biotechnology. This system uses Cas nucleases to precisely cut DNA sequences by relying on a protospacer adjacent motif (PAM). Cas9, especially SpCas9 from *Streptococcus pyogenes*, has been widely studied for genome engineering due to its well-understood biochemistry and short PAM sequence. However, its limitations in the recognition of PAM sequences have led researchers to look for natural orthologs of Cas9. This study focuses on the remote areas of the polar regions, where extreme conditions result in unique environments. During three expeditions - PI-ICE (2019) and ANTOM-2 (2022) in Antarctica, and QikIce in the Arctic (2023) – seawater, sea ice and brine samples were collected. Subsequent cultivation showed differences in bacterial growth and taxa enrichment between seawater and sea ice heterotrophic bacteria. Furthermore, the taxonomic analysis of 199 colonies revealed similarities and variations in their biodiversity across habitats, depths and regions. In particular, in ANTOM-2, seawater samples from different depths showed a prevalence of Gammaproteobacteria in deeper layers, while Alphaproteobacteria dominated the photic zones. Dominant genera included *Sulfitobacter*, *Pseudoalteromonas* and *Shewanella*. Five out of seven deep-sea genera were found in photic zones, including *Bacillus*, *Pseudoalteromonas*, *Pseudomonas*, *Psychroserpens* and *Winogradskyella*. In contrast, sea ice isolates from PI-ICE showed an absence of Alphaproteobacteria, with Flavobacteriia and Gammaproteobacteria dominating. Notable genera were *Flavobacterium*, *Psychrobacter* and *Chryseobacterium*. During the QikIce expedition, Bacilli and Flavobacteriia were predominant, while three genera differed from those found in Antarctica: *Planomicrobium*, *Maribacter* and *Octadecabacter*. In the final phase of the study, the design of two primer pairs led to the amplification of potential Cas9 sequences from three *Flavobacterium* isolates, opening up new avenues for future efforts. This research highlights the importance of exploring extreme environments, such as polar regions, for biotechnological discoveries and expanding the CRISPR-Cas toolbox.

Keywords: Bacterial diversity, Polar regions, Photic layers, Deep layers, Sea ice, Cas9

2.1. Introduction

Blue biotechnology aims to explore the potential of marine organisms for societal benefit. Marine microbes in particular have emerged as a promising avenue, accounting for 73% of patented sequences in the World Register of Marine Species (WoRMS) (Blasiak et al., 2018). The significance of their potential was further highlighted by the discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in the 1990s, a pioneering finding by Francisco Juan Martínez Mojica (Mojica et al., 1993).

CRISPR-Cas systems, which have been described as the adaptive immune system of prokaryotes, are powerful tools for genome editing. Cas nucleases, the heart of this revolutionary technology, play a key role in precisely binding to and cleaving DNA at specific genomic locations (Maccarrone, 2019). Among these nucleases, Cas9, and in particular SpCas9 derived from *Streptococcus pyogenes*, has been widely used. This is primarily due to its well-understood biochemistry and the presence of a short PAM sequence, 5′-NGG-3′, where 'N' represents any nucleotide base (Jinek et al., 2012).

However, the ability of Cas9 to target sequences is limited by the recognition of PAM sequences, requiring careful site selection to mitigate unintended binding and cleavage (Anzalone et al., 2020; D. Kim et al., 2019). To overcome these limitations, researchers are actively exploring naturally occurring Cas9 orthologs, which offer the potential to expand the range of editing possibilities and increase the adaptability of CRISPR-Cas systems (Gasiunas et al., 2020).

As a result, the search for organisms harboring commercially valuable molecules and genes, such as new CRISPR-Cas9 orthologs, is increasing in parallel with the exploration of marine biodiversity. This quest not only underscores the promise of blue biotechnology but also represents a dynamic frontier in genetic research and biotechnology innovation.

Polar regions, which include the Arctic and Antarctic, provide a fitting backdrop for this investigation due to their extreme conditions, characterized by low temperatures, limited carbon sources and highly seasonal light conditions (Hodson et al., 2008; Verde et al., 2016). These regions are home to unique microbial species, known as psychrophiles, which have developed adaptive strategies to thrive in these harsh environments (Tehei & Zaccai, 2005). These strategies enable them to withstand various stressors such as desiccation, radiation, excessive

UV exposure, pH fluctuations, osmotic pressure changes and limited nutrient availability (Morgan-Kiss et al., 2006; Tehei & Zaccai, 2005).

These challenging environmental conditions drive significant seasonal variations in microbial properties and community composition (Alonso-Sáez et al., 2008; Ghiglione et al., 2012), for instance, affecting the diversity of the upper water column (John Wiley & Sons, 2016). During winter, low light availability limits primary productivity and favors the growth of heterotrophic bacteria and archaea (Boetius et al., 2015; Riedel et al., 2007). Conversely, spring and summer, characterized by increased light and higher temperatures, favor the growth of phytoplankton. These blooms, which thrive under ice cover and in ice-melting regions, cause major shifts in bacterioplankton communities, in particular leading to significant increases in certain bacterial groups such as Flavobacteriia and several Gammaproteobacteria (Williams et al., 2012).

In the realm of sea ice, bacterial communities in both Arctic and Antarctic sea ice consist primarily of Flavobacteriia and Gammaproteobacteria, with smaller contributions from Alphaproteobacteria, Verrucomicrobia and Bacilli. Important genera within these classes include *Polaribacter*, *Psychrobacter*, *Psychroflexus*, *Flavobacterium*, *Glaciecola* and *Colwellia* (Bowman et al., 2011; Brinkmeyer et al., 2003a; Cowie et al., 2014; Han et al., 2014).

Despite shared bacterial genera between polar regions, only a few species, such as *Polaribacter irgensii* (Eric Collins et al., 2010; Murray & Grzymski, 2007) and *Colwellia psychrerythraea*, have been identified at the species level. As a result, the full extent of diversification within polar sea ice microbial communities remains largely unknown (Staley & Gosink, 1999).

This preliminary study, part of the "Ecogenomics and Evolution of Key Polar Uncultivated Prokaryotes" (Polar EcoGen) project, aims to **1)** investigate variations in cultivability among bacteria from samples collected at different depths and habitats during three different expeditions (ANTOM-2, PI-ICE and QikIce) from 2019 to 2023; **2)** use a combination of culture-dependent and culture-independent methods to assess microbial diversity in polar regions; **3)** assess the degree of sequence similarity among isolated strains; and **4)** develop primers for amplification of Cas9 genes within the genomes of cultured bacteria.

These efforts offer an exciting avenue for exploring the potential of these extreme bacteria in the context of blue biotechnology, particularly in the search for new CRISPR-Cas9 orthologues adapted to these unique environments.

2.2. Methods

2.2.1. Study Area and Sampling

The study area covered both polar regions: Antarctica and the Arctic. In Antarctica, a total of 27 samples were collected during two cruises. The first cruise, ***PI-ICE***, was conducted from 4 February to 11 March 2019. During this expedition, two sea ice samples (SI9 1B and SI9 4B) were collected at Station 9 (63°24'09.4"S 56°49'01.6"W), located near the entrance to the Weddell Sea from the Bransfield Strait (Fig. 2.1 (A)).

The second cruise, ***ANTOM-2***, took place from 27th January to 5th February 2022. Sampling stations 5, 7, 10, 11 and 12 in the Bellingshausen Sea (between 57°18'W and 102°20'W), along the western side of the Antarctic Peninsula, were selected (Fig. 2.1 (B)). A total of 12 photic layers, 10 mesopelagic and 3 bathypelagic samples were collected during this expedition. Based on the depth at which the seawater was collected, five distinct vertical profiles were identified: Surface (SRF), Deep Chlorophyll Maximum (DCM), Depth 3 (DE3), Depth 4 (DE4) and Depth 5 (DE5) (Fig. 8 (B)).

Finally, during the ***QikIce*** expedition in the Arctic, a total of 6 samples were collected southwest of Qikiqtarjuaq (67°28'55.0"N 63°58'36.7" W), known as Broughton Island, at station QI0200 (Fig. 2.1 (C)). These samples included slices from sea ice core, brine, and seawater. The collection took place from 22nd to 25th April 2023. The sea ice core samples were collected from three distinct depths: “1-10 cm”, in direct contact with seawater, the depth range of “10-40 cm”, and the depth range of “70-100 cm”. In addition, two brine samples were taken and their salinity was measured, with one sample having a salinity of 76‰ and the other 62‰ (Fig. 2.1 (D)).

Seawater, sea ice, and brine were prefiltered during the sample collection process using a 200 µm mesh filter to remove larger plankton cells. After prefiltering, each sample was collected in triplicate and placed in 2ml cryovial tubes with dimethyl sulfoxide (DMSO) at a final concentration of 7.5%. The cryovial tubes were then stored at -70 °C until processing in the laboratory.

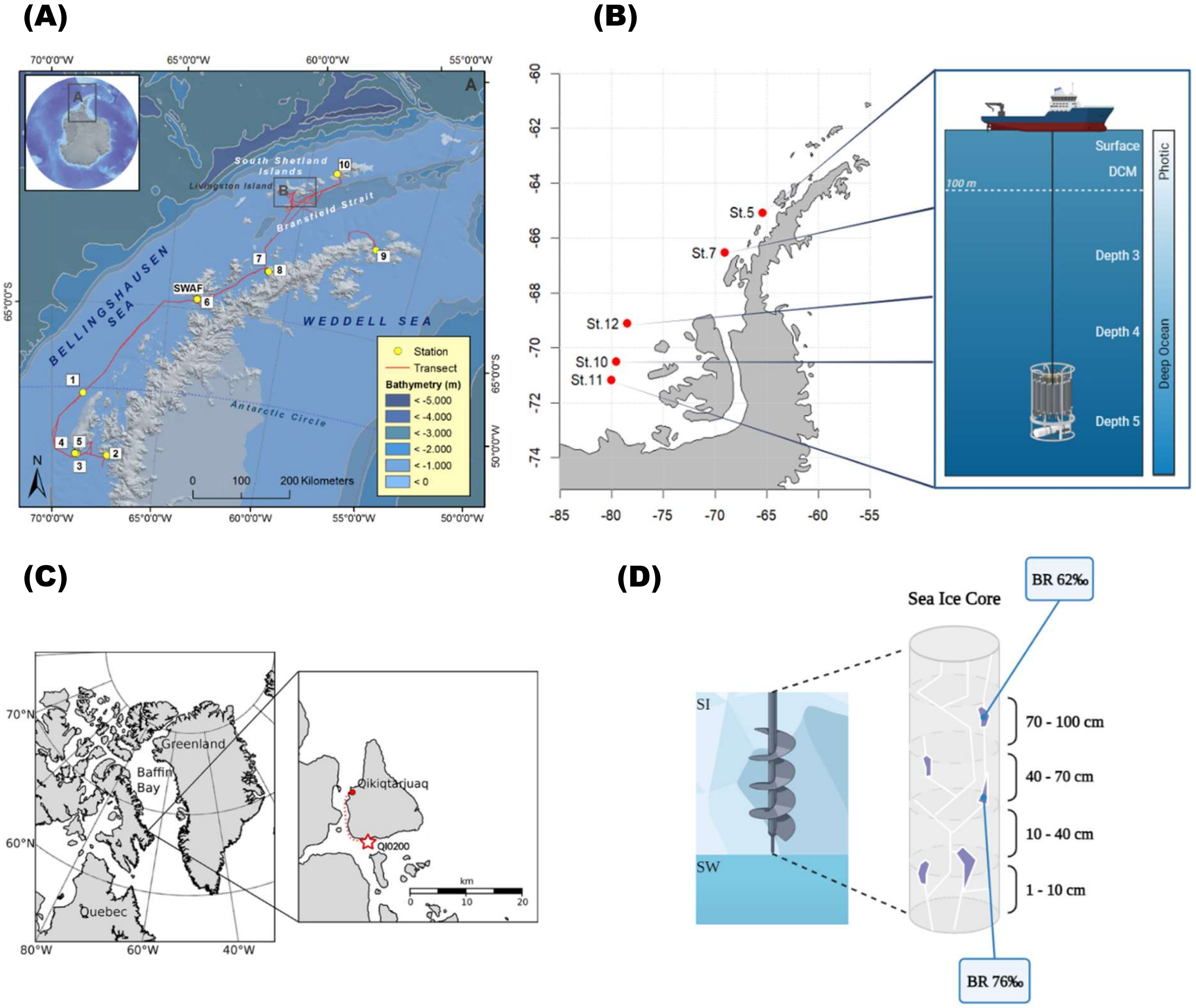


Figure 2.1. *(**A**) Map of the PI-ICE Expedition. Source: Created by Ana Sotomayor-Garcia. (**B**) Map of selected stations of the ANTOM-2 expedition and the five vertical profiles. Source: Created by the author using RStudio and Biorender. (**C**) Location of station QI0200 near the island of Qikiqtarjuaq. Source: Adapted from* Vérin et al., 2022 *using Biorender. (**D**) Sea ice core, brine (from sea ice, SI) and seawater (SW) from which samples were collected during the QikIce expedition. Source: Created by the author using Biorender.*

2.2.2. Culturing and isolation of bacteria

2.2.2.1. Conventional Culturing Strategy in Medium

Three different media were prepared for bacterial growth. For seawater bacteria, 37.4 g of Marine Broth 2216 powder and 15 g of Bacto Agar (Difco™) powder were suspended in 1L of sterile Milli-Q water (Power & Johnson, 2009). For sea ice bacteria, the culture medium was prepared by adding 18.2 g of R2A Agar (Difco™) to 1L of MQ water (Power & Johnson, 2009). Given the high salinity of the brine samples, the third medium was prepared by mixing Marine

Broth with Sodium Chloride to a salinity of approximately 70‰, followed by the addition of Bacto Agar. The three media were then heated and stirred frequently before boiling for 1 minute to fully dissolve the powdered media. Then, they were autoclaved at 121°C for 20 minutes, thoroughly agitated and then autoclaved again at 121°C for 20 minutes. The media was poured into sterile petri dishes and left to cool for at least 30 minutes, until complete solidification. The use of two different types of media, Marine Broth and R2A, was influenced by the distinct environmental conditions in which the samples were collected.

The elevated levels of Peptone and Yeast extract and salt in Marine Broth means it provides nitrogen, vitamins, minerals and high salt and resembles the natural marine habitat. This medium offers the necessary nutrients to support the proliferation of marine microorganisms and facilitates the retrieval of a wide array of marine bacteria. In contrast, R2A media is specifically formulated as a low-nutrient agar to restrict the growth of rapidly multiplying bacteria. It enables the cultivation of nutritionally demanding or slower-growing bacterial species, incorporating proteins, yeast extract, and peptone, but at lower levels compared to Marine Broth. The lower-nutrient composition of R2A media mimics the nutrient limitations found in a sea ice environment, creating a growth environment that resembles the conditions within the sea ice matrix.

2.2.2.2. Isolation of bacteria

Isolates were obtained by plating 100 µL of undiluted and diluted (1:10) samples in triplicate. Plates containing Marine Broth were inoculated with seawater samples from the ANTOM-2 and QikIce expeditions and brine samples from the QikIce expedition. Plates containing R2A were inoculated with sea ice samples from the PI-ICE and QikIce expeditions. In addition, sea ice samples from the QikIce expedition were also tested on plates containing Marine Broth, while plates containing Marine Broth and NaCl were inoculated with samples from the brine. All the plates were then stored and incubated at approximately 4 °C.

Depending on the rate of bacterial growth, three different counts were made at regular time intervals, while the abundance was calculated by determining the number of colony-forming units (CFU/ml):

$$CFU/ml \ = \ (N^{\underline{o}} \ colonies * Dilution \ factor)/Volume \ of \ culture \ plate$$

Bacterial colonies were selected based on their different phenotypic characteristics, such as colour and morphology and colonies well-separated from each other were chosen to avoid contamination with adjacent microorganisms. Then, following the Streak Plate Method (Katz, 2008), each of the picked colonies was streaked onto a new plate using an inoculation loop to isolate pure cultures. After 10 days, a portion of each colony's biomass was transferred into one well containing Marine Broth (900 µL) in a 12-well plate. The plates were then incubated at 4°C for approximately 5 days and the growth of colonies was detected by the turbid appearance of the Marine Broth.

2.2.3. Bacterial cell lysis and 16S rRNA PCR amplification from colony cultures

For each isolate, 10 µL was used for 16S rRNA PCR amplification, and the remaining portion was preserved in cryovials with a 1:1 ratio of glycerol, and stored at -70°C. The isolates were stored as part of the bacterial colony collection of the Institut de Ciències del Mar (ICM) - CSIC for future studies. To prepare the samples for PCR, 10 µL of each culture was diluted 1:4 (10 µL of culture + 30 µL of sterile MQ water) and subjected to heat treatment (95°C, 15 minutes) to induce cell lysis.

The partial 16S rRNA gene sequences were amplified using specific bacterial primers. The forward primer used was 358F [5'-CCT ACG GGC AGC AG-3'] (Muyzer et al., 1993), and the reverse primer was 907R [5'-CCG TCA ATT CMT TTG AGT TT-3'] (Lane et al., 1985).

Each PCR mix had a final volume of 25 µL and contained the following components: 2 µL of template DNA (from cell lysis), 0.5 µL of dNTPs (deoxynucleotide triphosphate) at a concentration of 10 mM, 0.75 µL of $MgCl_2$ to reach a final concentration of 1.5 mM, 1.25 µL of each primer to reach a final concentration of 0.5 µM, 0.125 µL of Taq DNA polymerase (Invitrogen), 2.5 µL of PCR buffer (Invitrogen), and 16.6 µL of sterile MQ water. The PCR reactions were performed using a Bio-Rad thermocycler with the following program: an initial denaturation step at 94ºC for 5 minutes, followed by 30 cycles of 1 minute at 94ºC, 1 minute at 55ºC, and 2 minutes at 72ºC. The reaction was then concluded with a final extension step of 10 minutes at 72ºC.

The PCR products were analyzed and quantified by agarose gel electrophoresis using a standard low DNA mass ladder (Invitrogen). A 1.2% (w/v) agarose gel was prepared by adding 1.2 g agarose in 100 ml Tris-Acetate-EDTA buffer (TAE) 1x. The solution was then boiled in a microwave until the agarose was completely dissolved. To visualize the DNA bands, 5 µL of SYBR Safe DNA gel stain (Invitrogen) was added to the solution before pouring the gel. The gel was left to solidify for approximately 20 minutes. For each electrophoresis procedure, 5 µL of the ladder (EasyLadder, Bioline No. BIO-33045) was loaded. Each well was loaded with 4 µL of PCR product mixed with 2 µL of loading buffer, to impart colour and density to the samples. Electrophoresis was performed at 100 V for 20 minutes. Images of the electrophoresis gels with samples were captured using an iBright CL1500 Imaging System. Successful PCR amplification was expected to yield a single band of approximately 600 bp on agarose gels after electrophoresis.

2.2.4. Sequencing of the 16S rRNA gene and bioinformatics analysis

Purification and OneShot Sanger sequencing of 16S rRNA gene products was performed by GenoScreen (Lille, France) using primer 358F. To ensure the accuracy of the sequences they were manually curated and the quality was controlled using Chromas software. The sequences were provided in two formats: FASTA and ABI. The FASTA format consisted of a text file with the base sequences indicated, while the ABI format contained the chromatograms. Both file formats showed the sequence of the DNA bases (A, T, G, C) and indicated ambiguous bases with N. The start and end regions of the supplied sequence files were removed since they are often of poor quality with many Ns. Ambiguous bases within the sequences were manually replaced with the appropriate base, identified by scrutinizing the chromatogram to ensure accuracy.

2.2.5. Taxonomy and phylogenetic analyses of isolated bacteria

The purified sequences of the isolated strains were used to determine their taxonomy through a homology search using the 'ACT: Alignment, Classification and Tree Service' of the SILVA rRNA database, an online resource that provides regularly updated datasets of aligned ribosomal RNA (rRNA) sequences for Bacteria, Archaea and Eukarya. The ACT page permits the submission of sequence data for processing by the SINA alignment software. For each query sequence submitted, the classifications associated with the matched sequences were aggregated using a lowest common ancestor (LCA) approach. When a match was found, the program provided information about the organism that had a sequence match with the query sequence. This information includes:

- Identity: the highest degree of identity that the aligned sequence shared with any sequence present in SEED, a vector sequence database and a compilation of non-chimeric sequences (Quast et al., 2013).
- Score: a measure of alignment quality.

Moreover, the Randomized Accelerated Maximum Likelihood (RAxML) program was used for neighbour identification via the ACT page. The parameters selected were as follows: one neighbour per query sequence, with a minimum sequence identity threshold (the ratio of shared bases to the length of the query sequence) set at 0.70.

To determine the possible phylogenetic relationship between the sequences obtained from the bacterial isolates and from the neighbours found, an alignment was performed using the bioinformatics program MEGA11 (Molecular Evolutionary Genetics Analysis). The alignment data was used to construct a phylogenetic tree with the Neighbour-Joining (NJ) method, originally developed by Saitou & Nei, 1987. This algorithm involves joining the two closest neighbour sequences based on their genetic distance. The process continues until all terminals are incorporated into the final phylogenetic tree. To assess the reliability of the phylogenetic relationships in the tree, a bootstrap value of 100 was incorporated using the bootstrapping method. Bootstrapping involves random sampling with replacement to estimate the sampling distribution of the desired estimator and is commonly used to evaluate the reliability of sequence-based phylogenies (Ojha et al., 2022).

2.2.6. Primer design for the detection of Cas9 in the cultured bacteria

2.2.6.1. Analysis of Cas9 Genes in Public Uncultured Metagenomic Assembled Genomes (MAGs)

For this analysis, the most representative genera of cultivated bacteria from polar regions were selected in the National Center for Biotechnology Information database (NCBI). A homology search was performed by comparing the sequences of the isolated bacteria with those of bacterial strains available on NCBI, using the Basic Local Alignment Search Tool (BLAST) algorithm. Specifically, the Megablast algorithm was used to identify the most similar sequences.

The list of Tara Arctic MAGs with Cas9 genes was then examined to see if Cas9 genes had been identified in bacteria of the same genera as the colonies isolated and sequenced in the present study. In addition, the Integrated Microbial Genome (IMG) system was consulted for the same purpose, as it integrates user-provided genome and metagenome datasets with publicly available datasets.

In particular, using the IMG platform, genomes were selected and filtered using the 'Function Search' feature, which allows the identification of functions using a keyword search. The filtering process involved the selected keyword ‘COG3513’, which represents the CRISPR-Cas system type-II protein Cas9. The selected genomes that contained Cas9 were filtered according to their habitat and origin, giving priority to those of polar regions and with taxonomic similarity to the isolated bacteria in the present study. Subsequently, CRISPR-CasFinder, a web-based tool for identifying clustered regularly interspaced short palindromic repeats, was used to confirm the presence of Cas9 proteins (Couvin et al., 2018). This process involved specifying the genus of interest and then filtering by selecting the 'Cas9' tag.

2.2.6.2. Primer Design for PCR

Multiple Sequence Alignment (MSA) of the Cas9 sequences belonging to the selected isolated bacteria from each genus was performed using CLUSTALW, and the output format chosen was CLUSTAL. The next step involved designing primers based on specific regions that were identical in two or more individuals in MSA. The primer design process was based on the following criteria; a) the desired primer length (18-24 bases), b) base composition (40-60% G+C), c) the 3'-ends of primers including G, C, CG, or GC to enhance priming efficiency and prevent annealing of ends and d) primer self-complementarity, which could lead to the formation of secondary structures like hairpins, was avoided (*Primer Design Guide - 5 Tips for Best PCR Results*, n.d.).

2.2.7. DNA Extraction and Cas9 Quantification

PowerSoil Pro Kit, typically used for extracting and purifying soil DNA, was adapted to extract DNA from the selected colonies of interest for the detection of the Cas9 protein. Some modifications were introduced to make the kit applicable to liquid samples. Specifically, the entire volume of each well from the 12-well multi-well plate was transferred to 2 mL microcentrifuge tubes. Subsequently, centrifugation was performed at 4000 rpm for 10 minutes to separate the supernatant from the pellet. Then the supernatant was discarded, and another centrifugation step was carried out for 1 minute at 4000 rpm, to ensure the complete removal of any residual supernatant. The resulting pellet was retained, and the kit protocol was carried out. The genomic DNA purification process uses six buffers/solutions called CD1, CD2, CD3, EA, C5, and C6, each serving a specific function in the extraction process as outlined below.

First, each sample was mixed with 800 µL of CD1 solution and homogenized in a PowerBead Pro tube to induce lysis of the bacterial pellet and protect nucleic acids from degradation. After centrifugation at 15000 RCF for 1 min, the supernatant (approximately 600 µL) was transferred to a clean tube. 200 µL of CD2 solution was added to the bacterial lysate to remove all organic and inorganic contaminants that could affect the purity of the DNA. After another centrifugation at 15000 RCF for 1 min, up to 700 µL of the supernatant was transferred to another microcentrifuge tube.

600 µL of CD3, a highly concentrated salt solution, was added, and the tubes were vortexed to facilitate DNA binding to the MB Spin Column membrane. Then, 650 µL of lysate was loaded onto an MB Spin Column tube and centrifuged for 1 min at 15000 RCF. During this step, the DNA adheres to the silica membrane in the supplied column due to the high salt concentration, while contaminants pass through, leaving only the DNA bound to the membrane. The filtrate was discarded and these purification steps were repeated for the remaining lysate.

The MB Spin column, containing the purified bacterial DNA, was transferred to a new 2 mL tube, and 500 µL of EA solution, an ethanol-based wash buffer, was added to further clean the membrane-bound DNA. After a centrifugation step of 1 min at 15000 RCF, the remaining liquid was discarded. 500 µL of C5 solution, an ethanol-based wash solution, was then added to further purify the membrane-bound DNA by removing residual salt, humic acid, and contaminants. The centrifugation step was repeated twice to remove the residual wash solution before being transferred to an Eppendorf tube. The final centrifugation steps are critical as they remove all traces of the C5 solution, and avoid interference of ethanol with subsequent DNA applications such as PCR or gel electrophoresis.

In the final step, 100 µL of C6 solution, a sterile salt-free elution buffer, was added to the MB Spin Column membrane, which was centrifuged at 15000 RCF for 1 min to elute the bacterial DNA. The purified DNA was quantified and purity assessed using a NanoDrop One spectrophotometer and then stored at -70 ºC.

The NanoDrop One spectrophotometer was used to determine the concentration of the DNA isolated (ng/µL), and the purity by assessing the A260/280 ratio. A ratio of 1.8 was considered indicative of good DNA purity. A schematic representation of the DNA extraction and purification process is provided in Figure 2.2.

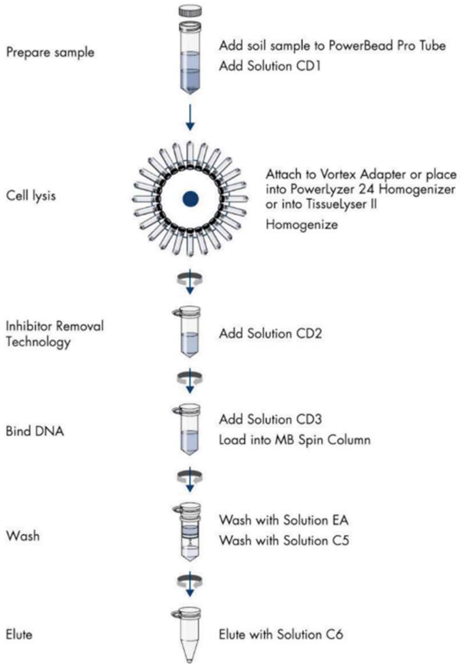


Figure 2.2. Graphical overview of DNA extraction process with PowerSoilPro Kit. *Source: DNeasy PowerSoil Pro Kit Quick-Start Protocol - QIAGEN, n.d.*

2.2.8 PCR amplification using the Primers designed

2.2.8.1 Primers Rehydration

The primers were received dried and in dark conditions. Their reconstitution was as follows: a) the primer pellets were briefly spined, b) each pellet was dissolved in sterile MQ water to give a concentration of 100 µM, c) the dissolved primers in tubes were vortexed, incubated for two minutes to fully rehydrate them, and d) diluted 1:10 to obtain the final working dilution of 10 µM (= 10 pmol μl^{-1}). The reconstituted primers were then stored in aliquots at -20 °C.

2.2.8.2. PCR amplification for the detection of Cas9

For the PCR amplification of the isolated bacterial DNA, an NZYTaq II 2× Colourless Master Mix was used. This pre-assembled solution incorporates the DNA polymerase NZYTaq II

(MB354), a member of the new generation of Taq-derived DNA polymerases. The master mix includes dNTPs, reaction buffer, and additives, with a final $MgCl_2$ concentration of 2.5 mM (*NZYTaq II 2x Colourless Master Mix*, n.d.). Each PCR reaction, had a final volume of 25 µL and contained the following reagents: 1 µL of template DNA (from DNA extraction), 12.5µL of NZYTaq II 2× Colourless Master Mix, 1.25 µL of each of the designed primer pairs to give a final concentration of 0.5 µM and then 9µL of MQ water. PCR amplifications were conducted according to the protocol used for 16S rRNA PCR, with the annealing temperature adjusted to be appropriate for the primers designed. Agarose gel electrophoresis was then performed and gel images were captured.

2.3. Results

2.3.1. Divergent cultured bacteria abundance and colony forming units (CFUs) in seawater and sea ice samples from the Antarctic Peninsula

Bacterial colony forming units (CFU) were estimated from seawater and sea ice samples collected during the ANTOM-2 and PI-ICE expeditions. A total of 162 plates were counted, including both undiluted and 1:10 diluted samples and three different counts were made at regular time intervals. However, the 33 plates inoculated with undiluted seawater and sea ice samples collected during the QikIce expedition were not counted over time since no colonies grew.

ANTOM-2 expedition

For the ANTOM-2 expedition, CFU/ml measurements were obtained from different stations at different depths. The mean CFU/ml was calculated from triplicates after 30, 45 and 60 days of incubation due to the slow growth of these bacteria at 4°C (Fig. 2.3).

In the case of direct dilution, there was a gradual and slight increase in CFU/ml from the initial to the final count. However, there was a consistent decrease in CFU/ml as one moved from the photic layers to the deeper layers. This decreasing trend was observed at all stations.

The error bars, representing the standard deviation (STD) of the CFU/ml measurements between the three plates for each depth, varied in size. The largest error bars were mainly associated with Station 11 and to some extent with Station 7, but only up to depth DE3.

Colonies grown from the undiluted solution collected at Station 11 were significantly more numerous than those at the other stations, and their mean CFU/ml remained relatively stable over time: SRF from 647 to 653 CFU/ml, DCM from 397 to 407 CFU/ml, DE3 120 CFU/ml, and DE5 3 CFU/ml throughout the observation period, with no colonies found in DE4. This trend was followed by Station 7, where the mean CFU/ml increased steadily: SRF from 53 to 63 CFU/ml, DCM from 67 to 87 CFU/ml, with no colonies found from the deep layers. In contrast, colonies from Station 5 showed slow growth: SRF from 20 to 27 CFU/ml, DCM from 17 to 30 CFU/ml, DE4 from 3 to 7 CFU/ml and DE5 from 10 to 13 CFU/ml, with no colonies found from DE3.

Colonies from Station 12 showed a consistent, flat trend with few colonies throughout the incubation period: SRF 27 CFU/ml, DCM 7 CFU/ml, DE4 from 3 to 7 CFU/ml and DE5 3 CF/ml, with no colonies found from DE3. Meanwhile, colonies from Station 10 showed a maximum of 7 CFU/ml found from SRF and DE4 plates, with no colonies found from DE3 and DE5. Replication of plates for this station and the deep layers gave similar results. The CFU/ml values for the 1:10 diluted samples are not shown, as they exhibited a lower growth tendency at all stations, except for St.11: SRF 900 CFU/ml, DCM 367 CFU/ml and DE3 67 CFU/ml with no colonies found from DE4 and DE5.

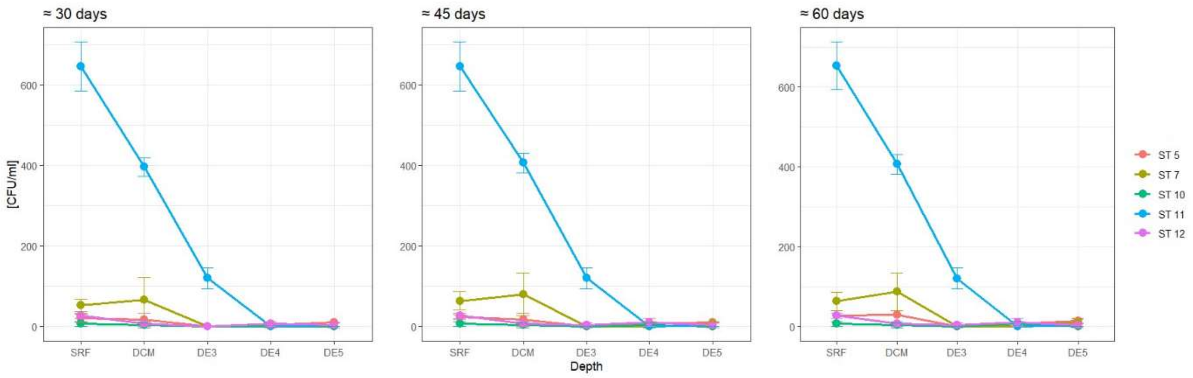


Figure 2.3. CFU/ml variation at different depths from ANTOM-2 plates. *Graph of CFU/ml in undiluted plates between sampling stations at different depths over three counts (30, 45 and 60 days). Source: Prepared by the author using RStudio*

PI-ICE expedition

For the PI-ICE plates, CFU/ml measurements were obtained from two sites, SI9 1B and SI9 4B. The mean CFU/ml was calculated from triplicates at 10, 20 and 30 days (Fig. 2.4).

The CFU count was higher at site SI9 4B, showing a faster growth rate, going from 5083 to 6007 CFU/ml, while at site SI9 1B the measurement was from 513 to 843 CFU/ml. The error bars, representing the STD of the CFU/ml measurements between the three plates, were more significant for site SI9 4B. A 1:10 dilution significantly reduced the number of colonies from both sites. This dilution was performed primarily to facilitate the selection of colonies for further taxonomic identifications.

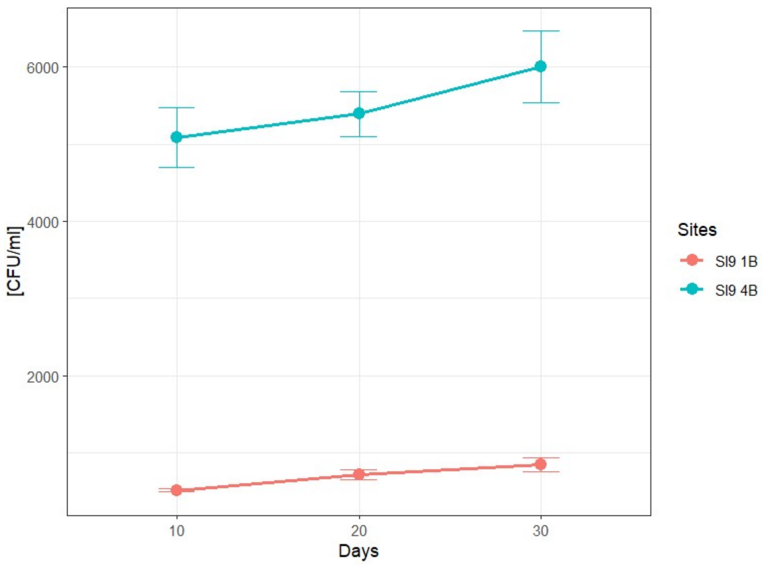


Figure 2.4. CFU/ml variation at different sites from PI-ICE plates. *Graph of CFU/ml in undiluted plates between sampling sites over three counts (10, 20 and 30 days). Source: Prepared by the author using RStudio*

QikIce expedition

In the case of the QikIce plates, no colonies were observed on the 33 plates examined after 60 days incubation.

Specifically, a total of 5 colonies were found on plates inoculated with the seawater sample. For samples from the 1-10 cm sea ice slice, 12 colonies were identified only on plates containing Marine Broth as the culture medium. One colony was detected in the sample taken from the 10-40 cm slice. For samples taken from the 70-100 cm slice, 4 colonies were found on plates with Marine Broth and 3 colonies on plates with R2A as the culture media. Finally, only one colony was observed on plates inoculated with brine samples at 64 ‰ salinity, and this occurred on a plate with Marine Broth as the culture medium. No colonies were observed on plates with Marine Broth and NaCl medium inoculated with brine samples.

2.3.2. Diversity of cultured bacteria from seawater and sea ice from polar regions

2.3.2.1. Taxonomic identification by 16S rRNA gene analyses

A total of 199 isolates were selected from the three expeditions: 119 isolates from the samples collected during the ***ANTOM-2*** expedition, 64 from the ***PI-ICE*** expedition and 16 from the ***QikIce*** expedition.

ANTOM-2 expedition

The isolation of 119 bacterial strains from ANTOM-2 allowed us to investigate the differences of taxa enriched between the photic versus aphotic ocean layers in 5 different Antarctic stations. Out of the 119 isolates, 86 were from photic layers (surface and DCM; 72.26 %) and 33 were from deep layers (mesopelagic and bathypelagic, 27.73 %). The results obtained from the ANTOM-2 isolates showed the presence of four classes of bacteria in both the surface (SRF) and deep layers: Alphaproteobacteria, Bacilli, Flavobacteriia and Gammaproteobacteria. Three classes were identified in the DCM layer, which included all the above classes except Bacilli (Fig. 2.5 (A)).

Gammaproteobacteria, with 57 isolates (47.90 % of the total), dominated both the SRF layer (44 %) and the deeper layers (66.67 %). On the other hand, their prevalence was slightly lower in the DCM layer (36.11 %), where Alphaproteobacteria (52.78 %) dominated. This last class, consisting of 39 isolates (32.77 % of the total), was significantly abundant in the SRF layer (38 %), while it was less represented in the deep layers (3.03 %). The class Flavobacteriia, with 17 isolates (14.28 % of the total), was detected in all three layers, although to a lesser extent in SRF (12 %) and DCM (11.11 %). Conversely, it was the second most abundant class in the deeper layers (21.21 %). Finally, the class Bacilli, with only six isolates (5.04 % of the total), showed a limited abundance in the SRF (6 %) and in the deep layers (9.09 %) (Tab.1).

A closer look at the genus level revealed, in order of abundance, a total of 12 main genera: *Pseudoalteromonas*, *Sulfitobacter*, *Shewanella*, *Bacillus*, *Flavobacterium*, *Winogradskyella*, *Psychroserpens*, *Colwellia*, *Halomonas*, *Pseudomonas*, *Ulvibacter* and *Psychrobacter* (Fig. 2.5 (B)). The genus *Pseudoalteromonas* emerged as the most dominant taxon, representing 70.18 % of the Gammaproteobacteria class and 33.61 % of the total isolates. This genus showed a distribution across all vertical profiles. It was followed by *Sulfitobacter*, which represented 97.44 % of the Alphaproteobacteria class and 31.93 % of the total isolates present in the SRF and DCM layers. *Shewanella* (17.54 % of the Alphaproteobacteria and 8.40 % of the total isolates), *Colwellia* (3.51 % of the Alphaproteobacteria and 1.68 % of the total isolates) and *Flavobacterium* (29.41 % of the Flavobacteriia and 4.20 % of the total isolates) were also detected in these two layers.

Furthermore, *Bacillus* (100 % of the Bacilli and 5.04 % of the total isolates) and *Winogradskyella* (29.41 % of the Flavobacteriia and 4.20 % of the total isolates) were isolated on few occasions within the SRF and deep layers. *Psychroserpens* (17.65 % of the

Flavobacteriia and 2.52 % of the total isolates) and *Pseudomonas* (3.51 % of the Gammaproteobacteria and 1.68 % of the isolates) were found in the DCM and Deep layers. Meanwhile, *Psychrobacter* (1.75 % of the Gammaproteobacteria and 0.84 % of the total isolates) was only identified at the surface and *Halomonas* (3.51 % of the Gammaproteobacteria and 1.68 % of the total isolates) and *Ulvibacter* (11.76 % of the Flavobacteriia and 1.68 % of the total isolates) were detected in the deep layers. Three colonies remained unidentified at the genus level (Tab. 2.1), two from the class Flavobacteriia and one from the class Alphaproteobacteria.

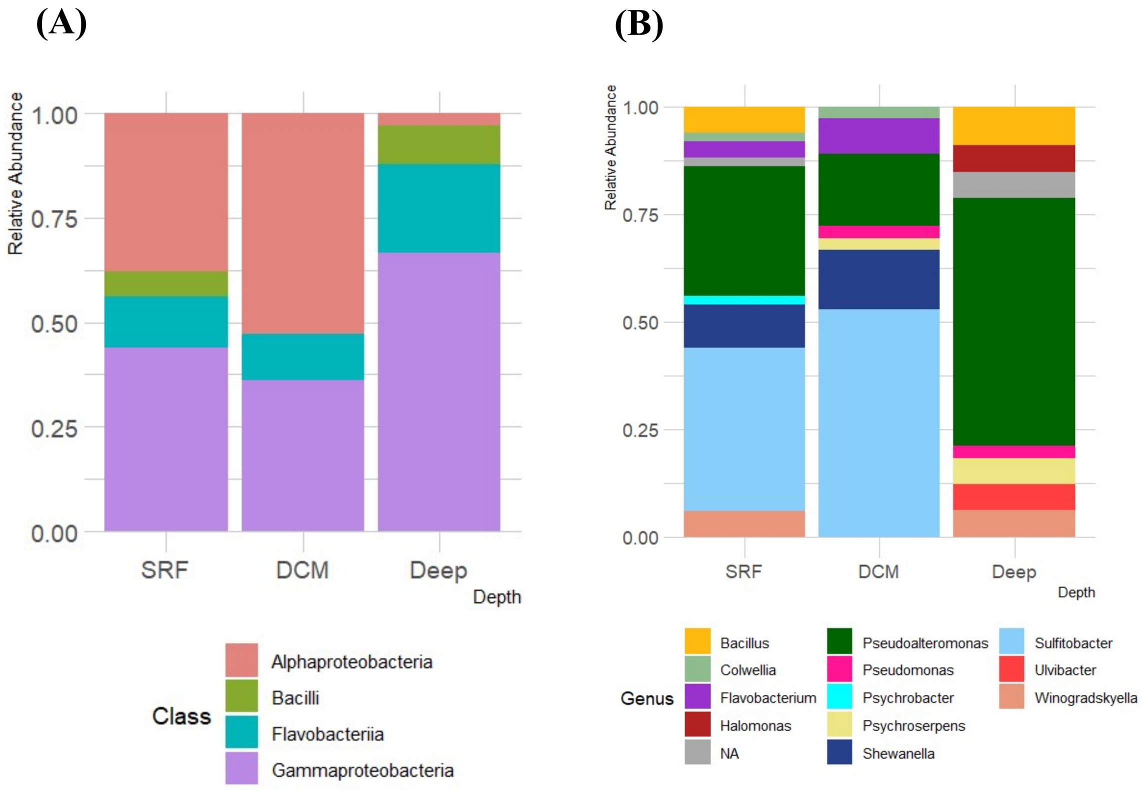
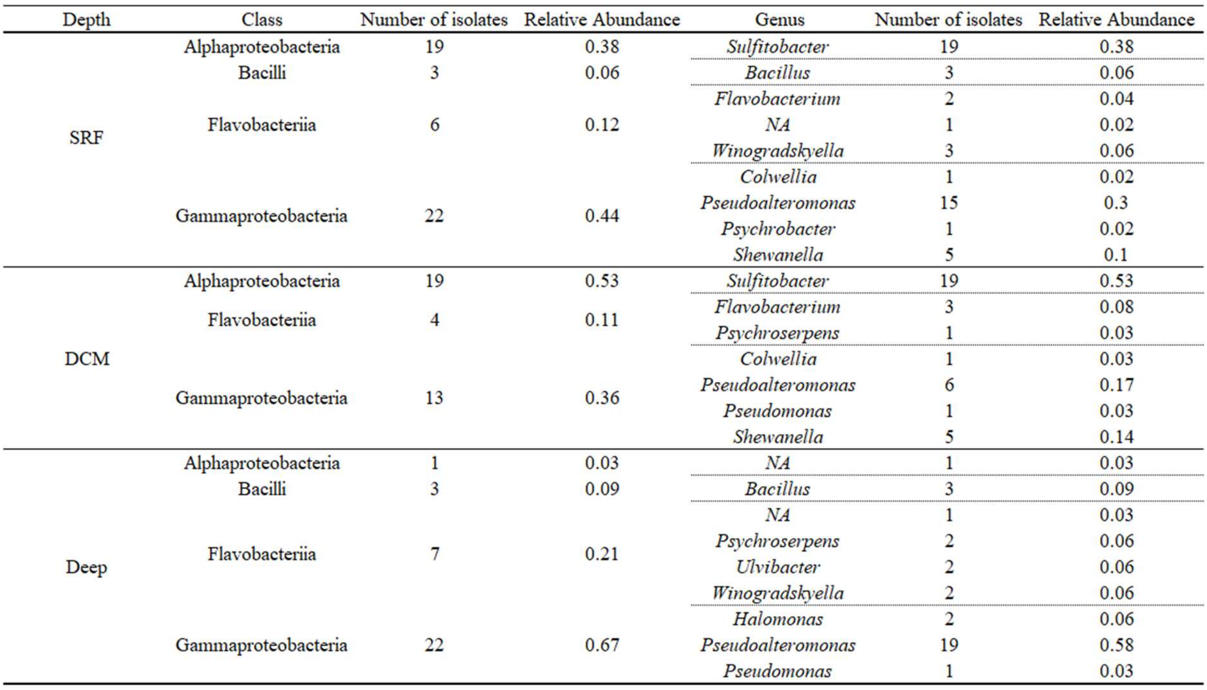


Figure 2.5. Distribution of bacterial isolates from ANTOM-2 expedition by Class and Genus based on depth. *Graph showing the proportion (out of 1) of (**A**) organisms categorized by class and (**B**) by each of the 12 genera, with 'NA'(in grey) denoting organisms not detected. Data are divided by depth: SRF, DCM and Deep. Source: Prepared by the author using RStudio.*

Table 2.1. Relative Abundance of Classes and Genera at different depths during ANTOM-2. *The table displays the relative abundance of Classes and Genera at various depth levels (SRF, DCM and Deep). Each row represents a different depth, and the columns show the proportion of organisms belonging to specific classes and genera. 'NA' indicates the percentage of organisms not detected. Source: Prepared by the author using Excel.*



Depth	Class	Number of isolates	Relative Abundance	Genus	Number of isolates	Relative Abundance
SRF	Alphaproteobacteria	19	0.38	*Sulfitobacter*	19	0.38
	Bacilli	3	0.06	*Bacillus*	3	0.06
	Flavobacteriia	6	0.12	*Flavobacterium*	2	0.04
				NA	1	0.02
				Winogradskyella	3	0.06
	Gammaproteobacteria	22	0.44	*Colwellia*	1	0.02
				Pseudoalteromonas	15	0.3
				Psychrobacter	1	0.02
				Shewanella	5	0.1
DCM	Alphaproteobacteria	19	0.53	*Sulfitobacter*	19	0.53
	Flavobacteriia	4	0.11	*Flavobacterium*	3	0.08
				Psychroserpens	1	0.03
	Gammaproteobacteria	13	0.36	*Colwellia*	1	0.03
				Pseudoalteromonas	6	0.17
				Pseudomonas	1	0.03
				Shewanella	5	0.14
Deep	Alphaproteobacteria	1	0.03	*NA*	1	0.03
	Bacilli	3	0.09	*Bacillus*	3	0.09
	Flavobacteriia	7	0.21	*NA*	1	0.03
				Psychroserpens	2	0.06
				Ulvibacter	2	0.06
				Winogradskyella	2	0.06
	Gammaproteobacteria	22	0.67	*Halomonas*	2	0.06
				Pseudoalteromonas	19	0.58
				Pseudomonas	1	0.03

We also looked at the diversity of genera by sampling stations. We highlighted the abundance of each genus, which is the number of isolates found belonging to that genus and the occurrence of each genus across all stations, which is the number of stations in which that genus was found (Fig. 2.6).

The highest occurrence, observed in three stations, concerned two genera: *Flavobacterium* and *Pseudoalteromonas*. *Flavobacterium* was detected at stations 10, 11 and 12, while Pseudoalteromonas was detected at stations 5, 11 and 12. A significant proportion of the *Pseudoalteromonas* isolates, 37 out of 40 to be precise, originated exclusively from Station 11, spanning all three strata: SRF, DCM and Deep.

In addition, *Sulfitobacter* appeared mainly in stations 5 and 7. *Bacillus* was also identified in two stations, occurring in both the SRF and the deep layers of station 10. Similarly, *Winogradskyella* was consistently found in two stations, most notably in Station 7, where it was found in both the SRF and Deep layers.

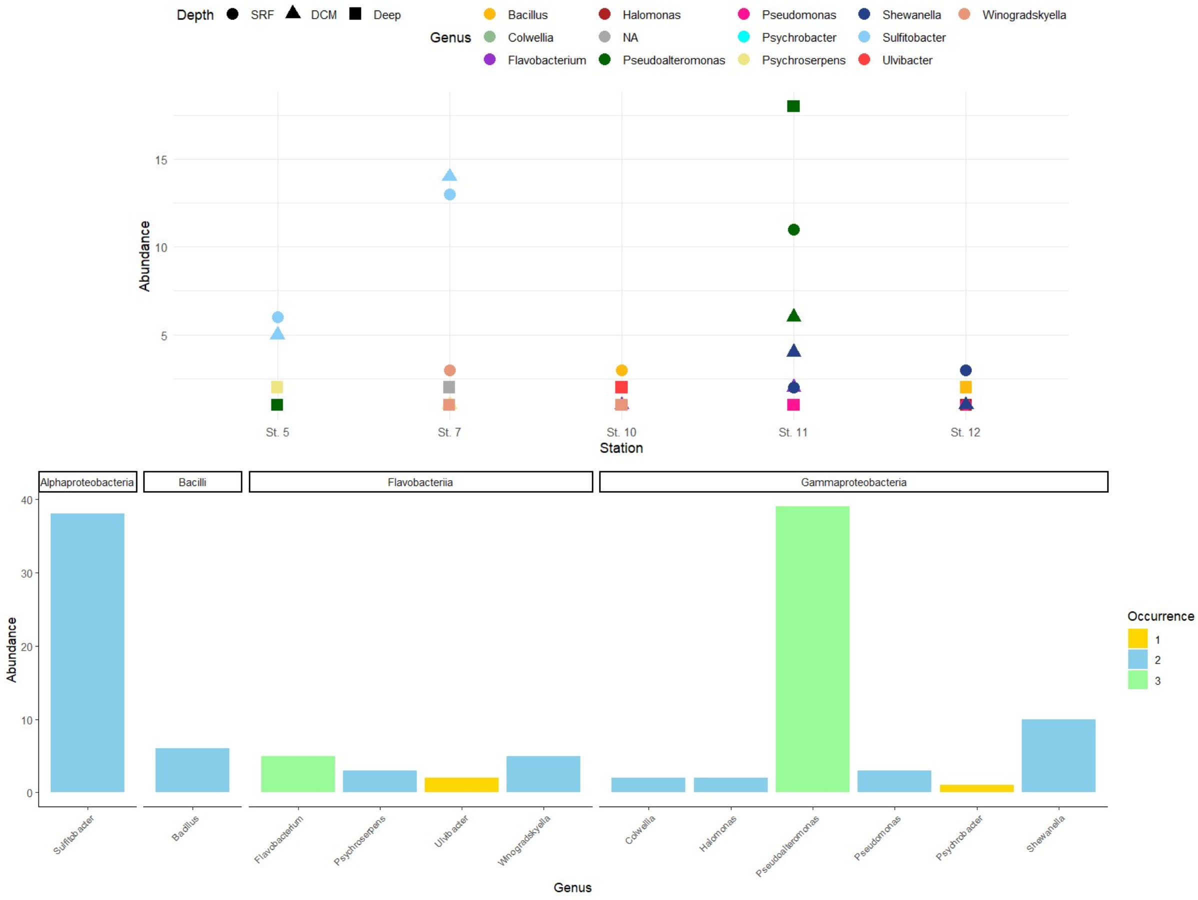


Figure 2.6. Comparison of Genus Abundance and Occurrence Across Antarctic Sampling Stations. *The upper graph shows the abundance of genera between stations at different depths. The lower graph shows the abundance of genera versus the occurrence of genera among stations. Source: Prepared by the author using RStudio.*

To explore the range of genetic diversity represented by our isolates, the genera of cultured colonies represented by more than four isolates were selected to highlight the range of percentage identity. The genera considered were *Bacillus* (with a total of 6 isolates), *Flavobacterium* (with a total of 5 isolates), *Pseudoalteromonas* (with a total of 40 isolates), *Shewanella* (with a total of 10 isolates), *Sulfitobacter* (with a total of 38 isolates) and *Winogradskyella* (with a total of 5 isolates).

The percentage identity range within these genera was as follows: *Bacillus* from 97.56 % to 99.63 %, *Flavobacterium* from 99.02 % to 99.29 %, *Pseudoalteromonas* from 99.62 % to 100 %, *Shewanella* from 99.43 % to 100 %, *Sulfitobacter* from 98.99 % to 100 % and *Winogradskyella* from 98.7 % to 100 % (Fig. 2.7).

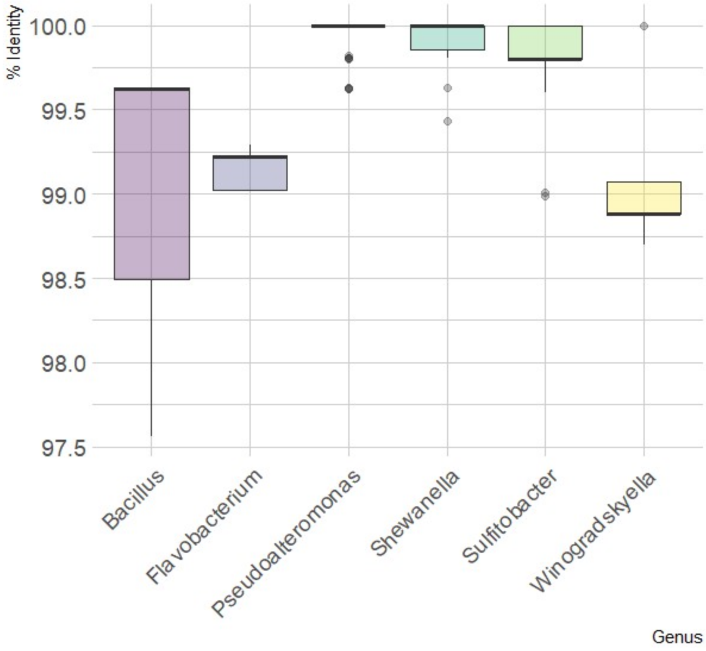


Figure 2.7. Percentage Identity Range Within Genera of Interest from ANTOM-2. *Boxplot graph illustrating the range of percent identity within the genera of interest obtained from the ANTOM-2 expedition. The y-axis represents the percentage identity, while the x-axis represents the genera of interest. Each boxplot shows the range of percent identity observed within each genus. Source: Prepared by the author using RStudio.*

PI-ICE expedition

The PI-ICE isolates from the Antarctic Peninsula, obtained from sea ice samples, permitted comparisons with the seawater from ANTOM-2 and to explore the connectivity between the two biomes. The PI-ICE isolates revealed the presence of four distinct classes of bacteria at the SI9 1B site: Actinobacteria, Bacilli, Flavobacteriia and Gammaproteobacteria and with the exception of Bacilli, the other three classes were identified at the SI9 4B site as well (Fig. 2.8 (A)).

The class Flavobacteriia, with 31 isolates (48.44 % of the total), was the most prevalent at the SI9 4B site (55.56 %). Together with Gammaproteobacteria, they were the most abundant at SI9 1B (43.24 %). Gammaproteobacteria, with 27 isolates (42.19 % of the total), was most present at SI9 1B (43.24 %), and the second most abundant at SI9 4B (40.74 %). Actinobacteria, with 5 isolates (7.81 % of the total), represented 10.81 % of the isolates at SI9 1B and 3.70 % at SI9 4B. Finally, Bacilli, with one isolate (1.56 % of the total), represented 2.70 % at SI9 1B (Tab. 2.2).

A total of 7 genera were then identified, in descending order of prevalence: *Flavobacterium* (with 39.06 % of the total isolates), *Psychrobacter* (with 37.5 % of the total), *Chryseobacterium* (with 7.81 % of the total), *Pseudomonas* (with 4.69 % of the total), together with *Cellulophaga*, *Staphylococcus* and *Arthrobacter* (each with 1.56 % of the total) (Fig. 2.8 (B)).

In particular, *Flavobacterium* was found at both SI9 1B and SI9 4B sites and represented 80.65 % of the Flavobacteriia. Similarly, the genus *Psychrobacter* was present at both sites and represented 88.89 % of the Gammaproteobacteria. *Pseudomonas*, although present at both sites, was less abundant and represented 11.11 % of the Gammaproteobacteria.

Site SI9 1B had several genera that were absent from the other site, including *Chryseobacterium* (16.13 % of the Flavobacteriia), *Cellulophaga* (3.23 % of the Flavobacteriia) and *Staphylococcus* (100 % of the Bacilli). Conversely, the genus *Arthrobacter* occurred exclusively at SI9 4B, representing 100 % of the Actinobacteria. In addition, at site SI9 1B, four isolates could not be classified at the genus level (Tab. 2.2).

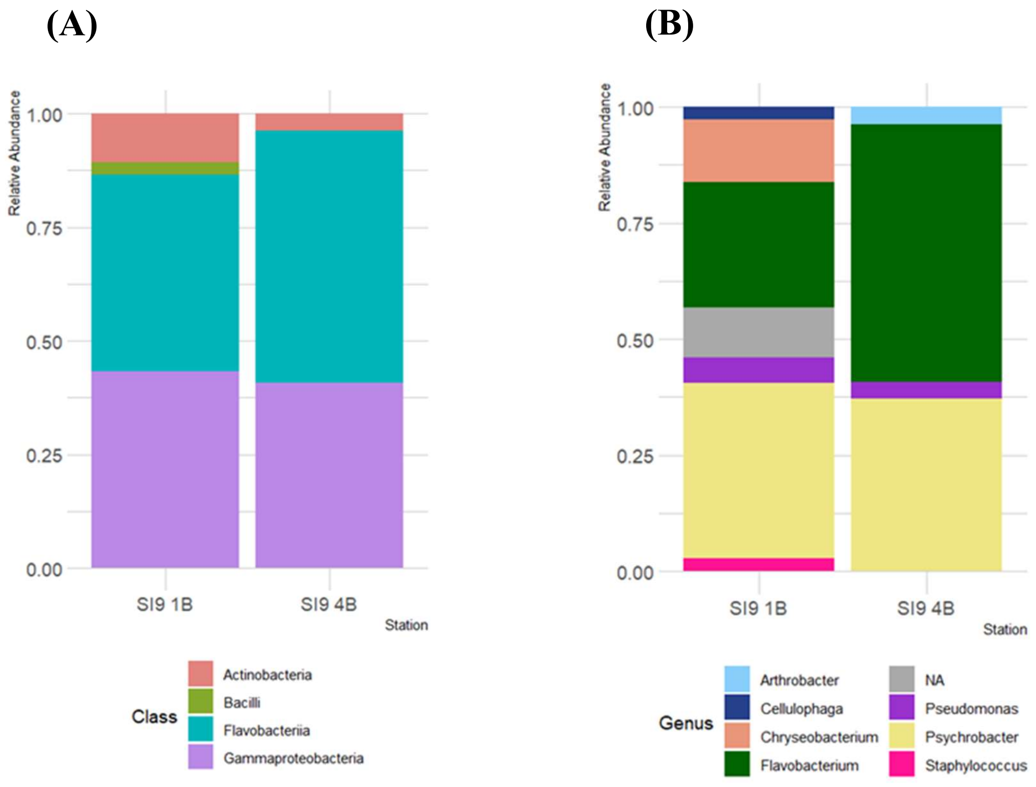


Figure 2.8. Distribution of isolates from PI-ICE expedition by Class and Genus Based on the Station. *The graphs show the proportion (out of 1) of **(A)** organisms categorized by class and **(B)** by each of the 7 genera, with 'NA' in grey denoting organisms not detected. Data are divided by Stations: SI9 1B and SI9 4B. Source: Prepared by the author using RStudio.*

Table 2.2. Relative Abundance of Classes and Genera at two Stations during PI-ICE. *The table displays the Relative Abundance of Classes and Genera at two stations: SI9 1B and SI9 4. Each row represents a different station, and the columns show the proportion of organisms belonging to specific classes and genera. 'NA' indicates the percentage of organisms not detected. Source: Prepared by the author using Excel.*

Site	Class	Number of isolates	Relative Abundance	Genus	Number of isolates	Relative Abundance
SI9 1B	Actinobacteria	4	0.11	*NA*	4	0.11
	Bacilli	1	0.03	*Staphylococcus*	1	0.03
	Flavobacteriia	16	0.43	*Cellulophaga*	1	0.03
				Chryseobacterium	5	0.14
				Flavobacterium	10	0.27
	Gammaproteobacteria	16	0.43	*Pseudomonas*	2	0.05
				Psychrobacter	14	0.38
SI9 4B	Actinobacteria	1	0.04	*Arthrobacter*	1	0.04
	Flavobacteriia	15	0.56	*Flavobacterium*	15	0.56
	Gammaproteobacteria	11	0.41	*Pseudomonas*	1	0.04
				Psychrobacter	10	0.37

Genera with more than four isolates from cultured colonies were chosen to analyse the range of percentage identity. *Chryseobacterium* (with 5 isolates), *Flavobacterium* (with 25 isolates) and *Psychrobacter* (with 24 isolates) were the genera examined. Within these genera, the percent identity ranges were as follows *Chryseobacterium*, 96.95 % to 98.86 %, *Flavobacterium*, 97.72 % to 100 % and *Psychrobacter*, 98.13 % to 100 % (Fig. 2.9).

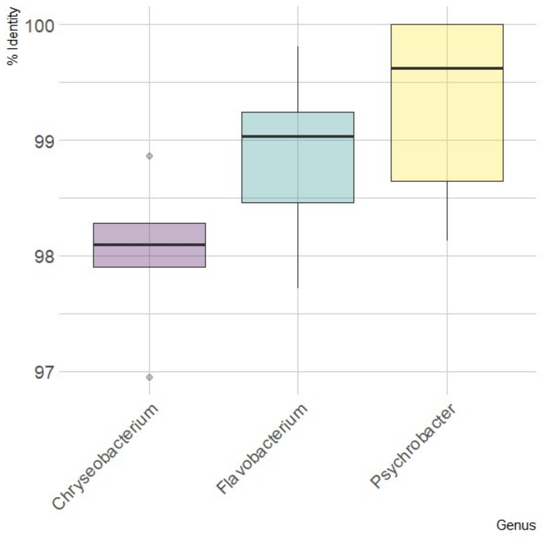


Figure 2.9. Percentage Identity Range within Genera of Interest from PI-ICE. *Boxplot graph illustrating the range of percent identity within the genera of interest obtained from the PI-ICE expedition. The y-axis represents the percentage identity, while the x-axis represents the genera of interest. Each boxplot shows the range of percent identity observed within each genus.*

QikIce expedition

The QikIce isolates from polar Arctic sea ice and seawater were relevant to enable comparisons with the Antarctic representatives and to explore potential microbial cosmopolitanism by identifying organisms distributed in both poles, and those strictly restricted to one of the poles. The 16 QikIce isolates showed the presence of different classes, with different abundances depending on the location of the sea ice core section and the seawater in which the samples were collected (Fig. 2.10 (A)).

Isolates belonging to the classes Flavobacteriia and Bacilli were the most numerous, with 7 isolates each (together representing 82.36 % of sampled organisms). From the seawater, there were 2 isolates belonging to the class Flavobacteriia (66.67 %), while in the 1-10 cm sea ice section slice, there were 5 isolates (71.43 %). Bacilli were more widespread among the sampling locations. They were found in the seawater with one isolate (33.33 %), in the 1-10 cm slice (14.29 %), in the 70-100 cm slice with 4 isolates (75 %) and in the brine at 62 ‰ with one isolate (100 %), which was the only one present at this site. As for the other two classes, Alphaproteobacteria had a total of two isolates (11.76 % of the total): one was present in the 1-10 cm slice (14.29 %), the other in the 70-100 cm slice (25 %). In total, 7 genera were identified, in descending order of prevalence: *Planomicrobium*, *Maribacter*, *Bacillus*, *Octadecabacter*, *Pseudomonas*, *Psychroserpens* and *Winogradskyella* (Fig. 2.10 (B)).

Among the cultured isolates, the genus *Planomicrobium* was the most prevalent, comprising 85.71 % of the Bacilli. In seawater, the two isolates of the genus *Maribacter* accounted for 28.57 % of the Flavobacteriia. A single isolate from the genus Bacillus (14.29 % of the Bacilli) was exclusively found in brine. In seawater, the genus *Octadecabacter* was represented by one isolate, making up 50 % of the Alphaproteobacteria.

Two different isolates belonging to the genus *Psychroserpens* and *Winogradskyella* were observed in Seawater, each contributing 14.29 % to the Flavobacteriia. It was not possible to determine the genus of four isolates selected from the 1-10 cm sea ice section slice that belonged to the Flavobacteriia and Alphaproteobacteria classes. (Tab. 2.3).

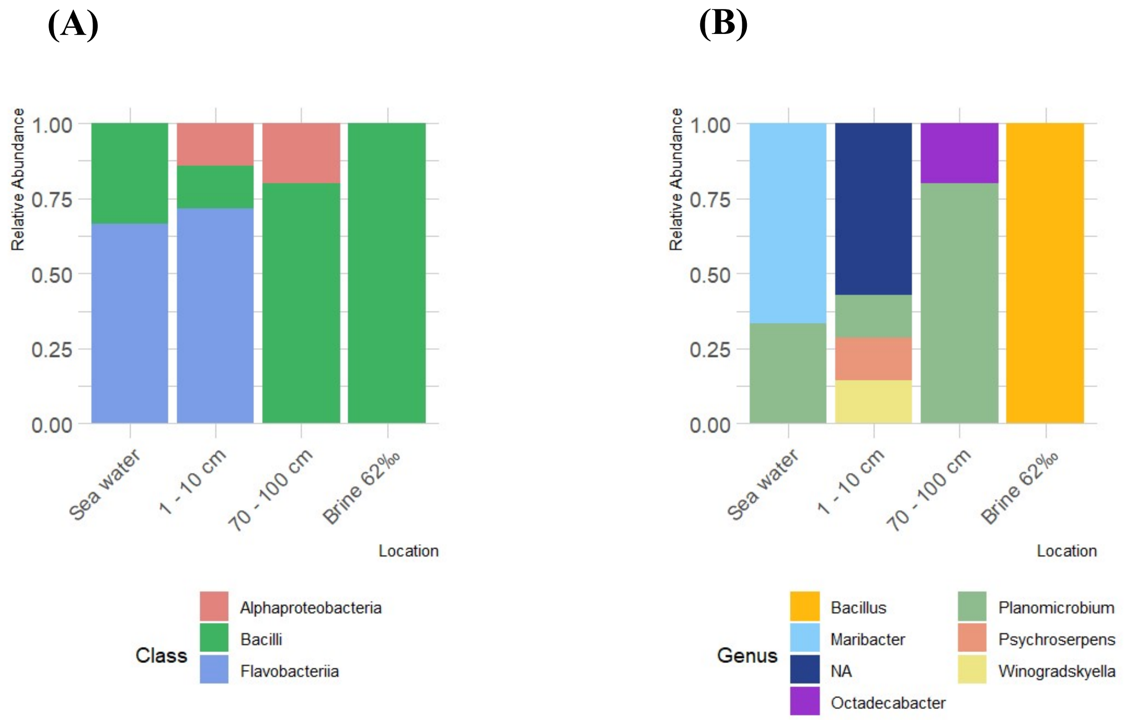


***Figure 2.10. Distribution of isolates from the QikIce expedition by Class and Genus Based on Location. The** graph shows the proportion (out of 1) of organisms (**A**) categorized by class and (**B**) by each of the 7 genera, with 'NA' in greay denoting organisms not detected. Data are divided by Location: Seawater, 1-10 cm slice, 70-100 cm slice and Brine 62 ‰. Source: Prepared by the author using RStudio.*

***Table 2.3. Relative Abundance of Classes and Genera at different Locations during QikIce.** The table presents the Relative Abundance of Classes and Genera at different locations: Seawater, 1-10 cm slice, 10-40 cm slice, 70-100 cm slice and Brine 62 ‰. Each row represents a different station, and the columns show the proportion of organisms belonging to specific classes and genera. 'NA' indicates the percentage of organisms not detected. Source: Prepared by the author using Excel.*

Location	Class	Number of isolates	Relative Abundance	Genus	Number of isolates	Relative Abundance
Sea water	Bacilli	1	0.33	*Planomicrobium*	1	0.33
	Flavobacteriia	2	0.67	*Maribacter*	2	0.67
1 - 10 cm	Alphaproteobacteria	1	0.14	*NA*	1	0.14
	Bacilli	1	0.14	*Planomicrobium*	1	0.14
				NA	3	0.43
	Flavobacteriia	5	0.71	*Psychroserpens*	1	0.14
				Winogradskyella	1	0.14
70 - 100 cm	Alphaproteobacteria	1	0.2	*Octadecabacter*	1	0.2
	Bacilli	4	0.8	*Planomicrobium*	4	0.8
Brine 62‰	Bacilli	1	1	*Bacillus*	1	1

The only genus represented by more than four isolates in QikIce samples was the genus *Planomicrobium* and the percent identity ranged from 98.69 % to 98.88 % (Tab. 2.4). Furthermore, within the pool of isolates selected from the current expedition, one isolate (ELE_QIK_6) had a percent identity of 95.4% and a score of 96%. This was the lowest percent sequence identity observed between the isolates from all three expeditions (Tab. 2.4).

Table 2.4. Percentage Identity of Each Genus from QikIce. *Table showing the percent identity of each genus identified in the QikIce expedition. The highlighted portions in green indicate isolates with the lowest percentage identity between the three expeditions.*

Location	Isolate	Identity	Score	Class	Genus
Sea water	*ELE_QIK_8*	98.47	99	Flavobacteriia	*Maribacter*
	ELE_QIK_11	98.88	99	Bacilli	*Planomicrobium*
	ELE_QIK_14	98.48	99	Flavobacteriia	*Maribacter*
1 - 10 cm	*ELE_QIK_5*	99.43	99	Flavobacteriia	*NA*
	ELE_QIK_6	95.4	96	Alphaproteobacteria	*NA*
	ELE_QIK_7	99.43	99	Flavobacteriia	*NA*
	ELE_QIK_10	97.72	99	Flavobacteriia	*Winogradskyella*
	ELE_QIK_12	98.88	99	Bacilli	*Planomicrobium*
	ELE_QIK_13	97.92	99	Flavobacteriia	*NA*
	ELE_QIK_15	98.86	99	Flavobacteriia	*Psychroserpens*
70 - 100 cm	*ELE_QIK_2*	98.69	99	Bacilli	*Planomicrobium*
	ELE_QIK_3	98.87	99	Bacilli	*Planomicrobium*
	ELE_QIK_4	98.69	99	Bacilli	*Planomicrobium*
	ELE_QIK_16	99.8	99	Alphaproteobacteria	*Octadecabacter*
	ELE_QIK_1	98.88	99	Bacilli	*Planomicrobium*
Brine 62‰	*ELE_QIK_17*	99.63	99	Bacilli	*Bacillus*

2.3.2.2. NCBI BLAST Analysis: Closest matches for sequences belonging to the most abundant genera

ANTOM-2 expedition

Based on the above results, the most commonly identified genera from the ANTOM-2 expedition were *Pseudoalteromonas*, *Sulfitobacter* and *Shewanella*. Specifically, the closest matches for sequences belonging to these genera were determined by NCBI BLAST analysis.

Of the 38 isolates within the genus *Sulfitobacter*, 36 showed predominant matches with ***Sulfitobacter sp. strain ACBC066*** (MK128994.1). These matches have a query cover ranging from 99% to 100% and a percent sequence identity ranging from 99.40% to 100%.

Of the 40 isolates within the genus *Pseudoalteromonas*, 33 isolates were most closely related to ***Pseudoalteromonas sp. W11*** (MN889233.1). In addition, 3 isolates were closely related to ***Pseudoalteromonas elyakovii*** (strain I 84, FJ200650.1), with a sequence identity of 100 % and a query cover of 99 % to 100 %.

All ten sequences of the genus *Shewanella* isolates predominantly matched ***Shewanella sp. strain BC1-22B*** (MT350303.1), with query cover ranging from 99% to 100% and percentage identity ranging from 99.43% to 100%.

PI-ICE expedition

The most common genera among the isolates selected from the PI-ICE expeditions were *Flavobacterium*, *Psychrobacter* and *Chryseobacterium*.

Of the sequences obtained from 25 isolates within the genus *Flavobacterium*, 5 showed the highest sequence identity with ***Flavobacterium sp. strain 28A*** (MN007166.1), while 4 isolates showed more sequence similarity with ***strain 14A*** (MN007165.1). In addition, 4 isolates shared sequence identity with ***strain 138H-11*** (MK143220.1), 3 isolates with ***strain KJF11-14*** (JQ800183.1) and 3 isolates with ***strain 92HB87*** (MG263464.1). In particular, two isolates showed a remarkable match to the species ***Flavobacterium frigidarium*** (strain FEB2-04, MG780342.1) with a percent sequence identity of 100 % and a query cover of 99 %.

Of the 24 sequences derived from isolates within the genus *Psychrobacter*, 6 showed a closer relationship to ***Psychrobacter sp. strain DZ-01-08-car*** (MK577348.1), while another 6 showed a higher similarity to ***Psychrobacter sp. strain DZ-03-13-aga*** (MK577361.1). Three sequences also matched ***Psychrobacter sp. strain TaseBurcu001*** (MN923049.1). In addition, five sequences showed a relationship with the species ***Psychrobacter glaciei*** (strain 4_KNBR_Sed_Z2, MT309522.1) with a percentage sequence identity of 98.69 % and a query cover between 99 % and 100 %, while one isolate exhibited a 100 % match with the species ***Psychrobacter aquimaris*** (strain SYR26, MH259944.1).

Finally, when the 5 sequences belonging to the genus *Chryseobacterium* were examined, 3 showed proximity to ***Chryseobacterium sp. strain M24_16S*** (MN480554.1) with a percent identity of 99.42 % to 99.81 % and a query cover of 99 %. While isolate *ELE_SI9_54* was related to ***strain M15_16S*** (MN480553.1) and *ELE_SI9_60* to ***strain 139*** (MT585933.1).

QikIce expedition

Of the 17 isolates selected from the QikIce expedition, 6 belonged to the genus Planomicrobium. 5 sequences were close to ***Planomicrobium sp. strain P148-L015c*** (MN043863.1), with percentages of sequence identity ranging from 99.62 % to 100 % and a query coverage of 99 %. Isolate QIK_1 was close to ***Planomicrobium sp. MN01-02*** (KM349904.1).

In addition, isolates *ELE_QIK_8* and *ELE_QIK_14* were closest to ***Pibocella sp. FI 1-6*** (KF365469.1), while isolate *ELE_QIK_16* matched ***Octadecabacter arcticus strain 238*** (NR_102905.1) with 100 % identity and 90 % query cover.

2.3.2.3. Association of morphological characteristics with some genera of interest

After obtaining the results of the taxonomic analyses and photographs of some colonies belonging to the PI-ICE and QikIce expeditions, the morphological characteristics observed were compared with the taxonomic data to determine the identity of the colonies based on the observable features. This was done to investigate potentially significant morphological differences between colonies of the same genus that may belong to different species, to validate the taxonomic classification based on molecular data, and to gain a more complete understanding of their biodiversity.

Certain phenotypic morphologies were recurrent within colonies belonging to the same genus. This was the case for isolates *ELE_SI9_42*, *ELE_SI9_48, ELE_SI9_56* and *ELE_SI9_67* (Fig. 2.11 (D, E, F, I)) belonging to the genus *Psychrobacter*, which were all round and whitish in colour. In terms of colony outer edge, *ELE_SI9_48* seemed to have irregular edges, while *ELE_SI9_42* had transparent edges and *ELE_SI9_56* and *ELE_SI9_67* had lighter edges. On the other hand, *ELE_SI9_49* (Fig. 2.11 (D)), which also belonging to the same genus, appeared slightly pinkish, regularly shaped and with a well-defined border.

The two isolates, *ELE_SI9_54* and *ELE_SI9_55* (Fig. 2.11 (A)), belonging to the genus *Chryseobacterium*, shared some characteristics. More specifically both were circular in shape and yellow in colour. However, *ELE_SI9_54* had a shiny appearance with a well-defined outer border, while *ELE_SI9_55* appeared opaquer with a light coloured outer border.

Of the three colonies of isolates from the genus *Flavobacterium* (Fig. 2.11 (G, H, I)), both *ELE_SI_37* and *ELE_SI_43* was yellowish in colour, had wide and transparent edges and a granular texture. In contrast, *ELE_SI9_63* appeared yellowish, with concentric, circular colonies with distinctly, lighter-coloured edges and a shiny appearance.

Finally, the two colonies, *ELE_QIK_1* and *ELE_QIK_3*, obtained during the QIKICE expedition (Fig. 2.11 (B, C)) and belonging to the genus *Planomicrobium*, showed contrasting characteristics in terms of colour and surface appearance. *ELE_QIK_1* had an orange colour, a shiny texture, a regular shape and a regular, well defined outer boarder, whereas *ELE_QIK_3* appeared whitish, had a regular shape and was almost transparent.

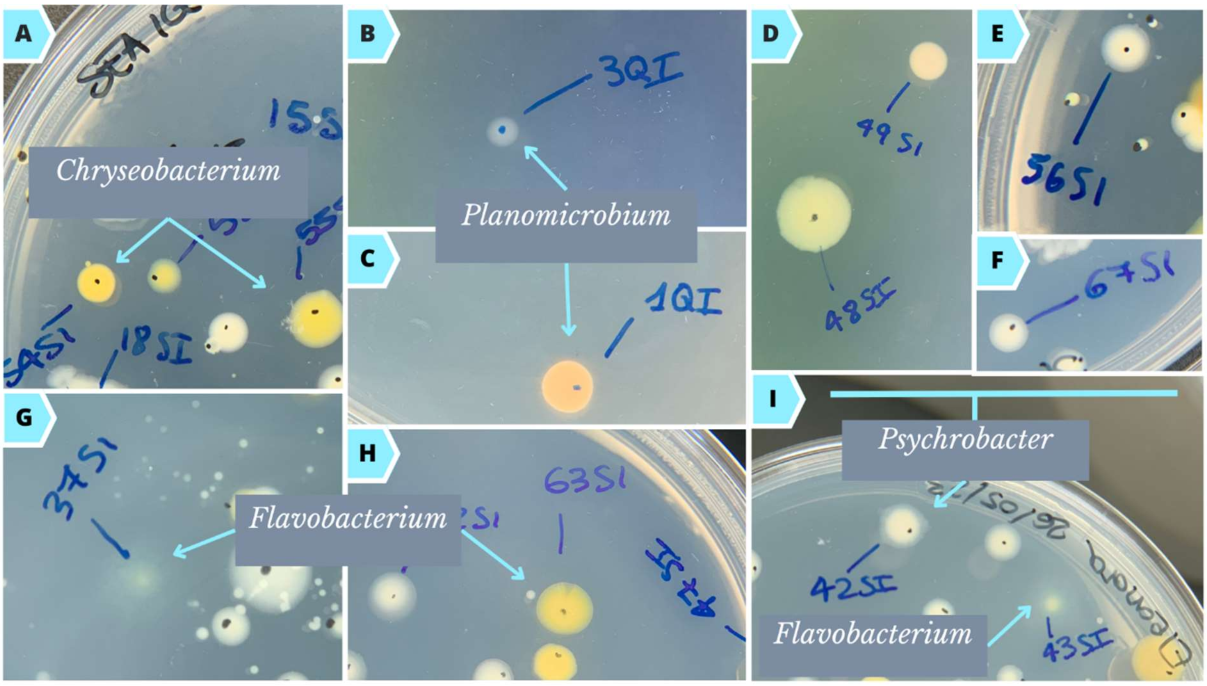


Figure 2.11. Morphology of colonies from PI-ICE and QikIce expeditions. *The figure shows the morphology of the cultivated colonies from PI-ICE and QikIce expeditions, classified by genus. Source: Prepared by the author.*

2.3.3. Phylogenetic analyses of the 199 isolates

A phylogenetic tree was constructed using the Neighbour-Joining (NJ) method to assess the degree of similarity between the 16S rRNA sequences obtained from 199 isolates (Fig. 2.12). These isolates were colour-coded according to their genus. In addition, the origin of each isolate in terms of polar region, habitat and expedition was highlighted. The reliability of sequence similarity within groups was determined using bootstrap values (shown as blue circles).

Independent clusters of isolate sequences were found in both Antarctic seawater and Antarctic sea ice. The seawater isolates belonged to the genera *Pseudoalteromonas*, *Sulfitobacter*, *Shewanella*, *Colwellia* and *Ulvibacter*, and the sea ice isolates belonged to the genus *Psychrobacter*.

Some seawater isolates from Antarctica clustered with the sea ice isolates, for the genera *Flavobacterium* and *Pseudomonas*. In particular, six *Pseudomonas* isolates (3 from seawater and 3 from sea ice) formed a single monophyletic clade. For *Flavobacterium*, the tree revealed a major clade of 22 isolates, including 18 sea ice and 4 seawater isolates, and minor clades containing only 4 sea ice isolates.

For the six *Planomicrobium* isolates from the QikIce expedition, it was possible to visualize five isolates from the sea ice that clustered independently with one isolate from seawater. Moreover, four *Psychroserpens* (3 from ANTOM-2 and 1 from QikIce) and five *Bacillus* isolates (4 from ANTOM-2 and 1 from QikIce) from Antarctic seawater and Arctic sea ice clustered. However, one of the *Bacillus* isolates from Antarctic seawater was outside the main cluster and in the phylogenetic tree was distant from the other isolates of the same genus.

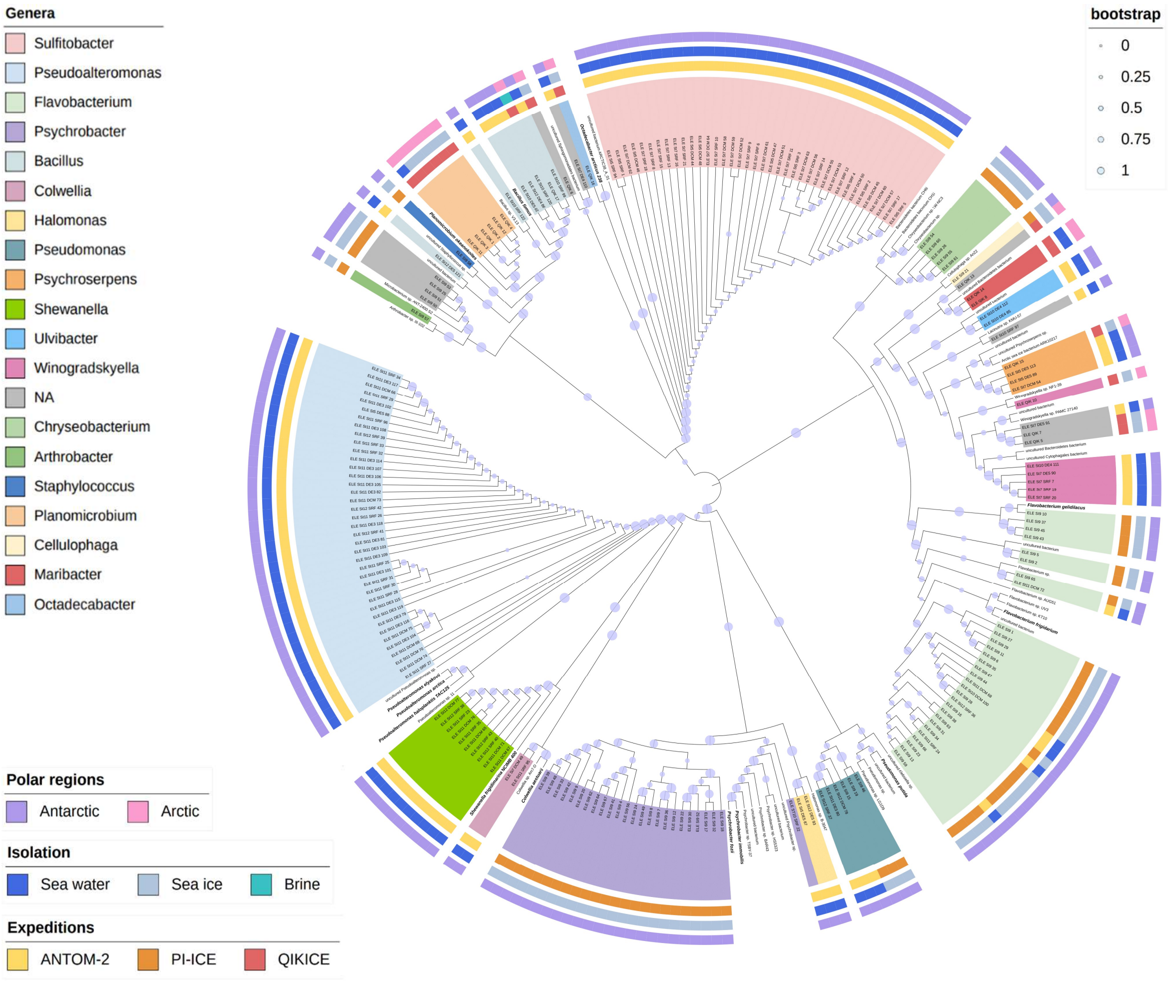


Figure 2.12. Circular neighbour-joining phylogenetic tree. *Tree based on 16S rRNA gene sequences, showing the relationship between strains from ANTOM, PI-ICE and QikIce, the genera represented in different colours, the expedition, the habitat, and the polar region to which each strain belongs. Bootstrap values are shown as blue circles. Source: Prepared by the author with MEGA11 and ITOL.*

A closer examination of the 119 Antarctic seawater isolates from different stations and depths revealed that there were distinct groups of isolates within the same genus (Fig. 2.13). In the case of *Flavobacterium*, one isolate from the DCM depth appeared to be genetically distant from the others within the same genus. For the genus *Bacillus*, three groups were identified. The largest group contained both surface and deep isolates, while two smaller groups contained one deep isolate and one from SRF. *Sulfitobacter* showed two distinct groups - one larger and one smaller. Similarly, *Pseudomonas* showed two distinct groups: one consisting of both deep and surface isolates, and another consisting of a DCM isolate. *Colwellia* also showed a two-group pattern, with one isolate from the surface and another from DCM. Finally, *Pseudoalteromonas* displayed a high number of closely related strains from different depth layers and a similar situation was observed for *Sulfitobacter*.

Figure 2.13. Rectangular neighbour-joining phylogenetic tree. *Tree based on 16S rRNA gene sequences from ANTOM-2 expedition, showing the relationship between strains, the genera found in different colours, the sampling station and the depth to which each strain belongs. Bootstrap values are shown as blue circles. The tree scale is shown. Source: Prepared by the author with MEGA11 and ITOL.*

2.3.4. Design and testing of primers for Cas9 gene detection in polar bacterial taxa

The final phase of our study focused on designing and testing primers to amplify the Cas9 genes potentially present in the sequences of some of our isolates. First, we examined the list of *Tara* Arctic MAGs to determine if any Cas9 genes were present within the same genera as our sequenced isolates. The closest matches were found for two MAGs belonging to the Flavobacteriaceae family. We then analyzed the FASTA files of both MAGs using the CRISPR-finder tool. The programme identified some spacers and Cas genes for both MAGs, although none of them turned out to be a Cas9 gene.

We then adopted a different approach by focusing on the genera *Flavobacterium*, *Psychrobacter* and *Colwellia*. We aimed to identify species within these genera that met three criteria: **1)** isolation from polar regions or cold habitats, **2)** presence of a recognized CRISPR-Cas system, and **3)** taxonomic proximity to the isolates sequenced in our study. To confirm the presence of Cas9, these species were then filtered using the CRISPR-finder tool. The results showed the presence of Cas9 only in species belonging to the genus *Flavobacterium*. Among these, sequences from *Flavobacterium frigidarium* and *Flavobacterium pectinovorum* were selected. In addition, *Flavobacterium psychrophilum* was also included due to the similarity of its Cas9 sequence to that of *F. frigidarium*.

Two pairs of primers were designed for the PCR amplification of Cas9. The first pair was designed by aligning the *F. frigidarium* and *F. psychrophilum* sequences and selecting the regions flanking the putative Cas9 gene. The designed primers consisted of a forward primer [5'- GACGGAATGAAAATTGCC -3'] of 18 bp containing 8 G+C's and a reverse primer [5'- CGTTTCTAGTTACTCTAGG -3'] of 19 bp with 8 G+C's. The expected PCR amplification size (bp) was approximately 1600 bp (Table 2.5). As for the primers designed based on the Cas9 sequence of *F. pectinovorum*, the forward primer [5'- GTAGAACTGGTGCAAGAGGAG -3'] was 21bp long, while the reverse primer [5'- CGCCTCCCATTACAACTGC -3'] was 19 bp long, both with 11 G+C's. The expectedsize of the PCR amplicon (bp) was approximately 1100bp (Table 2.5).

Table 2.5. Primers designed for Cas9 detection. *Table showing the two pairs of primers (forward and reverse) designed to amplify putative Cas9 genes in Flavobacterium isolates. The first pair targeted the Cas9 sequence of F. frigidarium and F. psychrophilum, while the second pair targeted the Cas9 sequence of F. pectinovorum.*

	Flav_frig_psy_F/Flav_frig_psy_R	**Flav_pect_F/Flav_pect_R**
Forward (5´- 3´)	GACGGAATGAAAATTGCC	GTAGAACTGGTGCAAGAGGAG
Forward length	18bp	21bp
Reverse (5´- 3´)	CGTTTCTAGTTACTCTAGG	CGCCTCCCATTACAACTGC
Reverse length	19bp	19bp
Target (Cas9)	*F. frigidarium & F. psychrophilum*	*F. pectinovorum*
G+C (%) F/R	8 G+C	11 G+C
PCR amplification size (bp)	1600	1100

A total of 22 *Flavobacterium* isolates from Antarctic seawater and sea ice samples were then selected to assess for the presence or absence of Cas9 using the designed primers. In preliminary PCR reactions, the annealing temperature chosen for the designed Cas9 primers was 55 °C, which is the standard temperature used for 16S rRNA PCR (Fig. 2.14). The DNA tested with the primers 'Flav_frig_psy_F' and 'Flav_frig_psy_R' was extracted from three seawater isolates from ANTOM-2 and fifteen sea ice isolates from PI-ICE. Meanwhile, the DNA tested with 'Flav_pect_F' and 'Flav_pect_R' were exclusively from four sea ice samples. In addition, to confirm the quality of extracted DNA from the selected samples they were tested with primers for 16S rRNA amplification in PCR. Reaction products were assessed by agarose gel electrophoresis and this confirmed the presence in all samples of an amplicon of the expected size for 16S rRNA. However, no amplicons were observed for the sequences tested with the 'Flav_frig_psy_F' and 'Flav_frig_psy_R' primers. On the other hand, with the 'Flav_pect_F' and 'Flav_pect_R' primers, a weak band greater than 600 bases was observed, and corresponded to isolate *SI9_23* (the closest match on NCBI with ***Flavobacterium sp.*** **strain 138H-11**).

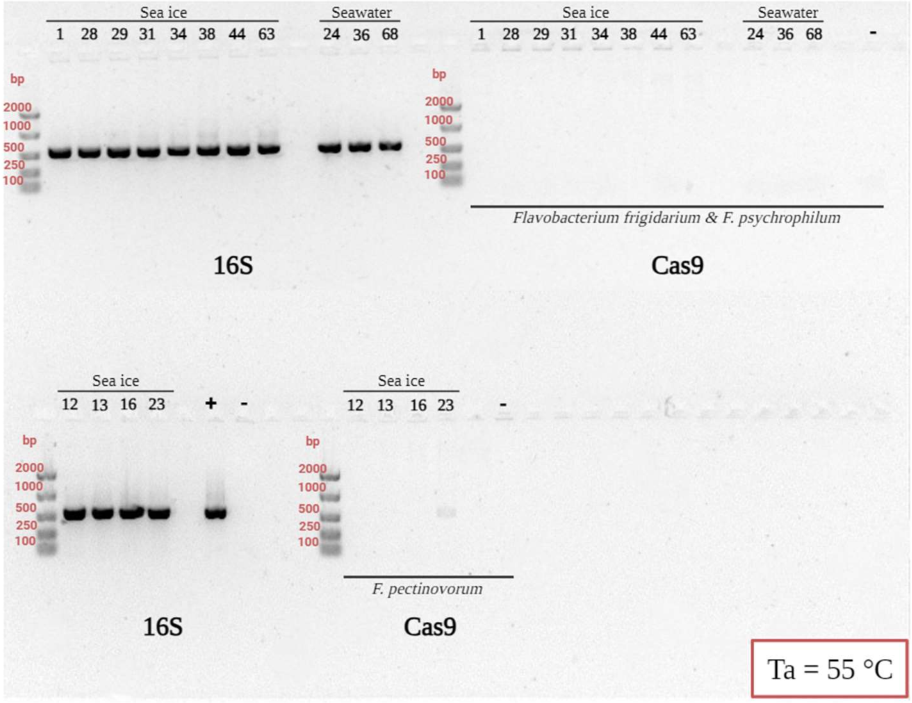


Figure 2.14. Electrophoretic gel showing PCR products amplified with 16S rRNA primers (positive control) and the primers designed to amplify Cas9 at an annealing temperature of 55 °C. *The image displays the reaction products obtained using 16S rRNA primers for PCR amplification, and Cas9 primers for PCR amplification. Information about the origin of the samples, "sea ice" and "sea water," is included. The ID codes for the tested isolates, the size markers in base pairs (bp) for the ladder and the Cas9 targeted by the primers are indicated. The annealing temperature was set at 55 °C.*

Taking into consideration the failure to amplify Cas9 with an annealing temperature of 55 ºC despite the strong reaction product obtained with the same samples using primers that amplify 16S rRNA a PCR was run with an annealing temperature set at 50 °C (Fig. 2.15). In this case, we focused on seven sea ice isolates obtained during the PI-ICE expedition. Specifically, representatives of each possible species or strain were selected for analysis by PCR. On examination of the electrophoretic gel, no amplicons for 16S rRNA was obtained with an annealing temperature of 50 ºC. However, some of the isolates amplified with the primers ('Flav_frig_psy_F' and 'Flav_frig_psy_R') designed against the Cas9 gene gave a very weak product. Specifically a PCR reaction product was detected with isolate *SI9_1* that gave the closest match in NCBI with ***Flavobacterium sp.* strain 28A** and *SI9_28*, which gave the closest match with ***Flavobacterium sp.* strain 14A**. The amplified products for isolate *SI9_1* appeared to be greater than 1000 base pairs long, while the PCR product for isolate *SI9_28* was approximately 1000 bases long. In addition, a single product was amplified in the isolates that gave a positive reaction in PCR. In the case of isolate *SI9_23*, tested with the 'Flav_frig_psy_F' and 'Flav_frig_psy_R' primers, a distinct band of more than 500 base pairs in length was observed.

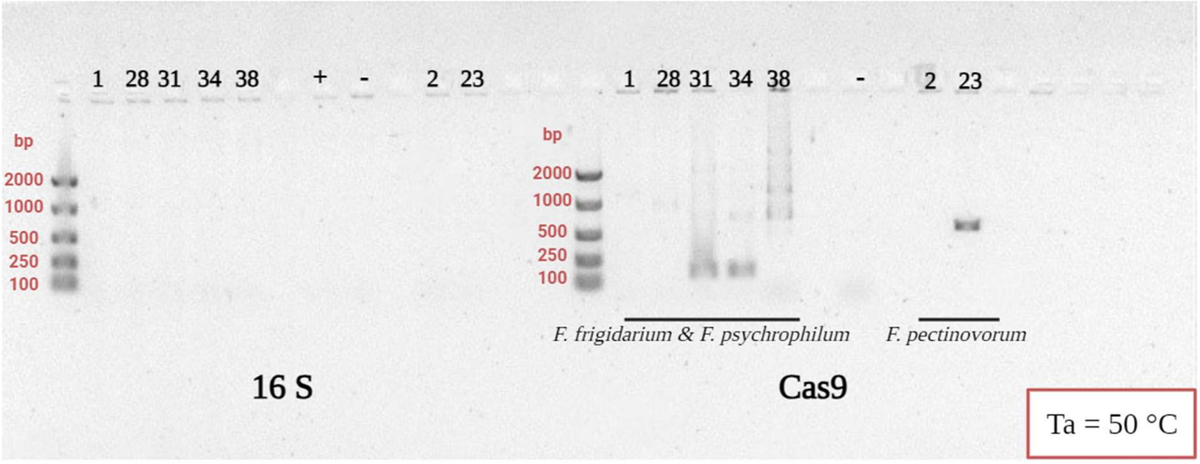


Figure 2.15. Electrophoretic gel showing the PCR products amplified with designed primers at an annealing temperature of 50 °C. *The image reveals the reaction products obtained from both 16S rRNA PCR amplification, used for validation purposes, and Cas9 PCR amplification using the designed primers. ID codes for the tested isolates, the length in base pairs (bp) for the ladder and the Cas9 targeted by the primers are indicated. The annealing temperature was set at 50 °C.*

2.4. Discussion

2.4.1. Higher cultivability in Antarctic sea ice than in seawater and in photic layers

Microbial cultivation in the laboratory is often challenging due to a variety of factors. Some reasons are straightforward and are related to the omission of essential nutrients (Köpke et al., 2005), inappropriate pH levels, inadequate incubation times (Davis et al., 2011; Janssen et al., 2002) or the use of nutrient-rich media that favour the proliferation of fast-growing microorganisms, potentially outcompeting slower growers (Pulschen et al., 2017). Our study revealed a significant difference in cultivability between seawater samples collected during the ANTOM-2 expedition and sea ice samples from the PI-ICE expedition. In particular, the seawater samples showed few colonies, with a maximum of 653 CFU/ml found in the surface layer from Station 11 (Fig. 2.3), in contrast to the abundance found in the sea ice samples, where a maximum of 6007 CFU/ml was found at site SI9 4B (Fig. 2.4). These results highlight that a higher fraction of the diversity can be retrieved in culture from sea ice samples.

In the present study, we exceeded the incubation time because psychrophilic bacteria, which thrive at temperatures of 7.2°C or lower, are generally expected to form visible colonies on plates incubated for ten days (Witter, 1961). In our case, the inoculated plates were incubated at 4°C in the dark until no more colonies appeared. The incubation times ranged from 10 to 30 days for PI-ICE samples and 30 to 60 days for ANTOM-2 samples. In addition, different culture media were used for the two habitats studied, as described in section 2.2.2.1. Seawater samples were grown on Marine Broth, a nutrient-rich medium, whereas sea ice samples were grown on R2A, a nutrient-poor medium, which probably favoured the growth of slow-growing bacteria. Analysis of the results revealed high variability in the number of colonies between the replicate plates and this presumably was a consequence of using the samples directly without dilution or effective mixing to produce a homogeneous suspension of the bacteria.

Another disparity was observed between the same sampling stations of the ANTOM-2 expedition. In particular, seawater samples from Station 11 had significantly higher colony counts than those from other stations. The cause of this phenomenon was unclear, especially when compared to neighbouring Station 10, where colony counts were consistently low at all depths. Moreover, samples from Station 11 differed from the general trend observed in seawater samples, which was a decrease in CFU/ml between samples from the photic layers and deeper layers. The complexity of these variations could potentially be explained by a combination of environmental factors, such as nutrient availability and microbial interactions within marine ecosystems. Several studies have shown that physical processes (eddies, tides, etc.) and factors such as soil runoff, wind transport of land-derived particles, vertical mixing and wind-driven water mixing do indeed affect both nutrient gradients and microbial growth (see e.g., Hewson et al., 2006; Lindh et al., 2015; Nelson et al., 2014). In addition, microbial interactions (molecular exchange, grazing, etc.) can shape community composition (Amin et al., 2012; Moran, 2015; Parsons et al., 2011). Seasonal patterns have also been found to vary with proximity to the coast, resulting in pronounced gradients - from coastal areas to open ocean surface waters - in inorganic nutrient levels and the ratio of allochtonously to autochtonously produced dissolved organic matter (DOM), which supports bacterial growth (Bunse & Pinhassi, 2017).

2.4.2. Diversity of cultured bacteria from seawater and sea ice from polar regions

The present study revealed both differences and similarities between the bacterial populations sampled from different environments. These include in the ANTOM-2 expedition bacteria from different depths at five different stations in the Bellingshausen Sea, which spans the western extent of the Antarctic Peninsula. In addition, the bacterial composition of both the Antarctic seawater and sea ice environments was examined by combining the results of the ANTOM-2 and PI-ICE expeditions. Furthermore, the scope of the study was expanded by comparing Antarctic bacterial communities from both the ANTOM-2 and PI-ICE expeditions with Arctic bacterial communities from the QikIce expedition.

2.4.2.1 Differences between bacteria across ocean layers in seawater (ANTOM-2 expedition)

During the ANTOM-2 expedition, significant taxonomic differences were observed between bacteria isolated from different depths (Fig. 2.5). In particular, the class Gammaproteobacteria was abundant in the photic layers and was the predominant class in the deep layers. This observation was in line with expectations, as bacteria belonging to this class are well-documented, easy to cultivate and commonly found in marine environments (Fuhrman & Hagström, 2008). The second most abundant class, Alphaproteobacteria, dominated the photic layers, while only one isolate was identified in the deep layers. Furthermore, from a comparative perspective using isolates from different depths revealed that *Psychrobacter* was only present in SRF, whereas *Halomonas* and *Ulvibacter* were only present in the deep layers. Nonetheless, five out of the seven genera identified in deep waters were also present in the photic layers (SRF and DCM). These genera include *Bacillus*, *Pseudoalteromonas*, *Pseudomonas*, *Psychroserpens* and *Winogradskyella*. This overlap may be due to the relatively low number of isolates obtained from the deep layers, which grew poorly on plates and exhibited slower growth and lower abundance compared to those from the surface layers. This suggests that there may be other populations less suited to conventional marine media and therefore under-represented in our study. However, the results of the present study are consistent with Sanz-Sáez et al., 2020, who found 99% clustering similarity, accounting for 52.7 % of a total of 1561 isolated sequences from different oceans and depths. This indicates common genera (and shared sequences) across these ocean layers, suggesting potential vertical connectivity within heterotrophic bacteria and highlighting their adaptability. They are likely to have versatile metabolic capabilities that allow them to thrive in different environments with fluctuating nutrient levels, ultimately facilitating their success across ocean gradients (Jones & Lennon, 2010).

A plausible explanation for the vertical connectivity between bacteria in the oceans could be explained by the capacity of bacteria to adhere and grow on particles in the photic layers. When such particulate matter sinks into the deep ocean, they may still retain the capacity for continued growth. Indeed, a recent study suggested that particle colonization by bacteria in the photic layers has a significant impact on the bacterial composition of deeper layers, particularly in bathypelagic communities (Mestre et al., 2018). This was confirmed in a recent study by Sanz-Sáez et al., 2023 that highlighted the recruitment of isolates was higher in the particle-attached fraction across all ocean layers compared to free-living communities, particularly in the larger sized fractions of the bathypelagic ocean.

More specifically, in the present study, a difference in genera was found between the different sampling stations (Fig. 2.6). However, the significance of the data is unclear since some stations had a much higher colony abundance (CFU/ml) than others, as in the case of Station 11. Nevertheless, according to Luria et al., 2014 the distance between sampling sites should not strongly influence the community composition of surface waters along the west coast of the West Antarctic Peninsula. However, it was also suggested that horizontal homogeneity could be disrupted by shifts in environmental factors linked to season. This hypothesis is supported by the observations of Signori et al., 2014, who found different surface bacterial communities at different spatial intervals when examining samples from a nearby area during the transition to autumn.

At the genus level, *Sulfitobacter* appeared to be most abundant in the photic layers and was found in samples collected at stations 5 and 7. *Sulfitobacter* is a member of the class Alphaproteobacteria and was originally proposed by Sorokin, 1995. to categorize a group of aerobic, gram-negative bacteria, mostly found in marine and hypersaline environments. It is involved in the degradation of algal dimethylsulfoniopropionate (DMSP), the main source of organic sulphur in the oceans and a precursor of dimethylsulphide (DMS), the main natural source of sulphur in the atmosphere, which contributes to cloud formation (Zeng, Qiao, et al., 2016). Specifically, for isolates belonging to this genus, the best match in NCBI (from 99.40 to 100 % percent identity) corresponded primarily to ***Sulfitobacter sp.* strain ACBC066** (MK128994.1). This strain was isolated from the sea surface during the Antarctic Circumnavigation Bacterial Collection (ACBC) expedition led by Fourquez & Hassler, 2021.

Pseudoalteromonas was found in both the photic and deeper ocean layers. This genus, proposed and formally documented by Gauthier et al., 1995, belongs to the group of gram-negative Gammaproteobacteria and is widespread throughout the marine environment. These rod-shaped bacteria are typically aerobic, chemoheterotrophic and motile. *Pseudoalteromonas* species are commonly found in polar marine samples and are very adaptable to cold environments, and even thrive under freezing conditions (Cristóbal et al., 2011; Giudice et al., 2012; E. H. Kim et al., 2010). The members of the genus *Pseudoalteromonas* are of high interest as they produce a wide range of bioactive compounds and are interesting candidates for biotechnology. Factors already identified from *Pseudoalteromonas* include antibiotics, toxins/antitoxins, anti-tumour agents and broad-spectrum enzymes, all of which exhibit remarkable specificity even at low temperatures (Holmström & Kjelleberg, 1999; Isnansetyo & Kamei, 2003; Xie et al., 2012), highlighting their considerable potential for a wide range of applications. The strain ***Pseudoalteromonas sp.* W11** (MN889233.1) in NCBI shared high similarity (from 99.62 to 100% percentage identity) with the 33 isolates from all three layers, collected at Station 11. The strain of *Pseudoalteromonas* in NCBI was isolated from the marine sponge genus *Axinella* and was used in a research study by Gutiérrez-Barranquero et al., 2019, aimed at screening for compounds that disrupt quorum sensing (QS), a vital cell-cell signaling process. QS disrupting compounds are interesting candidates for next generation antimicrobials, since they target virulence and the persistence of clinically relevant pathogens without limiting their growth.

Finally, the third most abundant genus identified in the present study was *Shewanella*, which was found exclusively in the photic layers. *Shewanella*, first described by MacDonell & Colwell, 1985, is a genus of gram-negative, rod-shaped bacteria that primarily inhabit extreme aquatic environments characterized by low temperatures and high pressures. These bacteria are known for their adaptable respiratory capacity, including the use of electron acceptors other than oxygen. *Shewanella* species are efficient in reducing and detoxifying toxic substances and heavy metals such as uranium, chromium and iron, rendering them less harmful (Nealson & Scott, 2006). This strong detoxification capacity positions *Shewanella* as a promising candidate for bioremediation (Friend, 2007). The isolates belonging to the genus *Shewanella* shared high sequence similarity to ***Shewanella sp.* strain BC1-22B** (MT350303.1) (from 99.43 to 100 % percentage identity) in NCBI. This strain was originally isolated from the brines of perennially ice-covered Antarctic lakes in the northern part of Victoria Land, specifically at the Boulder Clay site and exhibited several interesting characteristics (Rizzo et al., 2020). These included the ability of ***Shewanella sp.* strain BC1-22B** to oxidize polychlorinated biphenyls (PCBs), its resistance to copper (Cu) and its ability to resist the effects of two different metals simultaneously.

2.4.2.2. Differences between bacteria in different Antarctic polar habitats (seawater and sea ice)

In the Antarctic polar regions studied, significant differences were found between bacteria isolated from seawater and sea ice during the ANTOM-2 and PI-ICE expeditions respectively. While seawater showed a predominance of bacteria belonging to the classes Gammaproteobacteria and Alphaproteobacteria, as seen above, sea ice showed a different taxonomic composition (Fig. 2.8). Specifically, the isolates selected from the sea ice were not assigned to Alphaproteobacteria and this observation is consistent with the prevalence of Flavobacterial and Gammaproteobacterial sequences in sea ice studies. Despite seasonal variations, this general trend in relation to taxa abundance should result from the higher concentrations of dissolved organic matter (DOM) in sea ice pores compared to seawater. These conditions, therefore, favour bacteria adapted to exploit these resources, while discouraging organisms better adapted to nutrient-poor environments, such as Alphaproteobacteria, which tend to dominate the sea surface (Boetius et al., 2015). Conversely, no significant differences were observed between the isolates from the two collection sites, SI9 1B and SI9 4B. However, these two sites differed in the genus *Arthrobacter*, which was only present at site SI9 4B and the genera *Staphylococcus*, *Cellulophaga*, *Chryseobacterium* which were only present at site SI9 1B. The greater diversity of genera found at site SI9 1B may be due to the higher and faster bacterial growth observed on plates inoculated with both undiluted and 1:10 diluted samples, which may have limited the development of rarer or slower-growing organisms. Further dilution, e.g. 1:100, may be required to encourage the growth of other colonies. In particular, the most abundant genera found in sites SI9 1B and SI9 4B were *Flavobacterium* and *Psychrobacter*, followed by *Chryseobacterium.*

The name *Flavobacterium* was first proposed by Bergey et al., 1923 to categorize a genus within the family Bacteriaceae. *Flavobacterium* species are characterized as gram-negative rods, non-spore-forming, strictly aerobic and motile. These pigmented bacteria are commonly found in freshwater and soil environments (Holmes, 1992). Most of these bacteria are considered harmless as they feed primarily on inert organic matter and play an important role in biogeochemical cycles (Lee et al., 2023). Of the isolates belonging to the genus *Flavobacterium* selected in the present study, two shared 100% sequence identity with ***Flavobacterium frigidarium*** (MG780342.1), strain **FEB2-04** in NCBI. This psychrophilic, yellow-pigmented bacterium was originally isolated from the shallow waters around Adelaide Island in Antarctica (Humphry et al., 2001). Notably, the isolate *ELE_SI9_63*, which shared high sequence identity with this strain, showed a similar yellow pigmentation (Fig. 2.11 (F)). However, other isolates from the present study share sequence identity with other strains in NCBI such as ***Flavobacterium sp.*** **strain 138H-11** (MK143220.1) and ***Flavobacterium sp.*** **strain KJF11-14** (JQ800183.1). The two strains in NCBI were isolated from the subarctic region: **138H-11** during an expedition to Iceland (Costa et al., 2019) and **KJF11-14** from the waters of Kongsfjorden, a subarctic fjord in the Svalbard archipelago (Prasad et al., 2014), suggesting a possible link with the Arctic region.

The second most common genus found in Antarctic sea ice samples was *Psychrobacter*, originally described by Juni and Heym, 1986. Members of this genus are gram-negative, psychrophilic microorganisms that can be spherical or rod-shaped. They are strictly aerobic, chemo-heterotrophic, non-motile, cold-adapted, and osmotolerant. Most species within the genus can thrive at temperatures ranging from -10ºC to 42ºC, giving them a wide range of adaptability. However, they are generally isolated from cold environments such as Antarctic sea ice, sediments, deep sea waters, and permafrost (S. J. Kim et al., 2012). In the present study, five isolates showed high sequence similarity (98.69 to 100 %) with ***Psychrobacter glaciei*** (MT309522.1), specifically the **4_KNBR_Sed_Z2 strain.** This strain was originally isolated from the ice core of Austre Lovénbreen in Ny-Ålesund, Svalbard (Zeng, Yu, et al., 2016). The colonies belonging to this species appeared cream-coloured, circular, convex, smooth, and opaque, with completely continuous margins. The described morphology of the strains in NCBI is similar to the characteristics of the colonies with high sequence similarity (*ELE_SI9_56* and *ELE_SI9_67*) observed in the present study. Twelve of the isolates in the present study shared high sequence similarity with ***Psychrobacter* strains DZ-01-08-car** (MK577348.1) and **DZ-03-13-aga** (MK577361.1) deposited in NCBI, which are part of the microbiota of alga enriched in macroalgal polysaccharide-degrading bacteria in King George Island. The sequence of a further three isolates in the present study shared high identity with ***Psychrobacter sp.* strain TaseBurcu001** (MN923049.1) in NCBI, first isolated from the island of Svalbard. Members of *Psychrobacter* have shown promise in mitigating the harmful effects of herbicides in agriculture, making them of interest for agricultural and environmental biotechnology (Maciej Serda et al., 2013).

Finally, the third most abundant genus among the selected sea ice isolates was *Chryseobacterium*. It was proposed by Vandamme et al., 1994 to categorize a diverse group of gram-negative microorganisms previously included in the genus *Flavobacterium*. These bacteria are aerobic, non-fermenting and test positive for oxidase and catalase. They produce characteristic yellow pigments as was observed for the isolates *ELE_SI9_54* and *ELE_SI9_55* in the present study. *Chryseobacterium* species are widely distributed in different environments, including water, soil, plants and animals (Guiu et al., 2014). In the present study, isolates belonging to the *Chryseobacterium* genus shared sequence identity with two strains: one isolate was 100% identical to ***Chryseobacterium sp.* strain M15_16S** (MN480553.1) in NCBI, while three other isolates displayed sequence identities ranging from 99.42 to 99.81 % with **strain M24_16S** (MN480554.1) in NCBI. Both strains were isolated from guano samples collected from Adélie penguins (*Pygoscelis adeliae*) at the Point Thomas penguin rookery on the western shore of Admiralty Bay, King George Island, Antarctica (Grzesiak et al., 2020).

2.4.2.3. Comparative analyses of Antarctic vs Arctic bacterial isolates

Finally, a comparative analysis of bacteria isolated from the two polar regions, Antarctica and the Arctic, revealed taxonomic differences. However, it is important to note that the results cannot be considered statistically significant due to the limited number of isolates analysed from the QikIce expedition. This limitation was a consequence of the poor colony growth. Further research with a larger sample size is needed to confirm the results obtained in this study.

Nevertheless, among the sixteen isolates retrieved (3 from seawater, 12 from sea-ice and 1 from brine), the classes Bacilli and Flavobacteriia appeared to be the most abundant among the Arctic isolates (Fig. 2.10). In particular, these two classes were predominant among the isolates from seawater. In addition, the class Alphaproteobacteria appeared in the 1-10 cm and 70-100 cm sea ice core slices, while it was completely absent in the sixty-four isolates selected from the Antarctic sea ice during the PI-ICE expedition, as noted above.

Despite the limited number of isolates analysed, at the genus level, six different genera were identified. The diversity detected may be due to the time of sample collection, spring, since studies have shown that bacterial recruitment to sea ice is mainly facilitated by their attachment to microalgae or particles (Grossmann, 1994; Grossmann & Dieckmann, 1994). In addition, three genera were identified in both the photic and deep layers of the ANTOM-2 expedition but were absent from the sea ice samples of the PI-ICE expedition. These genera include *Psychroserpens* and *Winogradskyella*, which were found in the sea ice slice immediately adjacent to seawater, and *Bacillus*, which was found in brine with a salinity of 62 ‰. Comparing the bacteria present in the Antarctic and Artic, only three genera were unique to the Arctic and were, in order of abundance, *Planomicrobium*, *Maribacter* and *Octadecabacter*. The genus *Planomicrobium*, proposed by Yoon et al., 2001, comprises gram-stain-positive to gram-stain-variable, aerobic, motile, non-endospore-forming bacteria. Colonies of *Planomicrobium* species are typically characterized by their yellow to orange or pale orange colour (Yoon et al., 2001; Zhang et al., 2009). This morphological feature was consistent with the appearance of the isolate *ELE_QIK_1* (Fig. 2.11 (C)), but not with *ELE_QIK_3* (Fig. 2.11 (B)), which had a whitish and translucent coloration which highlight a phenotypic diversity within our isolates. Sequence analysis revealed that *ELE_QIK_1* shared good sequence similarity (99.81 %) with ***Planomicrobium sp. MN01-02*** in NCBI, while *ELE_QIK_3* shared sequence similarity (99.6 %) with ***Planomicrobium sp. strain P148-L015c*** in NCBI.

The isolates *ELE_QIK_8* and *ELE_QIK_14*, which belong to the genus *Maribacter* according to SILVA taxonomy analysis (Table 4), were closely related to the strain ***Pibocella sp.* FI 1-6** (KF365469.1) in NCBI with a sequence similarity between 99.42 to 99.43 %. This specific strain was originally isolated from the coast of King George Island and was used in a research study aimed at exploring the potential of certain Antarctic microorganisms as a source of omega-3 polyunsaturated fatty acids (Bianchi et al., 2014). Finally, another of the isolates was closely related to ***Octadecabacter arcticus*** (NR_102905.1), **strain 238**, and shared 100% sequence identity. This species is particularly abundant in Arctic sea ice, where it makes up a significant proportion (23 %) of the microbial community (Brinkmeyer et al., 2003b). ***Octadecabacter arcticus*** is a psychrophilic, heterotrophic bacterium isolated from the lower 20 cm of annual sea ice of the Arctic (Gosink & Staley, 1995). Its genome exhibits high plasticity caused by an unusually high density and diversity of transposable elements. Moreover, it appears to share 99 % sequence similarity with the 16S rRNA gene to the strain *Octadecabacter antarcticus 307* (Gosink et al., 1997). *Octadecabacter arcticus* and *Octadecabacter antarcticus 307* both possess unique xanthorodopsins that are adapted to icy environments and function as light-driven proton pumps (Vollmers et al., 2013).

The results of the present study revealing common bacterial isolates in both poles and in both habitats (seawater and sea ice), together with previous studies, support the idea of connectivity between the two polar regions at the level of culturable bacterial taxa, as has been demonstrated for microbial communities and uncultured genomes in several papers (e.g. Ghiglione et al., 2012; Royo-Llonch et al., 2021; Sul et al., 2013).

2.4.3. Phylogenetic analyses revealed potential different species and ecotypes

Our study used the relationships between the 16S rRNA sequences of the 199 isolated bacterial strains to identify monophyletic clusters. These clusters helped us identify related strains likely to have a similar evolutionary history and find links between isolates from different habitats and polar regions. It was not possible to run a Maximum Likelihood (ML) tree due to technical issues, so a Neighbour-Joining (NJ) tree was constructed. NJ trees are considered to not accurately represent evolutionary relationships but are often used when species are unidentified since it provides insight into the degree of similarity between alignments, allowing species to be grouped (Crisci & López Armengol, 1983). These groups are essentially made up of highly similar members. In the present study, we identified links between isolates from different habitats and different polar regions (Fig. 2.12). Specifically, for Antarctic isolates, some seawater isolates belonging to the genera *Flavobacterium* and *Pseudomonas* showed independent clustering with sea ice isolates of the same genera, highlighting a connection between these two habitats. It suggests that certain bacterial species in these genera may have adaptive mechanisms allowing them to thrive in seawater and sea ice environments. A similar pattern of behaviour can also be hypothesized for isolates of the genus *Planomicrobium* found in the Arctic. Again, a cluster was found between sea ice isolates and a single seawater isolate, further suggesting the presence of adaptive strategies to survive in both biomes. A link was observed between isolates from Antarctic seawater belonging to the genera *Psychroserpens* and

Bacillus and their counterparts found in Arctic sea ice, suggesting a cosmopolitan polar taxa. In addition, the genetic distance observed in one of the *Bacillus* isolates from deep Antarctic seawater compared to others within the same genus should be validated at the genome level and subjected to further analysis.

Finally, the Antarctic seawater isolates from different depths and sampling stations, the presence of three distinct groups among the isolates belonging to the genus *Bacillus* indicates a higher level of diversity within this genus compared to other taxa (Fig. 2.13). This diversity was further supported by the wider range of sequence identity observed within the genus (Fig. 2.7). In addition, the substantial number of closely related strains from different depth layers observed for both *Pseudoalteromonas* and *Sulfitobacter* underlines their microdiversity.

2.4.4. Detection of the Cas9 gene in polar bacterial isolates

In the final phase of our study, primers were designed to investigate the potential presence of Cas9 genes in our polar bacterial isolates. To begin, the list of MAGs from Tara Arctic was examined and only two uncultured MAGs were found that appeared to be taxonomically close to some of the isolates in the present study. These MAGs belonged to the Flavobacteriaceae family and contained CRISPR-Cas systems but did not have Cas9 genes. However, it should be noted that the list of MAGs is not exhaustive and their genomes in many cases are not full length and there may be Cas9 genes that were not detected. Two pairs of primers were designed to amplify potential Cas9 genes in selected isolates belonging to the genus *Flavobacterium* from Antarctic sea ice and seawater environments. The first primer pair was designed using Cas9 sequences from *F. frigidarium* and *F. psychrophilum* as templates, while the second pair was designed based on Cas9 sequence identified in *F. pectinovorum*. Using the first pair of primers, PCR reaction products were obtained at an annealing temperature of 50°C. The isolate *SI9_1* (the closest match on NCBI to ***Flavobacterium sp.*** **strain 28A**) and *SI9_28* (the closest match to ***Flavobacterium sp.*** **strain 14A**) had weak reaction products between 1000 and 1500 bp when the PCR reactions were analysed by agarose gel electrophoresis. These lengths were slightly shorter than the expected 1600 bp, indicating that the primers need further optimization, but they appear to be suitable for amplifying a potential Cas9 gene.

Regarding the potential Cas9 sequence from isolate *SI9_23* (the closest match with ***Flavobacterium sp.*** **strain 138H-11**), amplified with the 'Flav_frig_psy_F' and 'Flav_frig_psy_R' primers, a product slightly larger than 500 bp was identified for PCRs in which the annealing temperature was 55°C and at 50°C. However, the reaction products were considerably smaller than the expected length of about 1100 bp. The smaller size of the reaction product may indicate that they were the outcome of non-specific amplification. To address this issue, further experiments are needed, including sequencing and analysis of the product, better optimization of the PCR conditions and even the design of new primers. In addition, a new list of MAGs from the ANTOM-2, PI-ICE and QikIce expeditions is currently being prepared. Establishing a direct link between these MAGs and the isolates from our study will facilitate the identification of potential Cas9 genes. By using the link between uncultured MAGs and cultivated isolates, future research efforts can focus on elucidating the presence and functionality of Cas9 genes with greater accuracy and efficiency. This approach not only provides a more direct route to gene discovery but also allows for a more comprehensive understanding of CRISPR-Cas systems in the context of these polar environments.

2.5. Conclusions

Culturing remains an important tool in microbial ecology, facilitating the study of marine community diversity. Despite the limitations of our study, significant progress was made to improve understanding of bacterial taxonomy, phylogenetic diversity, and distribution in polar environments. In particular, differences in cultivability and diversity were identified between sea ice and seawater samples. Depth-dependent variations and disparities between bacterial communities in the Antarctic and Arctic regions were uncovered and at the same time polar cosmopolitan taxa (in both Antarctic and Arctic environments) and in both biomes (seawater and sea-ice) were identified reflecting surprising genetic adaptation to broad ranges of environmental polar conditions (salinity, light, nutrients). Finally, primers were designed to detect Cas9 genes in *Flavobacterium* isolates, suggesting the potential presence of Cas9 genes in organisms uniquely adapted to extreme cold conditions. This highlights the untapped research opportunities for studies of the CRISPR system in polar regions.

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Annexes

Appendix I – Tables CFU/ml from ANTOM-2 and PI-ICE and Number of colonies from QikIce

ANTOM-2 expedition

Vertical Profile	Station	Mean CFU/ml	STD	Mean CFU/ml	STD	Mean CFU/ml	STD
		1st count (≈ 30 days)		2nd count (≈ 45 days)		3rd count (≈ 60 days)	
Station 5	SRF	20	10	23	5.77	27	5.77
	DCM	17	15.28	17	15.28	30	0
	DE3	0	0	0	0,00	0	0
	DE4	3	5.77	7	5.77	7	5.77
	DE5	10	0	10	0	13	5.77
Station 7	SRF	53	15.28	63	23.09	63	23.09
	DCM	67	55.08	80	51.96	87	47.26
	DE3	0	0	0	0	0	0
	DE4	0	0	0	0	0	0
	DE5	0	0	7	5.77	10	10
Station 10	SRF	7	5.77	7	5.77	7	5.77
	DCM	3	5.77	3	5.77	3	5.77
	DE3	0	0	0	0	0	0,00
	DE4	0	0	3	5.77	7	5.77
	DE5	0	0	0	0	0	0
Station 11	SRF	647	61.10	647	61.10	653	60.28
	DCM	397	23.09	407	25.17	407	25.17
	DE3	120	26.46	120	26.46	120	26.46
	DE4	0	0	0	0	0	0
	DE5	3	5.77	3	5.77	3	5.77
Station 12	SRF	27	5.77	27	5.77	27	5.77
	DCM	7	5.77	7	5.77	7	5.77
	DE3	0	0	3	5.77	3	5.77
	DE4	7	5.77	10	10	10	10
	DE5	3	5.77	3	5.77	3	5.77

PI-ICE expedition

Site	Culture days	Mean CFU/ml	STD
SI9 1B	≈ 10 days	513	25.17
SI9 1B	≈ 20 days	717	66.58
SI9 1B	≈ 30 days	843	90.74
SI9 4B	≈ 10 days	5083	389.40
SI9 4B	≈ 20 days	5393	288.85
SI9 4B	≈ 30 days	6007	465.44

QikIce expedition

Isolation	Location	Culture medium	N. colonies		
			R1	R2	R3
Sea water	Surface	Marine Broth and Agar	3	1	1
Sea ice	1-10 cm	Marine Broth and Agar	1	8	3
		R2A	0	0	0
	10-40 cm	Marine Broth and Agar	1	0	1
		R2A	0	0	0
	70-100 cm	Marine Broth and Agar	1	2	1
		R2A	2	0	1
Brine	Brine 62‰	Marine Broth, NaCl and Agar	0	0	0
		Marine Broth and Agar	1	0	0
	Brine 76‰	Marine Broth, NaCl and Agar	0	0	0
		Marine Broth and Agar	0	0	0

Appendix II – Tables Taxonomy by 16S rRNA gene analyses (SILVA)

ANTOM-2 expedition

ID SEQUENCE	Depth	Station	Identity %	Score	Class	Genus
ELE_St5_SRF_1	SRF	St. 5	98.99	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St5_SRF_2	SRF	St. 5	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St5_SRF_3	SRF	St. 5	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St5_SRF_4	SRF	St. 5	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St5_SRF_5	SRF	St. 5	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St5_SRF_94	SRF	St. 5	99.01	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_10	SRF	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_11	SRF	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_12	SRF	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_13	SRF	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_14	SRF	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_15	SRF	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_16	SRF	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_17	SRF	St. 7	99.61	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_18	SRF	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_19	SRF	St. 7	98.88	99	Flavobacteriia	*Winogradskyella*
ELE_St7_SRF_20	SRF	St. 7	98.7	98	Flavobacteriia	*Winogradskyella*
ELE_St7_SRF_21	SRF	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_6	SRF	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_7	SRF	St. 7	98.88	99	Flavobacteriia	*Winogradskyella*
ELE_St7_SRF_8	SRF	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_SRF_9	SRF	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St10_SRF_120	SRF	St. 10	99.63	99	Bacilli	*Bacillus*
ELE_St10_SRF_122	SRF	St. 10	97.56	99	Bacilli	*Bacillus*
ELE_ST10_SRF_22	SRF	St. 10	100	100	Gammaproteobacteria	*Psychrobacter*
ELE_St10_SRF_97	SRF	St. 10	99.05	99	Flavobacteriia	*NA*
ELE_St10_SRF_99	SRF	St. 10	99.62	99	Bacilli	*Bacillus*
ELE_St11_SRF_23	SRF	St. 11	100	100	Gammaproteobacteria	*Shewanella*
ELE_St11_SRF_24	SRF	St. 11	99.22	99	Flavobacteriia	*Flavobacterium*
ELE_St11_SRF_25	SRF	St. 11	99.63	98	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_SRF_26	SRF	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_SRF_27	SRF	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_SRF_28	SRF	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_SRF_29	SRF	St. 11	99.8	99	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_SRF_30	SRF	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_SRF_31	SRF	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_SRF_32	SRF	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_SRF_33	SRF	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_SRF_34	SRF	St. 11	99.62	98	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_SRF_35	SRF	St. 11	99.81	99	Gammaproteobacteria	*Shewanella*
ELE_St11_SRF_95	SRF	St. 11	99.24	99	Gammaproteobacteria	*Colwellia*
ELE_St11_SRF_96	SRF	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St12_SRF_36	SRF	St. 12	99.02	99	Flavobacteriia	*Flavobacterium*
ELE_St12_SRF_37	SRF	St. 12	100	100	Gammaproteobacteria	*Pseudomonas*
ELE_St12_SRF_38	SRF	St. 12	100	100	Gammaproteobacteria	*Shewanella*
ELE_St12_SRF_39	SRF	St. 12	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St12_SRF_40	SRF	St. 12	100	100	Gammaproteobacteria	*Shewanella*
ELE_St12_SRF_41	SRF	St. 12	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St12_SRF_42	SRF	St. 12	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St12_SRF_43	SRF	St. 12	100	100	Gammaproteobacteria	*Shewanella*
ELE_St5_DCM_44	DCM	St. 5	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St5_DCM_45	DCM	St. 5	99.8	99	Alphaproteobacteria	*Sulfitobacter*

ELE_St5_DCM_46	DCM	St. 5	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St5_DCM_47	DCM	St. 5	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St5_DCM_48	DCM	St. 5	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_49	DCM	St. 7	100	100	Gammaproteobacteria	*Colwellia*
ELE_St7_DCM_50	DCM	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_51	DCM	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_52	DCM	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_53	DCM	St. 7	99.6	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_54	DCM	St. 7	98.85	98	Flavobacteriia	*Psychroserpens*
ELE_St7_DCM_55	DCM	St. 7	99.6	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_56	DCM	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_57	DCM	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_58	DCM	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_59	DCM	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_60	DCM	St. 7	99.8	99	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_61	DCM	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_62	DCM	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_63	DCM	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St7_DCM_64	DCM	St. 7	100	100	Alphaproteobacteria	*Sulfitobacter*
ELE_St10_DCM_100	DCM	St. 10	99.29	98	Flavobacteriia	*Flavobacterium*
ELE_St11_DCM_65	DCM	St. 11	100	100	Gammaproteobacteria	*Shewanella*
ELE_St11_DCM_66	DCM	St. 11	99.82	99	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DCM_67	DCM	St. 11	100	100	Gammaproteobacteria	*Shewanella*
ELE_St11_DCM_68	DCM	St. 11	99.23	99	Flavobacteriia	*Flavobacterium*
ELE_St11_DCM_69	DCM	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DCM_70	DCM	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DCM_71	DCM	St. 11	100	100	Gammaproteobacteria	*Shewanella*
ELE_St11_DCM_72	DCM	St. 11	99.02	99	Flavobacteriia	*Flavobacterium*
ELE_St11_DCM_73	DCM	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DCM_74	DCM	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DCM_75	DCM	St. 11	99.81	99	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DCM_76	DCM	St. 11	99.43	98	Gammaproteobacteria	*Shewanella*
ELE_St12_DCM_77	DCM	St. 12	99.63	99	Gammaproteobacteria	*Shewanella*
ELE_St12_DCM_78	DCM	St. 12	99.43	99	Gammaproteobacteria	*Pseudomonas*
ELE_St5_DE5_113	Deep	St. 5	99.43	99	Flavobacteriia	*Psychroserpens*
ELE_St5_DE5_87	Deep	St. 5	99.81	99	Gammaproteobacteria	*Halomonas*
ELE_St5_DE5_88	Deep	St. 5	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St5_DE5_89	Deep	St. 5	98.49	98	Flavobacteriia	*Psychroserpens*
ELE_St7_DE4_110	Deep	St. 7	100	100	Alphaproteobacteria	*NA*
ELE_St7_DE5_90	Deep	St. 7	99.07	99	Flavobacteriia	*Winogradskyella*
ELE_St7_DE5_91	Deep	St. 7	99.43	99	Flavobacteriia	*NA*
ELE_St10_DE4_111	Deep	St. 10	100	100	Flavobacteriia	*Winogradskyella*
ELE_St10_DE4_112	Deep	St. 10	97.51	99	Flavobacteriia	*Ulvibacter*
ELE_St10_DE4_85	Deep	St. 10	97.85	99	Flavobacteriia	*Ulvibacter*
ELE_St10_DE5_92	Deep	St. 10	99.62	99	Bacilli	*Bacillus*
ELE_St11_DE3_101	Deep	St. 11	99.81	99	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_102	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_103	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_104	Deep	St. 11	99.62	99	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_105	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_106	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_107	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_108	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_109	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_114	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_115	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_116	Deep	St. 11	99.81	99	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_117	Deep	St. 11	99.63	98	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_118	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_119	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_79	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_80	Deep	St. 11	99.81	99	Gammaproteobacteria	*Pseudomonas*

ELE_St11_DE3_81	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St11_DE3_82	Deep	St. 11	100	100	Gammaproteobacteria	*Pseudoalteromonas*
ELE_St12_DE4_86	Deep	St. 12	99.63	99	Bacilli	*Bacillus*
ELE_St12_DE5_121	Deep	St. 12	98.12	99	Bacilli	*Bacillus*
ELE_St12_DE5_93	Deep	St. 12	100	100	Gammaproteobacteria	*Halomonas*

PI-ICE expedition

ID SEQUENCE	**Site**	**Identity %**	**Score**	**Class**	**Genus**
ELE_SI9_1	SI9 1B	99.43	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_10	SI9 1B	100	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_11	SI9 1B	99.04	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_12	SI9 1B	99.81	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_13	SI9 1B	98.27	98	Flavobacteriia	*Flavobacterium*
ELE_SI9_15	SI9 1B	99.62	99	Gammaproteobacteria	*Pseudomonas*
ELE_SI9_16	SI9 1B	98.46	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_17	SI9 1B	98.49	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_18	SI9 1B	98.31	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_19	SI9 1B	98.12	99	Gammaproteobacteria	*Pseudomonas*
ELE_SI9_2	SI9 1B	98.85	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_20	SI9 1B	99.62	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_21	SI9 1B	99.43	99	Flavobacteriia	*Cellulophaga*
ELE_SI9_22	SI9 1B	98.31	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_23	SI9 1B	98.28	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_24	SI9 1B	99.06	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_25	SI9 1B	99.03	97	Actinobacteria	*NA*
ELE_SI9_26	SI9 1B	98.28	99	Flavobacteriia	*Chryseobacterium*
ELE_SI9_36	SI9 1B	100	100	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_4	SI9 1B	100	100	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_49	SI9 1B	98.84	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_5	SI9 1B	98.11	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_50	SI9 1B	99.81	99	Actinobacteria	*NA*
ELE_SI9_51	SI9 1B	99.81	99	Actinobacteria	*NA*
ELE_SI9_52	SI9 1B	98.5	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_53	SI9 1B	99.81	99	Actinobacteria	*NA*
ELE_SI9_54	SI9 1B	96.95	99	Flavobacteriia	*Chryseobacterium*
ELE_SI9_55	SI9 1B	98.1	99	Flavobacteriia	*Chryseobacterium*
ELE_SI9_56	SI9 1B	99.44	98	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_58	SI9 1B	99.44	99	Bacilli	*Staphylococcus*
ELE_SI9_59	SI9 1B	97.9	98	Flavobacteriia	*Flavobacterium*
ELE_SI9_6	SI9 1B	100	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_60	SI9 1B	98.86	99	Flavobacteriia	*Chryseobacterium*
ELE_SI9_61	SI9 1B	97.9	99	Flavobacteriia	*Chryseobacterium*
ELE_SI9_7	SI9 1B	100	100	Gammaproteobacteria	*Psychrobacter*

ELE_SI9_8	SI9 1B	100	100	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_9	SI9 1B	100	100	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_27	SI9 4B	99.43	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_28	SI9 4B	99.03	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_29	SI9 4B	99.43	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_30	SI9 4B	98.32	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_31	SI9 4B	98.28	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_32	SI9 4B	98.13	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_33	SI9 4B	100	100	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_34	SI9 4B	98.47	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_35	SI9 4B	99.04	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_37	SI9 4B	99.43	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_38	SI9 4B	98.85	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_39	SI9 4B	100	100	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_41	SI9 4B	99.07	98	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_42	SI9 4B	100	100	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_43	SI9 4B	99.05	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_44	SI9 4B	99.04	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_45	SI9 4B	99.24	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_46	SI9 4B	97.92	98	Gammaproteobacteria	*Pseudomonas*
ELE_SI9_47	SI9 4B	99.04	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_48	SI9 4B	100	100	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_57	SI9 4B	99.62	99	Actinobacteria	*Arthrobacter*
ELE_SI9_62	SI9 4B	99.62	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_63	SI9 4B	98.67	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_64	SI9 4B	99.79	99	Gammaproteobacteria	*Psychrobacter*
ELE_SI9_65	SI9 4B	98.67	99	Flavobacteriia	*Flavobacterium*
ELE_SI9_66	SI9 4B	97.72	98	Flavobacteriia	*Flavobacterium*
ELE_SI9_67	SI9 4B	98.69	96	Gammaproteobacteria	*Psychrobacter*

Appendix III – Tables Closest matches for sequences belonging to the most abundant genera (NCBI)

ANTOM-2 expedition

Sequence ID	Closest Match NCBI	Strain	Identity %	Accession Number
ELE_St11_DCM_66, ELE_St11_SRF_34, ELE_St11_DE3_117	*Pseudoalteromonas elyakovii*	I 84	100	FJ200650.1
ELE_St11_SRF_27	*Pseudoalteromonas sp.*	1643	100	MT585886.1
ELE_St11_DCM_74	*Pseudoalteromonas sp.*	C9_6	100	OQ300297.1
ELE_St11_SRF_26, ELE_St11_SRF_28, ELE_St11_SRF_29,	*Pseudoalteromonas sp.*	W11	100	MN889233.1
ELE_St11_SRF_30, ELE_St11_SRF_31, ELE_St11_SRF_32,			100	
ELE_St11_SRF_33, ELE_St12_SRF_39, ELE_St11_DCM_69,			100	
ELE_St11_DCM_70, ELE_St11_DCM_73, ELE_St11_DE3_79,			100	
ELE_St11_DE3_81, ELE_St11_DE3_82, ELE_St11_SRF_96,			100	
ELE_St5_DE5_88, ELE_St11_DE3_102, ELE_St11_DE3_103,			100	
ELE_St11_DE3_105, ELE_St11_DE3_106, ELE_St11_DE3_107,			100	
ELE_St11_DE3_108, ELE_St11_DE3_109, ELE_St11_DE3_114,			100	
ELE_St11_DE3_115, ELE_St11_DE3_116, ELE_St11_DE3_118			100	
ELE_St11_DE3_119			100	
ELE_St12_SRF_41, ELE_St11_DE3_101, ELE_St11_DE3_104			99.81	
ELE_St11_SRF_25			99.63	
ELE_St12_SRF_42			99.62	
ELE_St11_DCM_75	*Pseudoalteromonas sp.*	WN2	99.62	MN889235.1
ELE_St5_SRF_2, ELE_St5_SRF_3, ELE_St5_SRF_4	*Sulfitobacter sp.*	ACBC066	100	MK128994.1
ELE_St7_SRF_8, ELE_St7_SRF_9, ELE_St7_SRF_11			100	
ELE_St5_SRF_12, ELE_St7_SRF_13, ELE_St7_SRF_15			100	
ELE_St7_SRF_16, ELE_St7_SRF_18, ELE_St7_SRF_21			100	
ELE_St5_DCM_44, ELE_St5_DCM_45, ELE_St5_DCM_48			100	
ELE_St7_DCM_56, ELE_St7_DCM_60, ELE_St7_DCM_62			100	
ELE_St7_DCM_63, ELE_St7_DCM_64, ELE_St7_SRF_10			100	
ELE_St7_SRF_14, ELE_St7_DCM_50, ELE_St7_DCM_52			100	
ELE_St7_DCM_55, ELE_St7_DCM_57, ELE_St7_DCM_58			100	
ELE_St7_DCM_59, ELE_St7_DCM_61			100	
ELE_St5_SRF_5, ELE_St5_DCM_46, ELE_St5_DCM_47			99.80	
ELE_St7_DCM_51, ELE_St7_SRF_17, ELE_St7_DCM_53			99.80	
ELE_St7_SRF_6			99.40	
ELE_St11_SRF_23, ELE_St12_SRF_38, ELE_St12_SRF_40	*Shewanella sp.*	BC1-22B	100	MT350303.1
ELE_St12_SRF_43, ELE_St11_DCM_65, ELE_St11_DCM_67			100	
ELE_St11_DCM_71, ELE_St11_SRF_35			100	
ELE_St12_DCM_77			99.82	
ELE_St11_DCM_76			99.43	

PI-ICE expedition

Sequence ID	Closest Match NCBI	Strain	Identity %	Accession Number
ELE_SI9_38, ELE_SI9_63	*Flavobacterium frigidarium*	FEB2-04	100	MG780342.1
ELE_SI9_34			99.42	
ELE_SI9_10	*Flavobacterium sp.*	IMCC25616	100	KU199721.1
ELE_SI9_37, ELE_SI9_43, ELE_SI9_45	*Flavobacterium sp.*	KJF11-14	100	JQ800183.1
ELE_SI9_16,	*Flavobacterium sp.*	138H-11	100	MK143220.1
ELE_SI9_13, ELE_SI9_23			99.81	
ELE_SI9_28, ELE_SI9_35, ELE_SI9_44	*Flavobacterium sp.*	14A	100	MN007165.1
ELE_SI9_47			100	
ELE_SI9_1, ELE_SI9_6, ELE_SI9_27	*Flavobacterium sp.*	28A	100	MN007166.1
ELE_SI9_29			100	
ELE_SI9_11			99.62	
ELE_SI9_2	*Flavobacterium sp.*	2W20Y	100	MZ067624.1
ELE_SI9_5			99.81	
ELE_SI9_66, ELE_SI9_31	*Flavobacterium sp.*	92HB87	99.81	MG263464.1
ELE_SI9_59			99.62	
ELE_SI9_65	*Flavobacterium sp.*	EXB-L-2291	100	MK670552.1
ELE_SI9_8	*Psychrobacter aquimaris*	SYR26	100	MH259944.1
ELE_SI9_4	*Psychrobacter glaciei*	4_KNBR_Sed_Z2	100	MT309522.1
ELE_SI9_56			99.43	
ELE_SI9_64			99.38	
ELE_SI9_41			99.25	
ELE_SI9_67			98.69	
ELE_SI9_24	*Psychrobacter sp.*	Nj-36	100	AM491455.1
ELE_SI9_49			99.81	
ELE_SI9_17, ELE_SI9_18, ELE_SI9_30	*Psychrobacter sp.*	DZ-01-08-car	100	MK577348.1
ELE_SI9_52			100	
ELE_SI91B_22			99.81	
ELE_SI9_32			99.62	
ELE_SI9_20, ELE_SI9_9, ELE_SI9_33	*Psychrobacter sp.*	DZ-03-13-aga	100	MK577361.1
ELE_SI9_39, ELE_SI9_48, ELE_SI9_62			100	
ELE_SI9_42	*Psychrobacter sp.*	P151-L01a	100	MN043902.1
ELE_SI9_7, ELE_SI9_12, ELE_SI9_36	*Psychrobacter sp.*	TaseBurcu001	100	MN923049.1
ELE_SI9_54	*Chryseobacterium sp.*	M15_16S	100	MN480553.1
ELE_SI9_55, ELE_SI9_61	*Chryseobacterium sp.*	M24_16S	99.81	MN480554.1
ELE_SI9_26			99.42	
ELE_SI9_60	*Chryseobacterium sp.*	139	99.62	MT585933.1

QikIce expedition

Sequence ID	Closest Match NCBI	Strain	Identity %	Accession Number
ELE_QIK_11, ELE_QIK_12	*Planomicrobium sp.*	P148-L015c	100	MN043863.1
ELE_QIK_2, ELE_QIK_4			99.81	
ELE_QIK_3			99.62	
ELE_QIK_1	*Planomicrobium sp.*	MN01-02	99.81	KM349904.1
ELE_QIK_14	*Pibocella sp.*	FI 1-6	99.43	KF365469.1
ELE_QIK_8			99.42	
ELE_QIK_13	*Maribacter sp.*	I2216E04	98.86	MK841148.1
ELE_QIK_17	*Bacillus sp.*	JZDN18	100	DQ658998.1
ELE_QIK_16	*Octadecabacter arcticus*	238	100	NR_102905.1
ELE_QIK_10	*Winogradskyella sp.*	0KQ16	99.23	MH929572.1